Bioprocess Studies on Ethanol Production in Simultaneous Saccharification and Fermentation with Novel Fermenting *Mucor* spp.

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General introduction

Fossil fuel consumption has been increasing every year by using of car and airplane *etc*. It has released carbon dioxide to atmosphere approximately 392 billion metric tons since 1751. The half of these fossil-fuel CO_2 emissions has occurred since the middle 1980s. Then, the 2013 global CO_2 emission by fossil fuel burning was estimated to 9776 million metric tons of carbon; represents an all-time high and a 1.1% increase over 2012 emissions. Moreover, it was being an increase of about 7% just in the beginning of the current decade [1]. Some people point out that excessive consumption of fuel triggers depletion of fossil fuels and CO_2 emissions are a global problem contributing to global warming. From these current states, biofuels as renewable energies has emerged and is expected source replacing fossil fuels that will benefit the environment for natural resources preservation and greenhouse gases mitigation.

Today, 1st- and 2nd-generation biofuel have been increasingly understood. The former is produced from food crops such as corn in USA, sugar cane in Brazil, and oil seeds rape biodiesel in Germany. These have been characterized by mature commercial markets and well-understood manufacturing techniques. However, we have found that there are limits to the ability to achieve goals for petroleum product substitution, climate change mitigation and economic growth. The production is also concern contribution of higher food prices due to competition with food supply. Furthermore, it needs an expensive option for its energy development taking into account total production costs. Many serious problems which associated with 1st-generation biofuels can be addressed by the manufacturing from lignocellulosic biomasses such as agricultural and forest residues; 2nd-generation biofuels. Since lignocellulosic feedstocks are produced on the same land of crops grown and higher yield than crops, the biofuels have good potential for cost reduction and increase of production efficiency. Additionally, these 2nd-generation biofuels processes are fairly immature at this moment so they have enough space to enhance the production technology. Depending partly on oil price, this development is likely to be a solution to the challenge of shifting the transport sector to a more sustainable energy source. Although the lower initial investments on infrastructure required for the fossil fuel based energy system when compared to the renewable technologies of biofuels, it

is absolutely imperative for development on CO_2 emission mitigation technologies. The fuels production is expected not only for these environmental problems but also many amount of abundant agricultural residues that are undisposed and deposit on ground can be reduced the volume and converted valuable materials. 2nd-generation biofuels could avoid many of the concerns facing that of 1st-generation and potentially offer greater cost reduction potential in the longer term. However, major technical and economic hurdles are still faced before it can be deployed widely. The main effort normally focuses on advancing technologies that enable the cost reduction required for each process of biofuel production, as well as the research for fuels with lower carbon content and technology systems able to capture and store CO_2. In these manufacturing facilities, 2nd-generation bioethanol particularly has been recognized as a viable contender to replace a fossil-based fuel, gasoline, in many countries. It can be produce easily by well-known process similar with brewage. Strategy of bioethanol production was shown in Fig.1. Lignocellulosic biomasses as feedstock require pretreatment first, then are hydrolyzed by enzymes and finally converted to ethanol by fermentation using microorganisms.

Bioethanol is appropriate for mixing in gasoline because of the high octane number, broader flammability limits, flame speeds, and heats of vaporization hinder self-ignition [2]. Some properties of alcohol fuels are shown in Table 1. These properties allow for a higher compression ratio, shorter burn time and leaner burn engine which lead to theoretical efficiency advantages over gasoline in an internal contribution engine [3]. Lower flame temperature of bioethanol than that of gasoline benefits atmosphere by being impeded emissions of NOx and SOx. Disadvantage of bioethanol is the lower energy density than gasoline (66% energy compared to gasoline), corrosiveness on aluminum engine, low flame luminosity, lower vapor pressure (difficulty of cold start), miscibility with water, and toxicity to ecosystems [4]. Ethanol is an oxygenated fuel that contains 35% oxygen, which reduced particulate emissions from combustion. Hydrocarbon and carbon monoxide generated by combustion can also be reduced by the bioethanol. However, oxygenated fuels tend to increase nitrogen oxide emissions. Besides its use as an oxygenate, ethanol has also been used as a major fuel component. Considering these points, bioethanol has also used as blended fuel with gasoline in various countries. The most popular blend is known as E85 that contains 85% bioethanol and 15%

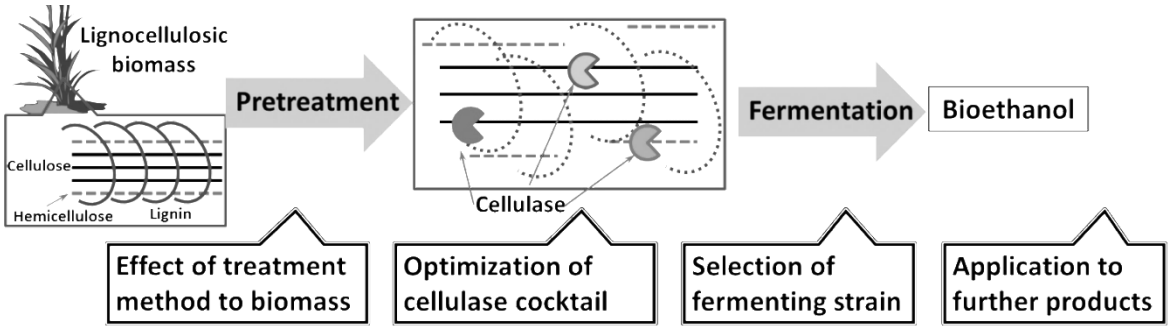

Fig. 1 Strategy of bioethanol production from lignocellulosic biomass

Table 1 Some properties of alcohol fuels

Fuel property	Isooctane	Methanol	Ethanol
Cetane number	—	5	8
Octane number	100	112	107
Auto-ignition (K)	530	737	606
Latent heat of vaporization (MJ/kg)	0.26	1.18	0.91
Lower heating value (MJ/kg)	44.4	19.9	26.7

Source: Ref. [2]

gasoline. In Brazil, sugar cane derivative bioethanol is used pure or blended with gasoline in a mixture called “gasohol” (24% bioethanol, 76% gasoline) [5]. Moreover, bioethanol blended in gasoline at 10% volume known as E10 is used in several states of the United States. While worldwide bioethanol production has been increasing year by year, the increase of amount produced in Brazil (2009-2015) was no more than 8%, and it was responsible for 28% of world ethanol production, behind USA with the expressive portion of 58% with 35% increase in 2015 [6]. Whereas bioethanol production in these countries, that on a commercial basis in Japan has not been implemented because of the poor feedstock and immature production technology. Japanese food-sufficiency of crops is the minimum for maintain the people’s life and depend on import from other countries; that is procurance of surplus grain to produce bioethanol is hard unlike enough corn and sugar countries of US and Brazil. Therefore, the target of Japanese-bioethanol feedstock has been lignocellulosic biomass such as agricultural waste, forestry residue, waste paper and industrial waste, from the beginning of development.

Lignocellulosic biomass has been recognized as an ideal inexpensive and abundantly available source of fermentable-sugars in Japan for the production of sustainable transportation fuel ethanol. It is mainly composed of cellulose (insoluble fibers of β-1,4 glucan), hemicellulose (non-cellulosic polysaccharides including xylans, mannans, and glucans), and lignin (a complex polysaccharide structure). Table 2 presents biochemical compositions for several feedstocks. Cellulose and hemicellulose occupy the large part of each biomass up to 50% and they have enough potential converted to ethanol. Particularly, whereas xylose is the predominant pentose derived from hemicellulose, general fermenting-microorganisms could not assimilate and ferment it. Effective extraction of glucose from cellulose and xylose *etc.* from hemicellulose and conversion of all sugars are very important process in bioethanol production. The cost of bioethanol production from such as lignocellulosic materials is fabulously high when based on current technologies which is production of ethanol from crops. Main challenges are improvement of the low yield and high cost of the hydrolysis process and increase of fermentation efficiency.

Table 2 Compositions of lignocellulosic content in several types of biomass resources

Biomass	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Corn stover	35-39	21-31	11-19
Switch grass	35-45	25-30	15-23
Sugar cane bagasse	25-43	28-32	15-25
Rice straw	29-37	23-26	17-19
Rice husk	23-33	12-29	14-20

Source: Ref. [7]

The first step in bioconversion of lignocellulosic biomass is pretreatment for size reduction and raveling the cellulose fiver. Therefore, many kinds of pretreatment have been studied on chemical and physical approach. The goal of some technology is to alter or remove structural and compositional impediments to hydrolysis by enzymes for improvement of fermentable sugar production from cellulose and hemicellulose [8]. A large number of pretreatment approaches have been investigated on a wide variety of feedstocks types. They can be classified into biological, physical, chemical and physicochemical pretreatments, according to the different forces or energy consumes in the pretreatment process.

Biological pretreatments take advantage of microorganism mainly brown-, white- and soft-rot fungi that can degrade lignin, a little hemicellulose and cellulose [9-11]. Among the cellulolytic fungi, white- and brown-rot fungi are considered the most prevalent and efficient decomposers of lignocellulosic materials. For carbon recycling, white-rot fungi accounts for 90% and brown rot fungi constitutes about 7% of all wood rotting basidiomycete fungi in nature [12]. Biological methods are selective in lignin and lignin-hemicellulose biodegradation and disturbing the three components, and altering or removing hemicellulose and lignin. Environmentally friendliness, low energy consumption, and no chemicals requirement are advantage of the method. However, the main drawback to develop the methods is very slow rate of degradation and delignification compared to other technologies [13].

Physical pretreatment can reduce the particle size and crystallinity of lignocellulose in

order to increase the specific surface and decrease the degree of polymerization. Energy source of the methods are mechanical comminution (milling, grinding, and compression), irradiation (electron beam, microwave), and pyrolysis [14, 15]. Effect of the method can disrupt plant cells and increase pore size for partially altering the structure of cellulose and hemicellulose. These require simple equipment and process but high power consumption is mainly disadvantage. Mechanical crushing is particularly required before any type of other pretreatment.

The objectives of chemical pretreatments vary widely depending on the type of chemical reagents. Alkali pretreatment affects lignin solubilization and is exhibiting minor denaturation of cellulose and hemicellulose than acid or hydrothermal processes [16]. Depending on catalyst used, alkaline pretreatments can be divided into two major groups: i) sodium, potassium, or calcium hydroxide; and ii) ammonia. NaOH causes swelling, increase of internal surface of cellulose and decreasing the degree of polymerization and crystallinity, which induces disruption of lignin structure [17]. $Ca(OH)_2$ has also widely studied on the removal of amorphous substances such as lignin, which increases the crystallinity index. It can removes acetyl groups from hemicellulose reducing steric hindrance of enzymes and enhancing cellulose digestibility [8]. One of ammonia pretreatment is ammonia fiber explosion (AFEX), which is alkaline physicochemical method. In this process, the biomass is exposed to liquid ammonia at relatively high temperature of e.g. 90-150°C for a period of e.g. 5-30 min, followed by immediate reduction of pressure [18]. This rapid drop of pressure results in physical disruption of the biomass structure; thus, exposing the cellulose and hemicellulose fibers. The AFEX process requires efficient ammonia recovery to be economical due to the high cost of ammonia as a solvent. Easiness of recover and reutilization is advantage of ammonia comparing with other alkali reagents on economical viewpoint.

Acid pretreatment approaches solubilization of hemicellulose fraction and is making the cellulose more accessible to enzymes. Delignification and swelling effect of the technology is however lower than that of alkali method. This type of pretreatments can be performed with concentrated or diluted acid but utilization of former is less attractive for ethanol production due to the formation of inhibitor. The main reagents used for the pretreatment are HCl and H_2SO_4. Corrosion of equipment and acid recovery are important drawback when using concentrated

acids. High operational and maintenance costs are problem of the method. Diluted acid pretreatment have been studied because of the applicability to wade range of lignocellulosic biomass and appears as more favorable method for industrial application. Many types of reactors such as percolation, pulp flow, shrinking-bed, batch, and countercurrent can be applied for the pretreatment [17]. High temperature reaction is operated during a short period of time; or that of low temperature for longer retention time. Solubilization of hemicellulose, mainly xylan, impacts for conversion to fermentable sugars. However, some sugar degradation compounds such as furfural, HMF and aromatic compounds are detected depending on the process temperature and they greatly affect fermenting microorganisms [19].

As one of chemical pretreatment, ionic liquid treatment has recently received much attention. Ionic liquids are salts; typically composed of large organic cations and small inorganic anions, which exists as liquid at room temperature [20]. Solvent properties of them can be varied easily by adjusting the anion and the alkyl constituents of the cation. The interesting properties include chemical and thermal stability, non-flammability, low vapor pressure, and a tendency to remain liquid in a wide range of temperature [21]. The ionic liquids treatment can effectively disrupt hydrogen-bond network in lignocellulose, correspondingly enhance its susceptibility for further transformation. It can dissolve carbohydrate and lignin simultaneously with anion activity because it forms hydrogen bonds between the non-hydrate chloride ions in the ionic liquid and the sugar hydroxyl protons in a 1: 1 stoichiometry. However, to eliminate the lignin derivatives form lignocellulose is quite difficult by ionic liquid treatment in contrast to carbohydrate [22]. Negative effect on cellulase activity and fermentation by microorganisms is occurred mostly by ionic liquid residues. Removal of the liquid would be essential to be required to prevent decrease of final sugar concentration. Furthermore, recycle use of ionic liquid is important in terms of economic because the chemical is expensive and recovery methods have not been fully developed.

Physicochemical pretreatment is furthermore also performed in order to make biomass more accessible to cellulase attack and improve the efficiency of downstream processing. The main technologies of pretreatment are combination of heating and pressuring such as steam explosion and hot water treatment. Simple operation for a short period and unnecessity of

chemicals are advantage of these technologies whereas the requirement of high cost due to high energy consumption is unavoidable [13]. Steam explosion is the most widely employed the type of pretreatment for lignocellulosic biomass [23]. The biomass is subjected to pressurized steam for a period of time ranging from seconds to several minutes, and then suddenly depressurized. The technology is combination of mechanical forces and chemical effects due to the auto-hydrolysis of acetyl groups present in hemicellulose. The mechanical effects are caused by reduction of pressure suddenly and make fibers separated owing to the explosive decompression. Auto-hydrolysis of lignocellulose takes place when high temperature promotes the formation of acetic acid and then environmental water can act as acid [20]. The main drawback of the method is partially hemicellulose over-degradation due to generate some toxic compounds that could affect the following steps to produce ethanol. The main furan derivatives generated by the operation are furfural and 5-HMF derived from pentoses and hexoses. Robust strategic approaches against these compounds are necessary in the subsequent process [24].

The second step subsequent pretreatment is enzymatic hydrolysis of exposed cellulose and hemicellulose to fermentable sugars. The enzymes are isolated from specific microbial broths, which called cellulase and hemicellulase. The cost of these enzymes remains the bottleneck for bioethanol production and remains one of the main challenges for large scale production of 2^{nd}-generation biofuels from lignocellulosic waste [25]. In order to complete breakdown of pretreated polysaccharides, enzymatic hydrolysis requires excessive use of high-cost enzymes [26]. A necessary association of cooperative action for bioconversion from cellulose to glucose is at least three cellulase; that is combination of *endo*-1,4-β-glucanase (E.C. 3.2.1.4), cellobiohydrolase (E.C. 3.2.1.91), and β-glucosidase (E.C. 3.2.1.21) [27]. The endoglucanase acts on amorphous cellulose regions, attacking the glucose-polymer chain randomly, which releases small chains consisting of more reducing and non-reducing ends. Then, these ends are exposed to the activity of cellobiohydrorase that cut off disaccharides from the chains and produce cellobiose. The third essential component in cellulase cocktail is β-glucosidase, which hydrolyzes cellobiose and produces glucose as a final product. In contrast to cellulose, the reaction mechanism of hemicellulose is more heterogeneous; hence, more specific enzymes are required to achieve complete biodegradation of hemicellulase into

pentoses and hexoses. Hemicellulases are combined with, for instance, *endo*-1,4-β-xylanase (E.C. 3.2.1.8), xylan 1,4-β-xylan esterases, ferulic and *p*-coumaric esterases, α-1-arabinofuranosidases, α-glucuronidase (E.C. 3.2.1.139), α-arabinofuranosidase (E.C. 3.2.1.55), acetylxylan esterase (E.C. 33.2.1.72), and α-4-*O*-methyl glucuronosidases xylosidase (β-xylosidase) (E.C. 3.2.1.37). These are all requires in specific ratios for the complete hydrolysis of hemicellulose biopolymer [28]. Any microbial enzymes possess differing degree of degradation potential so that could not have all activity mentioned above. The cost of the enzymatic hydrolysis depends on the efficiency and yield of fermentable-sugar production. A wide variety of enzymes are needed to act synergistically in the deconstruction and hydrolysis of lignocellulosic complex completely by optimizing cocktail consists of multiple types of enzymes. Recently, cell-surface technologies have been advanced as a promising genomic technique that uses microbial functional components to locate enzymes or peptides on the cell exterior of microorganisms [29]. Remodeling yeast that displayed cellulolytic enzymes such as endoglucanase, cellobiohydrolase and β-glucosidase together has possibility to achieve economical process without addition of extraneous enzyme, which is hydrolysis of lignocellulose and subsequent fermentation of the produced sugars simultaneously.

The main step of bioconversion from lignocellulose to bioethanol is fermentation by microorganisms using the fermentable-sugars induced from the degradation step. A variety of microbes are capable of fermenting hexose and pentose both separately as well as simultaneously. Ability of fermentation not only glucose from cellulose but also xylose from hemicellulose enable to complete bioethanol production in high yield. Bacterial genera like *Aerobacter*, *Bacillus*, *Klebsiella*, *Thermoanerobacter*, *Aeromonas*; yeasts like *Candidashehatae*, *Pichiastipitis*, *Pachysolen tannophilus* and fungi like *Fusarium*, *Mucor*, *Neurospora*, *Monilia*, *Rhizopus* are successfully capable of fermenting both glucose and xylose to ethanol [30]. In recent years, genetically modified organisms advanced effective production of bioethanol using molecular tools. The hosts obtain one or more ability such as xylose fermentation and cellulase expression by insertion of genomes from other organisms [31-34]. These strains have been modified by means of evolution strategies and recombinant tools such as direct mutagenesis, genetic and metabolic engineering to enhance ethanol yield as well as tolerance. However, no

single commercially viable strain has been reported yet due to restrictions such as the Cartagena Protocol, Moreover, the recombinant strains are avoided using in equipment without stringent sequestration system that should not flow out the strain to environment. Native strain that can ferment any sugars and also secrete hydrolyzing enzymes would be desirable for industrial use.

Monomeric sugars of hexose and pentose are formed as a result of pretreatment and saccharification of lignocellulose, and subsequently fermented by variety of fermentable microorganisms to convert into bioethanol. The fermentation is carried out via any bioprocess of as follows; separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), simultaneous saccharification and fermentation (SSCF), and consolidated bioprocessing (CBP) [35]. SHF is a two stage process wherein hydrolysis and fermentation operate distinctly. The main advantage of this process is that enzymatic hydrolysis and fermentation can operate their respective optimum conditions. Sugars inhibition to enzyme activity remains a major drawback that ultimately affects the rate limiting and ethanol yield [36]. SSF combines the saccharification by enzymes with fermentation of released sugars simultaneously in a single reactor. The key feature of this process is that as soon as the sugars are formed from biomass, they are rapidly converted into ethanol. Thus, accumulation of sugars is reduced and sugar inhibition on enzyme activity is greatly reduced. This operation requires low equipment compared with SHF and the presence of ethanol in the broth making the medium less vulnerable to contamination [37]. The main bottleneck of this approach is difficulty in optimization of process parameters considering both hydrolysis enzymes and microorganisms at the same time. For instance, enzymes for lignocellulose hydrolysis work best at around 50°C but the optimal conditions for general microbes for ethanol fermentation is around 30°C. Lowing temperature for enzymes causes decrease of their activities, and therefore thermotolerant strains are required efficient ethanol production at high temperature [38]. SSCF is co-culture process using two strains that possess different ability on ethanol production from lignocellulose. This integration is oriented to the microbial assimilation of all the sugars formerly formed during pretreatment and hydrolytic processes of lignocellulosic biomass. The problem of the process is faster growing of hexose-utilizing strains than pentose-utilizing strains. Researches in this technology have attracted in the term of low cost, shorter operation time,

lower contamination risk, and fewer inhibitory effects during enzymatic hydrolysis [39]. CBP, leading-edge fermentation technology, is direct microbial conversion of lignocellulosic biomass into ethanol without extraneous addition of hydrolysis enzymes. In order to accomplish this process, particular microorganism is absolutely imperative, that can secrete hydrolysis enzymes and ferment the sugars released. This process has several advantages since no capital or operational costs are required for purchase or production of enzyme due to production of cellulase by fermenting-microorganisms themselves. Moreover, the enzymatic and fermentation processes are entirely compatible because of both process is carried out only one strain. CBP-achievable microbes have a distinct advantage over conventional yeasts as they can directly use number of inexpensive biomass feedstocks and also withstand high temperatures [40]. Unfortunately, no valuable CBP organism has yet been constructed with capability of current genomic mutagenesis technologies.

In this article, some challenges on effective conversion of lignocellulosic or future feedstocks into ethanol or organic acid were carried out using fermenting fungi, and novel technologies corresponding to these challenges were developed.

Chapter 1 describes selection of the best fermenting fungus belonging in up to 80 species of Zygomycota *Mucor* fungi by comparison of conversion ability from both glucose and xylose to ethanol as previous step essential to effective bioconversion of lignocellulosic biomass. While most of the strain could produce ethanol from glucose only, a few strains could also ferment xylose. Especially, *Mucor circinelloides* NBRC 4572 demonstrated that it can produce the highest ethanol from xylose as well as glucose.

Chapter 2 describes production of biofuel from rice straw as a model lignocellulosic waste based on energy saving viewpoint. The degradation conditions about pretreatment and enzymatic hydrolysis were investigated to obtain effectively fermentable-sugars. Rice straw was pretreated by steam explosion selected based on the lowest energy requirement. Suitable cellulase reagents for the biomass hydrolysis were selected and that mixing ratio was optimized. Practical ethanol production from pretreated rice straw by using SSF method with *M.circinelloides* NBRC 4572 strain and the cocktail was carried out.

Chapter 3 covers the optimization of cellulase cocktail by statistical approach for

bioconversion to alkali treated rice straw. In order to hydrolyze the substrate to fermentable sugar in the process, some desirable commercial enzyme reagents were selected based on regression analysis by using enzymatic activities and then mixing ratio of them were optimized the design of experiments (DOE) with response surface method (RSM). The optimized enzyme cocktail was used in bioconversion of alkali-treated rice straw to fermentable sugar and subsequent ethanol by SSF system with *M.circinelloides*.

Chapter 4 describes direct ethanol production from rice straw by co-culture with two high-performing fungi. *Mucor* sp. fungi possess ability not only ferment sugars but also secrete hydrolysis enzymes. Ethanol-producing fungi, *M.circinelloides* NBRC 4572, could also secrete a few enzymes. Moreover, *M.ciecinelloides* NBRC 5398 found obviously higher secretion of cellulases. The SSF of mechanically crushes rice straw by co-culture with two strains was achieved without extraneous commercial cellulase reagent.

Chapter 5 discusses two new β-glucosidases (BGLs 1 and 2) from *M.circinelloides* NBRC 4572. These were purified to homogeneity from the extracellular enzyme preparations of the fungus statically grown on rice straw. These functional characterization and molecular cloning of the gene were investigated. The research indicated molecular masses, enzyme characteristics, amino acid sequences, and expression profiles of BGLs 1 and 2.

Chapter 6 describes direct ethanol production from *N*-acetylglucosamine (GluNAc) and chitin substrates as next generation biomass by *Mucor* species. Native *Mucor* sp. examined the possibility of direct ethanol fermentation from GluNAc as carbon sources. Moreover, ethanol production from colloidal chitin was demonstrated by SSF combining chitinase extracted by chitinase-prodcuing *Mucor* sp. and an ethanol-producing fungus, *M.circinelloides* NBRC 4572.

Chapter 7 suggests application of SSF process to lactic acid production from paper sludge (PS) with thermotolerant *Rhizopus* sp. PS was used as industrial lignocellulosic waste and removal method of inorganic substances in PS was investigated for exposure of cellulosic fiber. In order to dissolve optimum temperature gap between hydrolysis enzymes and fermenting microorganism, thermotolerant strain was selected among *Rhizopus* sp. that can produce lactic acid. The treated PS was converted to lactic acid by high temperature SSF with collulase cocktail and thermotolerant strain.

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Chapter 1

Selection of a desirable fermenting fungus belonging to *Mucor* spp. for ethanol production from glucose and xylose

1.1. Introduction

Fermentation has been well known from ancient time and intimately connected with people's life. It was described that alcohol drinks such as wine, beer and whisky have been brewed before B.C.3000. However, in comparison with the history of fermentation, the elucidation of the fact that alcohol fermentation is caused by the behavior of microorganisms has come to the nineteenth century. Louis Pasteur found that fermentation is caused by work of microorganism. He described ethanol production occurred by yeasts and each fermentation, such as ethanol or lactic acid, induced by respective microorganism. Yeast and bacteria have been used as starter for fermentation in Western Europe of dry climate whereas fungi in Asia because of warm and wet climate which is suitable for mycotic growth. *Mucor* spp. and *Rhizopus* spp. have been utilized in China and Indonesia, and *Aspergillus* sp. in Japan for production of fermented foods and alcohol drinks.

Among these fermenting microbes, *Mucor* is one of the most varied and least studied phylum. *Mucor* is classified in zygomycete and genus of about 3000 members that are widely found in soil, plant surfaces, decomposed vegetables and fruits. The genus has several species of *Mucor amphibiorum*, *M. circinelloides*, *M. hiemalis*, *M. indicus*, *M. racemosus*, *M. ramosissimus etc.* It has widely studied and utilized as producing sources for various extracellular enzymes such as amylase, lipase, protease and rennet *etc.* [1-5]. Because the fungi are primarily saprophytic microbes in nature, it possesses assimilation capability of various sugars of monosaccharides, disaccharides, and also polysaccharides. This suggests that fungi can produce a wide variety of hydrolytic enzymes. Moreover, *Mucor* spp. has attracted attention by the ethanol-producing ability particularly on bioethanol production from lignocellulosic biomass [6, 7]. Industrially practiced ethanol production has been carried out by yeast, mainly *Saccharomyces cerevisiae*, which is the most commonly microorganism used in alcohol fermentation due to the fast ethanol production rate and the high yield. However, some

problems with utilizing yeast are limited ethanol production on 2nd-generation biofuel. The first one is that it can ferment only confined sugar, glucose, and lacks capability of fermentation of neither pentose nor other sugars including oligosaccharides induced from lignocellulose degradation. As a solution of this problem, recombinant strains that inserted xylose metabolic enzymes have engineered for effective ethanol production [8]. The second problem is the weakness to toxic compounds derived from pretreatment of lignocellulose. They prevent yeast growth and are usually must be removed from the hydrolysate [9]. Furthermore, the yeast cannot produce extracellular enzymes such as cellulase, hemicelluase *etc*., for lignocellulose hydrolysis. Constructions of transformed yeasts that can express cellulases have recently performed to overcome the problem [10, 11]. Comparing the yeast to fungi, *Mucor* is expected to make lignocellulose fermentation more feasible by various factors related to inhibitor tolerance, enzyme secretion and ethanol production.

In this research, with the aim of highly efficient ethanol production from lignocellulose, ethanol production by 90 species of *Mucor* spp. was first performed with glucose and xylose as substrates for standard fermentation test. Then the optimum strain was selected considering the ethanol fermentation ability. In addition, various sugars were fermented by the selected fungus.

1.2. Materials and methods

1.2.1. Microorganisms

In this research, 90 kinds of the Zygomycota (*Mucor* and *Rhizomucor* genus) were used as shown in Table 1. The strains were purchased from NBRC (NITE Biological Resource Center, Chiba, Japan) and they were stored as Difco potato dextrose agar (PDA) (Becton, Dickinson and Company, Sparks, MD) slants at 4ºC in refrigerator in our laboratory.

1.2.2. Basic medium

The fungi were usually grown in basic medium: yeast extract (Oriental yeast Co., Tokyo, Japan) 5.0 g/L, $(NH_4)_3SO_4$ 7.5 g/L, $MgSO_4 \cdot 7H_2O$ 0.75 g/L, KH_2PO_4 3.5 g/L, $CaCl_2 \cdot 2H_2O$ 1.0 g/L, and glucose or xylose 100 or 50 g/L, respectively. Glucose, mannose, xylose, arabinose,

galactose, fructose, maltose, cellobiose lactose, sucrose, mannitol, sorbitol, glycerol, soluble starch, corn starch, Avicel, carboxymethyl cellulose (CMC), and xylan from beechwood were used as carbon sources. All reagents without yeast extract were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). The medium was autoclaved at 121ºC for 15 min after the pH of the medium was adjusted at 5.5 by using a NaOH solution and/or a HCl solution.

1.2.3 Batch culture for screening of the best fermenting strain

In order to examine that Zygomycetes mucorales can produce ethanol from glucose and xylose, all strains was cultured in liquid medium. The preculture was carried out on agar plate (90×15 mm) with modified basic medium for 3-5 days at 28 ºC by inoculating from PDA slant stored at 4ºC. Then, liquid batch cultures were carried out with each fungus under aerobic and anaerobic condition with respective two sugars in 100 mL flask. Fungi were inoculated into the flasks containing 25 mL of basic medium and cultured under aerobic and anaerobic condition with shaking at 120 rpm at 28ºC for 72 h (glucose culture) or 96 h (xylose).

1.2.4. Analytical method

After cultivation, these culture solutions were collected by filtration with filter paper (No.131 ADVANTEC Co., Tokyo, Japan). Dry weight of fungi on the paper was estimated after washing thoroughly with distilled water and drying at 90 ºC for 24 h. The amount of residual sugars and ethanol in the culture solutions were determined by HPLC (LC-10ADvp, Shimadzu Co., Kyoto, Japan) equipped with a refractive index detector (RID-10A, Shimadzu Co.) and a ICSep WA-1 Wine Analysis column (Transgenomic, NE, USA) operated at 40 ºC with 1.25 mM sulfuric acid as mobile phase at flow rate of 0.6 mL/min.

1.3. Results and discussion

1.3.1. Screening of ethanol-producing fungi from glucose and xylose

In order to select a better fungus for lignocellulose conversion to ethanol, *Mucor* spp. strains listed in Table 1 were investigated about capability of producing ethanol from both

Table 1 *Mucor* and *Rhizomucor* strains

Species	NBRC number
M.abundans	**9398**
M. albo-ater	**7056, 9399**
M. ambiguus	**6742, 8092**
M. bacilliformis	**6414, 8638**
M. circinelloides	**4554, 4563, 4569, 4570, 4572, 4574, 5382, 5398, 5774, 6746, 30470**
M. flavus	**9560,9561**
M. fragilis	**6449, 9402**
M. genevensis	**4585, 6415**
M. gulliermondii	**9403**
M. hiemalis	**5303, 5834, 6753, 6754, 8448, 8449, 8565, 8567, 9261, 9400, 9401, 9404, 9405, 9406, 9407, 9408, 9409, 9410, 9411, 9412**
M. inaequisporus	**8624, 8635, 8636**
M. indicus	**5773**
M. mucedo	**5776, 6750, 7684**
M. oblongiellipticus	**9258**
M. oblongisporus	**7058, 9259**
M. odoratus	**7102, 8637**
M. petrinsularis	**6751**
M.piriformis	**9413,9414,9415,9416**
M. plasmaticus	**7059, 9260**
M. plumbeus	**4575, 5317**
M. racemosus	**4555, 4556, 4581, 5403, 6745, 9255**
M. recurvus	**8093**
M. saturninus	**9562**
M. strictus	**9563**
M. subtilissimus	**6338, 6755**
M. tuberculisporus	**9256, 9257,100663**
R. miehei	**9740, 9741, 9742, 9743**
R. pusillus	**4578, 4579, 4580, 9744, 9745, 9856**

glucose and xylose. These cultures were carried out under anaerobic (glucose) and aerobic (glucose and xylose) conditions. Dry cell weight, residual sugars, and produced ethanol were shown in Figs. 1A-1C: A, 100 g/L glucose anaerobically for 72 h; B, 100 g/L glucose aerobically for 72 h; C, 50 g/L xylose aerobically for 96 h. Though almost all strains were able to grow three conditions, a part of these strains were able to produce ethanol and the amounts varied according to strain. Five strains of NBRC 4554, 4569, 4572, 8092, and 30470 produced over 40 g/L of ethanol from glucose under anaerobic condition as shown in Fig. 1A. These yields were more than 78% based on theoretical amount of ethanol from 100 g/L glucose. On the cultivation with glucose under aerobic condition as shown in Fig. 1B, dry cell weight of strains was higher with enhancement of glucose assimilation than that of anaerobic condition. However, ethanol production was low and the maximum yield was 27.1 g/L on NBRC 8092 strain. Anaerobic condition enhanced ethanol production from assimilated glucose whereas aerobic condition induced glucose metabolism to cell growth rather than ethanol production. These results indicate that *Mucor* spp. can produce ethanol under both aerobic and anaerobic conditions. The fungi have a unique capability of ethanol producing under completely aerobic condition because general fermenting microorganisms have been well known on requirement of slight aeration for ethanol production. Moreover, when the strains cultivated in xylose medium under aerobic condition, they could grow with consumption of xylose in varying amount as shown in Fig. 1C. However, a few strains could produce ethanol from xylose and these amounts were less than those from glucose. Additionally, these strains could not grow with xylose under anaerobic condition. The highest ethanol was produced by NBRC 4572 and the amount was 10.6 g/L. The strain was also able to produce ethanol from glucose in higher yield than other strains. Therefore, *M.circinelloides* NBRC 4572 was selected for the best strain for ethanol production from lignocellulosic biomass.

1.3.2. Characterization of fermentation capability of various sugars by NBRC 4572

Lignocellulosic biomass contains polysaccharide consisting of various sugars such as glucose, mannose, xylose, arabinose, and their oligosaccharides. Ethanol production from the biomass requires wide ability of fermentation for microorganisms. Since *Mucor* fungi is

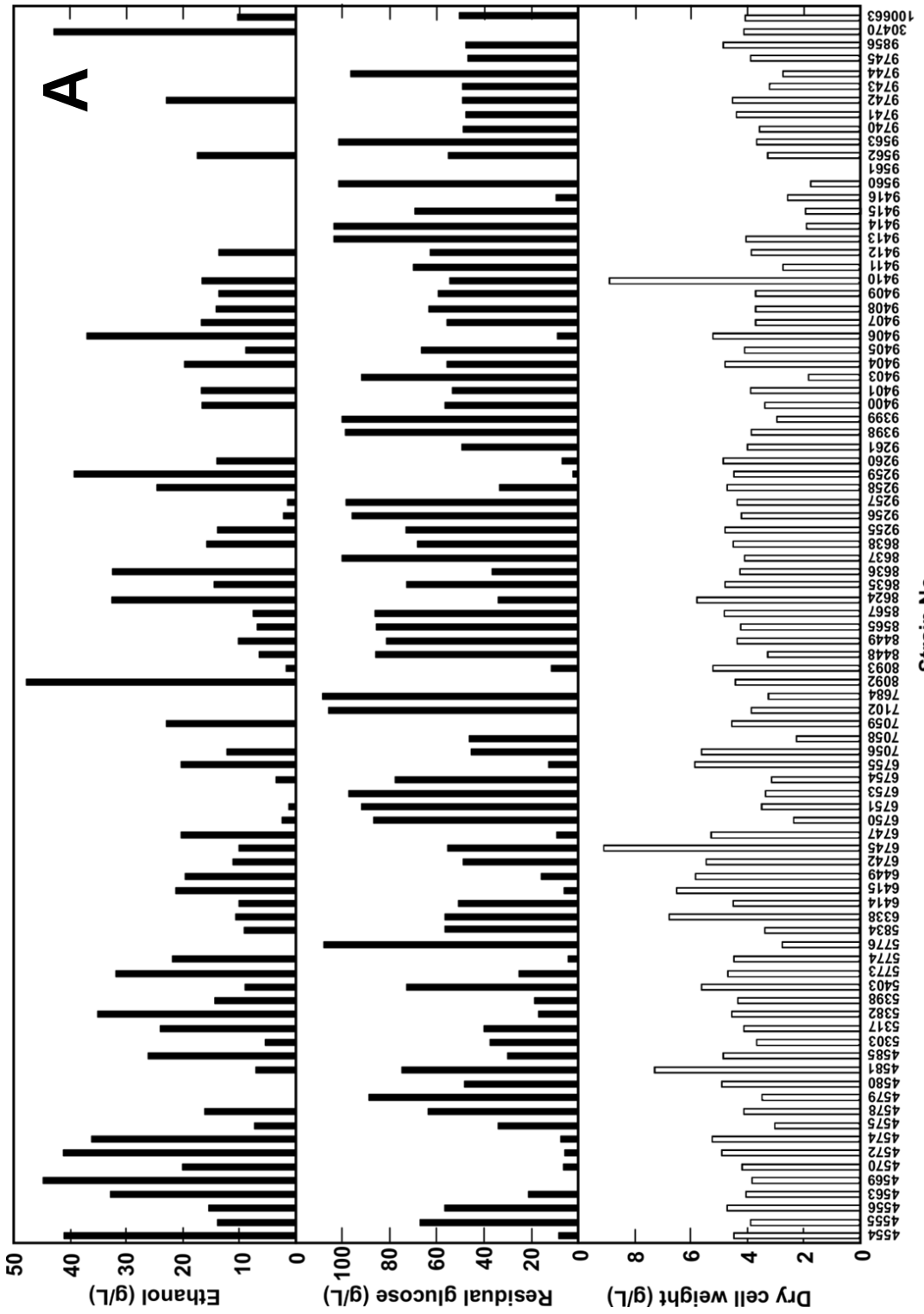

Fig. 1A Ethanol production by *Mucor* strain from glucose under anaerobic condition

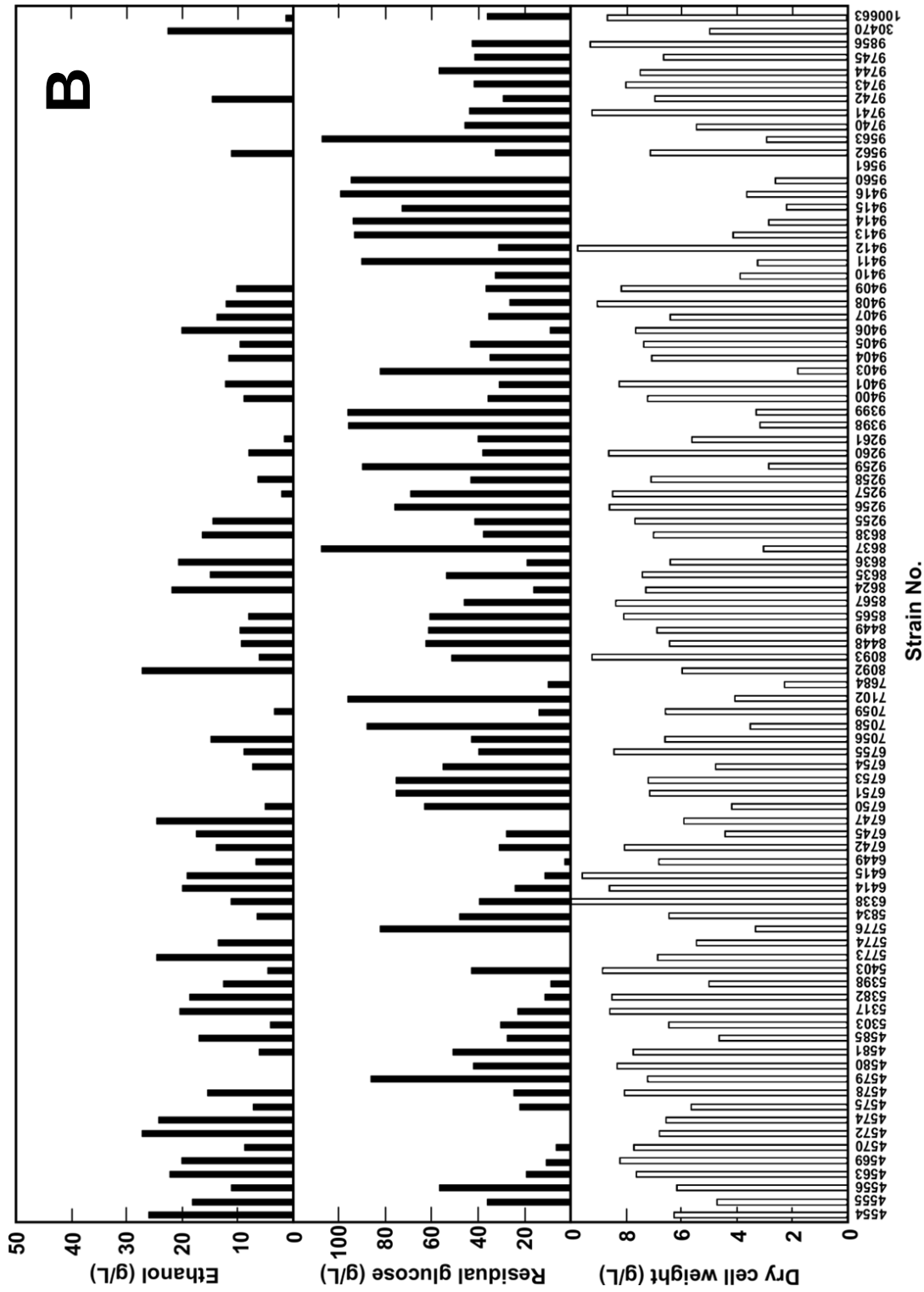

Fig. 1B Ethanol production by *Mucor* strain from glucose under aerobic condition

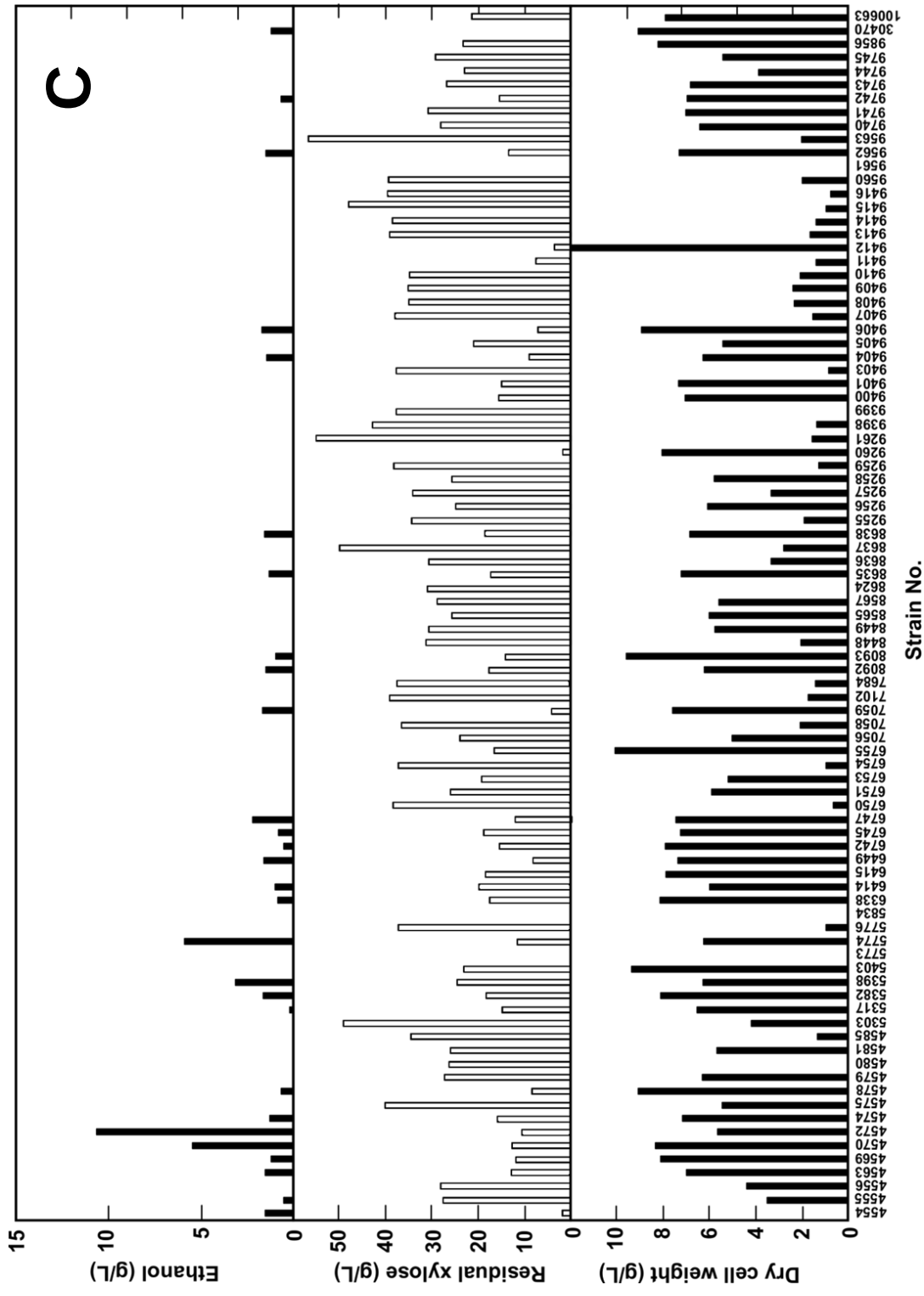

Fig. 1C Ethanol production by *Mucor* strain from xylose under aerobic condition

saprophytic genus, it should potentially have assimilation ability of the various sugars. The capability of their fermentation of NBRC 4572 was investigated by using 18 kinds of sugars from monosaccharides to polysaccharides. The strain was cultivated with 50 g/L of respective sugars as substrates for 96 h and produced ethanol was comparatively estimated. As a result, as shown in Fig. 2, the strain was able to ferment various sugars such as hexose, pentose (mainly xylose), disaccharides, and even polysaccharides directly. That indicated *Mucor* spp. can ferment general hexoses obtained from hydrolysis of lignocellulose. The produced ethanol from only xylose under aerobic condition was higher than that of anaerobic condition while other sugars were more effectively fermented anaerobically than aerobically. Moreover, anaerobic condition resulted in direct polysaccharides fermentation of soluble starch and corn starch. That suggests hydrolase secretion of the strain was able to secret α-, β-glucosidase, and α-amylases, glucoamylase *etc.* Cellulosic-polysaccharides were not able to be converted directly to ethanol though growth of the strain was confirmed on these sugars. The fact indicates the stain can also secret a little cellulases such as *endo*-β-glucanase and cellobiohydrolase, which were enough to grow. From these results, *M.circinelloides* NBRC 4572 can not only ferment various monomer sugars consisting lignocellulose but also secret hydrolases, which advantage for bioconversion of lignocellulosic materials.

1.4. Conclusion

Mucor spp. is one of available zygomycota which can ferment various materials by the conversion and enzyme production abilities. The microorganisms like this species are required for bioethanol production from lignocellulosic biomass because it contain intricate construction of cellulose, hemicellulose, and lignin. Moreover, many *Mucor* spp. have various useful abilities, and many researchers have confirmed the effectiveness as various enzyme origin, biocatalysts for fermented food production *etc.* In this research, demonstrations of comparison on ethanol production from glucose and xylose by *Mucor* fungi were performed. Fungi that were capable of producing high ethanol were limited to a part of this genus though almost all strains were able to grow in both glucose and xylose medium. From these evaluations, *M.circinelloides* NBRC 4572 was selected as required ethanol producing strain by which produced the highest

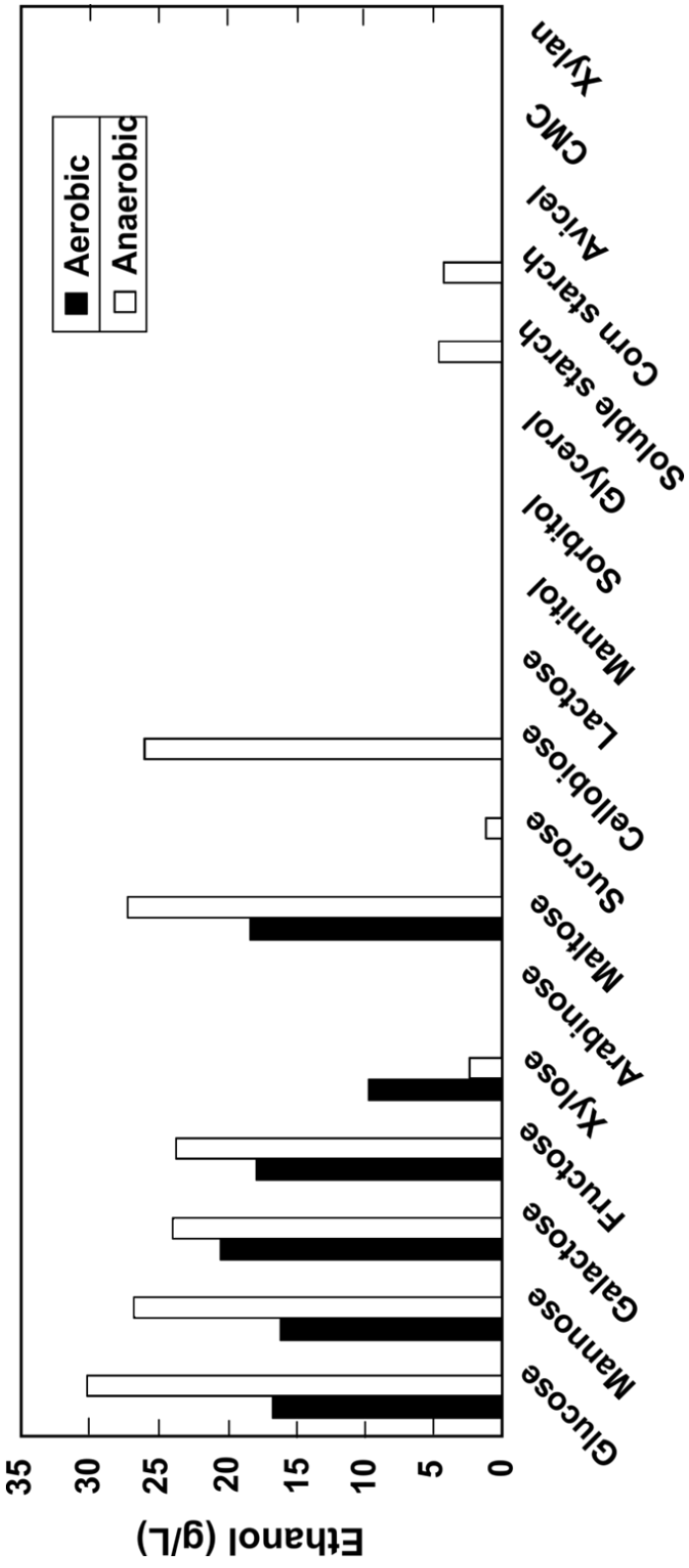

Fig. 2 Ethanol producing profile of *M.circinelloides* NBRC 4572 on various sugars

amount of ethanol from xylose. Furthermore, in order to evaluate the possibility of ethanol fermentation from lignocellulose hydrolyzate, culture using various sugars was carried out and thefermentation ability was examined. The selected strain can assimilate and ferment from various monosaccharides to polysaccharides as substrate. These results indicated it can secrete enzymes for hydrolysis of polysaccharides. This research on selection and characterization of *Mucor* sp. showed that bioethanol production could be successfully achieved by using selected strains. However, direct ethanol production from lignocellulosic biomass is quite difficult at present because of a little amount of these cellulases secreting from the strain. In actual bioprocess such as SSF of lignocellulosic biomass is further necessary to be established with variety technologies for its pretreatment and hydrolysis.

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Chapter 2

Production of biofuel from waste lignocellulosic biomass materials based on energy saving viewpoint

2.1. Introduction

There is worldwide demand of renewable fuels such as bioethanol. Rice straw is one of waste lignocellulosic material which the produced majority is abandoned. Though the greater part has been always used as cattle feed *etc.*, it is nowadays expected as substrate for ethanol production economically and environmentally. Rice straw has complex structure consisting of cellulose, hemicelluloses, and lignin. Ethanol is produced from sugars in rice straw by the following successive reactions; i) formation of fermentable sugars executing the structural fracture by pretreatment and enzymatic hydrolysis, ii) ethanol production from them by fermenting-microorganism. Since the rate determining step of these processes is the hydrolysis step, effective pretreatment of rice straw is required preceding the step [1]. Therefore, many methods were proposed as pretreatments of rice straw such as physical, chemical, and thermal method [2-4]. However, the most suitable method for rice straw has not determined. Although pretreatment has been mentioned as the highest cost process requirement of large energy, it is also absolutely necessary for good performance of subsequent enzymatic process [5]. Meanwhile, the commercial cellulase agent contains some activities which have different reaction mechanisms, and balance of them strongly influences production of fermentable sugars. In this research, to obtain economically fermentable sugars, characterization of 5 kinds of pretreatment and several commercial cellulase agents for rice straw degradation were investigated. Furthermore, the simultaneous saccharification and fermentation (SSF) from the most suitable pretreated straw with a cellulase cocktail and a fermenting-fungus was performed.

2.2. Materials and methods

2.2.1. Pretreatment of rice straw

Rice straw “Kinmaze”, which was harvested in Manto-City (Toyama, Japan), was used as cellulosic material in this study. The rice straw was air-dried, cut, and blended prior to pretreatment. The componential analysis of rice straws was carried out according to Klason lignin method [6]. The alkali treatment of rice straw was carried out as follows; 80 g of rice straw was soaked in 1 M NaOH solution of 1 L overnight and then autoclaved at 121ºC for 1 h. After neutralization by adding equivalent amount of 1M HCl solution, the solid separated from the mixture, washed thoroughly with tap water, and dried at 50ºC overnight. The sulfate treatment was carried out by soaking 100 g of rice straw into 0.5% sulfuric acid of 1 L overnight and then autoclaved at 121ºC for 30 min. After the solid was separated from the mixture, washed with tap water and dried at 50ºC overnight. The steam explosion treatment was performed as follows; the straw was introduced reaction vessel in the steam explosion apparatus (Japan Chemical Engineering and Machinery, Osaka, Japan) and exposed to saturated steam at 25 atm for 5 min. And then the valve at the bottom of the reactor was suddenly opened to bring it rapidly atmospheric pressure. The product containing solid and liquid materials was obtained as a pretreated material [4]. The hydrothermal treatment was carried out as follows; the straw set in holder with water at concentration of 10 wt% was autoclaved at 180ºC for 40 min. Then the mixture was cooled with stirring at 500 rpm in jacket with cooling water. The treatment by wet type atomization was carried out as follows; the straw was miniaturized down to nano-size by a novel wet type atomization system using ultrahigh-pressure jet flow (Starburst, Sugino Machine, Toyama, Japan).

2.2.2. Cellulases

Hydrolysis of rice straw was carried out by using commercial food-processing cellulase reagents as follows: Meicelase (adjective to A, Meiji Seika Pharma, Tokyo), Cellulase T “Amano” 4 (B, Amano Enzyme, Nagoya, Japan), Cellulase A “Amano” 3 (C, Amano Enzyme), Pectinase G “Amano” (D, Amano Enzyme), Hemicellulase “Amano” 90 (E, Amano Enzyme),

Cellulase Y-NC (F, Yakult Pharmaceutical Industry, Tokyo), Cellulase "Onozuka" 3S (G, Yakult), Cellulase "Onozuka" R-10 (H, Yakult), Sumizyme AC (I, Shin-Nihon Kagakukogyo, Anjyou, Japan), Sumizyme X (J, Shin-Nihon Kagakukogyo), Sumizyme SNX (K, Shin-Nihon Kagakukogyo), Cellulase *Trichoderma viride* (L, Sigma, St Louis, MO), Accellerase 1500 (N, Genencor Kyowa, Tokyo), Vecelex (O, Godo-Shusei, Tokyo).

2.2.3. Fermentable sugar production by enzymatic hydrolysis of rice straw

Hydrolysis reaction was performed in 100 mL bottles containing 100 g/L of each pretreated rice straw in 25 mL of 0.1M sodium acetate buffer (pH 5.5) or culture medium. It was previously autoclaved at 121°C for 15 min before reaction. Hydrolysis was started by addition of the filter-sterilized single or cocktail enzyme at the total protein concentration of 3 g-protein/L. These suspensions were incubated at 28°C and 120 rpm for 96 h. Hydrolysates were obtained by removal of solid fraction by filtration by filter paper (No.131, Advantec, Tokyo, Japan) and concentrations of sugars in the liquid phase were analyzed by HPLC system.

2.2.4. Ethanol production by simultaneous saccharification and fermentation

Mucor javanicus sp. was used as a fermenting fungus for simultaneous saccharification and fermentation (SSF) of rice straw. SSF was performed under aerobic condition in the 25 mL medium as described in the section 1.2.2. containing in 100 mL flask with pretreated rice straw of 100 g/L. The medium was adjusted at pH 5.5 and previously autoclaved at 121ºC for 15 min. Cultivations were started by inoculating 1 mL of the fungus suspension in normal saline and simultaneously adding the filter-sterilized cellulase adjusted to at 3 g-protein/L. These were shaken at 120 rpm at 28ºC for 120 h. Then, the cultures were filtrated and the liquid containing sugars and ethanol that produced by SSF were analyzed by HPLC.

2.2.5. Analysis methods

The filtrated supernatant was analyzed about ethanol, residual sugars, by-products, and cellulase and hemicellualse activities. Sugars, ethanol, and by-products in the reaction solutions were measured by a HPLC system as described in section 1.2.4. Enzymatic activities of each

commercial cellulase about *endo*-β-glucanase (EG), cellobiohydrolase (CBH), β-glucosidase (BG), xylanase (X) and β-xylosidase (BX) were measured by using Azo-CM-cellulose (Megazyme, Ireland), 4-nitrophenyl-β-D-Cellobioside, 4-nitrophenyl-β-D-glucopyranoside (Sigma-Aldrich), Azo-arabinoxylan (Megazyme) and 4-nitrophenyl-β-D-xylopyranoside, respectively. On analysis of enzymatic activities, EG and X activity were determined by reference to each standard curve drawn by using Meicelase. CBH, BG and BX activity were determined as the ability by which released 1 μmol of 4-nitrophenol per 1 minute.

2.3. Results and discussions

2.3.1. Comparison of component in pretreated rice straw

Figure 1 shows the changes of components in the rice straw by treatment of five kinds methods. The native rice straw was composed of cellulose (38%), hemicellulose (25%), Klason lignin (21%), acid soluble lignin (4%), and other materials such as ash (12%). Lignin contents in pretreated materials reduced and conversely the sugar contents increased compared with the untreated material. The contents of cellulose and hemicellulose in these straws were different depending on treated method. The steam explosion reduced the hemicellulose content from 8.2 to 6.5 %. The wet-type atomization, sulfate treatment, and alkali treatment were able to increase the content of cellulose in the rice straw. Especially, the alkali treated straw had the highest total sugar content (cellulose and hemicellulose), which was increased from 40 to 60%. The rice straw with high content of cellulose (glucose) is desirable as feedstock to perform ethanol production by using fermenting microorganism. Since ethanol production has to perform economically, low energy method of pretreatment is required. The energy demands to produce 1L-ethanol from rice straw pretreated by crush, steam explosion, wet type atomization, hydrothermal, and sulfate method are ca. 10, 20, 90, 25, and 33 MJ/L-ethanol, respectively. From these viewpoints, the steam explosion was desirable methods for rice straw pretreatment because of high cellulose content in the material increased to about 40% with low energy demand.

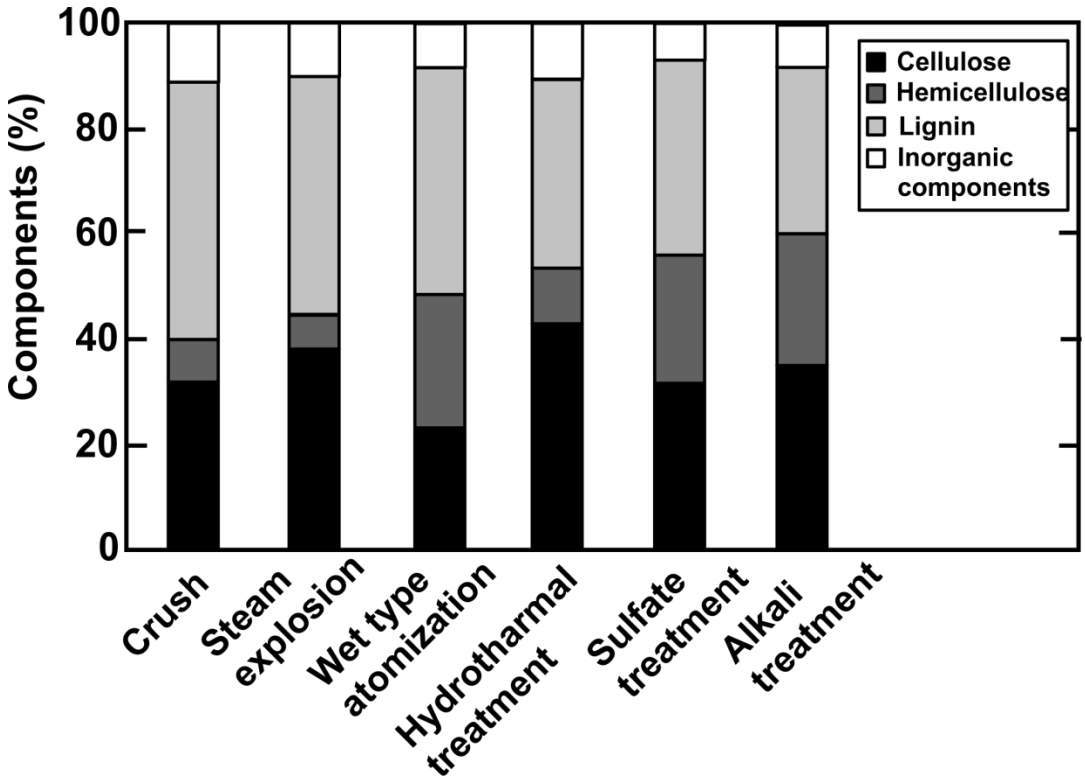

Fig. 1 Components ratio of rice straws pretreated by each method

2.3.2. Characterization of commercial cellulases based on enzymatic activity

Many kinds of commercial cellulases have used in food manufacture *etc*. The utilization of these enzymes on ethanol production was desirable for economical process since they are mass-produced enzymes and inexpensive. However, the reactivity of these enzymes to biomass is greatly different depend on its origin and the manufacturing method. Therefore, the reactivity of various commercial enzymes was estimated and compared on the basis of these specific activities of CBH, EBG, BG, X, and BX. Figure 2 shows protein contents per powder weight of commercially cellulase reagents and their specific activities. Each enzyme had various activities for hydrolysis of cellulose and hemicellulose. The activities of Cellulase Onozuka (G in Fig. 2) on CBH, EBG, and BG were very high (95.8, 263, and 551 U/g-protein, respectively), and in addition, it possessed the activity of X (98.3 U/g-protein). Efficient hydrolysis of material that has the complex structure like rice straw would be able to achieve by combining two or more enzymes. In regard to the activity for xylan hydrolysis, Pectinase (D) had high activities of X and BX, which were 157 and 68.3 U/g-protein, respectively. A cellulase cocktail prepared by combining D and G was desired as suitable mixture for efficient and economical hydrolysis of pretreated rice straw.

2.3.3. Effect of commercial cellulases to degradation of pretreated rice straws

The selection of a desired method for pretreatment is essential to obtain effectively fermentable sugars. Enzymatic degradations of the straws pretreated by five kinds of methods were performed with 15 kinds of commercial cellulases (A-O), and the degradabilities to the straws were evaluated as shown in Fig. 3. These activities were subjected to the pretreating methods in the following order: alkali treatment, steam explosion, hydrothermal treatment, wet type atomization, sulfate treatment, and untreated. Where, energy demands according to our estimation for production of 1 L ethanol from raw rice straw were about 33, 20, 25, 90, 33, and 10 MJ, respectively. Therefore, from an economical aspect, the steam explosion treatment was selected as a pretreating method for rice straw in this study.

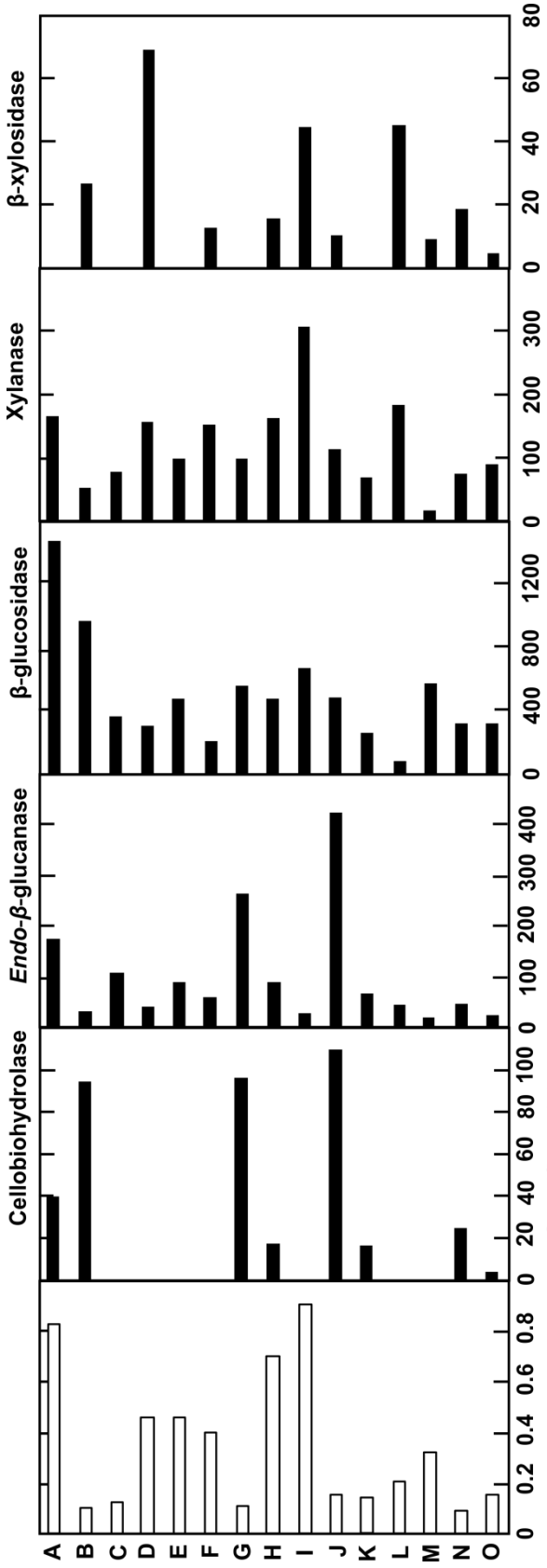

Fig. 2 Hydrolysis activities of commercial cellulases

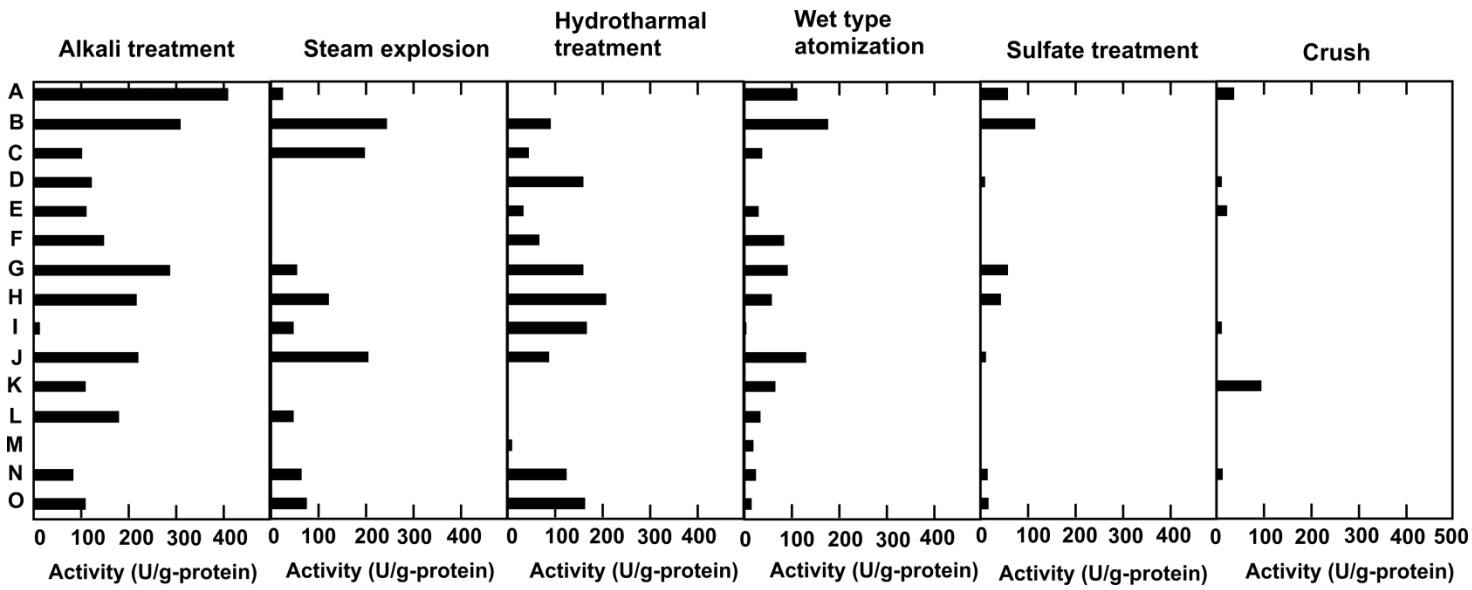

Fig. 3 Degradation activities of commercial cellulases to rice straws pretreated by each method

2.3.4. Hydrolysis of steam exploded rice straw by cellulase cocktail

In order to improve the degradability of steam exploded rice straw, a suitable compounding ratio of D and G was investigated. The concentration of protein in G was fixed at 2 g/L and that of D was changed from 0 to 1 g/L. Figure 4 shows the hydrolysis of 50 g/L steam exploded rice straw to produce fermentable sugars of disaccharides (cellobiose and xylobiose *etc*.), glucose, and xylose with different ratio of these enzymes at 28˚C and pH 5.0. At the reaction with G alone, many amounts of disaccharides were produced and achieved 8.4 g/L. Since hydrolysis of them progressed with increase of addition of D, accumulation of disaccharides were prevented at the ratio of 2:1. Xylose released from hemicellulose in the straw was less than 0.26 g/L under all conditions. Disaccharides hydrolyzed to glucose and the concentration at the ratio of 2:1 was 16.8 g/L at 72 h, which indicated hydrolysis efficiency of 88.1%. From these results, the steam exploded rice straw was effectively hydrolyzed by using the cellulase cocktail containing G of 2 g/L and D of 1 g/L.

2.3.5. SSF of steam exploded rice straw with fungus and cellulase cocktail

In order to produce ethanol from steam exploded rice straw, SSF was performed with combining a fermenting fungus and the cocktail containing cellulase reagents of D and G. Since the fungus was found as a particular microorganism that can convert to ethanol from both glucose and xylose formed by hydrolyzing the pretreated rice straw with the cocktail. The culture was performed under the aerobic condition with the pretreated rice straw of 100 g/L, and a desired cellulase cocktail of 3 g-protein/L. As shown in Fig. 5, cellulose and hemicellulose in the rice straw were hydrolyzed to glucose, xylose and disaccharide for 36 h. Then the fermentable sugars were rapidly assimilated, and apparent sugars were very low after 48 h. Ethanol was produced from 36 h and the concentration achieved maximum of 13.2 g/L at 96 h. The yield was 58% of the theoretical conversion based on all sugars in the rice straw. Additionally, accumulation of by-products of glycerol and xylitol kept low level. Almost all fermentable sugars were used to growth of the fungus and the ethanol production. Although steam exploded rice straw contained harmful substances such as furfural, the strain was able to grow and ferment; that is the strain has tolerance against these fermentation inhibitors. Efficient

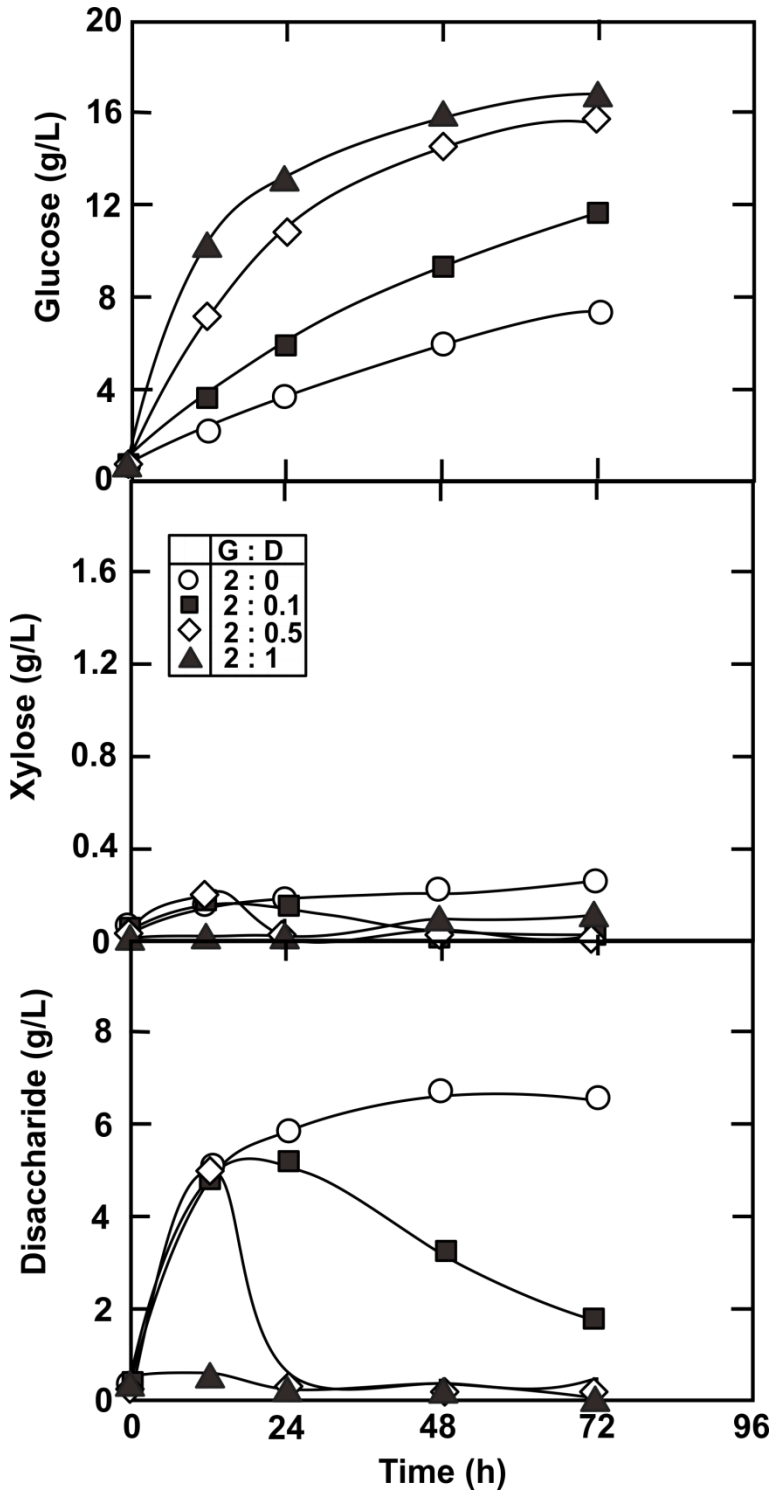

Fig. 4 Hydrolysis of steam exploded rice straw and production of fermentable sugars by cellulase cocktail at different ratio

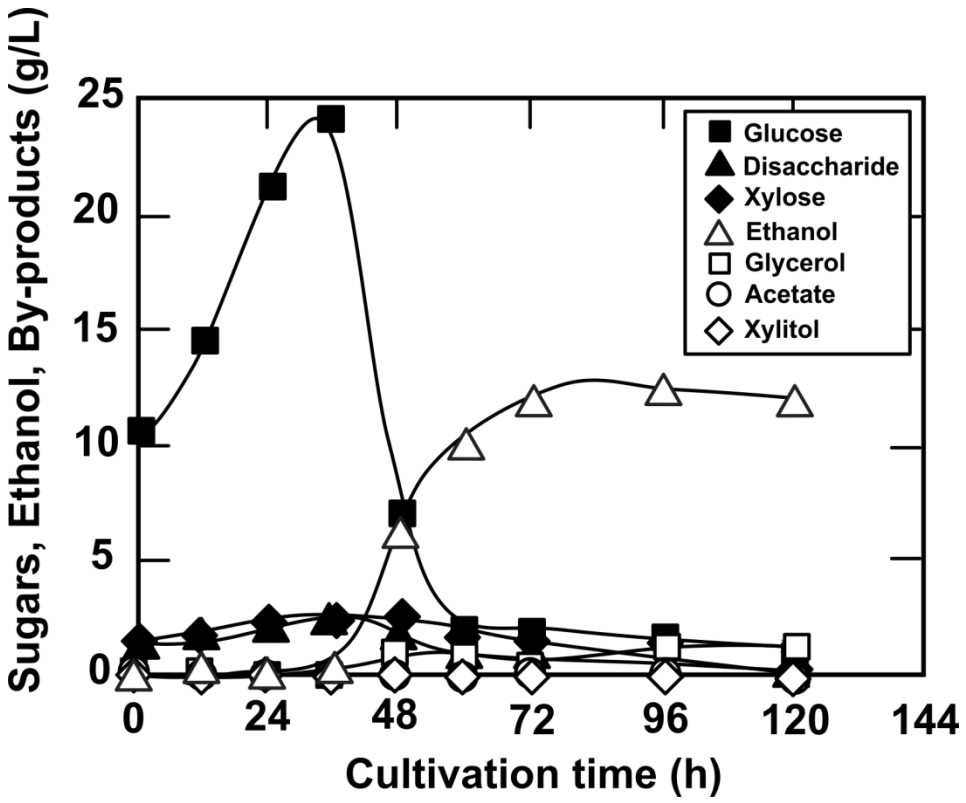

Fig. 5 Ethanol production from steam exploded rice straw by SSF with fermenting fungus and cellulase cocktail

ethanol production from steam exploded rice straw could achieve by SSF system with the optimized cellulases cocktail and the fermenting fungus.

2.4. Conclusion

The demand of biofuel production has increased on the environmental preservation and the energy supply problems. In order to develop biofuel production from waste lignocellulosic biomass, the rice straw was selected as desired biomass and the degradation condition was investigated about pretreatment and enzymatic hydrolysis to obtain effectively fermentable sugars. In the estimation of pretreatment methods of rice straw, steam explosion was selected because it requires relatively lower energy and has higher glucose component than other pretreatment methods. Hydrolysis of the pretreated straw was effectively performed with combination of two commercial cellulase reagents: Cellulase Onozuka 3S and Pectinase G. On the basis of these results, ethanol was able to be produced effectively when SSF of the steam exploded rice straw by the cellulase cocktail and a novel fermenting fungus was performed.

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Chapter 3

Bioethanol production from rice straw by simultaneous saccharification and fermentation with statistical optimized cellulase cocktail and fermenting-fungus

3.1. Introduction

Rice straw has heterogeneous complex structure in which hard cellulose fiber due to the crystal structure and entangled lignin and hemicellulose are contained. In the bioconversion to ethanol from lignocellulose, pretreatment by physical and/or chemical method is necessary to break the strong structure and obtain fermentable sugars easily by biocatalyst such as cellulase, xylanase, ligninase *etc.* Many researches have performed physical and chemical processing for pretreatment of lignocelluloses [1-9]. Especially, among the chemical treatments, alkali treatments using NaOH, KOH, CaOH and Na_2CO_3 are more effective than other chemical treatments because it is able to break the ester bonds between lignin, hemicellulose, and cellulose so that lignin and a part of hemicellulose are concurrently removed [7, 10]. Moreover, the pretreatment was able to reduce excessive degradation to furfural, 5-HMF, and vanillin more than acid treatment. These reactions result in cellulose fiber swelling and improve of cellulase contact to the fiber. As described in Chapter 2 (Figs. 1 and 3), alkali treated rice straw was consisted by many amount of sugars and was easily degradable material compared with the other pretreated straws. Therefore, this material was used in this chapter.

After pretreatment, biomass requires enzymatic hydrolysis on bioethanol production process. There are many kinds of hydrolysis enzyme reagent worldwide and any pretreated biomass demand the own reagents suitable for effective hydrolysis. However, few researches have performed both selection of the reagents and determination of the mixing ratio statistically whereas hydrolysis conditions have been mentioned by statistical optimization method [11]. The selection of appropriate cellulase is very difficult because of structural difference of each biomass and difference of enzymatic activities in commercial cellulase reagents.

The fungus *Mucor circinelloides* J used in this study is a mutant constructed by carbon ion-beam irradiation to improve xylose fermentation. *Mucor* sp. of parental strain is known as ethanol-producing fungus which is able to ferment not only glucose but also xylose and other

monosaccharides included *N*-acetyl glucosamine [12-14]. Moreover, it was characterized the ability to grow and ferment simultaneously under aerobic and anaerobic conditions. As lignocellulosic biomass such as rice straw contains a great deal of xylose, use of xylose-fermenting microorganism is necessary to achieve efficient ethanol production from such materials. The strain is also able to slightly secrete cellulase (*endo*-β-glucanse, cellobiohydrolase, β-glucosidase) and xylanase (*endo*-β-xylanase, β-xylosidase) and directly grow on polysaccharides [15]. Furthermore, it has tolerance for fermentation inhibitors such as furfural and 5-HMF produced by pretreatment with high temperature and pressure.

In this study, ethanol production was investigated by performing simultaneous saccharification and fermentation (SSF) of rice straw pretreated by alkali with thermal method. In order to hydrolyze the substrate to fermentable sugar in the process, some desirable commercial enzyme reagents were selected based on regression analysis by using enzymatic activities and then mixing ratio of them were optimized the design of experiments (DOE) with response surface method (RSM). The optimized enzyme cocktail was used in bioconversion of alkali-treated rice straw to fermentable sugar and subsequent ethanol by SSF system with *M.circinelloides*.

3.2. Materials and Methods

3.2.1. Rice straw

Rice straw used in this study was same and componential analysis was performed by Klason lignin method as described in the section 2.2.1.

3.2.2. Commercial enzyme reagents

The reagents of 15 kinds of enzymes were as listed in the section 2.2.2.

3.2.3. Microorganism for ethanol production

Mucor circinelloides J was used as a fermenting fungus in this study. The fungus is a mutant of *M.circinelloides* NBRC4572, which was mentioned in Chapter 1, constructed by carbon

ion-beam irradiation (Wakaswan Energy Research Center, Fukui, Japan) [13-16]. Then it was bred for efficient ethanol production from both glucose and xylose. The mutant was precultured for 3 days at 28°C on Difco™ Potato Dextrose Agar (PDA) plate after inoculation from PDA slant stored at 4°C.

3.2.4. Pretreatment of rice straw by alkali solution

Rice straw was soaked in 1M NaOH solution at the concentration of 80 g-dry straw/L-NaOHaq overnight at room temperature and then thermally treated by autoclave at 121°C for 1 h [17]. The treated material was neutralized by 1M HCl solution after cooling and then washed thoroughly by water several times. The washed material was dried in oven at 50°C till completely dried and then powdered using a pulverizer (BLENDER 7011 HB).

3.2.5. Optimization of cellulase reagent

Some cellulase reagents for effective hydrolysis of alkali-treated rice straw were first selected from 15 kinds of commercial cellulase reagents based on the calculation of regression analysis method on Excel solver, which is one of multiple classification analysis. The relational expression was estimated by statistical method on explanatory variable and objective variable about hydrolysis efficient by each enzyme. The explanatory variables were derived by enzymatic activities of *endo*-β-glucanase (EBG), cellobiohydrolase (CBH), β-glucosidase (BGL), xylanase (XN), β-xylosidase (BX), Filter paper degradation activity (FPU) and unwoven cellulosic cloth (BEMCOT®) degradation activity (BCU). The objective variables (hydrolysis activity) were derived by hydrolysis activities analyzed with 10 g/L pretreated straw by each enzyme reagent of 1 g-protein/L at 30°C, pH 5.5, and 120 rpm. The activities were determined as reducing-sugar production per one minute. These enzymatic activities as variables were fed to regression analysis tool in Excel and regression expression was obtained from coefficients in displayed analysis of variance (ANOVA) table containing significant value (F-value), coefficients, and probability value (P-value), *etc*. Three kinds of reagents were selected based on the high coefficients of activity in the expression. Next, an optimum ratio of these reagents mixture was constructed by design of experiments (DOE) method using

Design-Expart® 8. The software planned 14 kinds of mixing pattern that was necessary experiments for calculation of optimum ratio using the central composite design. Hydrolysis reactions were performed by using these mixtures of enzyme reagents on the basis of the designed patterns with 100 g/L alkali-treated rice straw for 48 h. After the reactions, hydrolysates were separated by filtration (Filter paper No.131, Advantec Ltd., Japan) and the liquids were analyzed by HPLC about the concentrations of glucose and xylose. Then, these sugar concentrations was input to the software again and that resulted in drawing a response surface of desirability on fermentable sugar production from pretreated straw and simultaneously the software calculated candidate of optimum mixing ratio of reagents by ANOVA.

3.2.6. Enzymatic hydrolysis of rice straw

Hydrolysis reactions were performed in 100 mL bottles containing 100 g/L of the pretreated rice straw suspended in 25 mL of 0.1M sodium acetate buffer (pH 5.5) or culture medium. These were previously autoclaved at 121°C for 15 min. Reaction was started by addition of the filter-sterilized single or cocktail enzymes at the total protein concentration of 2 g/L. The pretreated rice straw suspension was incubated at 28°C and 120 rpm for 96 h. Hydrolysates were obtained by removal of solid fraction by filtration and its sugars concentration was analyzed by HPLC system.

3.2.7. Simultaneous saccharification and fermentation (SSF)

SSF experiments were performed in 100 mL bottle containing 100 g/L pretreated rice straw suspension in 25 mL medium as described in the section 1.2.2. Cultivation was started by inoculating 1 mL fermenting fungus suspended in normal saline and simultaneously adding the filter-sterilized single of cocktail of cellulase that conditioned at 2 g-protein/L and then shaken at 120 rpm in an incubator at 28°C for 96 h. The cultures were filtrated and separated supernatants containing sugars and ethanol produced by SSF were analyzed by HPLC.

3.2.8. Analytical methods

Enzymatic activity existed in each commercial cellulase reagent about EBG, CBH, BGL, XN, and BX were measured by the same method as described in the section 2.2.5. Additionally, FPU, BCU and rice straw hydrolysis activities were measured by degradation of filter paper (Whatman® qualitative filter paper, Grade 1, Sigma-Aldrich) and BEMCOT® (Asahi Kasei Corporation, Japan), respectively, in acetate buffer (pH5.5) for 1 h at 28°C. Then, produced reducing-sugar was analyzed by 3,5-Dinitrosalicylic acid (DNS) method [18] and the activities were determined as the ability by which 1 μmol of reducing sugar per 1min released. The products such as glucose, xylose, xylitol, glycerol, and ethanol formed by hydrolysis or SSF were determined by a HPLC system as described in section 1.2.4.

3.3. Results and Discussion

3.3.1. Component of rice straw

Removal of lignin from biomass by pretreatment is essential to produce ethanol from rice straw efficiently by saccharification and fermentation. Among many kinds of pretreatment method for biomass, alkali pretreatment is profitable method because the treatment is able to remove lignin easily, fiberize, and break the crystal structure of cellulose [19]. Figure 1 shows the componential ratio of raw rice straw and the alkali-treated straw. Each result of the componential analysis was shown based on the mean value of three times. The pretreatment decreased lignin content from 29.6% to 11.6% and also reduced inorganic substances as ash containing silica from 14.1% to 8.5%. Since these cover the fiber, that reduction affected not only to increase cellulosic fiber content but also to be accessible to the fiber for enzymes and fermenting microorganism. Cellulose contents increased to 65.5% from 32.0%. However, the hemicellulose content decreased from 24.2% to 14.5% because of partially-solubilization of xylan by alkali treatment [20]. From these results, the fermentable sugar in the straw increased to 80.0% from 56.3% by the pretreatment. Considering total mass balance, cellulose recovery was 79.8% after the treatment. The treatment achieved production of the reformed material that contains 142% higher fermentable sugar per unit mass than that of raw rice straw. Therefore,

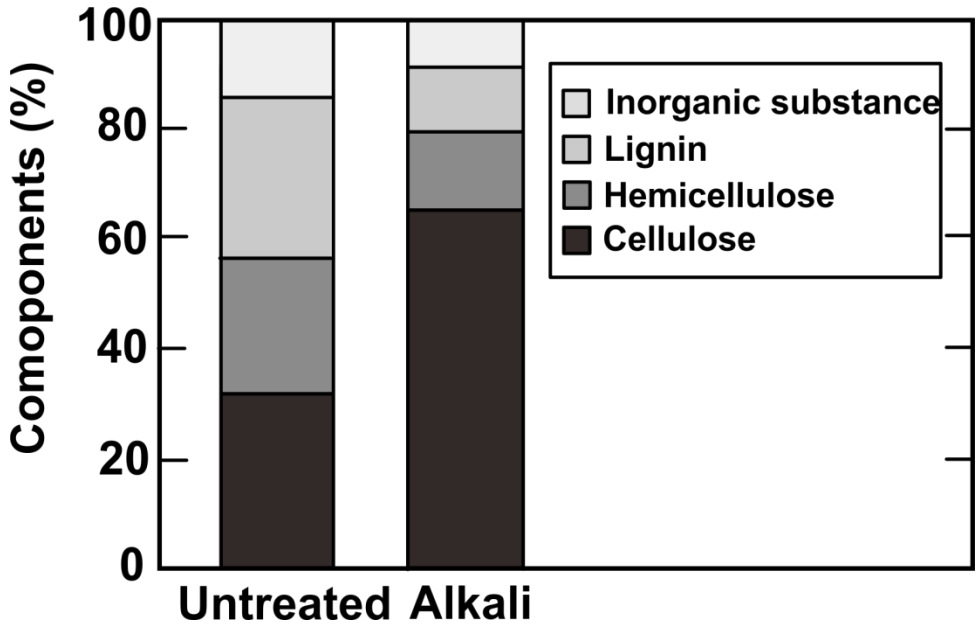

Fig. 1 Components ratio of raw and alkali treated rice straw

alkali pretreating method was a promising method for well removal of lignin from rice straw and obtainment of the cellulose fiber.

3.3.2. Selection and optimization of hydrolysis enzymes

Hydrolysis of lignocellulosic biomass requires effect of multiple enzyme activities such as EBG, CBH, BGL, XN, BX, FPU, and BCU. The hydrolysis rate of biomass is greatly controlled by the balance of these activities. This study chose commercial enzyme reagents with low price and quality for economical process. These possess particular activities because of the enzyme production origins and methods. Therefore, compounding several enzymes was expected to provide the synergy effect for enhancement of hydrolysis reaction and to cover the deficient activities each other under low dose condition. Figure 2 shows protein contents in these reagents per powder weight and their specific activities related to polysaccharide hydrolysis. Each reagent showed multiple protein contents and activities that act complicatedly on the cellulose and hemicellulose complex in biomass. Cellulase Onozuka 3S and Sumizyme X showed high activity on EBG, Cellulase T, Cellulase Onozuka 3S, and Sumizyme X on CBH, Meicelase on BGL, Sumizyme AC on XN, and Pectinase G on BX. Additionally, Fig. 3 shows hydrolysis activity of alkali-treated rice straw by these reagents. Meicelase, Cellulase T, and Cellulase Onozuka 3S indicated comparatively high degradation activity. However, determination of only one appropriate reagent was difficult for hydrolysis of the straw based on the results of various activities. Blending was desirable by combining the reagents having various enzyme activities for efficient hydrolysis of the rice straw. Therefore, suitable enzyme reagents were selected to combine them based on the multivariate analysis by setting enzymatic activities described in Fig. 2 as explanatory variables and hydrolysis activities in Fig. 3 as objective variable (Y). Regression expression obtained by the analysis was as follows;

$$Y= 1.562 - 2.578(CBH) + 1.193(EBG) + 1.549(FPU) + 0.057(XN) + 0.305(BCU) - 0.032(BGL) - 0.244(BX)$$

The coefficient in the expression showed the weight on degradation of the straw and large absolute value indicated grate requirement. This result is significant as evidenced by Fisher's F test, as the multiple correlation determination $R^2= 0.725$, and the very low significant value of

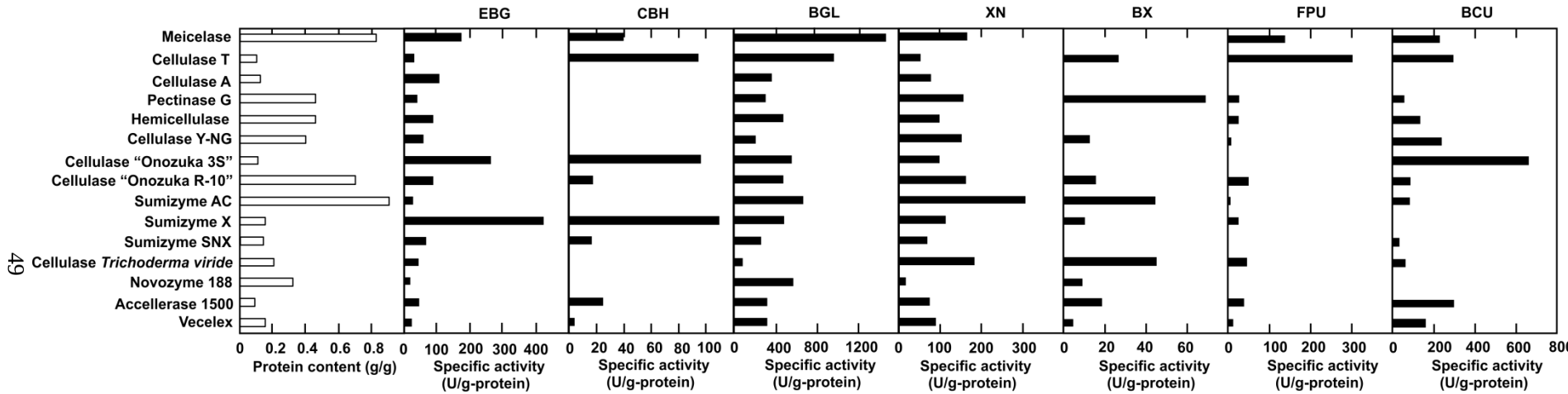

Fig. 2 Protein content and enzyme activities of commercial reagents for hydrolysis. Enzymatic activities was shown as follows; EBG: *Endo*-β-glucanase, CBH: Cellobiohydrolase, BGL: β-glucosidase, XN: Xylanase, BX: β-xylosidase, FPU: Filter paper degradation activity, and BCU: unwoven cellulosic cloth (BEMCOT®) degradation activity.

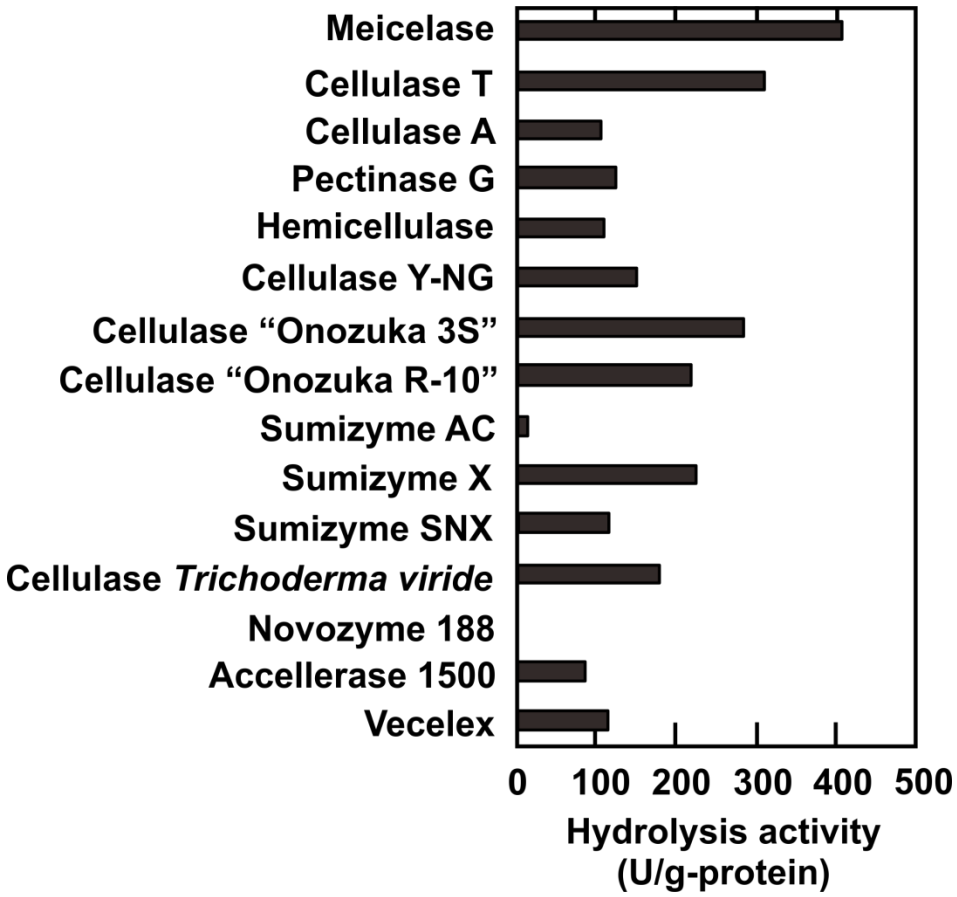

Fig.3 Hydrolysis activities of alkali-pretreated rice straw by commercial enzyme reagents.

F = 0.008. Here, P-value indicates the significance probability of the coefficient of each explanatory variable and this value is required to be less than 0.05 which shows variation of less than 5%. Because the P-value on (XN), (BGL), and (BX) in this statistical analysis were 0.87, 0.59 and 0.84, respectively, which were considerably higher than 0.05, these parameters should involve poorly in the hydrolysis. Another multivariate analysis was performed again except for these three inputs and new regression expression was calculated. As a result, degradation activity was obtained as follows;

$$Y= - 3.431 - 2.416(CBH) + 1.155(EBG) + 1.418(FPU) + 0.283(BCU)$$

The analysis resulted in significant expression with R^2 = 0.791, F = 0.0002, and P-values of explanatory variables CBH, EBG, FPU, and BCU of 0.02, 0.001, 0.00003, and 0.0006, respectively. The expression confirmed the requirement of these four activities for hydrolysis of the treated straw. From these viewpoints, Cellulase T Amano 4 (denoted by Cellulase T) with high activities of CBH, FPU, and BCU, and Cellulase Onozuka 3S (Onozuka) with high CBH, EBG, and BCU were chosen as candidates for preparing a cocktail. In addition, Pectinase G Amano (Pectinase G) was selected for hemicellulose degradation activities of XN and BX since hemicellulose was still contained in the alkali-treated rice straw. The suggested analysis method can offer accurate response for selection of appropriate agents from various reagents for hydrolysis of any biomass or even any different pretreated substances from same material. Since there are many excellent reagents of commercial enzyme around the world, selection of appropriate one from them is complicated issue for effective hydrolysis of any substrate. Construction of database previously on enzymatic activities of reagents will make it possible to apply the analysis and that will help selection of hydrolysis enzyme reagents.

An optimal enzyme cocktail containing selected three reagents was constructed according to DOE with RSM in order to efficiently biodegrade alkali treated rice straw by enzymatic hydrolysis. Software Design Export 8® structured 14 kinds of mixture patterns of three reagents as shown in Table 1 and hydrolysis reactions of the straw with the mixture were carried out in culture medium at pH 5.5 for 48 h at 28°C. Then glucose and xylose concentrations obtained by these reactions were input and applied for ANOVA in the software. Figure 4 shows a3D-response surface graph obtained by the calculation. The response was desirability for the

Table 1 Design models on mixture ratio of 3 kinds of cellulase reagents for optimization of cocktail

Run	Enzyme 1 Onozuka (g/L)	Enzyme 2 Cellulase T (g/L)	Enzyme 3 Pectinase G (g/L)	Response 1 Glucose (g/L)	Response 2 Xylose (g/L)
1	1.833	0.833	0.333	25.2	6.54
2	2	0	1	28.6	6.66
3	2	1	0	17.4	6.53
4	2	0	1	28.1	6.57
5	1.5	1	0.5	27.0	6.97
6	1.833	0.333	0.833	26.7	6.37
7	2	1	0	17.6	5.84
8	1	1	1	25.3	7.17
9	2	0.5	0.5	27.7	6.65
10	1.333	0.833	0.833	27.7	7.58
11	1	1	1	26.1	7.15
12	1.5	0.5	1	27.5	7.19
13	1.5	0.5	1	27.9	7.40
14	1.667	0.667	0.667	25.6	6.40

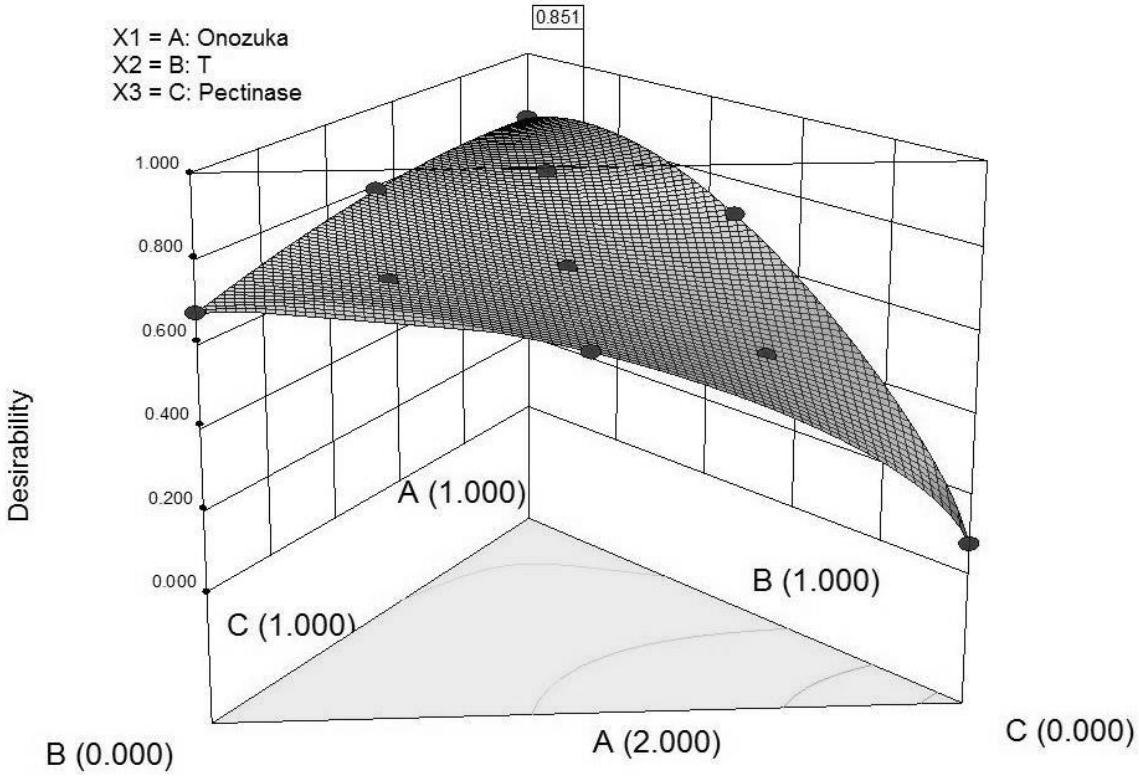

Fig. 4 Response surface of desirability of hydrolysis for optimization of mixture ratio on enzyme cocktail

production of both glucose and xylose together from the straw, and A, B, and C show the set range of Onozuka (1-2 g/L), Cellulase T (0-1 g/L), and Pectinase G (0-1 g/L), respectively. Although the analysis also drew another response graphs of expected glucose and xylose each (data not shown), we adopted the desirability for effective hydrolysis to produce both of the sugars. From the analysis, an optimum ratio of cellulase cocktail was calculated by ANOVA of the response function as follows: Cellulase T : Onozuka : Pectinase G = 0.333 : 0.388 : 0.286. Hydrolysis with the optimal cocktail was promised the largest amount of fermented sugar (total glucose and xylose) from alkali-treated rice straw. DOE method can propose how combine several types of reagent not only the enzymes like this study but also other parameters on medium components or culture conditions. The method will be better approach to adapt the reagents successfully by synergy of activities which affect to biomass hydrolysis because commercial enzymes contain multiple activities due to the low purification.

3.3.3. Fermentable sugar production from alkali-treated rice straw by three selected enzyme reagents or an optimal cocktail

Hydrolysis effect by the optimized cocktail designed by RSM was confirmed and was compared with that of each enzyme reagent alone on the alkali-treated rice straw. The treated straw of 100 g/L was reacted with these enzymes for 96 h at pH 5.5 and 28°C. Figure 5 shows sugars production from the straw with the each reagent or the cocktail at protein concentration of 2 g/L. In all hydrolysis reactions of the substrate, indicated pH were almost constant valuethrough the reactions. In the reaction with Cellulase T as shown in Fig. 5A, disaccharides, glucose, xylose, and arabinose were began to release in the early stage of the reaction and concentrations of them grew continuously for 96 h. Glucose kept increasing and achieved 30.1 g/L at 96 h, which was 46% of the cellulose content in the straw. This behavior was caused by high CBH and BGL activity in Cellulase T; that is CBH in the reagent behaved for disaccharides production, and immediately it converted to glucose by BGL activity with keeping apparent disaccharides on at low level. However, low EBG activity in the reagent caused scarce degradation of amorphous part of cellulose fiber and that suppressed glucose production though FPU activity was relatively high compared with other reagent shown in Fig. 2. Total

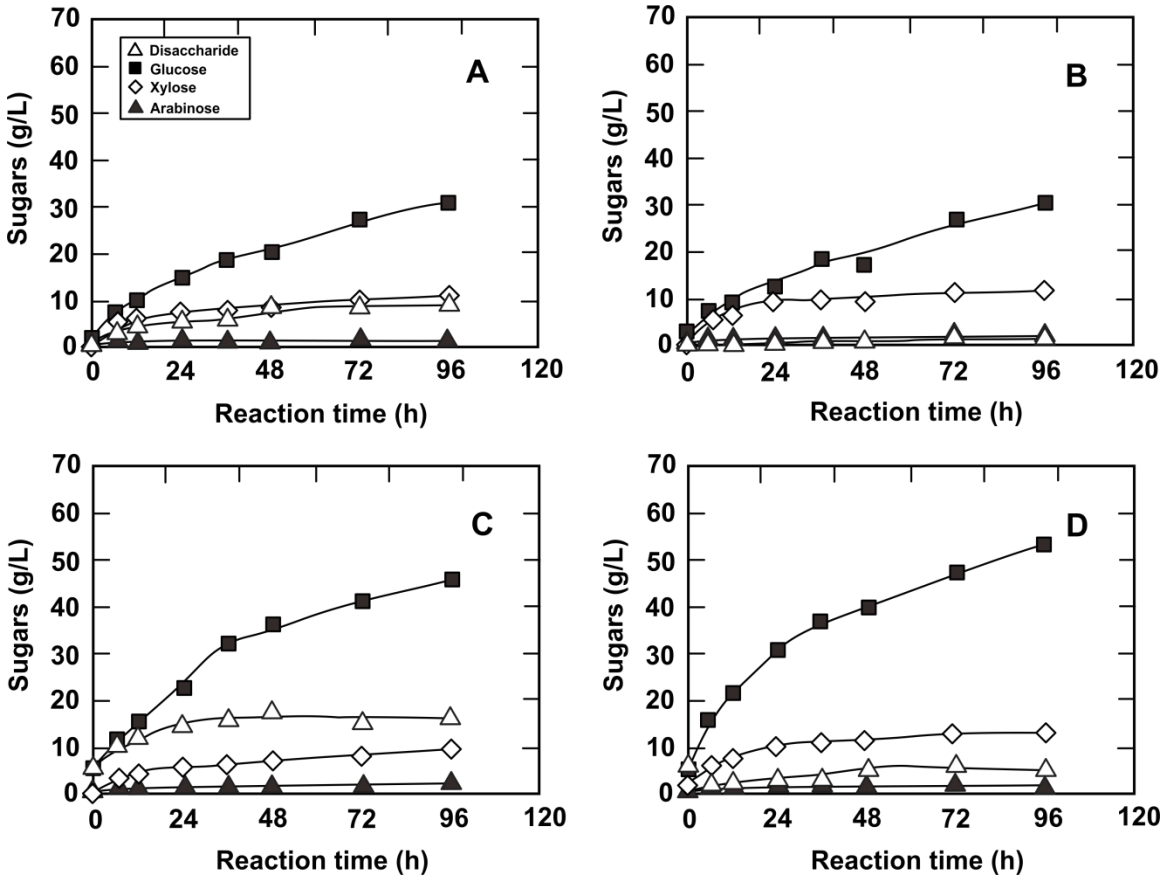

Fig. 5 Production of fermentable sugars from alkali-pretreated rice straw by enzyme reagents alone or optimized cocktail
A: Cellulase T, B: Pectinase G, C: Onozuka, D: cocktail

concentration of glucose and xylose was 42.4 g/L and the hydrolysis efficiency was 53% based on the total cellulose and hemicellulose content in the straw. In hydrolysis of the straw by Pectinase G, glucose and xylose could be produced without disaccharide accumulation through the reaction due to low CBH activity (Fig. 5B). The amount of produced glucose was 30.3 g/L which was same level of Cellulase T. At the same time, 12.2 g/L of xylose was able to be produced for 96 h and the yield was 84.1% of hemicellulose content in the straw. That was the highest yield among the three reagents alone due to particular high XN and BX activity. In the hydrolysis reaction of Onozuka as shown in Fig. 5C, glucose, xylose, disaccharide, and arabinose were produced from the straw. Glucose and xylose was produced at the concentration of 45.6 g/L and 10.0 g/L, respectively, for 96 h. The sum concentration of these sugars was 55.7 g/L that showed hydrolysis efficiency of 69.6%. The highest amount of disaccharide was produced by the reagent among the three kinds of reagents at the concentration of 15.9 g/L for 96 h. Disaccharides (mostly cellobiose) accumulation was caused by low BGL activity in Onozuka whereas EBG, CBH and BCU activities were high. In fact, oligosaccharides that had various lengths were detected by HPLC analysis (not identified). Accumulated disaccharides had to be degraded to glucose and xylose for fermentation to ethanol. Meanwhile, on hydrolysis of the treated straw by the optimized cocktail as shown in Fig. 5D, higher glucose and xylose concentrations achieved at 53.2 g/L and 13.5 g/L than that in the reactions with three single reagents, respectively. The yields of glucose and xylose from the straw were 81.2 and 93.1%, respectively. The hydrolysis efficiency on total fermentable sugars reached 83.3%. These were the highest value among the four patterns of reactions and that represented the cocktail greatly affected hydrolysis of the alkali-treated straw by interaction of the three reagents. Additionally, glucose production proceeded satisfactory without accumulation of disaccharides. Combination with Cellulase T that possesses high CBH, BGL and FPU and Onozuka that possess high EBG, CBH and BCU was able to progress degradation of disaccharides to glucose by synergy effect of these activities each other. Particularly, BGL in Cellulase T interacted with disaccharides produced by Onozuka activity. Moreover, since disaccharides contained not only cellobiose but also xylobiose, the BX activity in Pectinase G affected the xylobiose that produced by activity in Onozuka to induce xylose production.

Combination of several enzyme reagents complemented deficient activities in these reagents each other and effective fermentable sugar production from lignocellulosic substance was successfully demonstrated by using the optimized enzyme cocktail. Several studies have regarded optimization results of enzyme for fermentable sugar production by RSM [21, 22]. However, these reports arbitrarily selected several enzymes based on previous experiences or articles. The method in this study provided better approach that started from a selection method among many kinds of commercial reagents based on detailed investigation of activities and statistical examination than vague choice of them. No matter if some reagents have to be selected from more kinds of reagents than that of in this study (15 kinds), the application of proposed method will submit appropriate response for any biomass. And then optimizing these enzymes by DOE will be able to induce successful hydrolysis.

3.3.4. SSF of alkali-treated rice straw with fermentable-fungus and optimized cocktail

Ethanol production from 100 g/L of the alkali-treated rice straw was examined by simultaneous saccharification and fermentation (SSF) with an ethanol-producing fungus, *M.circinelloides* J, and the optimized cocktail at protein concentration of 2 g/L, pH 5.5, and 28°C for 96 h. As control experiments, three SSFs were performed using three selected reagents alone. In all SSFs as shown in Fig. 6, cellulose and hemicelluloses in the straw were hydrolyzed to disaccharides, glucose, and xylose at the early stage of cultivation. Then these were consumed by fungus and subsequently converted to ethanol by the fungus. Ethanol was started to produce after 12 h in all cultivations. In the SSF with Cellulase T as shown in Fig. 6A, 9.2 g/L glucose and 6.1 g/L xylose appeared at the initial stage of cultivation and then they were consumed immediately by the fungus. Then, ethanol reached 21.8 g/L at 48 h and the yield based on the fermentable sugar content in the substrate material and the productivity was 53.5 % and 0.454 g/L/h, respectively. Assimilation of the sugars by the fungus resolved product inhibition to enzyme caused by accumulation of them and consequently the sugars production in SSF was smoother than that in hydrolysis reaction without the fermenting strain. Moreover, the fungus converted oligosaccharides in the culture to ethanol directly. Because glucose production rate might be slower than fermentation rate, the fungus assimilated cellobiose *etc.* before

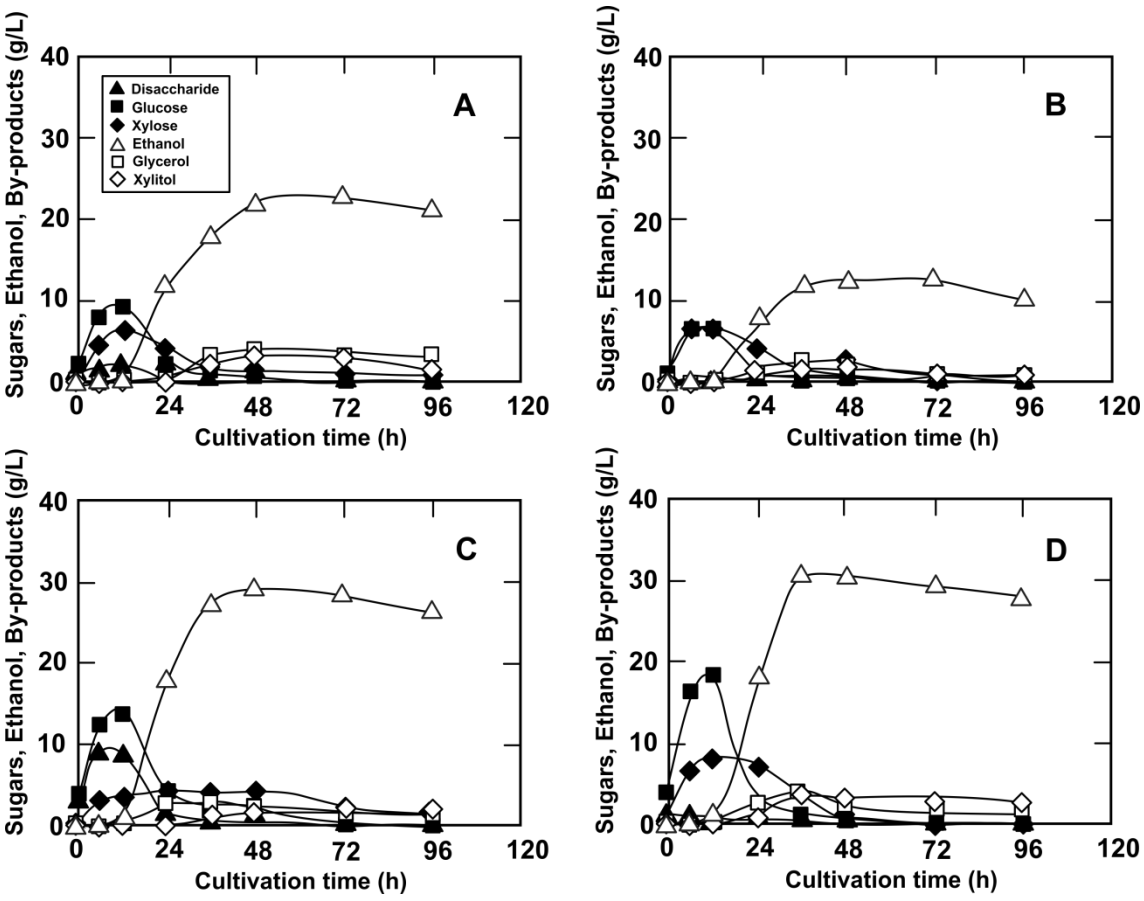

Fig. 6 Ethanol production by *M.circinelloides* J by SSF of alkali-pretreated rice straw
A: Cellulase T, B: Pectinase G, C: Onozuka, D: cocktail

enzymatic degradation to glucose by particular fermentation ability of the strain on these sugars. In the SSF with Pectinase G as shown Fig. 6B, 6.5 g/L glucose and 6.9 g/L xylose was produced for 12 h and then these sugars consumed with fermentation by the fungus to produce 12.3 g/L ethanol for 48 h. The produced ethanol was lower than that of SSF with Cellulase T whereas hydrolysis efficiency was same level as shown in Fig. 5. Because Pectinase G possesses scanty degrading activity to the straw such as FPU, the rate of fermentable sugar supply from the straw was not enough to produce high ethanol even though product inhibition was resolved by the fungus. When Onozuka was used for SSF as shown in Fig. 6C, 13.6 g/L glucose and 9.7 g/L disaccharides were produced quickly in the early stage of the culture, and the subsequent fermentation produced ethanol of 28.9 g/L for 48 h. The yield and productivity were 70.7% and 0.602 g/L/h, respectively. About 4.0 g/L xylose was produced and the sugar existed in the culture through the SSF because diauxie effect suppressed the fungus fermenting of xylose under presence of comfortable glucose. Moreover, appeared disaccharides at the early stage of SSF was decreased quickly for 24 h. Secretion of BGL and assimilation of disaccharides by the fungus helped the utilization of produced them for fermentation whereas the sugars accumulated in hydrolysis reaction bottle as shown in Fig. 5C. On the SSF with the optimized enzymes cocktail as shown in Fig. 6D, highest glucose and xylose were able to be produced at the concentration of 18.4 and 8.0 g/L without disaccharides accumulation, respectively. And subsequently, 30.6 g/L ethanol was able to be produced effectively for 36 h. The productivity and the fermentation efficiency achieved the highest value of 0.85 g/L/h and 90.0% of hydrolyzed fermentable sugar basis, respectively. The yield that cellulose content basis in the treated straw achieved 91.4 %. Synergistic effect led to efficient production of glucose and xylose by Cellulase T that including high CBH, BGL and FPU, Onozuka that including high EBG, CBH, and BCU, and Pectinase G including XN and BX. Although disaccharides were produced from the straw by CBH and other cellulase activities in Onozuka, BGL in Cellulase T and that secreted by the fungus could degraded them to glucose as soon as produced. Moreover, a few of them appeared in the culture were also assimilated by the fungus. These advantages induced the highest ethanol production compared to SSF with each reagent alone. Since the optimized cocktail improved hydrolysis from the estimation with three reagents alone, the

hydrolysis rate was faster comfortable for ethanol production rate. Moreover, resolution of product inhibition also increased the hydrolysis rate and that induced high ethanol production. Additionally, by-products such as xylitol in the SSF were barely formed from xylose metabolism because the highest xylose was produced by the cocktail from the straw, which was not used for ethanol production. At the same time, the SSFs decreased pHs from 5.5 to 3.2-4.3, and the lower pH was, the higher ethanol was produced. The secretion of various substances by the fungus with growth should decrease the pHs. Since optimum pH of general cellulases are mild acidity about 3-4, the lower pH could activate hydrolysis reactions for the rice straw degradation by enzyme reagents. More fermentable sugars were released under low pH condition in the medium from the straw by efficient hydrolysis with the cocktail, the more fungus was able to grow and to produce ethanol.

The proposed SSF system with the optimized cellulase cocktail and the novel ethanol-producing fungus is a promising method that was able to produce bioethanol directly from not only rice straw but also other lignocellulosic biomass. In order to utilize all sugars existing in rice straw, improvement of SSF system must be investigated about cultivation conditions: enzyme dose, medium composition, pH, temperature, surfactants addition and aeration. For example, high temperature can improve hydrolysis reaction more quickly than low temperature because that activates these enzymes at 40-60°C. However, the fungus can grow only in mild condition like room temperature. Construction of high-performing strain that can tolerate high temperature will achieve more effective SSF.

3.4. Conclusion

This study was presented a promising process for decision of enzyme reagents for hydrolysis of alkali-pretreated rice straw by statistical approach and efficient ethanol production by SSF. The pretreatment method of alkali (NaOH) with heating resulted in effective lignin removal from raw rice straw and making a high sugar content material. Multivariate analysis could suggest several required enzymatic activities and derive a selection of appropriate reagents for the pretreated rice straw. Three kinds of desired reagents were selected from 15 kinds of commercial enzyme reagents based on the analysis. DOE with RSM proposed an

optimum ratio of enzyme cocktail consisted of the three reagents for effective production of fermentable sugar from the alkali-treated straw. The enzyme cocktail constructed by these statistical approaches advanced hydrolysis efficiency to 83.3% at 2 g-protein/L from low efficiencies with each reagent alone. SSF of the alkali treated straw by using a novel pentose-fermenting fungus with the cocktail achieved high ethanol production with the fermenting efficiency of 90% of fermentable sugar basis. Further investigations suggest enhancement of ethanol production from lignocellulosic biomass by any improvement such as increase of temperature.

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Chapter 4

Direct ethanol production from rice straw by co-culture with two kinds of high-performing fungi

4.1. Introduction

Rice straw is an attractive resource for the bioethanol production. The annual rice production was about 689 million tons (FAO, 2008) and concomitant rice straw was estimated to be about 689-1034 million tons per year in the world but a goodly portion of rice straw is wildly discarded [1]. It is a lignocellulosic material containing cellulose, hemicellulose, lignin, and inorganic materials. The major fraction is cellulose and hemicellulose composed of glucose and xylose, respectively. To produce ethanol effectively and economically from such cellulosic materials, enzymatic hydrolysis of biomass by hyperactive cellulases and fermentation by high ethanol-producing microorganism are necessary. Therefore, the cellulase possessed high digesting-activity has been developed [2, 3]. In addition, bioprocesses for ethanol production have been developed about separation hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) methods by combining the commercial cellulase reagents and glucose/xylose fermenting microorganism [4, 5]. However, these processes have not been practically used due to the high prices of enzymes.

Mucor sp. is one of non-pathogenic filamentous fungi belong to Zygomaycete which has no dissepiment. It is commonly found in soil and plants *etc*., and capable to assimilate various sugars and grow under aerobically and anaerobically. The fungus is also known as a microorganism that secrete milk-clotting enzyme (Rennin) widely applied in the cheese-making industries [6] and that produce γ-linolenic acid which is an important fatty acid for human health and nutrition [7]. The genus has recently been received attention as an ethanol-producing microorganism which is capable of fermentation from cellulosic materials hydrolysate contained not only hexoses such as glucose, fructose, and mannose but also pentose such as xylose. For example, the production of ethanol by *M. indicus* from hydrolysate of rice straw, wood, and other lignocellulosic materials are investigated [8, 9]. The yield and productivity from dilute acid spruce hydrolysate by *M.indicus* were 0.45 g/g and 0.83 g/L/h, respectively [10]. Other

species of *Mucor* (*M. corticolous*, *M. hiemaris*, *M. miehei*, and *M. cirsinelloides* etc.) have also been demonstrated on capacity of ethanol production [11-14]. Moreover, *Mucor* sp. has also been known as microorganism which has cellulases secretion ability [15-19]. Especially, *M. circinelloides* has been attracted attention because of high ability for producing ethanol from hexose and pentose and further secretion of cellulases such as *endo*-β-glucanase (EBG), cellobiohydrolase (CBH), β-glucosidase (BGL), xylanase (XN), and β-xylosidase (BX) *etc*. [20-23]. Therefore, *M. circinelloides* has been selected as a good candidate for direct ethanol production from lignocellulosic materials. Although the fungus is expected achieving at the same time the cellulase secretion and the ethanol fermentation of glucose and xylose, they have been no report on such innovative wild fungus. Therefore, it is desirable under the present situation strategy to construct SSF by coculture with two kinds of fungi that possess different functions. In general, the operation of SSF by coculture with two different microorganisms is like pulling teeth because it is difficult to match the culture condition for each microorganism [24-30]. Based on this viewpoint, if the congeneric species are used in coculture, construction of better culture condition will be easily able to achieve. However, no research has been reported on bioconversion by coculture with congeneric fungi from lignocellulosic materials to ethanol.

In this study, two high-performing fungi on cellulase secretion and ethanol fermentation were selected from some strains belong to *M. circinelloides*. Further, direct conversion was investigated for lignocellulosic biomass, fine rice straw crashed mechanically, to bioethanol by using SSF system with coculture of two selected fungi.

4.2. Materials and Methods

4.2.1. Microorganisms and cultivation

Mucor circinelloides was used through this study for screening of cellulase-secreting fungus and ethanol-producing fungus. *Mucor circinelloides* f. *circinelloides* NBRC 4554, 4569, 4570, 4572, 4574, 5382, 5774, 30470, *M. circinelloides* f. *griseo-cyanus* NBRC 4563, *Mucor circinelloides* f. *janssenii* NBRC 5398, 6746 were purchased from the NBRC (NITE Biological Resource Center, Chiba, Japan). Each Hypha was put on a potato dextrose agar petri plate (9 cm

diameter) and incubated at 28ºC for 3 days as preculture. The mycelium on one plate was milled in 100 mL saline by using blender (Milser LM-Plus, Osaka chemical Co., Osaka, Japan) and used as inoculums. The fungi were cultivated in 100 mL flasks with liquid media as described in section 1.2.2 with carbon source of 50 g/L. Glucose, xylose, and rice straw were used as carbon sources. The media was adjusted at pH 5.5 and cultivation was started by inoculating 1 mL suspension of each fungus in the media and then shaken at 120 rpm at 28ºC.

The high-performing fungi were selected on the basis of the analysis of cellulase activity and ethanol concentration in the broth obtained after the cultivations for 72 h on glucose and 120 h on xylose and 120 h on rice straw. The direct fermentation of rice straw by co-culture using two kinds of fungi was performed under the same condition as described above. In coculture, each selected fungus was milled by the blender with saline by the method described above and then these were inoculated to medium at appropriate ratios. After cultivation, secreted enzymes, sugars, and ethanol in broth were analyzed.

4.2.2. Biomass and pretreatment

High-yielding rice straw named “Kinmaze” was used as cellulosic biomass in this study. The rice straw was harvested in Manto-City (Toyama, Japan) in September 2008, air-dried, and cut into about 1 cm length prior to pretreatment into a fine powder about 40 μm in diameter by a dry-mechanical crusher (Dry Burst, Sugino Machine Ltd., Toyama). The rice straw contained 27.4% cellulose (glucose), 14.4% hemicelluloses (12.6% xylose and 1.8% arabinose), 47.1% lignin, and 10.8% ash, which were measured according to Sun et al. [31].

4.2.3. Analysis methods

After cultivation, fungi were separated from media by filtration (Filter paper No.131, Advantec Co., Tokyo) and dry cell weight was measured after drying for 24 h at 90ºC. The broths were analyzed about ethanol, residual sugars, by-products, and cellulases and hemicellualse activity. Substances in broths were measured HPLC as described in section 1.2.4

4.2.4. Cellulase activity secreted by *Mucor circinelloides*

Activities of enzymes secreted in broth by fungi were analyzed by colorimetric method as described in section 2.2.5.

4.3. Results and discussion

4.3.1. Screening of high-performing fungi

Cellulase and hemicellulase-secreting ability of these fungi were estimated by measuring the enzymatic activity in culture broth. These fungi were cultured aerobically in media containing 50 g/L glucose or xylose at 28°C for 120 h. After the cultivation, broths were analyzed about CBH, EBG, XN, BGL, and BX activities. Especially, the relation between EBG and BGL was paid attention because cellulose is mainly the carbohydrate containing in rice straw as shown in Fig. 1. The fungus belong to *M. circinelloides* were able to secret various enzymes that degrade lignocellulosic material. However, CBH activity, which can hydrolyze microcrystal cellulose moiety, was not observed in the all broths of fungi (data not shown). The activities of EBG and BGL secreted by high ethanol-producing fungus, strain 4572, were not too high comparing with those of other strains because these secretions were not induced by other than polysaccharides: glucose and xylose. *M. circinelloides* NBRC 5398 showed obviously higher activity of both EBG and BGL at the culture of xylose compared with other strains, and these activities were 2.46 U/L and 2.94 U/L, respectively, though the fermentation ability on xylose by the strain was low. The secretions of these enzymes by the strain were induced at the presence of xylose.

Meanwhile, ethanol production abilities of fungus, *M. circinelloides*, based on the fermentation of glucose and xylose were investigated and a fungus was selected based on production of the highest amount of ethanol from both of sugars. These fungi were cultured aerobically in media containing 50 g/L glucose or xylose as a carbon source at 28°C and the broths were collected by filtering after 72 h on glucose or 120 h on xylose. Figure 2 shows the relation between ethanol production from glucose and xylose in order to compare the fermentation ability among 11 species of *M. circinelloides*. The point in upper right in the figure

means the strain that possesses high ethanol production ability. Almost strains were able to produce ethanol from both glucose and xylose. Among these strains, *M. circinelloides* NBRC 4572 was produced the highest ethanol of 18.3 g/L and 8.45 g/L from glucose and xylose, respectively. The microorganisms that ferment xylose are strongly required for effective process of biomass conversion. However, general fermenting microorganisms such as yeast are not able to ferment xylose from the hydrolysis of hemicellulose though lignocellulosic biomass contains not only cellulose but also hemicelluloses. These results proposed a possibility of direct ethanol production from rice straw by coculturing with combination of 5398 as hydrolase secreting strain and 4572 as ethanol producing strain.

4.3.2. Batch cultivation of two high-performing *M. circinelloides*

The strain 5398 as high cellulase-secreting fungus and the strain 4572 as high ethanol-producing fungus were cultivated aerobically in media containing 50 g/L glucose or xylose at 28°C in order to examine behavior of mycelia growth and the cellulase secretion of both strains in detail. Figure 3 shows the results of cultivation behaviors of two strains with each sugar. On the strain 4572 grown with glucose, it was consumed sharply and the dry cell weight reached 5.51 g/L when it was completely consumed after 36 h (Fig. 3A). Simultaneously, high ethanol was produced from glucose and the maximum concentration was 21.0 g/L at 36 h, which corresponds to the ethanol productivity of 0.583 g/L/h and the yield of 0.420 g/g. After depletion of glucose, the ethanol concentration slowly decreased to 11.0 g/L after 96 h, which was caused by consuming of ethanol as a carbon source to acquire energy by the strain. In the case of 50 g/L xylose, the growth rate was fast as well as glucose cultivation and the dry cell weight was 5.07 g/L at 24 h though it was consumed slower than glucose. Then it was completely consumed after 96 h (Fig. 3B). The strain might be able to obtain energy from xylose more effectively than glucose. Ethanol produced from xylose reached 17.8 g/L after 60 h, which was before depletion of xylose, and the productivity and the yield was 0.296 g/L/h and 0.478 g/g, respectively. However, the ethanol production stopped despite the presence of residual xylose and then the simultaneous consumption of xylose and ethanol was advanced for cell growth and maintenance. Moreover, the fungus was able to assimilate other

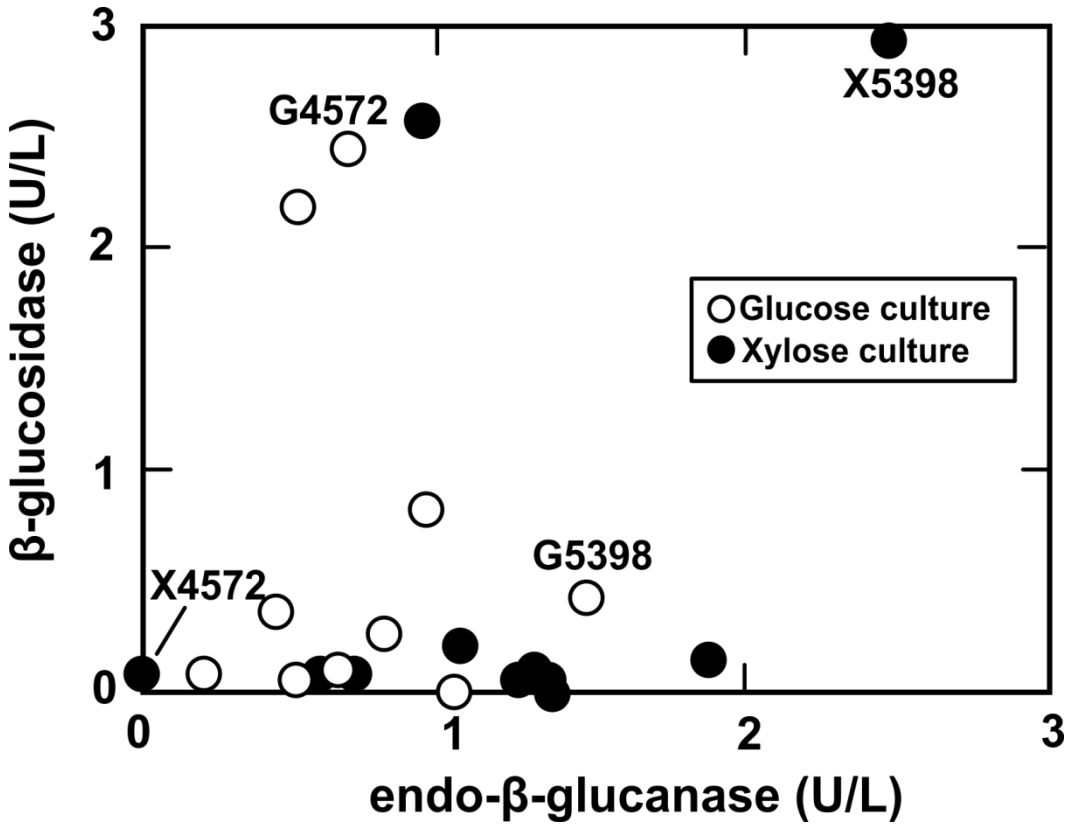

Fig. 1 Cellulase activities secreted in broth by several species of *M.circinelloides*

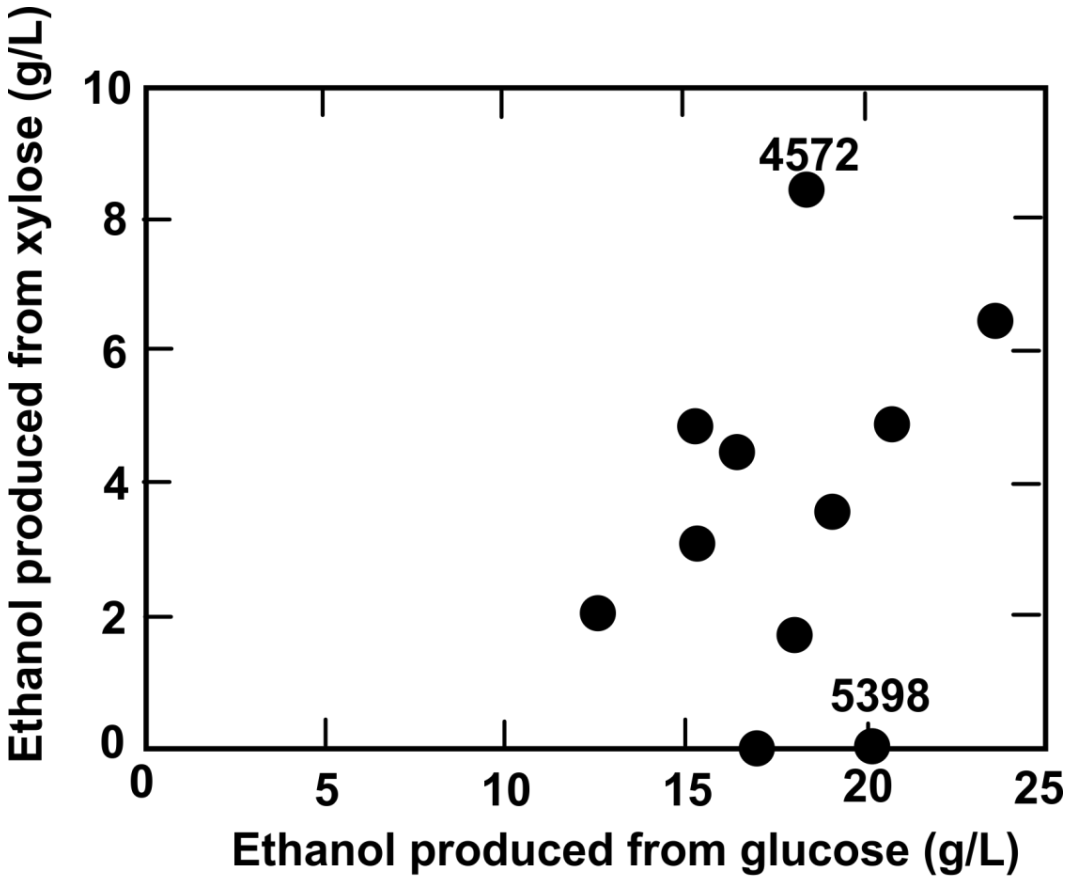

Fig. 2 Ethanol production from glucose and xylose by several species of *M.circinelloides*

monosaccharide, disaccharide, polysaccharide, and sugar alcohol and also ferment most sugars except a part of sugars. At the same time, growth rate of the strain 5398 was slower than that of the strain 4572, and the mycelium grew with consumption of glucose and maximum cell concentration reached 3.90 g/L after 72 h (Fig. 3C). Ethanol was also produced and the concentration, productivity, and yield reached 19.5 g/L, 0.340 g/g, and 0.202 g/L/h after 96 h, respectively. In contrast to 4572, the strain 5398 consumed few xylose, and its cell concentration was only 0.748 g/L after 96 h (Fig. 3D). However, even the strain can uptake a little xylose and grow slightly, it was able to induce cellulase secretion by the strain according to Fig.1. Because of the low growth ability in xylose, many amount of inoculum would be required in coculture for as enough cell weight as 4572.

4.3.3. Behavior for cellulase secretion of *M. circinelloides*

The performances of cellulase secretions by two kinds of *M.circinelloides* were investigated. Since the strain 4572 was able to assimilate polysaccharides (soluble starch, rice starch, CMC, Avicel, xylan, and rice straw), the strain also should slightly secret cellulase and hemicellulase. Two high-performing fungi were cultured aerobically with rice straw in order to induce the secretion of cellulases. The broths were analyzed about EBG, XN, BGL, and BX activities as shown in Fig. 4. As a result, the strain 4572 produced cellulases slightly (Fig. 4A). The activities of EBG, XN, and BGL increased slowly, and they were 0.578 U/L, 0.546 U/L, and 0.0231 U/L after 96 h, respectively. The strain 5398 secreted these hydrolases in higher activities than those of the strain 4572 (Fig. 4B). Especially, EBG activity increased rapidly to 2.11 U/L after 24 h and decreased to 0.584 U/L at 48 h, and then increased again. BGL activity began to increase at 36 h and reached 1.47 U/L at 72 h. The strain 5398 had higher secreting ability (4-fold on EBG and 20-fold on BGL) than the strain 4572. These results indicate that 5398 produce EBG in the initial cultivation and then started to secret BGL after accumulation of oligosaccharides by the EBG. Since 4572 can assimilate short oligosaccharides, combination of these strains in culture with biomass suggested the possibility of direct ethanol production from rice straw with degradation by 5398 and fermentation by 4572.

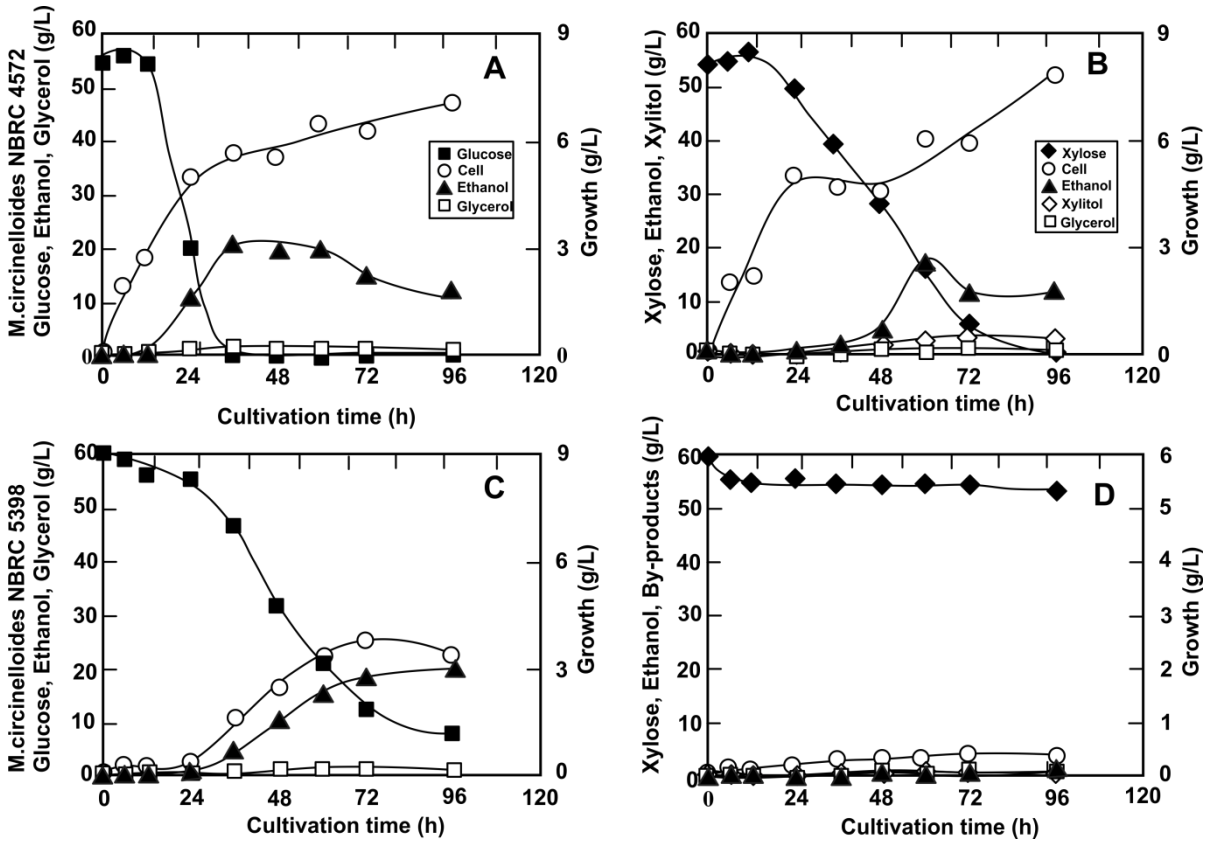

Fig. 3 Batch culture of *M.circinelloides* NBRC 4572 and 5398 by using glucose (A, C) and xylose (B, D) as carbon sources

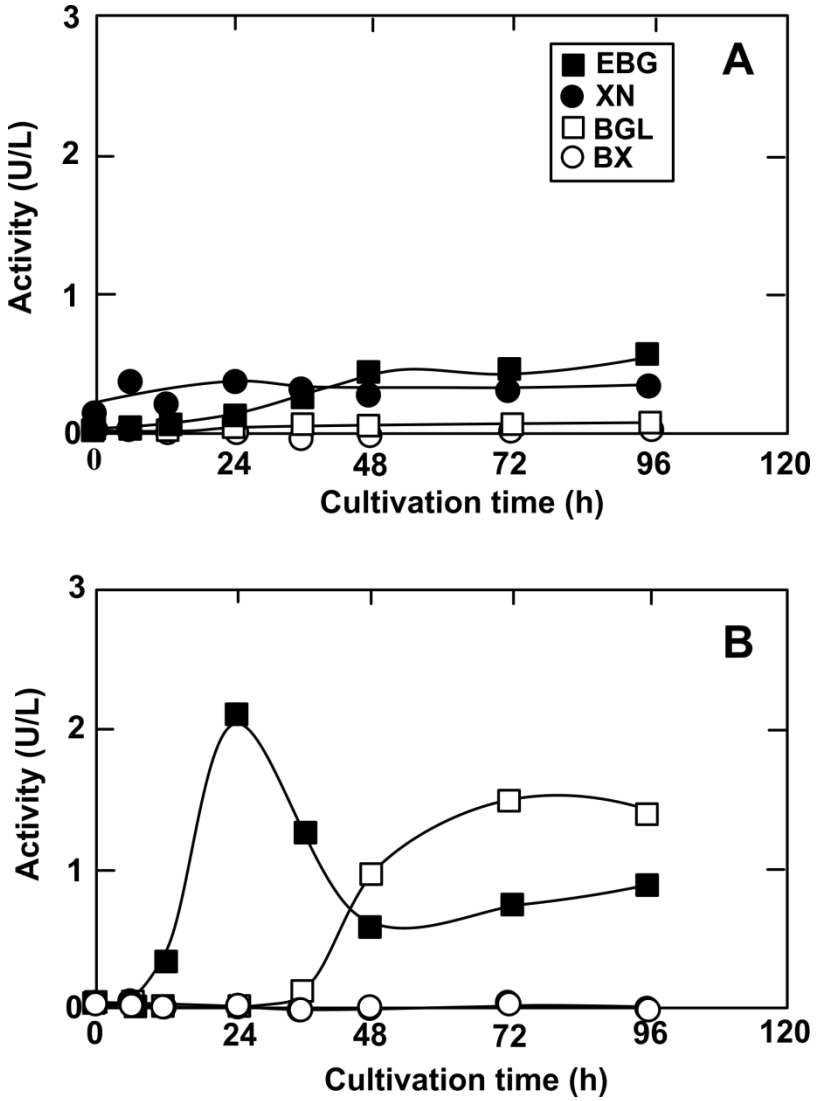

Fig. 4 Cellulase and hemicellulase secreted in broth by *M.circinelloides* NBRC 4572 (A) and 5398 (B) as batch sultures was performed with rice straw

4.3.4 Direct ethanol production by SSF with coculture of two high-performing fungi from rice straw

The possibility of direct fermentation of rice straw was examined by the strain 4572 or the strain 5398 only. These were cultured in basic medium with 100 g/L fine mechanically crushed rice straw as a carbon source (contained worth 50 g/L sugars). Each culture was started by inoculating milled fungus suspension to the medium and aerobically shaking at 28°C. As a result, neither ethanol nor other organic alcohols and acids were not detected for 96 h. Therefore, coculture of two kinds of high performing fungus was tried for ethanol production directly from rice straw powder. When the coculture was performed at the inoculation ratio of 5398 to 4572 of 1: 1 or 3: 1, ethanol was hardly produced from the rice straw. That was probably attributed to faster growth rate of 4572 than that of 5398 and slow secretion of cellulases; that is, though once sugars produced from rice straw, they were consumed immediately by 4572 and therefore that inhibited the growth of 5398. The sugars were not enough to ethanol production by 4572. Based on these results, coculture was performed at the inoculation ratio of 4572: 5398 = 1: 9 as shown in Fig. 5. Glucose and xylose eluted from rice straw were consumed completely for 24 h and then these sugars were not observed until 96 h. Subsequently, ethanol concentration increased slowly after 12 h and reached 1.28 g/L at 96 h. Since increase of inoculum volume of 5398 progressed cell growth and secretion of cellulase, cellulose and hemicellulose in the straw was degraded to enough amount of glucose and xylose for ethanol production. Therefore, direct ethanol production from rice straw was successfully achieved with two kinds of high-performing fungi.

Meanwhile, though the fungi grew and ethanol was produced by the coculture system, a little cellulase activities were detected in the broth as shown in Fig. 6. Only EBG activity was observed and it was about 0.5-0.8 U/L between 12 h and 96 h. The reason why little other cellulases activities were observed is that EG, XN, and XG might adsorbed on the surface of mycelia or rice straw. In fact, these enzyme activities were detected when cell was homogenized and then the supernatant was analyzed. Moreover, these enzymes should adsorb on the straw because cellulase has carbohydrate binding modules [32]. Ethanol production by the coculture suggested the existence of these cellulases and these degraded rice straw. At the same time,

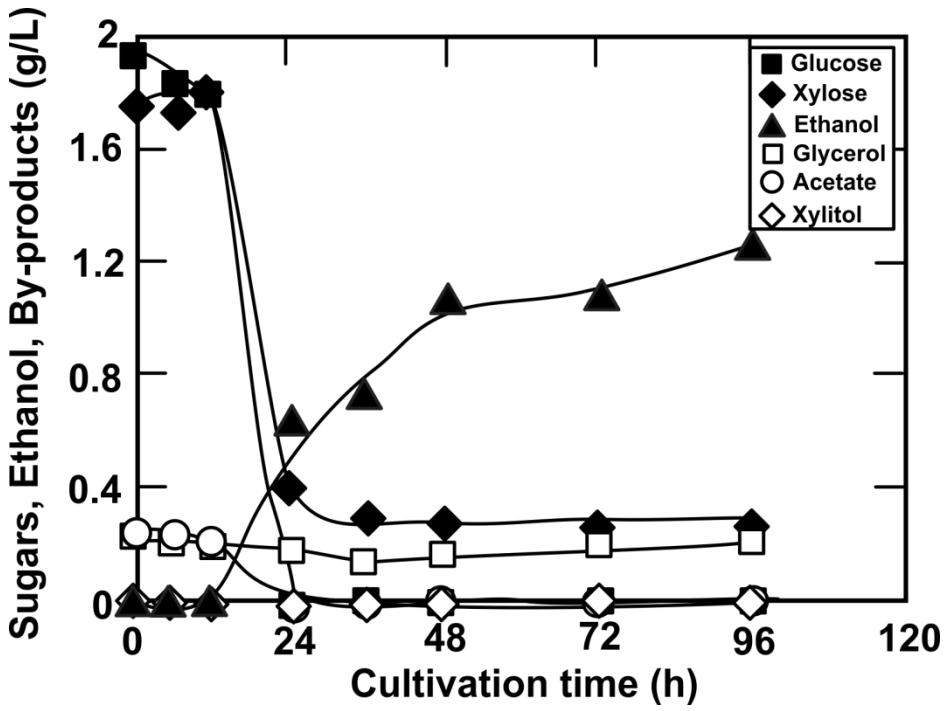

Fig. 5 Direct ethanol production from mechanical crushed rice straw by SSF with a coculture combined with cellulase secreting-fungus (strain 5398) and ethanol-producing fungus (strain 4572) at the ratio of 1:9

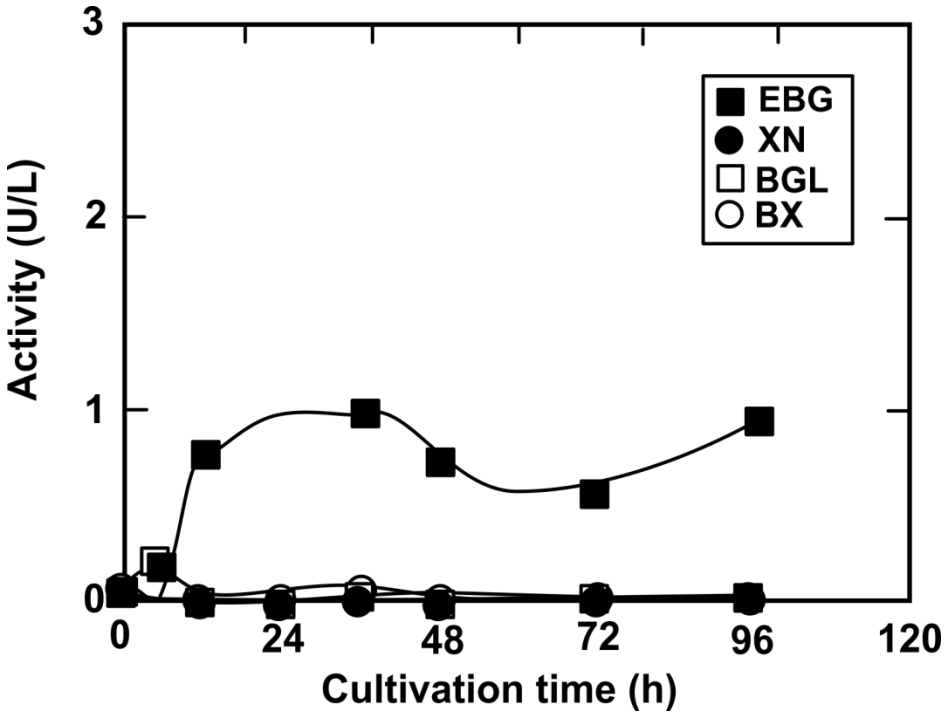

Fig. 6 Enzymatic activities existed in broth as direct ethanol production from fine mechanically crashed rice straw by SSF with coculture of strain 5398 and strain 4572

about 25 g/L ethanol should be obtained theoretically from initial concentration of 100 g/L rice straw (ca. 50% total sugar content). However, in the coculture system as showed in Fig. 5, ethanol was produced only 1.28 g/L. The difference of growth rates between two selected high-performing fungi was caused by imbalance of rate between fermentable sugar formation by cellulases secreted from both fungi and fermentation of these sugars. Improvement of secretion amount of various cellulases will achieve effective coculture system for direct ethanol production from rice straw.

4.4. Conclusion

Development of direct ethanol production process is recently demanded on the basis of economical view point, which is made from lignocellulosic biomass that abandoned voluminously all over the world. Therefore, we attempted the development of direct ethanol producing process from rice straw by coculture with only fungi that can hydrolyze or ferment belong to Zygomycetes, especially *Mucor circinelloides*. Among these fungi, *M. circinelloides* NBRC 5398 could secret cellulases and *M. circinelloides* NBRC 4572 could ferment glucose and xylose in high yield. In fermentation of fine mechanically crushed rice straw by the coculture system with them, ethanol directly produced and achieved 1.28 g/L after 96 h when inoculation ratio of the strain 5398 to the strain 4572 was 9:1. To improve the yield of ethanol using our proposed coculture system, the strong secretion of cellulases by *M. circinelloides* NBRC 5398 or other strains must be required. Since usage of congeneric species is advantageous at the point of not only culture condition but also fusion of these strains, the other process by using fused strain also has to be constructed for further efficient ethanol production.

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Chapter 5

Two new β-glucosidases from ethanol-fermenting fungus *Mucor circinelloides* NBRC 4572: enzyme purification, functional characterization, and molecular cloning of the gene

5.1. Introduction

Zygomycetes fungus, *Mucor circinelloides*, which can not only assimilate and ferment several types of sugars efficiently but also can produce several extracellular soft-biomass hydrolases [1, 2]. These properties enabled us to discover novel and efficient method for bioethanol production by simultaneous saccharification and fermentation (SSF) of rice straw only with the fungal strain. However, the addition of cocktails of commercial cellulases is indispensable for complete hydrolysis of rice straw because the production level of the hydrolases in the fungal strain is not sufficient [1]. In order to perform this process more economically, it is necessary to decrease the amount of the added expensive commercial enzymes. Here, we focused on a β-glucosidase (BGL; EC 3.2.1.21) of the strain because of its quite low production level (<0.1 U/L culture; [3]). BGL activity in commercial cellulases is generally quite low, which results in the overuse of expensive cellulases and/or use of BGL-enriched cellulases or exogenous BGL additives [3, 4]. Moreover, the performance of BGL is considered rate-limiting in the saccharification of cellulosic materials because the enzyme determines not only the rate of the hydrolysis but also the extent of cellulose hydrolysis by relieving end-product inhibition of soft-biomass hydrolases, such as cellobiohydrolase and endoglucanase [5, 6]. Hence, the enhancement of the BGL productivity and reactivity in the *Mucor* strain would be important for developing the bioethanol production process by SSF of rice straw.

Numerous studies on BGLs have demonstrated that they can be potentially employed not only for bioethanol production from cellulosic biomass but also for flavor enhancement, detoxification of cyanogenic glucosides, and oligosaccharide synthesis [7]. Filamentous fungi are promising candidates for BGL production because they produce higher amounts of cellulose-hydrolyzing enzymes than bacteria and because they secrete BGLs extracellularly [8].

It is very important to accumulate data on the relationship between enzyme characteristics and fungal BGL structure. In particular, among zygomycetes fungi, information on BGLs is extremely scarce except those from *Mucor miehei* [9], *M. racemosus* [10], *M. circinelloides* [11], *Rhizopus oryzae* [12], and very recently *Rhizomucor miehei* [13, 14]. Of those, only the primary structure of *R. miehei* BGL is available [13].

In the present work, we succeeded in the purification and characterization of two novel extracellular BGLs as well as in cloning their genes from the ethanol-fermenting fungus *M. circinelloides* NBRC 4572 statically grown on rice straw. Additionally, we discuss the suitability for the use of the BGLs in bioethanol production by SSF of rice straw with the fungal strain.

5.2. Materials and methods

5.2.1. Materials

Gels for DEAE- and Butyl-Toyopearl 650M column chromatography were purchased from Tosoh (Japan). Superdex 200, Resource Q columns, and standard proteins for gel filtration chromatography (Gel Filtration Calibration kit, HMW) were from GE Healthcare (USA). Standard proteins (low range) for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were obtained from Bio-Rad (USA). Enzymes and kits for deoxyribonucleic acid (DNA) manipulations were purchased from commercial suppliers and used according to the manufacturers' protocols. All other chemicals were from commercial sources and used without further purification. Rice straw of cultivar "Koshihikari" harvested in Imizu City (Toyama, Japan) in September 2011 was air-dried, cut into small pieces, and used as a carbon source for cultivation of *M. circinelloides*.

5.2.2. Protein analysis

Protein concentration was determined using a Protein Assay kit (Bio-Rad) with bovine serum albumin (BSA) as a standard. Detection of glycoproteins in SDS-PAGE gels was carried out by Glycoprotein Detection kit (Sigma, USA). Deglycosylation of N- and O-linked glycoproteins was achieved with PNGase F and O-glycosidase/neuraminidase (New England

Biolabs., USA), respectively, according to the manufacturers' protocols. The native and subunit molecular mass (*Ms*) of the enzymes was estimated as described previously [15].

5.2.3. Microorganisms and cultivation conditions

M. circinelloides NBRC 4572 (Biological Resource Center, NITE, Japan) was used as a source of BGL throughout this study. Basal medium was as described in section 1.2.2. The mycelia grown on a solid-agar plate of the basal medium supplemented with 2 % glucose at 30°C for 7 days were milled with a mortar and pestle in 100 mL of physiological saline per one plate and it was used as an inoculum. Five-hundred microliters of the inoculum were added to a 100-mL Erlenmeyer flask containing 0.5 g (dry weight, d.w.) of carbon source (i.e., glucose or rice straw cut into 2- to 3-cm length) were supplemented with 1.25, 2.5, 5, 10, and 25 mL of the basal medium and were incubated at 30°C for 3 days with rotary shaking at 80 rpm (aerobic) or without shaking (static). The total medium volume was adjusted to 25 mL by adding physiological saline, and the extracellular enzymes were extracted by gently suspending the mixture using a mortar and pestle. After centrifugation (14,000×*g*, 10 min), the supernatant obtained was directly used to measure BGL activity.

5.2.4. Enzyme assay

The standard assay contained 50 mM CH_3CO_2Na-CH_3CO_2H buffer (NaAc), pH 4.8, 5 mM *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG), and 5 μL of the enzyme at several dilutions in a total volume of 125 μL. After incubating at 40°C for 10 min, the reaction was terminated by addition of 25 μL of Tris-HCl buffer (0.5 M, pH 9.0). The enzyme activity was measured by the amount of *p*-nitrophenol (*p*NP) liberated using a calibration curve at 405 nm. One unit of the enzyme activity was expressed as the amount of enzyme required to release 1 μmole of *p*NP per minute under the assay conditions. Determination of substrate specificities and kinetic studies were carried out as follows. Enzyme activities toward *p*NP-glycosides type substrates were measured by monitoring the amount of released *p*NP as described above at various concentrations (0.2-50 mM) of substrates in 125 μL reaction mixture containing 50mM NaAc buffer, pH4.8, and the enzyme (5 μL) with several dilutions at 40°C for 30 min. Activities

toward oligosaccharides and alkyl-glucosides and aryl-glucosides, salicin and arbutin were determined by monitoring the amount of released glucose at various substrate concentrations (0.2-50 mM) in 25 μL reaction mixture containing 50 mM NaAc buffer, pH 4.8, and the enzyme (5 μL) at several dilutions at 40˚C for 1-20 h. Glucose concentrations were measured with LabAssay Glucose kit (Wako Pure Chemicals, Japan).

5.2.5. Purification of BGLs

All purification procedures were performed at 4°C K_2HPO_4-KH_2PO_4 buffer (KPi), pH 6.0, containing 5 mM 2-mercaptoethanol was used throughout the purification. Rice straw (55 g d.w.) cut into 3- to 4-cm length was put into a plastic container (17×28×9 cm) with 275 mL of basal medium to which 50 mL of the inoculum was added. After static cultivation at 30°C for 3 days, six sets of the culture were combined and mixed with 3 L of 10 mM KPi buffer. The extracellular enzymes were extracted by gently suspending the mixture with a blender (LBC15,Waring, USA) for 1 min. The rice straw was removed by passing through a stainless sieve followed by diatomite-pad (Radiolite #100, Showa Chemical Industry, Japan). DEAE-Toyopearl gels (250 mL) equilibrated with 10 mM KPi buffer were added to the filtrate (10 L), and the mixture was gently stirred overnight. The gels were packed into a column and successively washed with 10 and 100mMKPi buffers (1 L each). The active fractions eluted with the 100 mM KPi buffer containing 1 M NaCl were collected and then brought to 45 % ammonium sulfate saturation. The enzyme solution was placed on a Butyl-Toyopearl column (2.5×10 cm) that was equilibrated with 100 mM KPi buffer containing 45%saturated ammonium sulfate. The active fractions that were eluted with a linear gradient of ammonium sulfate (45-0 % saturation) in 100 mMKPi buffer were combined and dialyzed against 10 mM KPi buffer. The enzyme solution was loaded on a second DEAE-Toyopearl column (1×16 cm) equilibrated with 10 mM KPi buffer. The active fractions eluted with 100mM KPi buffer containing 100 mM NaCl were collected and dialyzed. The following purification step was carried out using the ÄKTA Explorer System (GE Healthcare). The enzyme solution was loaded on a Resource Q column (1 mL) equilibrated with 10 mM KPi buffer and eluted with a linear gradient of NaCl (0–300 mM) in the same buffer at a flow rate of 1 mL/min. The two separated

active enzyme fractions (BGLs 1 and 2) were collected.

5.2.6. Determination of an N-terminal amino acid sequence

Purified BGLs 1 and 2 were enzymatically deglycosylated, subjected to SDS-PAGE, and then transferred electrophoretically onto a polyvinylidene difluoride (PVDF) membrane (0.22 μm; ATTO). After Coomassie brilliant blue (CBB) staining, 70-kDa band was cut out from the blot and subjected to automatic Edman degradation on a peptide sequencer (model 494, Applied Biosystems, USA).

5.2.7. Effect of temperature and pH on the enzyme stability and activity

The effect of the temperature and pH on the enzyme stability was determined by measuring the enzyme activity in the reaction mixture (125 μL) containing 50 mM of NaAc buffer, pH 4.8, 5 mM *p*NPG, and 5 ng of the preincubated enzyme for 30 min at various temperatures (4-65°C) in 50 mM KPi buffer, pH 6.0, or at 30°C in the following buffers (100 mM) at various pHs: glycine-NaCl-NaOH, pH 3.0-4.0; NaAc, pH 3.5-6.0; KPi, pH 6.0-8.0; and Tris-HCl, pH 7.5-9.0. The effect of temperature and pH on the enzyme activity was determined by measuring enzyme activities in the reaction mixture (125 μL) containing 5 mM pNPG and 5 ng of the enzyme for 30 min at various temperatures between 4-65°C in 50 mM NaAc buffer, pH 4.8, or in several buffers (50 mM) at 40°C.

5.2.8. Effect of various compounds on the enzyme activity

Various chemicals and metal ions (1 mM) were added to the reaction mixture (125 μL) containing 50 mM NaAc buffer, pH 4.8, 5 mM *p*NPG, and 5 ng of the enzyme. The *p*NPG hydrolyzing activity was measured. The chemicals used were sulfhydryl reagents such as *p*-chloromercuribenzoic acid, 5,5'-dithiobis (2-nitrobenzonic acid), and iodoacetic acid; metal-chelating reagents such as EDTA, EGTA, o-phenanthroline, α,α'-bipyridyl, 8-hydroxyquinoline, and 1,2-dihydroxy-3,5-benzenedisulfonic acid; serine reagents, such as phenylmethanesulfonyl fluoride and *N*-ethylmaleimide; carbonyl reagents such as NaF, NaN3, KCN, NH_2OH, diphenylhydrazine, and hydrazine; reducing reagents such as 2-mercaptoethanol,

dithiothreitol, thioglycerol, thioglycolic acid, DL-penicillamine, L-cysteine, cysteamine, $Na_2S_2O_3$, $Na_2S_2O_4$, and $Na_2S_2O_5$; and coenzymes and vitamins such as NAD(P)(H), ATP, CTP, GTP, TTP, ADP, AMP, rivoflavin, FMN, FAD, pyridoxal 5'-phospahte, acetyl-CoA, Ca-panthothenate, phosphoenolpyruvic acid, glucose-6-phosphate, biotin, thiamine, glutathione (reduced and oxidized form), ascorbic acid, vitamin K_3, and vitamin B_{12}. The metal ions used were Li^+, B^{3+}, Na^+, Mg^{2+}, Al^{3+}, K^+, Ca^{2+}, V^{3+}, Cr^{3+}, Mn^{2+}, Fe^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, As^{3+}, Rb^+, Nb^{5+}, Mo^{6+}, Ag^+, Cd^{2+}, Sn^{2+}, Cs^+, Hg^{2+}, Tl^{2+}, Ba^{2+}, and Pb^{2+}.

5.2.9. Cloning of *bgl*1 and *bgl2* genes

Total RNA of *M. circinelloides* NBRC 4572 was isolated from the mycelia of the strain statically grown on rice straw for 3 days by RNeasy PlantMini kit (Qiagen, USA), followed by DNase I treatment and repurification by phenol-chloroform treatment and ethanol precipitation. Complementary DNA (cDNA) was synthesized from the purified RNA, using a SuperScript III first-strand synthesis SuperMix for RT-PCR kit (Invitrogen, USA), and subsequently used as template for PCR. The primers were designed based on the draft genome sequences of the *M. circinelloides* (unpublished data) and the enzymes' N-terminal sequences of which BGL1-F2, BGL1-R2, BGL2-F2, and BGL2-R2 were nested primers. For isolating bgl1 gene, the first PCR was performed as follows: 2 min at 94°C, followed by 35 cycles of amplification (30 s at 96°C, 1 min at 45°C, and 2 min at 72°C) in a 20-μL reaction mixture containing 2 ng of cDNA, 0.5 μM each of BGL1-F1 (5'-TGCTATCTCTATGCTAGTAGCTGCC-3') and BGL1-R1 (5'-AGCCTAGTAGTTTATTAAG-3'), 0.2 mM dNTPs, 1× reaction buffer, and 0.5 U of Blend Taq DNA polymerase (Toyobo, Japan). The second PCR was performed using 1 μL of diluted (1:50) first PCR reaction mixture as template under the same conditions as the first PCR with nested primer sets BGL1-F2 (5'-TGCTAGTAGCTGCCAATGCTGCTA-3') and BGL1-R2 (5'-GAAAATATCTAGAGGGTAAAG-3') changing the annealing temperature to 60°C. For isolating *bgl2* gene, the first PCR reaction was performed as for the isolation of *bgl1* gene changing the annealing temperature to 55°C with primer sets BGL2-F1 (5'-GCTATCTCTGTACTAGTTGCTGCAA-3') and BGL2-R1 (5'-GTTTAAGAGGTATGGAT TGT-3'). The second PCR was also performed as for *bgl1* with nested primer sets BGL2-F2

(5’-ACTAGTTGCTGCAAATGCCGCTACT-3’) and BGL2-R2 (5’-GAGGTATGGATTGTGC AGTG-3’). The two final PCRs yielded products around 2.2-kbp that were cloned into a pT7Blue T-vector (Novagen, USA) and sequenced, resulting in the isolation of coding sequences of *bgl1* and *bgl2* genes.

5.2.10. Immunoblot analysis

Two peptides, CKNFPETTLSSFTREDAK and CDKGFPKT SINAFHREKA, derived from BGLs 1 and 2, respectively, were chemically synthesized with an automatic batch synthesizer (Apex 396-DC, Advanced Chem. Tech.,USA) and crosslinked to BSA. The peptide-BSA conjugates were injected into a rabbit subcutaneously with complete Freund’s adjuvant. After 2 weeks, five booster injections with incomplete adjuvant were given at 7-day intervals. One week after the booster injections, blood was drawn and the antibodies were prepared. After SDS-PAGE, proteins were transferred electrophoretically onto a PVDF membrane. The membrane was thoroughly dried for blocking and then incubated in Tris-buffered saline (pH 7.5) plus 0.05 % (v/v) Tween 20 with 2,000-fold diluted antibodies overnight. Alkaline phosphatase-conjugated goat antibodies against rabbit IgG (Cell Signaling Technology, USA) were diluted 4,000 times for use as secondary antibodies. Immunodetection was performed using the one-step NBT/BCIP kit (Thermo Scientific, USA).

5.2.11. Binding of BGLs 1 and 2 onto rice straw

The mill-blended rice straw was pretreated with 0.5% H_2SO_4 (acid) or 1 M NaOH (alkaline) at 121°C for 0.5 or 1 h, respectively. The treated straws were washed with water until the pH of the filtrate reached 7 and were dried at 60°C overnight to constant weight. Forty mL each of BGL1 and BGL2 (40 mg/mL) were added to 10 mg of pretreated or nontreated rice straw that had been wetted with water and incubated overnight at 4°C. After centrifugation (14,000×g, 10 min), the obtained supernatant was separated and used as “unbound” fraction followed by addition of 40 μL of SDS-PAGE sample buffer and boiling. The precipitated rice straw was washed with water (30 μL) for three times, after which 40 μL of SDS-PAGE sample buffer and water was added and following the boiling afforded “bound” fraction. Each fraction

was electrophoresed by SDS-PAGE, blotted onto a membrane, and immunostained with the specific antibodies against BGLs 1 and 2 as described above.

5.2.12. Accession numbers

The cDNA sequences of *bgl1* and *bgl2* were deposited into GenBank with the accession numbers of AB703466 and AB703467, respectively.

5.3. Results

5.3.1. Conditions for efficient production of BGL in *M. circinelloides* NBRC 4572

The fungal strain was grown on rice straw and glucose as a control under various aeration conditions, and the extracellular *p*NPG-hydrolyzing (BGL) activities were measured. As shown in Table 1, the enzymes were efficiently expressed by culturing the strain on rice straw as a sole carbon source and their activity was gradually enhanced by lowering the medium volume added to the straw. The highest activity was seen when the strain was statically grown on rice straw using fivefold total medium volume (v/w), referred to as "solid-state cultivation conditions". When the strain was inoculated onto a piece of rice straw that was moistened with the basal medium and was cultivated statically on a solid agar plate containing 5 mM *p*NPG in phosphate buffered saline for 2 days at 30°C, the remarkable yellow color of free *p*NP developed around the rice straw, indicating that BGL was secreted in a soluble form from the cells of the strain.

5.3.2. Purification of BGLs

We succeeded in purifying two extracellular BGLs produced by the fungal strain grown in the solid-state culture on rice straw. The extracellular crude enzyme from 330 g (d.w.) of rice straw was subjected to purification by DEAE and Butyl-Toyopearl column chromatographies. Subsequent Resource Q chromatography gave two distinct protein fractions with BGL activity. The major and minor BGLs are hereafter referred to as BGLs 1 and 2, respectively, and have been purified 111- and 71.5-folds with 21.8 % and 2.30 % yields, respectively. The enzymes were judged homogeneous by SDS-PAGE and gel-filtration chromatography on Superdex 200

Table 1 Extracellular BGL activity in *M. circinelloides* NBRC 4572 grown under various cultivation conditions

Aerobiosis	C-Source	Added medium volume (mL)[a]	BGL activity (U/g C-Source)[b]	Fold[c]
Aerobic	Glucose	25	0.009 ± 0.0002	1
Static	Glucose	25	0.02 ± 0.001	2
Aerobic	Straw	25	0.10 ± 0.01	11
Static	Straw	25	0.14 ± 0.01	16
	Straw	10	1.1 ± 0.2	122
	Straw	5	2.0 ± 0.6	222
	Straw	2.5	2.6 ± 0.9	293
	Straw	1.25	1.3 ± 0.6	144

[a] The medium was added to 0.5 g of carbon sources.

[b] Values are means ± SE of triplicated experiments.

[c] Calculated from the mean values.

column, as each of these procedures yielded a single band or a single peak. The *Ms* of the denatured BGLs 1 and 2 were estimated to be around 78 kDa by SDS-PAGE (Fig. 1a), while those of the native BGLs 1 and 2 were calculated to be 91 and 83 kDa, respectively, according to gel filtration chromatography, indicating that the native enzymes are monomer. By staining the SDS-PAGE gel with the periodate-fuchsin method, both the purified BGLs 1 and 2 were shown to be glycoproteins (data not shown). To examine the type of linkage of sugar chain on the BGL molecules, both enzymes were treated with PNGase F and/or *O*-glycosidase/neuraminidase, which cleave N-linked and/or *O*-linked sugar chains, respectively, under denatured conditions. Both *N*- and *N*/*O*-deglycosylation treatments decreased the *Ms* of BGLs 1 and 2 from 78 kDa to about 70 and 68 kDa, respectively, whereas *O*-deglycosylation treatment only slightly decreased their *Ms* (Fig. 1b). These results suggest that large portions of the sugar chains present in the BGLs are in the form of N-linked residues whose *Ms* is approximately 10 kDa. Enzymatically deglycosylated BGLs 1 and 2 were blotted onto a PVDF membrane and subjected to automated Edman degradation, which clarified that their *N*-terminal amino acid sequences corresponded to KVNVL and KVKVL, respectively. This result suggests that both BGLs have different primary structures and are possibly encoded by different structural genes. The deglycosylation of the BGLs could also be achieved under nondenaturing conditions, under which though they completely lost their activities due to aggregation.

5.3.3. Effect of temperature and pH on the enzyme activity and stability

The stability of BGLs 1 and 2 at various temperatures and pH values was determined by incubating the enzymes for 30 min at different temperatures (4-65°C) in 50 mMKPi buffer, pH 6.0, or at different pH values (3.0-9.0) at 30°C, respectively. As shown in Fig. 2a, b, BGLs 1 and 2 were found to be stable for 30 min at 45 and 55°C, respectively, and at pH values in the ranges of 4.5-6.5 and 4.0-7.0, respectively, when maintained at 30°C. The BGL activities were measured using *p*NPG as substrate at various temperatures (4-65°C) in 50 mM NaAc buffer, pH 4.8, or at various pH values (3.0-9.0) at 30°C, respectively (Fig. 2c, d). The apparent maximum activity of BGLs 1 and 2 at 50 mMKPi buffer, pH 6.0, occurred at 50 and 55°C, respectively, and the apparent optimum pHs were 3.5-5.0 and 3.5-5.5, respectively.

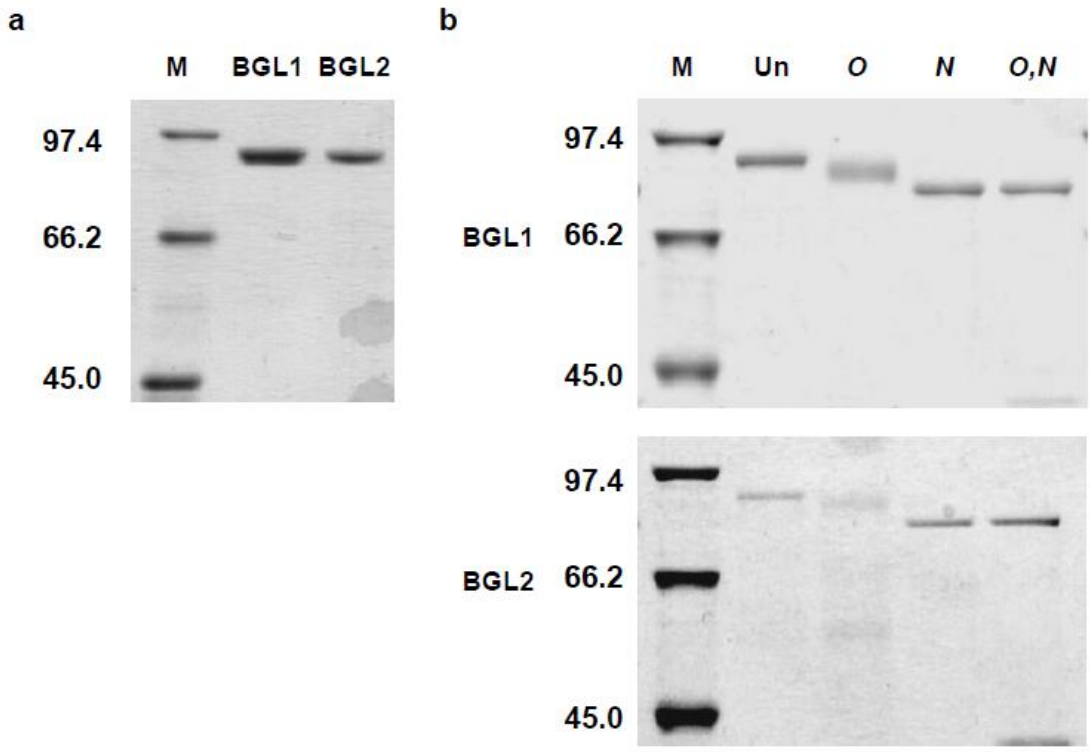

Fig. 1 SDS-PAGE of the purified BGLs 1 and 2. (a) The purified and (b) non- (Un), *O*-Glycosidase/Neuraminidase- (*O*), PNGase F- (*N*), and *O*-Glycosidase/Neuraminidase + PNGase F (*O*,*N*)-treated BGLs 1 and 2 were subjected to SDS-PAGE.

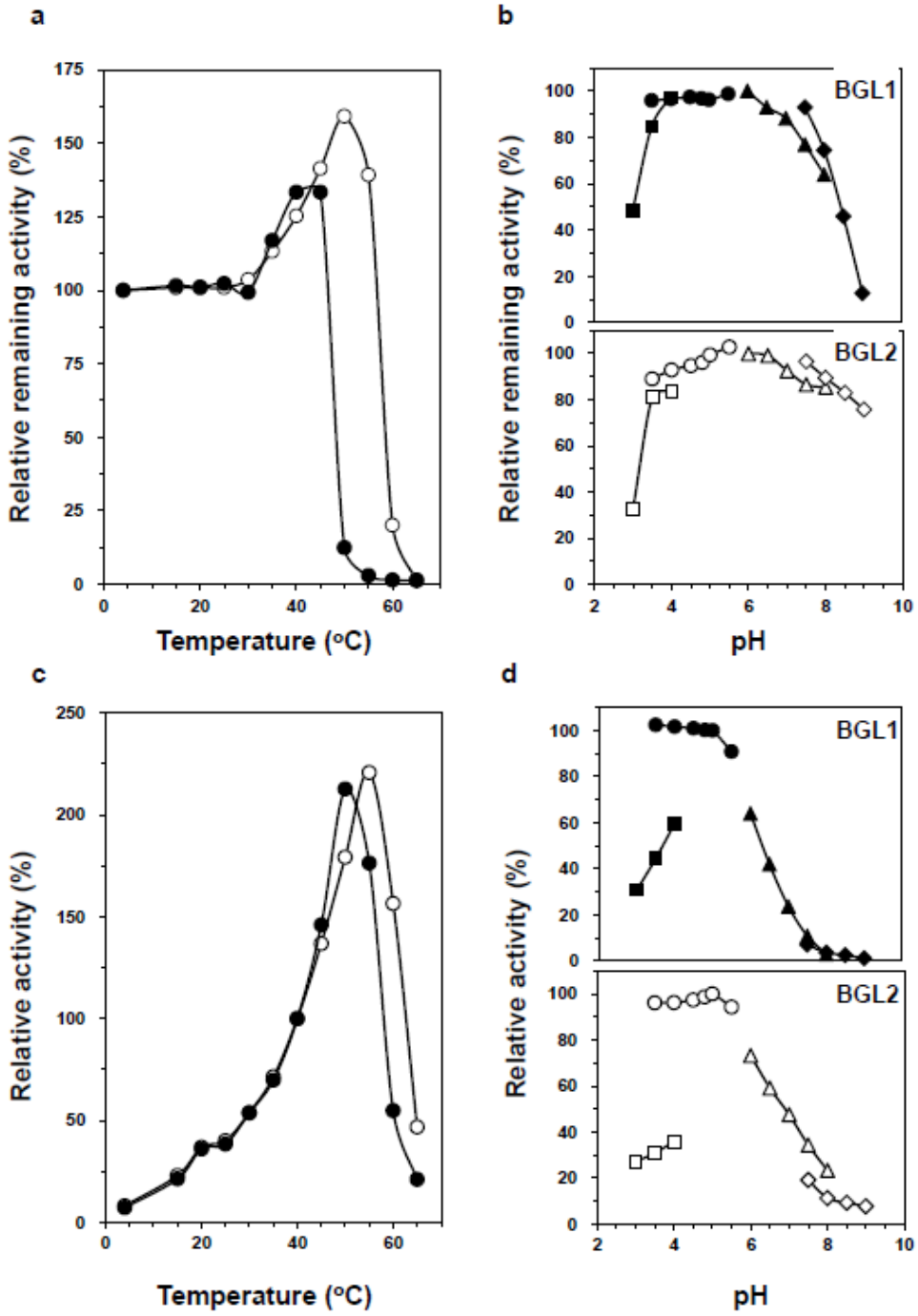

Fig. 2 Effect of (a) temperature and (b) pH on the stability and of (c) temperature and (d) pH on the activity of BGL1 (filled symbol) and 2 (open symbol)

5.3.4. Effect of various compounds on the enzyme activity

Various chemicals and metal ions were added to the reaction mixture and the *p*NPG-hydrolyzing activity was measured. Sulfhydryl reagents, metal-chelating reagents, serine reagents, carbonyl reagents, reducing reagents, vitamins, or coenzymes had no inhibitory or activation effects on the enzymes at a concentration of 1 mM. Heavy metal ions, Ag^{+} inhibited BGLs 1 and 2 activities by 68% and 74%, respectively, and Hg^{2+} did by 29% and 64%, respectively. Other metal ions did not inhibit the enzyme activities at 1 mM. Inhibition of BGL activities by glucose and gluconolactone, which are generally known to be potent competitive inhibitors of many BGLs, was also examined using *p*NPG as substrate. As shown in Fig. 3, BGL activity was gradually decreased by increasing glucose concentrations in the reaction mixture especially for BGL2; BGLs 1 and 2 activities were decreased by 43% and 82%, respectively, in the presence of 100 mM glucose. Glucose acted as noncompetitive and mixed-type inhibitor for BGLs 1 and 2 with K_i of 5.39 and 1.41 mM (obtained at the intersection of the lines on the Dixon plot analysis), respectively. On the other hand, gluconolactone acted as strong noncompetitive and competitive inhibitor for BGLs 1 and 2, with K_i of 42.9 and 14.9 μM, respectively. BGL1 was not inhibited by cellobiose, a natural saccharide type substrate, even at concentrations over 250 mM, while BGL 2 activity decreased by 50% at 10 mM. Moreover, BGLs 1 and 2 were markedly inhibited by the aryl-type substrate *p*NPG at concentrations over10 and 5 mM, respectively. Glucosamine and other sugars such as xylose, lactose, and sucrose did not cause any inhibition up to 100 mM.

5.3.5. Kinetic properties

The kinetic parameters of the BGLs were calculated by nonlinear fitting of the data to the Michaelis-Menten equation using SigmaPlot 2001 (Systat Software). K_m values of BGLs 1 and 2 for *p*NPG were 0.288±0.03 and 1.41±0.196 mM, respectively, and those for cellobiose were 6.49±0.359 and 4.07±0.674 mM, respectively. V_{max} values of BGLs 1 and 2 for *p*NPG were 309±9.80 and 275±19.9 U/mg, respectively, and those for cellobiose were 213±6.08 and 240±29.0 U/mg, respectively. The efficiency of substrate utilization was estimated on the basis of V_{max}/K_m ratio (U/mg/mM), indicating that BGL1 preferentially (30-fold) acted on aryl-type

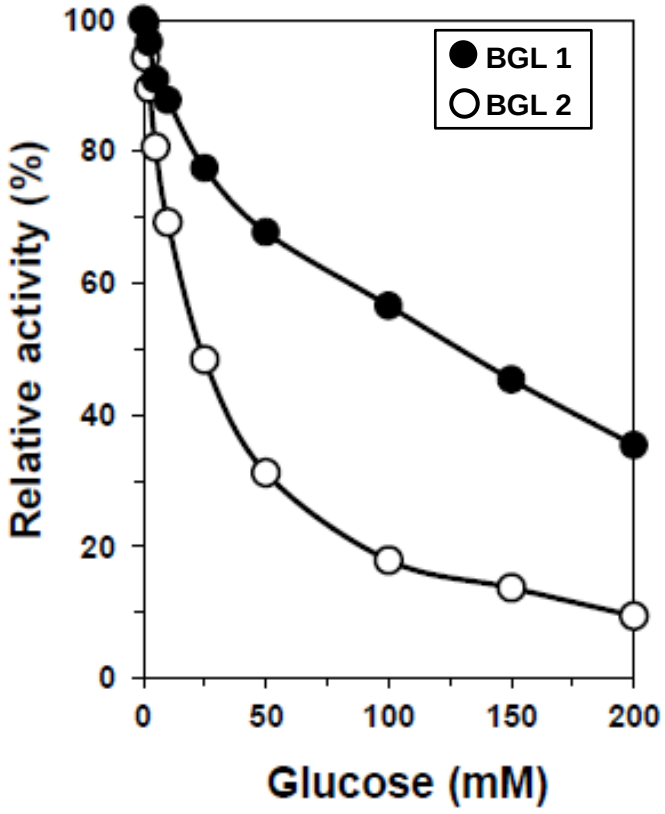

Fig. 3 Effect of glucose on BGL1 (filled circle) and 2 activity in the presence of various concentrations

substrates (1,070) than disaccharide type substrates (32.8), although V_{max} values were not very different, whereas BGL2 acted on both types of substrates with closer efficiency (195 and 58.9, respectively; threefold).

5.3.6. Cloning of the genes encoding BGLs 1 and 2

A genome-wide search of the total genomic sequence of *M. circinelloides* NBRC 4572 (unpublished results) for putative BGL genes was performed with "in silico Molecular Cloning" software (in silico Biology, Japan). This analysis identified two open-reading frames (ORFs), named bgl1 (gene_id: g1648) and bgl2 (gene_id: g4371) which encode polypeptide sequences that share a certain identity with those determined by *N*-terminal amino acid sequencing of the purified BGL1 (KVNVL) and BGL2 (KVKVL), respectively. To clone their complete genes, PCR was performed on the cDNA of the mycelia of the strain statically grown on rice straw that displayed high extracellular BGL activity, using primers designed based on their gene sequences. Fragments of approximately 2.2 kbp were obtained as *bgl1* and *bgl2*, which were sequenced and showed a high level of similarity to other fungal extracellular BGL sequences, especially with the 3-glycoside hydrolases (GH3) family. The genes *bgl1* and *bgl2* had 2,148 bp and 2,145 bp, respectively, encoding 716- and 715-residue proteins, with estimated Ms of 75,920 and 76,031 Da, respectively, which showed good agreement with those determined by SDS-PAGE analysis after enzymatic deglycosylation. The identity between the two translated sequences was 81%. By comparing the cDNA and the genomic sequences, the coding sequences of *bgl1* and *bgl2* genes were found to be interrupted by 5 (57, 65, 56, 57, and 57 bp in length) and 9 (55, 58, 63, 59, 53, 51, 59, 55, and 58 bp in length) introns, respectively. The amino acid sequences of BGLs 1 and 2 contained 6 and 5 potential *N*-linked glycosylation sites (N-X-S/T), respectively. Conserved domain search clearly showed that both BGLs 1 and 2 had conserved domains among GH3 BGL, Glyco_hydro_3 (GH3 *N*-terminal domain; pfam00933), and Glyco_hydro_3_C (GH3 C-terminal domain; pfam01915) that adopt $(\alpha/\beta)_8$-barrel and $(\alpha/\beta)_6$-sandwich folds, respectively [17]. Furthermore, BGLs 1 and 2 contain an additional Fn3-like domain (Fibronectin type III-like domain; pfam14310) at their C-terminus (634-716 for BGL1 and 633-715 for BGL2) similar to the BGL (β-D-glucan glucohydrolase) from

Hordeum vulgare [16]. The highest identity (56%) was seen with BGL from the closely related zygomycetes fungus, *R. miehei* NRRL 5282. The typical residues that act as catalytic nucleophile and proton donor, contained into all GH3 BGLs [17, 7], are conserved in BGLs 1 and 2: the nucleophiles situated in a consensus motif of S/TDW are D246 and D245, respectively, and acid/base that donate H^+ in the KHF/Y/L motif are H169 and H168, respectively. PLFPFGHGLS sequence, which is located at the C-terminal Fn3-like domain, also existed in BGLs 1 and 2 as *R. miehei* BGL. The residues that are capable of forming hydrogen bonds with the sugar hydroxyl groups as glucose-recognizing sites in GH3 BGLs [17, 7] are also well conserved in BGL1 (D72, R129, and K168) and 2 (D71, R128, and K167). Sequence analysis by SignalP, TargetP, TMHMM, SOSUI, and Wolf PSORT programs could not predict any typical signal peptide sequences at the *N*-terminus of the BGLs although both enzymes were secreted to outside of the cells. Takó et al. reported that BGL from *R. miehei* NRRL 5282, the most similar to our BGLs, has a typical 22-amino acid signal peptide (MFAKTALALLT AWSAMQGVAGG) at its N terminus, while the secreted BGL has N-terminal sequence starting with INFRSWDEAHEL, which is slightly similar to the *N*-terminal sequences of the purified BGLs 1 and 2 (KVN/KVLSWDDAYKK) [15]. We also examined the expression of the genes *bgl1* and *bgl2* in *Escherichia coli* under the control of general promoters (e.g., *T7*, *lac*, and *csp*), but the expression was unsuccessful under any cultivation conditions (media, temperature, aeration, or hosts) presumably due to the high hydrophobic behavior of BGLs without the sugar chains.

5.3.7. Expression profiles of BGLs 1 and 2 in *M. circinelloides*

We evaluated the expression profiles of BGLs 1 and 2 in the *Mucor* strain when it was statically grown on rice straw by performing immunodetection analysis. Peptide antibodies specific against BGLs 1 and 2 were prepared using the peptides CKNFPETTLSSFTREDAK and CDKGFPKTSINAFHRE KA, which correspond to residues 301-317 and 300-316 of BGLs 1 and 2, respectively, as antigens. Each antibody clearly reacted with the corresponding BGL that was electrophoretically blotted onto PVDF membrane, where a single band with an estimated *Ms* was observed, leading to the evaluation of the time course of their expression.

BGL1 was abundantly expressed compared to BGL2 when the strain was grown on rice straw without glucose, and the expression profiles did not change with the cultivation length. BGL1 is more likely to play an important role than BGL2 in saccharification of rice straw by the *Mucor* strain.

5.3.8. Binding of BGLs 1 and 2 onto rice straw

We evaluated the binding ability of BGLs 1 and 2 onto acid or alkaline-treated and nontreated rice straw to determine whether the BGLs could absorb onto insoluble cellulosic biomasses. BGL1 did not bind strongly to any type of rice straw, whereas BGL2 bound more tightly to non- and alkaline-treated rice straw than acid-treated one. This suggests that BGL2 has considerably higher affinity than BGL1 for the insoluble components of rice straw, especially hemicellulose that is degraded by acid treatment [18].

5.4. Discussion

Nearly 300-fold enhancement of the extracellular BGL enzymatic activity was achieved by optimizing the cultivation conditions compared with the previously reported cultivation conditions [1]. It is generally known that solid-state cultures of filamentous fungi result in significant higher enzyme production than liquid cultures because the former condition is closer to the natural growth conditions [19]. *M. circinelloides* NBRC 4572 had been originally isolated from yeast cake of Chinese local rice wine, kaoliangchiu, produced by solid-state fermentation of kaoliang (Sorghum plants). It is reasonable to consider that the strain adapted to the fermentation conditions to secrete cellulosic biomass hydrolases. Recently, according to the results of this study, Takó et al. reported that various zygomycetes fungal strains exhibited high extracellular BGL activity when they were grown under solid-state cultivation conditions [20]. The addition of glucose as the sole carbon source led to the detection of only low activity, probably because of the inhibitory effect of glucose on the enzyme synthesis [21].

BGLs 1 and 2 are monomeric enzymes with *Ms* of about 78 kDa harboring *N*-linked sugar chains having *Ms* of approximately 10 kDa: these are common characteristics of fungal BGLs [8]. Petruccioli et al. purified and characterized an extracellular BGL from *M. circinelloides* LU

M40 that they identified as linamarase [11]. Since the subunit and native *Ms* of linamarase are 94.8 and 210 kDa, respectively, BGLs 1 and 2 of this study are in fact different from that enzyme. The presence of the sugar moiety on BGLs 1 and 2 might be important for maintaining their structure, because enzymatic deglycosylation of the purified BGLs completely impairs their solubility and activity, and the BGLs heterologously expressed in E. coli quite easily aggregated, like other extracellular fungal BGLs did [8].

BGLs 1 and 2 were stable at optimum temperatures and pH similar to those needed for mesophilic fungal BGLs including zygomycetes BGLs, although *R. miehei* BGL required higher optimum temperature (~70°C; [14]) [8, 20]. Heavy metal ions and sulfhydryl reagents generally inhibit fungal BGLs [10]. However, the results shown in a study indicate that BGLs 1 and 2 are less susceptible to these reagents than the general fungal BGLs are, including linamarase from *M. circinelloides* LU M40 that was inhibited by 1 mM Co^{2+}, Cu^{2+}, andMn^{2+} [11]. High concentrations (>50 %) of ethanol, which is an organic solvent, usually denature BGLs, although ethanol enhances BGL activity by acting as acceptor in transglucosylation reactions [8]. Both BGLs 1 and 2 activities were enhanced appreciably in the presence of ethanol and the enhancement of BGL activities due to their transglucosylation ability. Since the maximal ethanol concentration produced by *M. circinelloides* by SSF is below 10% [2], the value is far below the concentration that inhibits the enzyme (i.e., >40 %), and therefore, the inhibition of BGLs by ethanol may not be taken into account during the SSF process. It is quite interesting that BGLs 1 and 2 were not inhibited by glucose in a competitive manner, which is rarely seen in fungal BGLs except for those from *Scytalidium lignicola* [22] and *Trichoderma viride* [23]. Since inhibition by glucose is a quite important factor for BGL because BGL is a key tool for saccharification of cellulose [5, 6], BGLs 1 and 2 are likely to be used as a model for studying inhibition mechanism of BGL by glucose. The identification of specific catalytic residues and/or structures responsible for this property would facilitate targeted approaches for modifying the glucose tolerance of existing fungal BGLs.

Both enzymes were active against a wide range of aryl-β-glucosides and cello-oligosaccharides with (1→2), (1→3), (1→4), and (1→6)-β-linkages, suggesting that the enzymes are not exclusively specific for the (1→4)-β-linkage. Some BGLs isolated from

Aspergillus fumigatus [24] and *Trichoderma koningii* [25] are much more active toward laminaribiose and sophorose than cellobiose, and therefore, the enzymes may not be involved in cellulose degradation. Since our BGLs acted on these disaccharides at similar rates, they are likely considered responsible for cellulose degradation unlike *A. fumigatus* and *T. koningii* BGLs. Fungal BLGs exhibit a great diversity in substrate specificity but can be broadly classified into three groups based on their relative activities toward synthetic *p*NPG and cellobiose: (1) aryl-BGLs, (2) cellobiases, and (3) broad substrate specificity BGLs. Although BGLs 1 and 2 displayed higher activity with *p*NPG than cellobiose when the substrate concentration was 5 mM, they acted on both *p*NPG and cellobiose. In particular, BGL1 more efficiently degraded cellobiose as substrate at 50 mM and hydrolyzed cello-oligosaccharides with various polymerization degrees at catalytic rates similar to cellobiose. Based on the results shown in this study, we can categorize BGLs 1 and 2 into the broad substrate specificity BGLs class. It has been reported that a number of fungal strains produce extracellular BGL isoforms belonging to the broad substrate specificity BGLs [8] except for *Aspergillus aculeatus* F-50 that produces one aryl-BGL and two cellobiases, which act on cello-oligosaccharides and are considered to play an important role in cellulolysis exerted by the strain [26]. It is of interest to clarify the physiological functions of the extracellular broad substrate specificity BGLs found in this study with a focus on the growth and utilization of rice straw by *M. circinelloides*. K_m values of BGLs 1 and 2 for *p*NPG and cellobiose are within the range of the reported values of other fungal BGLs, corresponding to 0.055-34 and 0.031-340 mM, respectively [8].

BGLs 1 and 2 had 81% identity with each other and exhibited less than 60% identity with the known GH3 family BGLs. A number of fungal strains are known to produce BGL isozymes: almost all of them are encoded by different structural genes [8], but some exceptions exist for which the same gene encodes enzymes that are subsequently posttranslation modified in different way, such as in *Schizophyllum commune*, *Aspergillus kawachii*, andthe zygomycetes fungus *M. miehei* [8]. Both the BGLs found in this study are encoded by different genes as is the case of general fungal BGLs. The fact that BGLs 1 and 2 were secreted by *M. circinelloides* NBRC 4572 statically grown on rice straw but had neither remarkable signal sequences nor homologies with the signal peptide of *R. miehei* BGL suggests that *M. circinelloides* has a

unique system for secreting BGLs 1 and 2. The BGLs identified in this study are phylogenetically similar to fungal GH3 BGLs but different from those from bacteria and plants. Although remarkable sequence identity was seen among the fungal GH3, a few gene products from *A. aculeatus* (P48825), *A. kawachii* (GAA83366.1), and *R. miehei* (CAP58431.2) were functionally identified as BGL, while others were defined as BGLs only by virtue of their homologies. Furthermore, only a few structures of GH3 BGLs were solved, such as BGLs from the bacterium *Pseudoalteromonas* sp. (3UT0, 3USZ, 3F95, and 3RRX) and from the fungi *Kluyveromyces marixianus* (PDB 3ABZ and 3AC0), *A. aculeatus* (PDB 4IIB–G), and *Hypocrea jecorina* (formally *Trichoderma reesei*; PDB 3ZZ1 and 3ZYZ), and the mechanically similar β-D-glucan glucohydrolase from the plant *H. vulgare* (PDB 1EX1) and exo-1,3/1,4-β-glucanase from the bacterium *Thermotoga neapolitana* (PDB 2X40-42). β-*N*-Acetylhexosaminidases, which utilize different types of substrates, from the bacteria *Vibrio cholera* (PDB 1TR9, 1Y65, 2OXN, 3GSM, and 3GS6) and *Bacillus subtilis* (PDB 3BMX, 3LK6, and 3NVD) are also included in the GH3 proteins whose structure is solved. Since BGLs 1 and 2 are phylogenetically distinct from these GH3 proteins, the three-dimensional structure elucidation of BGLs 1 and 2 will yield new insights into structure-based biochemical and functional characterization of GH3 proteins.

Table 2 summarizes the characters of BGLs 1 and 2. We evaluated the suitability of the enzymes for bioethanol production by SSF of rice straw by using *M. circinelloides* strain. Although BGL2 had relatively high temperature stability and catalytic properties, BGL1 seemed to be much more suitable for the process because of its wider substrate specificity for cellooligosaccharides, less inhibitory effects by the reaction substrate and product, and enhancement of the activity by ethanol that is present in the SSF system. Low transglucosylation activity against glucose is also an important factor as well as high cellooligosaccharide-hydrolyzing activity for use in SSF, because accumulation of cello-oligosaccharides in the reaction system might disturb the complete saccharification of celluloses. The low binding affinity of BGL1 onto insoluble cellulosic biomass materials is another factor that should be considered [3]. Furthermore, BGL1 is abundantly expressed in the wild-type strain. Based on these results, BGL1 is a suitable catalyst for bioethanol production

Table 2 Summary of the characters of *M. circinelloides* NBRC 4572 BGLs 1 and 2

Properties	BGL1	BGL2
Temperature stability (°C)	<45	<55
V_{max}/K_m for cellobiose	32.8	58.9
Substrate specificity	Wide	Moderate
Inhibition by cellobiose (mM)	>250	>10
Glucose inhibition type	Non-competitive	Mixed
Accumulation of cello-oligosaccharides	Low	Moderate
Activation by 10% ethanol (%)	132	116
Productivity in the wild type strain	Abundant	Minor
Binding onto cellulosic materials	Low	Moderate (hemicellulose)

by SSF of rice straw by *M. circinelloides*. Differential expressions of BGL isoforms are reported in response to the media components and cultivation conditions in some fungal strains [27, 28]. For example, we have found that the addition of rice powder markedly enhanced BGL1 formation during solid-state culture of the strain on rice straw (unpublished results). The final goal of our research was to establish highly efficient, environmentally friendly ethanol production method by SSF of rice straw using the *Mucor* strain without relying on genetic engineering approaches. The basic findings obtained in this study suggest that BGL1 is potentially an efficient catalyst for the saccharification of soft biomasses. We believe that the enhancement of the production of BGL1 might dramatically increase the saccharification ability of the strain. The optimization of the cultivation conditions for hyperexpression of BGL1 in the fungal strain as well as the manipulation of high-BGL1-producing mutants by heavy-ion beam irradiation, which is not a genetic engineering approach, is in progress.

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Chapter 6

Direct ethanol production from *N*-acetylglucosamine and chitin substrates by *Mucor* species

6.1. Introduction

The search for feasible petroleum substitutes among renewable resources has become a global priority as atmospheric carbon dioxide levels continue to rise. Chitin, which is a polymer of partially deacetylated β-(1-4) linked *N*-acetyl-D-glucosamine (GlcNAc) residues, is one of the most abundant renewable resources in nature, after cellulose [1]. It is a principal structural component of most fungi, yeasts and algae cell walls, insect exoskeletons, shells of crustaceans and the microfilarial sheath of nematodes [2]. Recent investigations confirmed the suitability of chitin and its derivatives in a broad range of applications for use in medicines, cosmetics, agriculture, food preservation, textile industry, sorbents and enzyme supports based on their polyelectrolyte properties, the presence of reactive functional groups, gel-forming ability, high adsorption capacity, biodegradability, bacteriostatic, fungistatic and antitumor influence [3]. An estimated annual production of chitin is in the order of 10^{10} to 10^{11} tons on earth [4]. At present, however, only small quantity of shell wastes are being utilized for animal feed or chitin isolation for the purposes mentioned above, and the processing of shellfish leads to environmental pollution [3]. Thus, if chitin and its derivatives can be converted to ethanol efficiently using microorganisms, it will become one of the strong favorites for petroleum substitutes. The most commonly used microorganism for ethanol production is *Saccharomyces cerevisiae*. It can utilize GlcNAc as a carbon source and produce ethanol by introducing four genes required for a GlcNAc catabolic pathway from *Candida albicans*, which can utilize GlcNAc as a sole carbon source [5-7]. However, fermentation of GlcNAc using these strains was slow and the obtained ethanol titers were low (around 3 g/l after 11 days). On the other hand, the conversion of chitinous wastes to hydrogen gas by *Clostridium paraputrificum* M-21 was reported [8]. Hydrogen is considered to be a potential source of alternative energy [9, 10]. However, based on our knowledge, no ethanol production from chitin or GlcNAc by native microorganism has been reported yet. The enzymatic hydrolysis of chitin to GlcNAc is carried out by chitinolytic

enzyme system, which consists mainly of endochitinase (EC 3.2.1.14) and β-*N*-acetylhexosaminidase (GlcNAcase) (EC 3.2.1.52). *Endo*-chitinase cleaves glycosidic linkages randomly along the chain to give chitooligomers, and GlcNAcase cleaves chitooligomers from the non-reducing end to give GlcNAc. These enzymes have been detected in a wide variety of organisms including fungi [11-14]. In fungi, chitinolytic enzymes play roles in autolysis, nutrition, morphogenesis, symbiosis and pathogenesis. Recently, it has been reported that various fungi can utilize GlcNAc as the sole carbon source, and several of them can also grow on chitin as the carbon source [15]. *Mucor* is a dimorphic zygomycota that can switch between yeast and filamentous growth modes depending on environmental stimuli [16], and have been recently presented as an ethanol-producing organism from various carbon sources including lignocellulosic hydrolyzates [17, 18]. In addition, *endo*-chitinase and GlcNAcase have been detected in some Mucor species [19, 20]. These reports pointed out the existence of *Mucor* strains capable of producing ethanol from chitin or GlcNAc.

In this study, we evaluated the ethanol production capacity of *Mucor* and its related genus *Rhizomucor* strains from GlcNAc and colloidal chitin. We successfully demonstrated the ethanol fermentation from GlcNAc and chitin substrates, and also the chitinolytic enzyme production using selected *Mucor* strains. This is the first report on bioethanol production from GlcNAc and chitin substrates by native organisms.

6.2. Materials and methods

6.2.1. Strains and culture media

Mucor and *Rhizomucor* strains, shown in section 1.3.1. (Table 1), obtained from NITE Biological Resource Center (NBRC) were used in this study. Difco potato dextrose agar (PDA) (Becton, Dickinson and Company, Sparks, Maryland) was used for the preculture medium. Ethanol production medium as described in section 1.2.2 was used for fermentations. Aerobic and microaerobic fermentation were performed using a 100-ml flask and a 100-ml bottle with screw cap, respectively.

6.2.2. Substrates

In this study, GlcNAc (Wako Pure Chemicals, Osaka, Japan), chitin powder, and colloidal chitin were used as substrates. Chitin from shrimp shells (≥95% acetylated, Sigma–Aldrich, St. Louis, MO, USA) was ground in a Waring blender for 1 min and sieved through a # 18 mesh. The obtained chitin powder was used as the substrate.

For colloidal chitin preparation, 20 g of chitin powder described above was added to 400 ml of ice-chilled 10 N hydrochloric acid under agitation and homogenized by ultrasonication. Then, the suspended chitin was dissolved by incubation at 37°C for 30 min and agitation at room temperature for 10 min. After centrifugation (3000×*g*) for 10 min at 4°C to remove insoluble matter, the supernatant was added to 4 l of water under agitation and kept in a refrigerator for 18 h. Subsequently, the white material formed was separated by vacuum filtration through No. 5C filter paper (Advantec, Tokyo, Japan). The retained cake was washed with water until the filtrate reached a pH of 5.5. The obtained colloidal chitin was used as a substrate for ethanol production and the endo-chitinase activity determination.

6.2.3. Screening of ethanol producer strains

For ethanol production from GlcNAc and colloidal chitin, 83 Mucor or Rhizomucor strains listed in Table 1 were inoculated on PDA plate medium and cultured aerobically at 28°C for 24-96 h. After cultivation, a portion of these agar media (5 mm×5 mm) embedded with the grown strains were inoculated into 10 ml of ethanol production medium containing 50 g/L of GlcNAc or colloidal chitin and cultured microaerobically at 28°C with shaking (120 rpm). After cultivation for 48 h (GlcNAc) or 96 h (colloidal chitin), the supernatants were collected by filtration using No. 131 filter papers (Advantec) (GlcNAc) or centrifugation (3000×*g*) for 5 min at 4°C (colloidal chitin) and used for the measurement of ethanol.

6.2.4. Ethanol fermentation from GlcNAc

M. circinelloides NBRC 4572, 6746 and *M. ambiguus* NBRC 8092 were inoculated on a PDA plate medium and cultured aerobically at 28°C. After cultivation for 96 h, the medium was

suspended in 100 ml of sterilized normal saline. Then, 0.8 ml of the suspension was inoculated into 20 ml of ethanol production medium containing 50 g/L of GlcNAc and cultured microaerobically at 28°C with shaking (120 rpm). Its supernatant was collected by filtration through No. 131 filter papers (Advantec) and used for measurements of GlcNAc, ethanol and acetate. For dry cell mass determination, the collected cells were washed with distilled water and dried to a constant mass at 90°C.

6.2.5. Ethanol fermentation from chitin substrates

After precultivation of *M. circinelloides* NBRC 4572, 6746 and *M. ambiguus* NBRC 8092 on PDA plate medium for 96 h, the medium was suspended in 100 ml of sterilized normal saline. After that, 0.8 ml of the suspension was inoculated into 20 ml of ethanol production medium containing 50 g/L of colloidal chitin or chitin powder and cultured aerobically or microaerobically at 28°C. The culture's supernatant was collected by centrifugation and used for measurements of GlcNAc, ethanol, and endo-chitinase and GlcNAcase activity. All ethanol fermentations from chitin substrates in this paper were performed simultaneously using the same chitin powder or colloidal chitin.

6.2.6. Extraction of protein from mycelium

Extraction of protein from mycelia of *Mucor* strains NBRC 4572, 6746 and 8092 was carried out by following the procedure reported previously [21] with minor modifications. Briefly, after microaerobic cultivation in ethanol production medium containing 50 g/L of colloidal chitin for 8 days at 28°C, the cells were washed twice with distilled water, defatted with acetone and airdried at room temperature. 10 mg of defatted and air-dried cells were suspended in 120 μl of 100 mM sodium acetate buffer, pH 5.5 with Triton X-100 (0.5%, w/v). The mixtures were stirred for 30 min at 4°C, centrifuged (13,000 × *g*) for 20 min at 4°C and the supernatants were used as cell extract solutions.

6.2.7. Enzyme assay

GlcNAcase activity was assayed with *p*-nitrophenyl-1-*N*-acetylglucosaminide

(pNPGlcNAc) as the substrate. The enzyme solution (10–20 l) was added to 500 l of 2 mM pNPGlcNAc solution dissolved in 100 mM sodium acetate buffer, pH 5.5. After incubation for 18 h at 30°C, 500 µl of 1.0 M sodium carbonate solution was added to terminate the reaction, and the *p*-nitrophenol liberated was determined based on absorbance at 420 nm. For these conditions, the molar extinction coefficient of 13,060 M^{-1} cm^{-1} was used to calculate the concentration of *p*-nitrophenol in the assay mixture. One unit of enzyme activity was defined as the amount of enzymes required to liberate 1 µmol of *p*-nitrophenol per minute.

The activity of *endo*-chitinase was determined by quantitative estimation of the reducing sugars produced with the colloidal chitin as the substrate of enzyme assay. Briefly, the reaction mixture consisted of 20 µl enzyme solution and 180 µl of 0.5% (w/v) colloidal chitin in 100 mM sodium acetate buffer, pH 5.5. After incubation for 18 h in the shaking incubator at 30°C, the reaction was stopped by immersing the mixture in boiling water for 5 min. The amount of reducing sugars released in the supernatant was measured by a method that uses dinitrosalicylic (DNS) acid reagent, and the absorbance was measured at 500 nm. One unit of endochitinase activity was defined as the amount of enzymes required to liberate 1 µmol of the reducing sugar per minute at the same condition using GlcNAc as the standard.

6.2.8. GlcNAc and fermentation product analysis

Ethanol was quantified using the GC-2010 gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and the AOC-20i automatic injector and sampler (Shimadzu). The separation of compounds was carried out with the InertCap WAX capillary column (30 m; 0.25 mm inside diameter; 0.25 µm film thickness; GL Sciences, Tokyo, Japan). The gas chromatograph oven temperature was initially held at 40˚C for 1 min and then raised with a gradient of 20˚C/min to 180°C, where it was maintained for 2 min. Helium was used as the carrier gas with 30 ml/min of inlet pressure. The injector and detector were maintained at 250 and 260°C, respectively and for the measurement, 1.0 µl of diluted culture supernatant was injected in the split-injection mode (1:12 split ratio). 1-Propanol was used as the internal standard. Other secreted metabolites and GlcNAc was analyzed by HPLC system as described in the section 1.2.4.

6.3. Results and discussion

6.3.1. The screening of ethanol production strain from GlcNAc and colloidal chitin

Mucor and *Rhizomucor* strains listed in Table 1 were evaluated to select strains which can produce ethanol from GlcNAc and colloidal chitin, as described in Section 5.2. The result of the evaluation is shown in Fig. 1. Wide variety of *Mucor* strains except *M. flavus*, *mucedo*, *oblongisporus*, *odoratus*, *strictus* and *tuberculisporus*, and *R. pusillus* NBRC 4578 produced ethanol from GlcNAc after 48 h (Fig. 1). Some of these strains also produced slight amount of ethanol from colloidal chitin (<1.2 g/L) after 96 h. Among them, *M. circinelloides* NBRC 4572, 6746 and *M. ambiguus* NBRC 8092, which produced more than 10 g/L and 1 g/L of ethanol from GlcNAc and colloidal chitin, respectively, were selected for further study. Among the 3 strains, *M. circinelloides* NBRC 6746 achieved the highest ethanol production from both GlcNAc (15.0 g/L) and colloidal chitin (1.2 g/L), respectively (Fig. 1).

6.3.2. Ethanol production from GlcNAc

Ethanol productions from GlcNAc using *M. circinelloides* NBRC 4572, 6746 and *M. ambiguus* NBRC 8092 were performed (Fig. 2). *M. circinelloides* NBRC 4572 produced 14.5±0.2 g/L of ethanol from GlcNAc after 72 h, and the maximum ethanol production rate between 12 and 24 h was 0.52±0.07 g/L/h (Fig. 2A). The theoretical maximum ethanol yield from GlcNAc by the GlcNAc catabolic pathway [6] and glycolysis pathway via fructose-6-phosphate, is 2 mol ethanol/mol GlcNAc consumed (0.417 g ethanol/g Glc-NAc consumed). In this fermentation, at 72 h, the maximumethanol yield was 81.3±2.1% (mol ethanol/mol GlcNAc consumed); however, this strain could not consume initial GlcNAc completely after 144 h.

M. circinelloides NBRC 6746 had the highest ethanol titer and ethanol production rate among these *Mucor* strains. This strain consumed 50 g/L of GlcNAc completely and produced 18.6±0.6 g/L of ethanol after 72 h (Fig. 2B). The maximum ethanol production rate reached0.75±0.10 g/L/h between 12 and 24 h, and the maximum ethanol yield was 87.1±3.1% (mol ethanol/mol GlcNAc consumed) at 72 h.

M. ambiguus NBRC 8092 also produced 16.9 ± 0.5 g/L of ethanol from GlcNAc after 96 h,

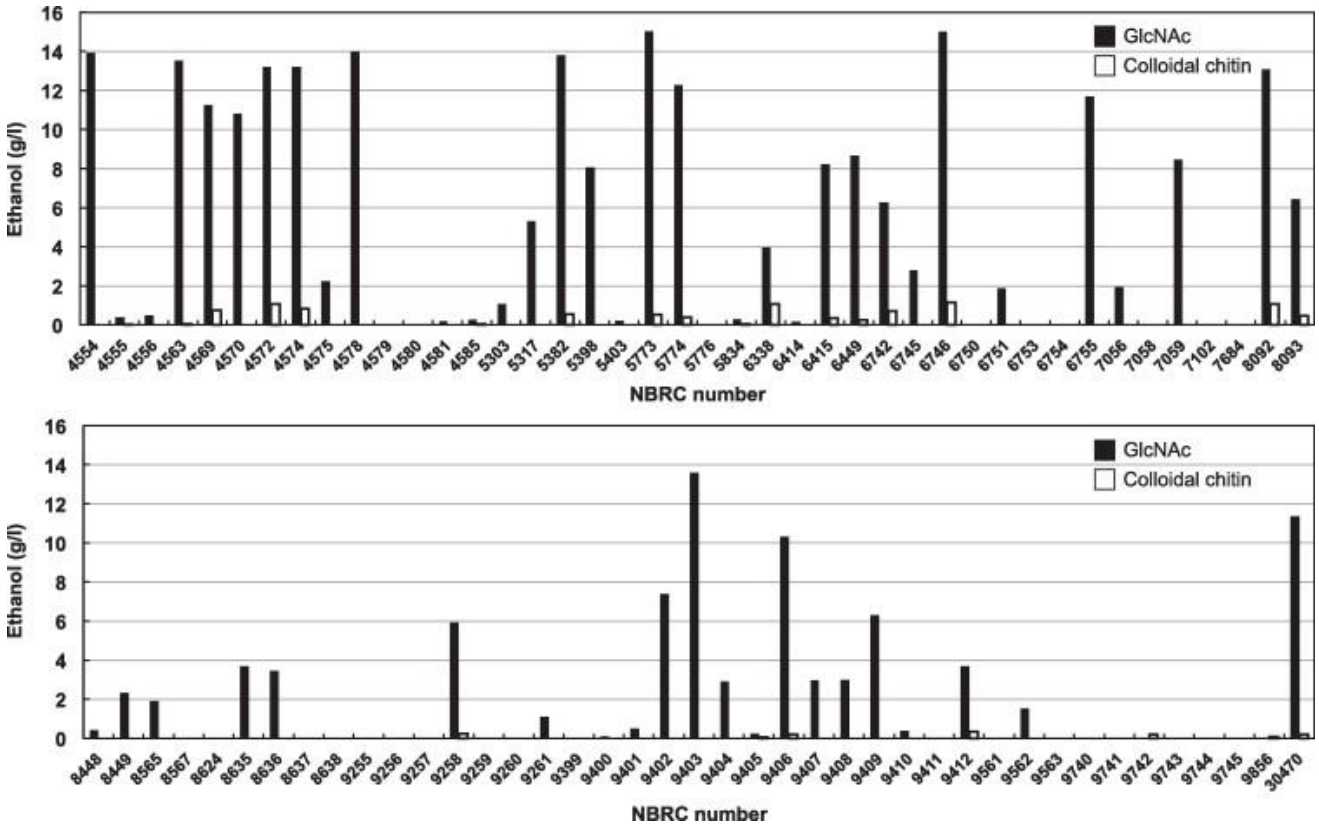

Fig. 1 The screening of ethanol production strain from GlcNAc and colloidal chitin

and the maximum ethanol production rate was 0.50±0.07 g/L/h between 12 and 24 h (Fig. 2C). The maximum ethanol yield was 86.6±3.3% (mol ethanol/mol GlcNAc consumed) at 96 h. To the best of our knowledge, this is the first report to produce ethanol from GlcNAc by native organisms. Although ethanol production from GlcNAc using genetically engineered *S. cerevisiae* strains (3 g/L after 11 days) was reported previously [5], the ethanol productivity of NBRC 6746 (18.6±0.6 g/L after 72 h) was far greater than that of these yeast strains. In addition, these *Mucor* strains converted GlcNAc into ethanol with high yields (>80%), which were about the same as that of metabolically engineered ethanologens *S. cerevisiae* 424A (LNH-ST) and Escherichia coli KO11 from 100 g/L of glucose (86.6±3.3 and 85.2±0.5%, respectively) [22]. These results suggest that these strains have a high uptake and catabolic pathway from GlcNAc to fructose-6-phosphate [5, 6], which can be converted to ethanol.

6.3.3. Ethanol production from chitin substrates

As in the case of lignocellulosic biomass [23], it is forecasted that bioconversion of chitin substrates to ethanol consists of chitin pretreatment, chitinase production, enzymatic saccharification and ethanol fermentation. In this system, if enzyme production, saccharification and fermentation can be integrated in a single step, it would be favorable to low cost ethanol fermentation by decreasing the number of processing steps [23]. Therefore, a microorganism which can not only produce ethanol but also saccharify chitin using its own chitinase is important in the simultaneous saccharification and fermentation (SSF) of chitin. Figs. 3 and 4 show the time course of the cultivation in medium containing 50 g/L of colloidal chitin or chitin powder as the carbon source. In fermentation using fungi with chitin substrates, mycelia cannot be separated completely from the substrates because they penetrate into the colloidal chitin and chitin powder [24]. Therefore, the accurate amount of consumed substrates and dry cell weight were unknown, but distinct mycelia growth was observed in all fermentations.

In microaerobic fermentation, *Mucor* strains, NBRC 4572, 6746 and 8092 produced 3.12 ±0.13, 1.32±0.12 and 2.54±0.10 g/L of ethanol from colloidal chitin, respectively (Fig. 3A, C and E). These strains also produced ethanol from chitin powder under microaerobic condition

(Fig. 3B, D and F), but the productivity was significantly lower than that from colloidal chitin

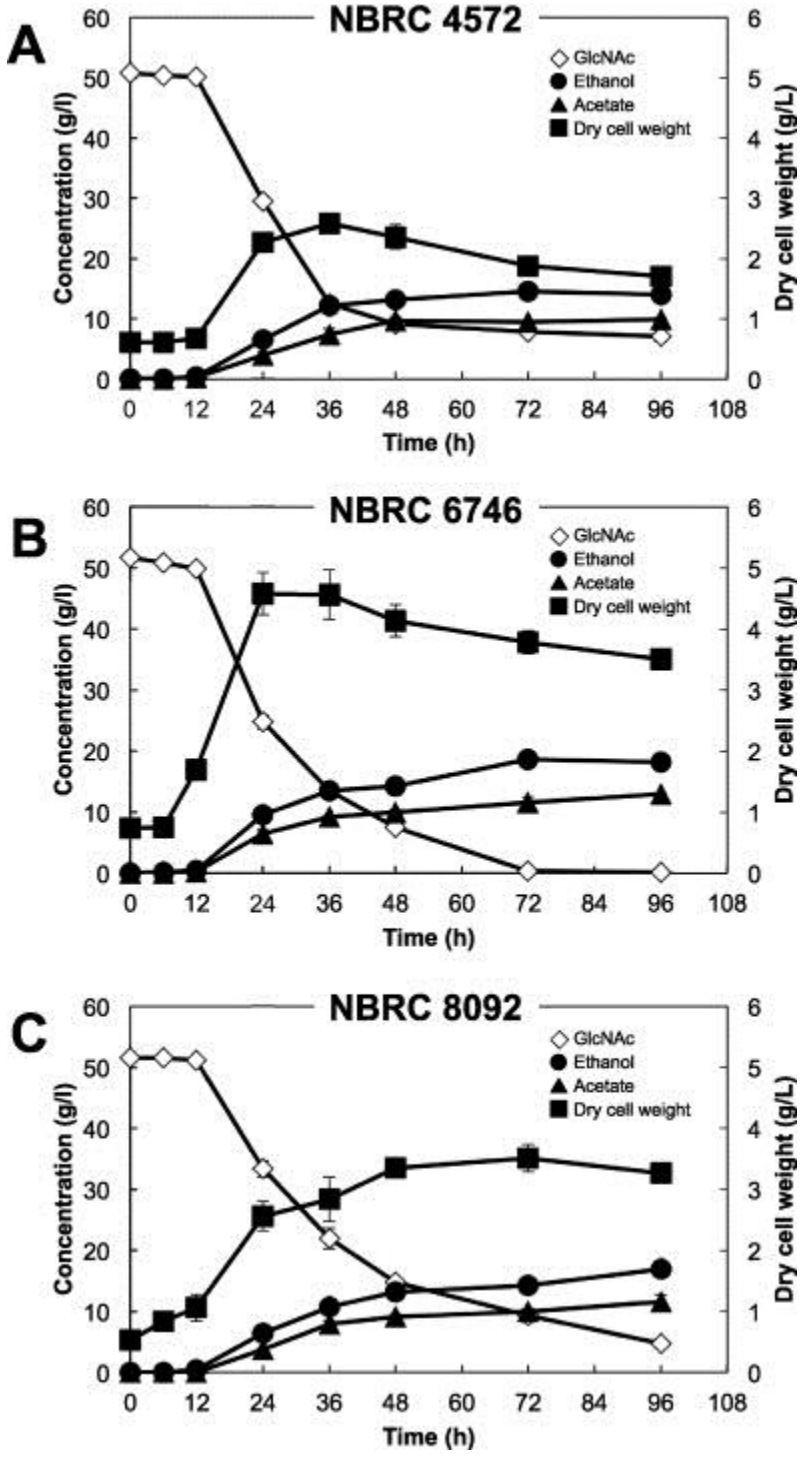

Fig. 2 Time course of ethanol production from 50 g/L of GlcNAc by *M. circinelloides* NBRC 4572 (A), 6746 (B) and *M. ambiguus* NBRC 8092 (C)

(0.09±0.03, 0.12±0.03 and 0.11±0.02 g/L, respectively). GlcNAcase activity was detected from these culture's supernatants, and the activity was higher in the culture with colloidal chitin than that with chitin powder (Fig. 3). On the other hand, the endo-chitinase activity was generally low (<1.5 U/L), and no significant activity was detected from the culture's supernatant using NBRC 4572 with colloidal chitin (Fig. 3A).

In aerobic fermentation, no significant ethanol accumulation was observed in the culture's supernatant (Fig. 4). This result might be due to ethanol volatilization from the culture. Instead, the chitinolytic enzyme activity was generally higher than that of microaerobic fermentation. Especially, remarkable *endo*-chitinase activity (22.7±2.5 U/L after 12 days) was detected from the culture's supernatant of NBRC 8092 with chitin powder (Fig. 4F). The results shown in Figs. 3 and 4 indicate that expression of extracellular chitinolytic enzyme of these *Mucor* strains is strongly affected by the dissolved oxygen (DO) and state of chitin substrate. Previously, Abd-Aziz et al. [24] investigated the effect of agitation and aeration rates on chitinase production using *Trichoderma virens* UKM1, and reported that there was no DO limitation during the highest chitinase production. On the other hand, the most inducible substrate varies with the individual report. It was reported that some bacterial and fungal extracellular *endo*-chitinase activities were induced by the addition of low-concentrated (0.2-0.5%) colloidal chitin [24-27]. Andronopoulou and Vorgias [28] also found out that treated (colloidal) chitin was a more efficient inducer for the chitinolytic system of *Thermococcus chitonophagus* than chitin powder or flakes. In contrast, Suresh and Kumar [13] reported chitinolytic enzyme production using *Penicillium monoverticillium* CFR 2, *Aspergillus flavus* CFR 10 and *Fusarium oxysporum* CFR 8 using commercial wheat bran (CWB) as the solid substrate, and 1% (w/w) of shrimp shell pure α-chitin powder was added as an inducer. Extracellular endo-chitinase activity of NBRC 8092 significantly increased in aerobic condition with 5% (w/v) of chitin powder (Fig. 4F). This result suggests the possibility that this strain has a different *endo*-chitinase expression mechanism from previously reported microorganisms described above. Further optimization of its culture condition for chitinolytic enzyme production is needed to evaluate its chitin degradation capacity. In addition, purification and characterization of individual enzymes would

also be necessary for more understanding.

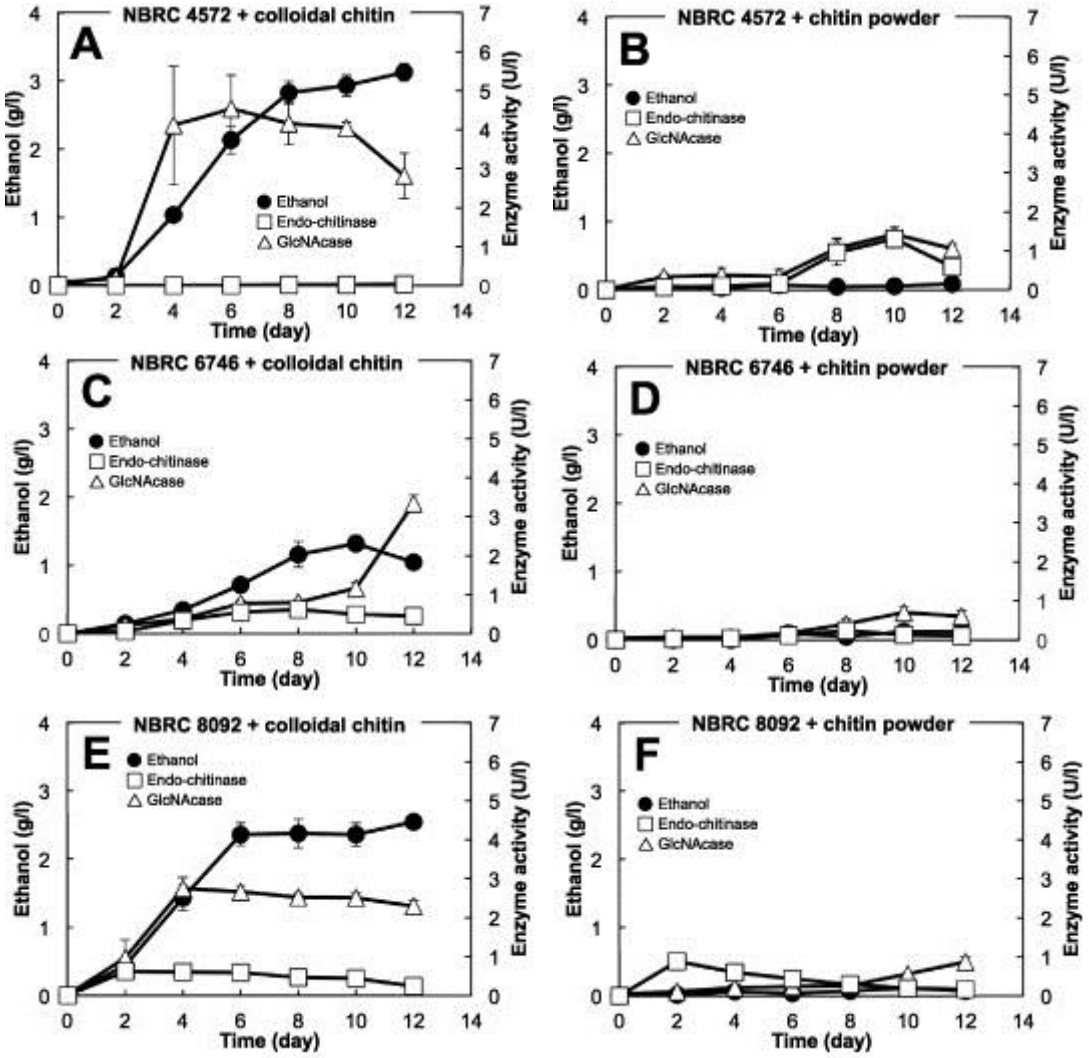

Fig. 3 Time course of microaerobic ethanol production from 50 g/L of colloidal chitin or chitin powder by *M. circinelloides* NBRC 4572 (A and B), 6746 (C and D) and *M. ambiguus* NBRC 8092 (E and F)

6.3.4. Intracellular and cell surface chitinolytic enzymes activities

To confirm intracellular and cell surface chitinolytic enzyme activity, *Mucor* strains, NBRC 4572, 6746, and 8092 were cultured in 20 ml of ethanol production medium containing 50 g/L of colloidal chitin microaerobically at 28°C for 8 days. After cultivation, the proteins were extracted from the mycelium of these strains in a way described in Section 2, and *endo*-chitinase and GlcNAcase activities were measured. However, no significant activities of these enzymes were detected from these cell extracts. As shown in Fig. 3, in the ethanol fermentation from colloidal chitin, the *endo*-chitinase activities in the supernatant of these strains were absent or very low. These results indicated the absence of significant *endo*-chitinase activity both in the supernatant of culture and in the cell, though these strains grew and produced ethanol using colloidal chitin as the substrate (Fig. 3A, B and C). This apparent absence of endo-chitinase activity might be due to the immobilization of the enzyme binding to the chitin substrates. Suresh et al. [29] previously pointed out the possibility that the chitinolytic enzymes were immobilized to chitin substrate during chitin degradation.

6.3.5. Ethanol fermentation with the addition of concentrated chitinolytic enzyme

Ethanol fermentation from chitin substrates added with 1.0 ml of concentrated chitinolytic enzyme was performed using *M. circinelloides* NBRC 4572 (Fig. 5). The concentrated enzyme was prepared from *M. ambiguus* NBRC 8092 and its *endo*-chitinase and GlcNAcase activities were 105 and 305 U/L, respectively. By the addition of this enzyme, the ethanol productivity of NBRC 4572 from colloidal chitin was significantly improved. The ethanol titer and maximum production rate reached 9.44±0.10 g/L after 16 days and 1.05±0.10 g/L/d between 2 and 4 days, respectively (Fig. 5A). Maximum ethanol yield from 50 g/L of colloidal chitin was 45.3± 0.5%. On the other hand, the ethanol productivity from chitin powder was not improved by the addition of the concentrated chitinolytic enzyme (Fig. 5B). In these fermentations, significant declines of *endo*-chitinase and GlcNAcase activities in the culture's supernatant were observed in the first 2 days, and then these activities were gradually increased (Fig. 5A and B). These results support the hypothesis of the immobilization of the chitinolytic enzymes binding to the

chitin substrates as described above. From these observations, it is suggested that *Mucor* strains

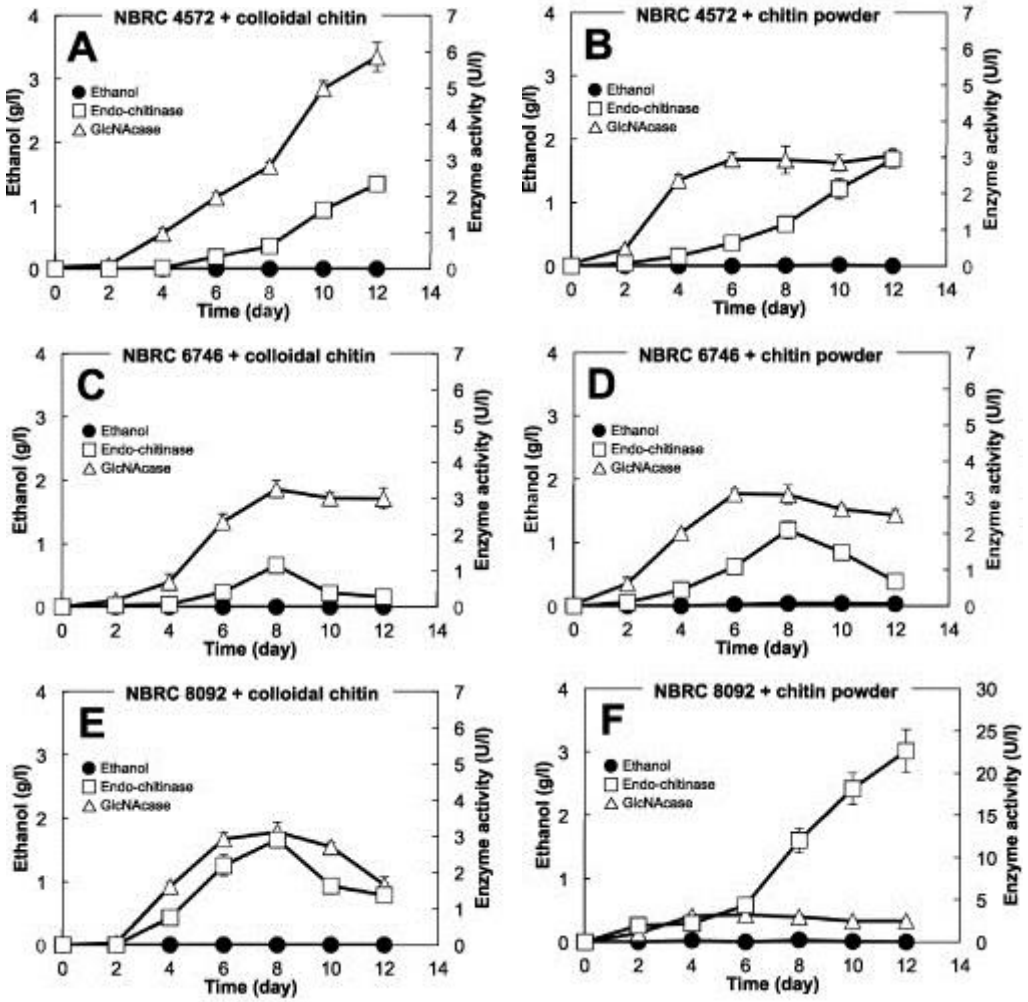

Fig. 4 Time course of aerobic ethanol production from 50 g/L of colloidal chitin or chitin powder by *M. circinelloides* NBRC 4572 (A and B), 6746 (C and D) and *M. ambiguus* NBRC 8092 (E and F)

used in this study produce secretory *endo*-chitinase and GlcNAcase, and most of these secreted enzymes are immediately immobilized on chitin substrates due to high substrate concentration (50 g/L). Karlsson et al. [30] reported that similar adsorption of proteins at a solid-liquid interface is a common phenomenon widely observed in various areas and the adsorption and desorption behavior on surfaces was affected by fundamental properties of protein. In order to control the immobilization of chitinolytic enzyme and maximize its activity, detailed characterization of individual enzymes would be needed.

To confirm the GlcNAc supplying capacity of the concentrated enzyme, chitin saccharification experiment were carried out under the same conditions of the fermentations shown in Fig. 6A and B without inoculation of fungal suspension. The result was shown in Fig. 6. The concentrated enzyme produced 20.8±0.5 g/L of GlcNAc from 50 g/L of colloidal chitin after 18 days. The maximum GlcNAc production rate was 2.08±0.38 g/L/d between 2 and 4 days. It is suggested that this GlcNAc supply of NBRC 4572 led to significant improvement of ethanol production in Fig. 5A. The enzyme produced only 4.1±0.2 g/L of GlcNAc from 50 g/L of chitin powder after 18 days and the maximum GlcNAc production rate was 0.87±0.07 g/L/d between 2 and 4 days. The difficulty of chitin powder saccharification is thought to be the reason of the unchanged ethanol productivity in Fig. 5B.

Mucor strains used in this study produced ethanol from high concentrated colloidal chitin directly (Fig. 3). Furthermore, ethanol titer and production rate from colloidal chitin of *M. circinelloides* NBRC 4572 could be improved by external chitinase addition (Fig. 5). These results suggest that NBRC 4572 is a promising strain for bioconversion of chitin substrates to ethanol. In lignocellulosic bioethanol production, the high cost of the hydrolytic enzyme has been a barrier to the commercialization [31]. This hydrolytic enzyme cost is particularlya serious problem for bioconversion of chitin because commercial exploitation of chitinolytic enzyme for the production of GlcNAc is currently limited owing to the high cost, their activity and stability [29, 32]. Therefore, the spontaneous chitin saccharification by NBRC 4572 is important to reduce the cost of external chitinase addition even if the saccharification capacity of this strain is insufficient to satisfy the GlcNAc demand of the cells. Optimization of

fermentation conditions (temperature, pH, chitinase inducer, initial substrate concentration and inoculum rate) of this strain with colloidal chitin would lead to a more economical chitin bioconversion. In addition, further evaluation on other *Mucor* strains, which showed relatively high ethanol producing capability during screening, would also be needed. Recently, productions of high chitinolytic enzyme activity using wide variety of microorganisms including various fungi have been reported. Wang and Hwang [33] reported that the maximum extracellular chitinase activities of Bacillus cereus J1-1, B. alvei E1, and B. sphaericus J1 using colloidal chitin as the substrate were 1.34, 1.34 and 1.29 U/mL, respectively after 2 days. Suresh and Kumar [13] reported that *P. monoverticillium* CFR 2, *A. flavus* CFR 10 and *F. oxysporum* CFR 8 produced 4.09, 2.64 and 1.33 U/mL of *endo*-chitinase using ethylene glycol chitin as the substrate and 19.54, 22.20 and 16.83 U/mL of GlcNAcase activities, respectively after 166 h. These *endo*-chitinase and GlcNAcase activities were significantly higher than that of concentrated enzyme from NBRC 8092 (0.105 and 0.305 U/mL after 10 days). If these enzymes can compensate for the deficiency of *Mucor* chitinase activity, the combination of these chitinolytic enzymes and ethanol fermentation using *Mucor* strains would be promising. One of the other means to solve the saccharification problem is heterologous chitinase genes expression in *Mucor* strains. A number of expression vectors, which are available for heterologous gene expression in *M. circinelloides* have been reported [34-37]. Takó et al. [37] constructed the expression vector and successfully expressed heterologous β-glucosidase gene from *R. miehei* in *M. circinelloides* and significantly increased the enzyme activity of the transformants. If genetically engineered *Mucor* strain producing extremely high chitinolytic enzyme activity can be constructed, SSF of chitin using the strain will progress toward the realization significantly. Colloidal chitin is chitin powder or flakes partially broken β-(1-4) linkages of its polysaccharide. The lipid and protein components are removed by the treatment of acid such as hydrochloric acid or phosphoric acid. Due to this treatment, colloidal chitin is easily utilized by fungi [38] and more accessible for hydrolytic enzymes than non-treated chitin [39]. The use of colloidal chitin as the substrate for ethanol fermentation have serious economic and environmental disadvantages compared to the use of non-treated chitin because colloidal chitin preparation involves high cost, reduction of chitin yield, and high acidic effluent. *Mucor* used in this study

produced ethanol from not only colloidal chitin but also chitin powder. However, the titer

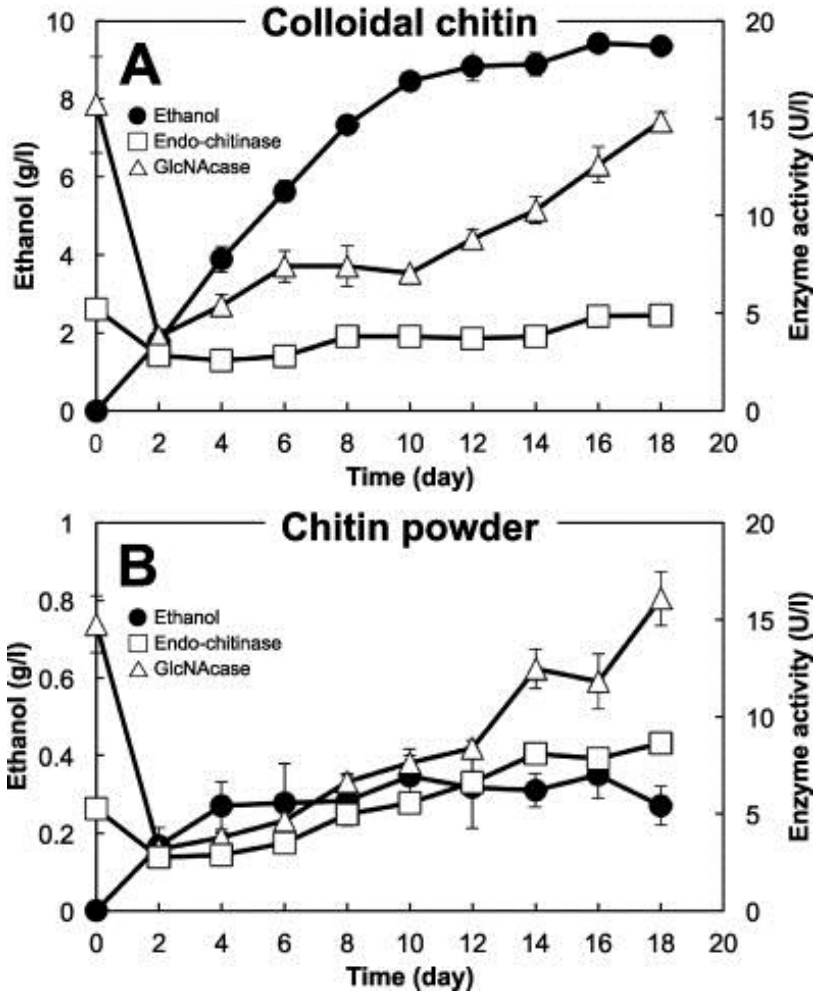

Fig. 5 Time course of ethanol production using both of five times initial inoculum rate of NBRC 4572 and the addition of concentrated chitinolytic enzyme secreted by NBRC 8092 from 50 g/L of colloidal chitin (A) and chitin powder (B)

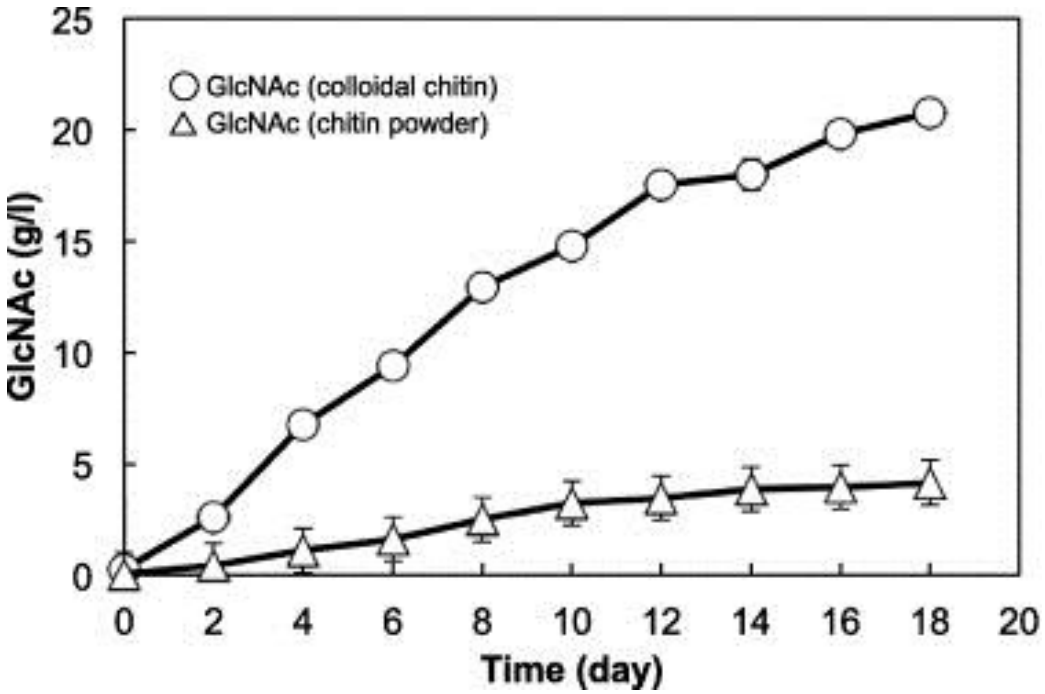

Fig. 6 The saccharification of 50 g/L of colloidal chitin or chitin powder using concentrated chitinolytic enzyme of *M. ambiguus* NBRC 8092

from chitin powder was extremely limited compared to that from colloidal chitin and no significant enhancement was observed by the addition of chitinolytic enzyme from NBRC 8092. These results indicated that pretreatment of chitin substrates is necessary for effective fermentation using these strains. Chitin pretreatment methods have been reported previously [40], but the detailed research has not been progressing compared with lignocellulosic materials [31]. Establishment of low-cost, environment-friendly, and effective chitin pretreatment method is urgent for commercially acceptable bioconversion of chitin to ethanol.

6.4. Conclusions

Chitin is one of the most abundant renewable resources in nature, after cellulose. At present, however, only small amount of chitin has been utilized for the limited purposes. In this study, we successfully demonstrated the efficient bioconversion of GlcNAc into ethanol, which is one of the alternative fuels using *Mucor* strains. Furthermore, these strains produced chitinolytic enzyme and could convert chitin substrate into ethanol directly under microaerobic condition. This is the first report on direct ethanol production from chitin substrates. This bioconversion means that abundant unused chitinous wastes could become available as a source of alternative fuels, and also suggest that these *Mucor* strains have great potential for industrial bioconversion of chitin to ethanol. However, the observed ethanol production rate from chitin substrates was low due to shortage of its chitinolytic enzyme activity. Therefore, in order to commercialize ethanol production using Mucor strains, optimization of all aspects of fermentation, characterization of individual chitinolytic enzymes, screening of chitinase for addition, construction of genetically engineered *Mucor* strain, and establishment of effective chitin pretreatment method are necessary.

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Chapter 7

Lactic acid production from paper sludge by SSF with thermotolerant *Rhizopus* sp.

7.1. Introduction

Petrochemical production has caused worries amid concern about depletion of fossil fuel. Additionally, disposal treatment of a lot of the waste plastic is also a major consideration. As one of the sustainable green plastic materials, polylactic acid synthesized from lactic acid has been used increasingly as an alternative to petrochemical plastics. It can be discarded into the soil because of its biodegradability; the matter may be decomposed by microorganisms and used as carbon sources for plants and microorganisms. Lactic acid and its derivatives are also important chemicals that have many applications in food, chemical and pharmaceutical products [1, 2]. While it is produced by chemical synthesis, this manufacturing method results in a racemic mixture of D- and L-lactic acids. As enantiopure lactic acid is required to produce polylactic acid which is stable structurally and does not contain impurities, lactic acid production by bioconversion has attracted attention because of its economical procedure compared with chemical synthesis. Moreover, the biological production method of lactic acid is valid because of the possibility of using lignocellulosic materials as feedstock.

Rhizopus fungi belonging to Zygomycota are well known microbes of enantiopure L-lactic acid from glucose and other carbon sources. The fungi are also able to ferment starch to lactic acid [3]. Furthermore, several researches have been reported about the fungus, by which cellulolytic and xylanolytic enzymes were secreted [4, 5]. The saprophytic fungus has high potential for lactic acid production from various abundant cellulosic biomasses, such as agricultural (rice straw, wheat straw), forestry (wood) and industrial waste (old paper, paper sludge) [6-10]. *Rhizopus* fungi can produce L-lactic acid from simple media with not only several monosaccharides, but also starch and lignocelluloses with lower production cost than that by other lactic acid bacteria though many kinds of microorganisms such as *Lactobacillus*, *Lactococcus* and *Enterococcus* [11, 12]. Additionally, *Rhizopus* can produce

three major end products of L-lactic acid, fumaric acid and ethanol, unlike homo-fermentative lactic acid bacteria. These products are affected by culture condition, and sufficient aeration induces efficient lactic acid [13].

Paper sludge (PS) is a solid residue arising from paper manufacture and the generated amount is more than 3.5 million ton per year in Japan, according to statistic data on industrial waste from paper manufacturing by the Japan Paper Association. Since it is mainly incinerated to reduce the volume and regenerate thermal energy, the use of a lot of fossil fuel for the discard treatment has been viewed with suspicion, because PS has high moisture content up to 50 % and incineration discharges a large amount of CO_2. Also, the utilization of PS ash derived from the incineration is limited and is used as filling materials for cement, asphalt and rubber, or as a material for manufacturing cement [14]. Though a small part of PS has been used as a soil conditioner and a fertilizer in agriculture, a large part of ash is disposed of in sanitary landfills [15, 16]. Therefore, new technology of effective utilization of PS should be developed for not only the production of sustainable materials as alternative to petrochemicals, but also the reduction of paper waste. PS is a promising material as one of a lignocellulosic feedstock for lactic acid production from the content of a large amount of short cellulosic fibers already treated by delignification of paper making. While PS has already been transmuted to cellulosic fiber in the pulping process by digestion with alkali and steam, general pretreatment of lignocellulosic biomasses needs high energy to delignify and expose the cellulose fiber. However, the PS contains a large amount of paper filler materials, such as $CaCO_3$, kaolin ($Al_2Si_2O_5(OH)_4$), talc ($Mg_3Si_4O_{10}(OH)_2$) and other inorganic materials, and it is possible to be an inhibitor of enzymatic reaction and metabolism of microorganism for the bioconversion of PS. The fillers and the other additives in PS envelop the cellulose fibers and obstruct the access of microbes and enzymes to the cellulose substrate [17]. Cellulase hydrolysis is especially inhibited by $CaCO_3$, because the pH of this reaction suspension (pH 6-8) is relatively higher than the optimum pH of cellulases [18]. Although $CaCO_3$ is essential for pH control for lactic acid production to suppress pH decrease by the acid, removal of ash in PS leads to effective hydrolysis of PS and lactic acid production. $CaCO_3$ can be removed by acid dissolution or CO_2 treatment [19].

In this study, lactic acid production by an appropriate *Rhizopus* fungus from PS that removed the inorganic materials was investigated using simultaneous saccharification and fermentation (SSF) method. In SSF, the enzymatic hydrolysis step of cellulose and the fermentative step by microorganisms are typically combined and the cultivation is operated on a one-batch system [20]. Unfortunately, appropriate temperatures for the enzymes and microbes are in general very different. As optimum temperature of cellulases are generally 40-60°C and the growth temperature of the common lactic acid bacteria is around 30 °C, SSF must be operated at the growth temperature which causes the cellulase activities to decrease. To achieve effective SSF, thermotolerant *Rhizopus* strain was selected. Several strains can grow and produce lactic acid up to 40°C [21]. Conversion of PS to lactic acid by SSF was studied with thermotolerant *Bacillus*; however, it was not able to secrete cellulases [22]. Since *Rhizopus* sp. is able to secrete cellulases and hemicellulases, SSF will be able to perform with addition of relatively low-dose cellulase economically. Therefore, this study investigated about the selection of thermotolerant *Rhizopus* strain, removal of $CaCO_3$ from raw PS and lactic acid production from the treated PS by SSF with the strain and cellulase cocktail.

7.2. Materials and Methods

7.2.1. Materials

To evaluate lactic acid production, 55 kinds of *Rhizopus* sp. was obtained from NBRC (NITE Biological Resource Center, Chiba, Japan). These strains were maintained on Difco™ potato dextrose agar (PDA) slants at 4°C.

The paper sludge (PS) used in this study was supplied from the final waste sludge discharge spout of deinked pulp manufacturing process in Chuetsu Pulp & Paper Co., Ltd. (Toyama, Japan). The moisture content of PS was around 50% by squeezing of the filler-pressed machine. PS was analyzed after oven drying at 50°C to constant weight. The PS components of cellulose, hemicellulose, lignin, acid-soluble lignin and ash were assayed by Klason lignin method.

Cellulases in this study used 15 kinds of commercial reagents were as listed in the section 2.2.2.

7.2.2. Removal of inorganic materials from raw PS by alkali and acid solution

Raw PS was suspended in 1 M NaOH solution with 200 g/L consistency and soaked overnight. After the PS fiber was separated from the alkaline liquid, it was resuspended in appropriate HCl solution of the required concentration for neutralization of both residual NaON and $CaCO_3$ contained in PS and soaked overnight. Then, it was separated from the solution and thoroughly washed with water. Final material was completely dried at 50°C, and then powdered finely in a mill.

7.2.3. Selection and determination of optimum cellulase cocktail

The treated PS of 50 g/L was hydrolyzed by 15 kinds of commercial cellulase each at the protein concentration of 1 g/L in 100 mL bottles containing 25 mL acetic acid buffer (pH 5.0). These were shaken in a rotary shaker at 120 rpm at 40°C for 48 h and then hydrolysates were separated by filtration (Filter paper No.131, Advantec Co., Tokyo, Japan) and analyzed by HPLC. Three cellulase reagents were selected based on the analysis and their blending ratio was determined by the design of experiment method using Design Expert 8 (Stat-Ease, Inc. MN).

7.2.4. Cultivation of *Rhizopus* fungi

Fungi were precultured on PDA plate at 40°C until they covered the overall surface. The mycelia on a plate were milled using a blender (Milser LM-Plus, Osaka chemical Co., Osaka, Japan) in 100 mL saline per plate and it was used as inoculums. The composition of the culture medium was 120 g/L glucose, 3 g/L $(NH_4)_2SO_4$, 0.3 g/L KH_2PO_4, 0.25 g/L $MgSO_4 \cdot 7H_2O$, 0.04 g/L $ZnSO_4 \cdot 7H_2O$, and 50 g/L $CaCO_3$. All reagents used were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). The medium of 25 mL was put in a 100 ml medium bottle and then sterilized at 121°C for 15 min. Cultivations of fungi were started by inoculation of 1 mL hypha suspension into the medium and then incubated in a

rotary shaker (120 rpm) at 40°C. After the cultivation, 5 ml of 5 M HCl was added to each bottle for dissolution of $CaCO_3$, and the broth was separated by filtration. Residual sugar and lactic acid in the culture were analyzed by HPLC.

7.2.5. Enzymatic hydrolysis and SSF of the treated PS

For lactic acid production from treated PS, 50 or 100 g/L of the treated PS was used as substrate instead of glucose. Medium components and the PS in 500 mL baffle flasks were sterilized at 121°C for 15 min. SSF was started by the addition of filter-sterilized cellulase cocktail into the medium and then shaking at 130 rpm for 144 h at 28 or 40°C. SSF was started by the addition of 8 mL of homogenized mycelium suspension and the cellulase cocktail, and then they were cultured at the same condition of hydrolysis. Hydrolysates and broths sampled from cultures were analyzed by HPLC.

7.2.6. Analytical method

Elemental composition in ash of PS was determined by X-ray fluorescence analysis (XRF) using wave length-dispersive X-ray spectroscopy (PW2404, Spectris Co., Ltd., Tokyo, Japan). The amount of glucose, xylose, and lactic acid produced from each culture were determined by HPLC as described in the section 1.2.4.

7.3. Results and discussion

7.3.1. Removal of inorganic materials from PS

PS contains many components with exception of cellulose and hemicelluloses fibers such as $CaCO_3$ from loading materials, reagents and surfactants by the deinking and the repulping. These materials have the possibility of becoming inhibitors of hydrolysis of enzymes and fermentation of microorganism. To remove these inhibitors for the effective hydrolysis and subsequent fermentation of PS, removal process by dissolution with alkali and acid solution was investigated.

First, raw PS was soaked in 1 N HCl solution to dissolve $CaCO_3$ in PS. The treatment

resulted in the decrease of the component ratio of inorganic materials from 41.9 to 28.7% with increase in the ratio of cellulose and other sugars of 14.0 and 8.4%, respectively, as shown in Table 1. While partial inorganic materials were removed from raw PS by the HCl treatment, the PS became strong water-shedding fibers because of the exposure of the hydrophobic surface of the fibers by uncovering of $CaCO_3$.

Next, raw PS was soaked in 1 N NaOH before HCl treatment in an effort to reduce the hydrophobicity with dissolution of reagents and surfactants used on repulping. The NaOH–HCl treatment resulted in the augmentation of hydrophilicity with removal of 24.5% inorganic materials and increase of cellulose and other sugar contents to 51.3 and 22.4%, respectively. The elemental components ratio in PS ash by X-ray fluorescence analysis is shown in Table 2. Ca, Al, and Fe elements were mainly reduced from PS and the decrease ratios were 29.1, 18.9, and 1.84%, respectively. From these results, the NaOH–HCl treatment was excellent method for the disposal of $CaCO_3$ from raw PS.

7.3.2. Selection of thermotolerant lactic acid-producing strain

To select the best strain that was able to grow at 40°C and concurrently to produce effectively lactic acid among 55 species of *Rhizopus* library, they were first cultured at 40°C on a PDA plate. Since 40 species well grew the condition, they were cultured in liquid medium under the aerobic condition at 40°C and produced lactic acid by them for 144 h were compared. Figure 1 shows that lactic acid production by the thermotolerant strain. It was able to produce by 22 kinds of species. Since *R. oryzae* NBRC 5384, 5413 and 31005 was produced more than 50 g/L lactic acid from 120 g/L glucose, these strains were cultured again to detail the production performance. As a result, three strains produced mainly lactic acid with a small amount of fumaric acid as shown in Table 3. Maximum lactic acid concentrations were about 66-74 g/L with slight fumaric acid of 0.22 g/L at 40 °C for 120 h. Additionally, only *R. oryzae* NBRC 5384 was able to grow up to 50°C. Therefore, the *R. oryzae* NBRC 5384 strain was selected as a thermotolerant fungus for the production of lactic acid from treated PS.

Table 1 Components ratio in raw and reach treated PS

Content (%)	Raw	HCl	NaOH-HCl
Cellulose	22.4	36.3	51.3
Other sugars	10.1	18.6	22.4
Inorganic materials	41.9	28.7	17.4
Others	25.5	16.3	8.88

Table 2 Elemental components ratio of ash in raw and treated PS

		Raw	Treated
	Ratio of ash	1	0.537
Elemental components (%)	Al	32.9	14.1
	Ca	30.8	1.66
	Si	16.4	24.3
	Mg	5.77	6.07
	Fe	3.55	1.71
	Na	3.24	2.53
	S	2.95	0.743
	P	1.99	0.905
	Ti	0.643	0.840
	Cl	0.490	0.216
	K	0.426	0.401
	Mn	0.412	0.0759
	Zn	0.244	0.0684
	Cu	0.114	0.115

*Based on content of inorganic material (ash) in raw PS.

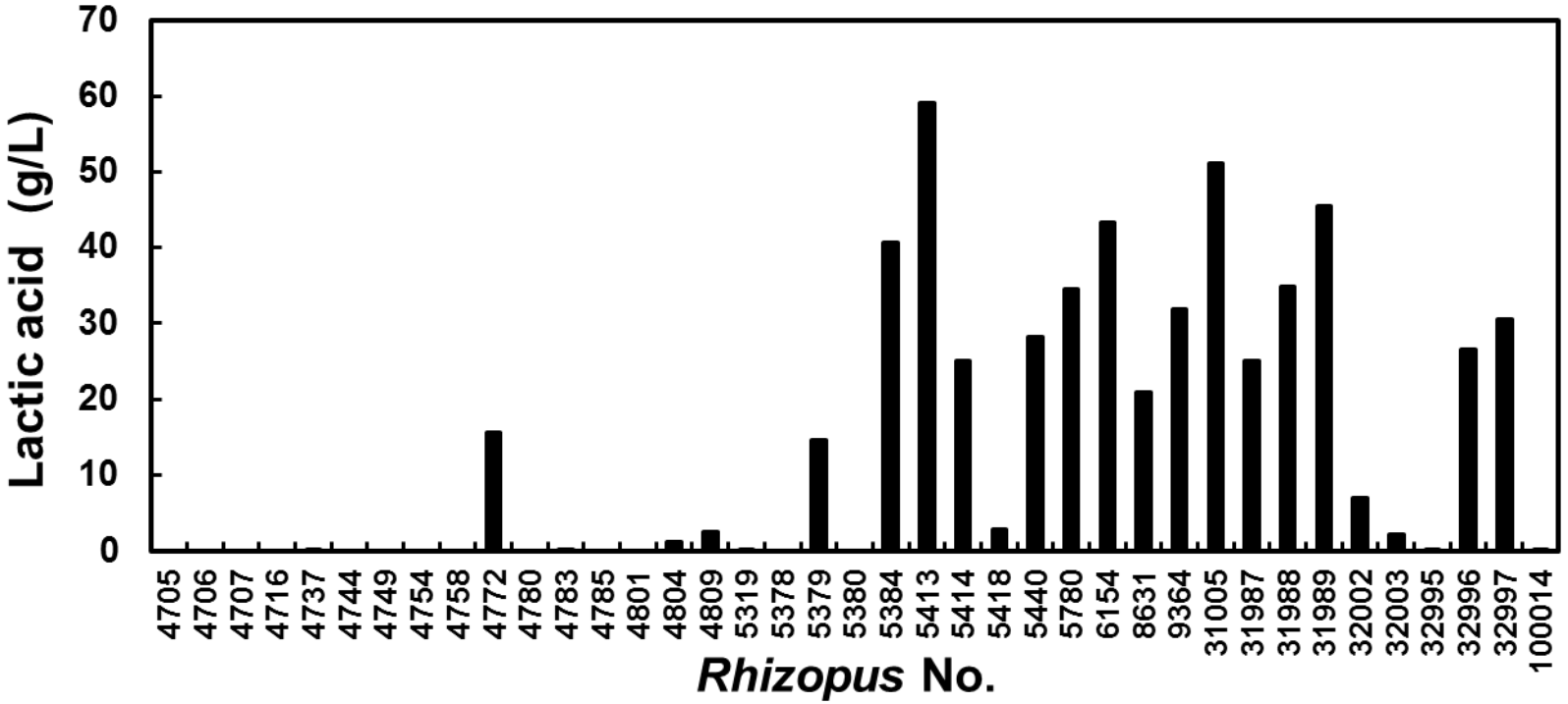

Fig.1 Comparison of lactic acid production from glucose by various *Rhizopus* strains at 40˚C

7.3.3. Lactic acid production by NBRC 5384 at various temperatures

R. oryzae NBRC 5384 was cultured with 120 g/L glucose as a carbon source at 28, 36, 40, and 42°C for 144 h and the results are shown in Fig. 2. In all cultures, glucose was almost consumed for 96 h (28 and 36°C) or 144 h (40 and 42°C) and lactic acid was produced by the strain. It was also able to produce lactic acid even at 42°C. It was produced up to 80 g/L for 120 h except for the culture at 42°C with different rates at each temperature. These rates increased with temperature rising until 36°C and decreased up to 42°C and were 0.894 (72 h), 1.11 (72 h), 0.692 (120 h) and 0.462 g/L/h (144 h), respectively. From these results, 5384 strain is applicable to lactic acid production at high temperature about 40°C. Although the most effective temperature was 36°C for the production, it in following cultures was decided at 40°C to maintain stable and effective fermentation because general cellulases require 40-60°C for high activity to hydrolysis of cellulose and hemicellulose.

7.3.4. Characterization of lactic acid production from several carbohydrates by *R. oryzae* NBRC 5384

Lactic acid productions by *R. oryzae* NBRC 5384 were carried out with 15 kinds of carbohydrates (hexoses, pentoses, sugar alcohol, disaccharides and polysaccharides) as carbon sources at 40°C to characterize lactic acid forming ability of the strain. The cultures were performed with 50 g/L of each carbon source for 120 h and produced lactic acid and the yields are shown in Table 4. The strain was able to convert efficiently hexoses such as glucose, galactose, mannose and fructose to lactic acid, and the yields based on the initial carbon sources were about 0.62-0.65. The strain was also able to ferment to lactic acid from not only hexoses, but also slightly from pentoses such as xylose and arabinose. Moreover, the strain was able to ferment sorbitol slightly though common lactic acid microorganism cannot use sugar alcohols for lactic acid production. Maltose and cellobiose were converted to lactic acid and lactose was not fermented by the stain. These results mean that the strain could produce α- and β-glucosidases though β-fructofuranosidase could not be produced because it could not ferment sucrose. Since soluble starch was directly fermented to lactic acid, the secretion of α-amylase and/or glucoamylase by the strain was also suggested.

Table 3 Comparison of lactic acid and fumaric acid produced by three strains of *Rhizopus*

Strain	Lactic acid (g/L)	Fumaric acid (g/L)
NBRC 5384	69.8	0.164
NBRC 5413	65.9	0.0581
NBRC 31005	73.9	0.167

All cultures were carried out 120 h at 40°C

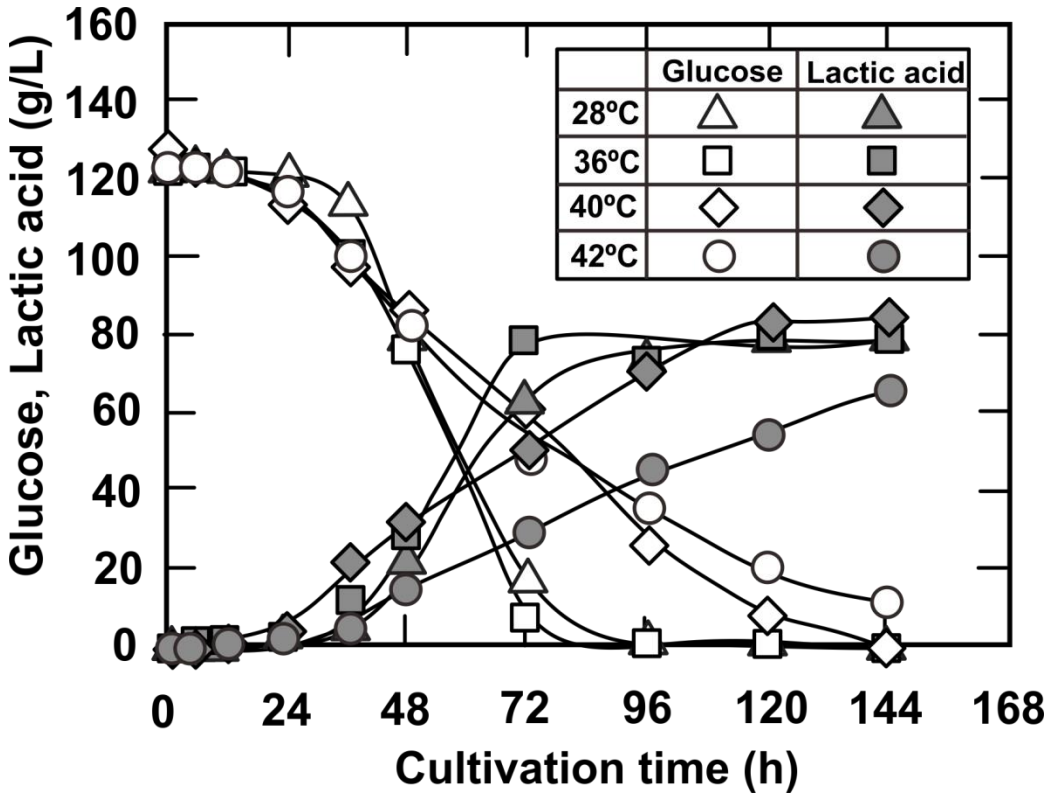

Fig. 2 Effect of temperature on lactic acid production from glucose by *R.oryzae* NBRC 5384

Table 4 Ability of lactic acid production by *R.oryzae* NBRC 5384 on various carbon sources

	Lactic acid (g/L)	Yield (g/g)*
Glucose	34.2	0.648
Galactose	32.7	0.654
Mannose	31.0	0.620
Fructose	32.9	0.658
Xylose	5.23	0.105
Arabinose	0.13	0.0026
Sucrose	0.243	0.00486
Maltose	36.5	0.730
Cellobiose	6.55	0.131
Lactose	0	0
Glycerol	0	0
Sorbitol	8.31	0.166
Mannitol	0	0
Soluble starch	6.80	0.136
α-Cellulose	0	0

*Based on initial concentration of each sugar (50 g/L).

The culture was carried out 120 h at 40°C

Hydrolysis of α-Cellulose by cellulases such as *endo*-β-gluconase, cellobiohydrolase, β-glucosidase was not enough for the lactic acid production though the strain was secreted the slightly several cellulases because mycelia growth was observed. To produce lactic acid from cellulosic biomass with the fungus, extra cellulases are needed into the culture. In order to execute effective lactic acid production from treated PS, high temperature SSF is required for which cellulases and thermotolerant fungus were combined.

7.3.5. Hydrolysis of treated PS by an optimum cellulase cocktail

Hydrolysis rate of the cellulosic fiber might be greatly controlled by the compounding ratio of these enzymatic activities which affect substrates. Since commercial cellulase reagents have various cellulase activities, selection of one cellulase reagent for hydrolysis of PS is difficult. Therefore, blending of several commercial reagents is a promising strategy to obtain fermentable sugars from treated PS. First, the hydrolysis reactions of treated PS by 15 kinds of commercial reagents were carried out at the concentration of 1 g-protein/L and 50 g-PS/L for 24 h to compare the initial hydrolysis rates. Among all the reagents, Cellulase Onozuka 3S (O) and Accellelase (A) were produced many amount of fermentable sugars (glucose and xylose) from the PS. Next, the optimization of mixture with these reagents was performed by Design of Experiment (DOE) method with Design Expert 8.0 for effective hydrolysis of treated PS. The ratio calculated by DOE was O: A = 0.157: 0.843. Additionally, 0.5 g-protein/L Pectinase G (P) was used as a supplement of β-xylosidase activity for xylose production from hemicellulose in treated PS because the activities of two selected reagents were weak. From these results, a desirable cellulase cocktail was constructed at the ratio of O: A: P = 0.47: 2.53: 0.50 (total concentration was 3.5 g-protein/L).

For confirmation of hydrolysis ability on treated PS of the cocktail, substantiative experiment was performed with the cocktail at 28 and 40°C. Figure 3 shows the fermentable sugars produced from 100 g/L treated PS by the cocktail (4.73 FPU/g-PS) at 28 and 40°C. The hydrolysis rate increased with rising of temperature. Maximum concentrations of glucose at these temperatures were 32.1 and 38.3 g/L for 72 h, respectively. Glucose yield at 40°C was 0.747 based on cellulose content in the treated PS. However, xylose production

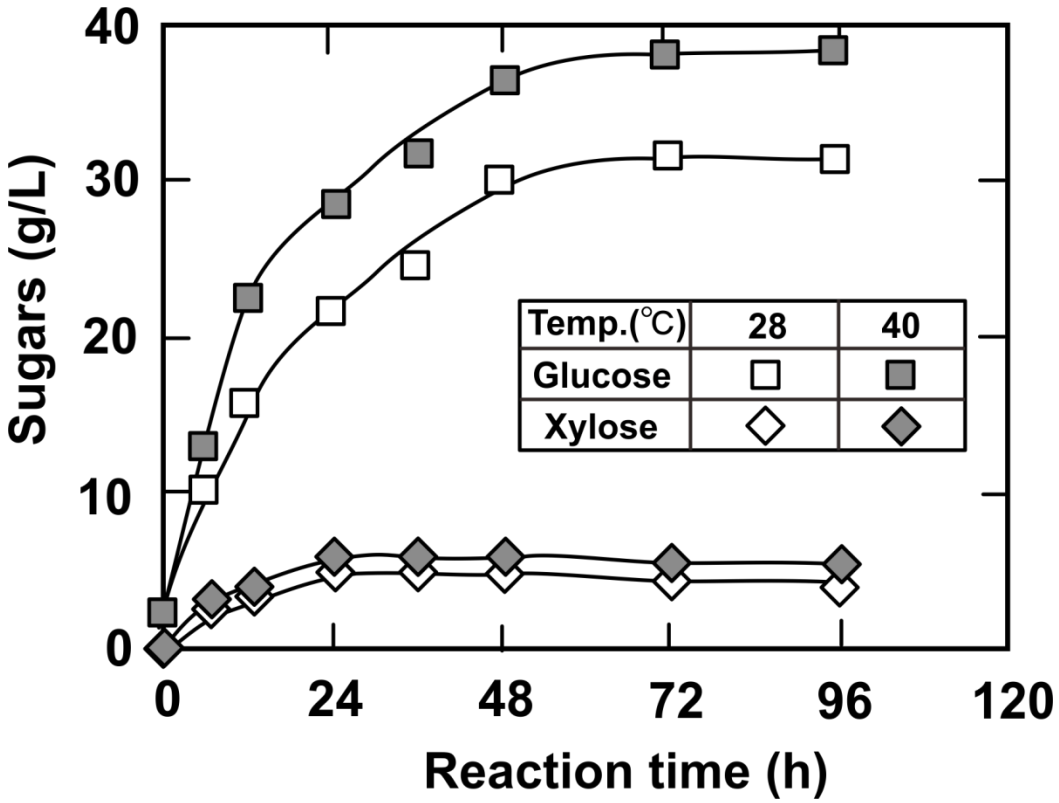

Fig. 3 Glucose and xylose production from the treated PS with optimum cellulase cocktail at 28 and 40°C

increased slightly by rising of temperature. Hydrolysis efficiency of cellulose achieved 70 % and was improved obviously by temperature rising even though existence of $CaCO_3$ in the reaction medium caused pH 6-8 and it inhibited the cellulase activity.

7.3.6. Lactic acid production from treated PS by SSF with thermotolerant fungus

Figure 4 shows the lactic acid production from treated PS by SSF at 40°C, which was combined a thermotolerant strain with the cellulase cocktail. Glucose and xylose were produced rapidly at the initial stage of culture and then consumed for 24 h. Lactic acid began to produce at 24 h and was kept producing. The concentration of it reached 9.33 g/L at 96 h and the yield based on the sugar produced from the treated PS was 0.475. The cellulase cocktail were able to produce fermentable sugars at an enough amount for lactic acid production by the strain at 40°C. Moreover, the strain could use xylose to growth or fermentation. Meanwhile, in this SSF, neither ethanol nor fumaric acid, common by-products of lactic acid fermentation by *Rhizopus* sp., was detected. Although lactic acid was produced from PS treated by the SSF, its productivity of 0.244 g/L/h was less than that of the culture in the glucose substrate (0.667 g/L/h). Therefore, improvement of the lactic acid production by increasing the substrate concentration was attempted.

Figure 5 shows SSF with 100 g/L treated PS at 40°C. During culture, lactic acid and ethanol were simultaneously produced from 24 h and then lactic acid (7.56 g/L, at 48 h) and ethanol (5.25 g/L, at 36 h) reached the maximum values. The yield of lactic acid at that point was 0.272, and that was less than that of SSF with 50 g/L treated PS. The low yield was attributed to decrease of mass transfer in culture and fluidity by the increase of initial substrate concentration because the treated PS was mainly composed of light fibers. Considering ethanol production at initial time of the culture, the environment in culture might induce suffocation to the strain through the SSF. Since the 5384 strain used in this study responds sensitively against dissolved oxygen, it switches lactic acid production to ethanol production by deficient oxygen supply. From these results, low concentration of substrate at the start of SSF is better than high concentration for effective lactic acid production. Fuethermore, a large amount of cell which initially accumulates enough energy

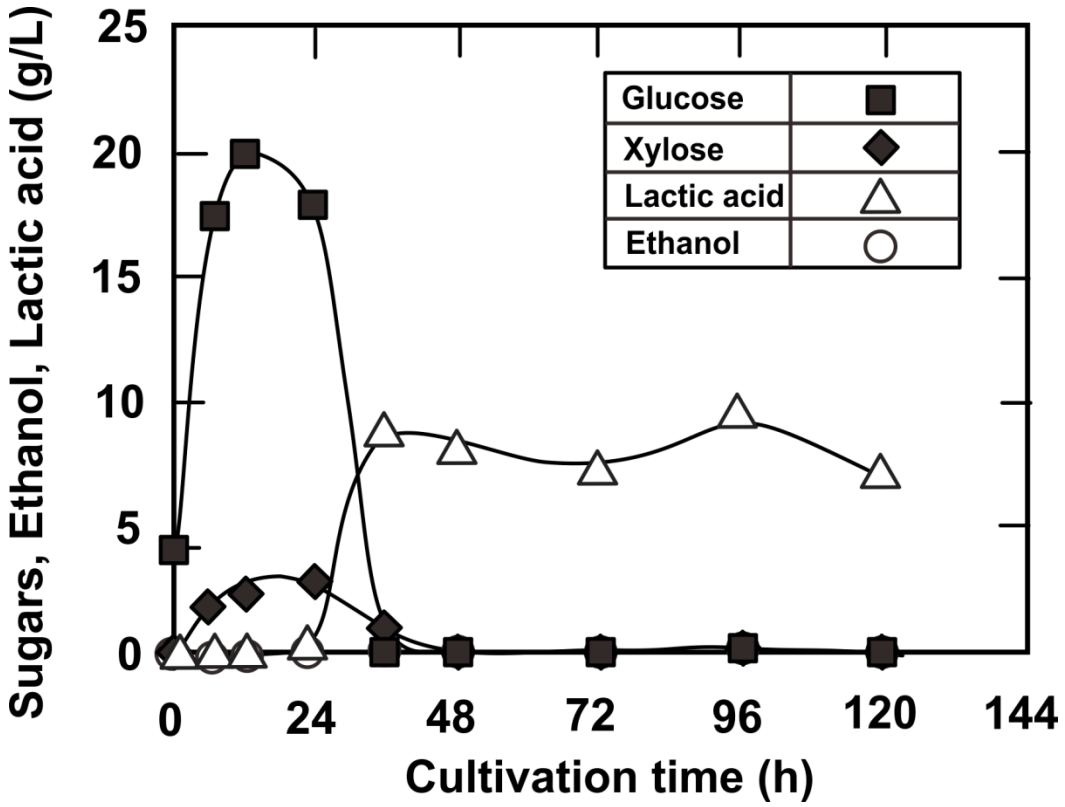

Fig. 4 Lactic acid production by SSF of the treated PS of 50 g/L with 5384 strain and cellulase cocktail

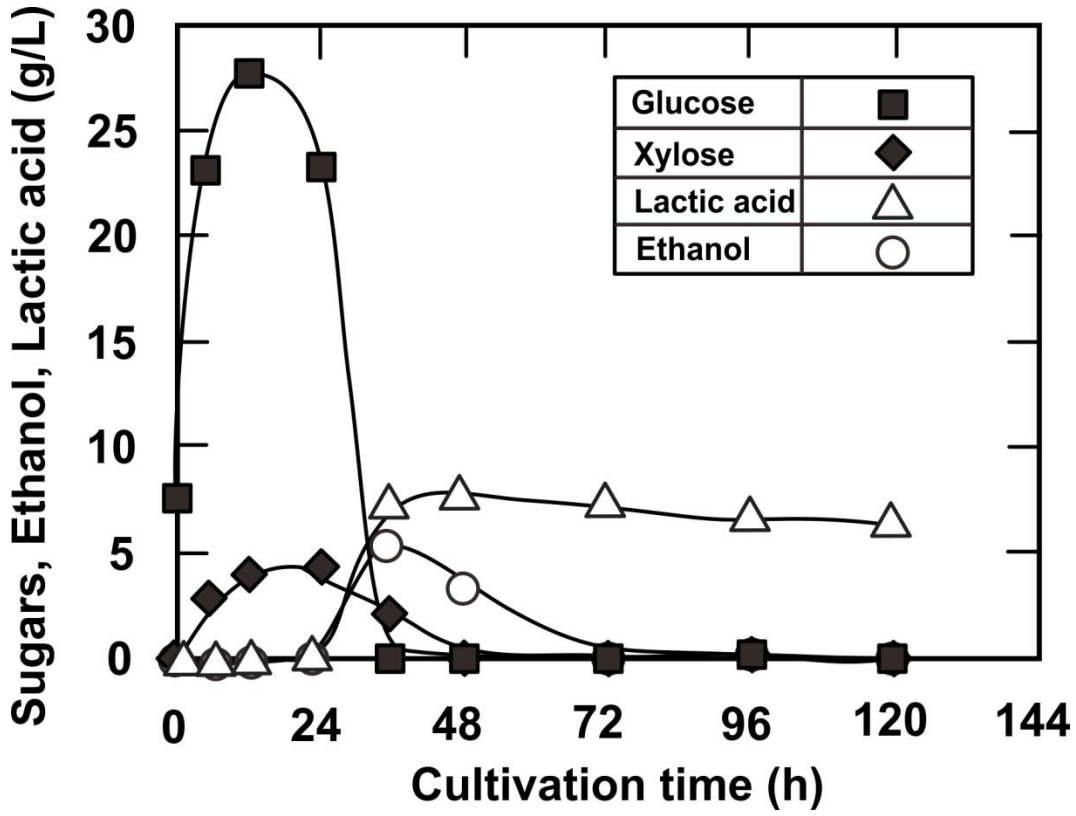

Fig. 5 Lactic acid production by SSF of the treated PS of 100 g/L with 5384 strain and cellulase cocktail

will produce lactic acid immediately, and that will also improve the delayed lactic acid production. Compared to other researches on lactic acid production from several feedstocks, the yields and the productivities were low in this research on PS. For example, SSF of starch such as cassava, rice bran and wheat by *Lactobacillus* sp. resulted in the yields of 0.66, 0.28 and 0.89 and the productivities of 0.41, 0.78 and 1.74 g/L/h, respectively [23-25]. In other researches of lactic acid from alfalfa fiber and cassava bagasse by the same species, the yields were 0.35 and 0.96 and the productivities were 0.75 and 1.40 g/L/h, respectively [26, 27]. While these feedstocks are pure materials obtained from agriculture, PS used in this study is industrial waste and contains a lot of harmful components such as inorganic materials and various reagents for repulping, which inhibit hydrolases and microorganism. Since pH in culture is rendered faintly alkaline with $CaCO_3$ which is the loading material for pulping and suppressor for pH decrease by the lactic acid produced, the activities of cellulases that have optimum pH at mild acid were depressed and the production of fermentable sugars was limited. If the SSF process is accomplished with high efficiency, as the PS is a complex material and difficult to ferment than the other general cellulosic biomasses, it must be able to apply to other biomasses.

7.4. Conclusion

The economical production of green plastic from lignocellulosic materials by bioconversion has recently been interest in terms of carbon-neutral. Polylactic acid is one of the green plastic and lactic acid is a raw material widely used in the field of food, chemical, pharmaceuticals, *etc*. In this research, paper sludge (PS) excreted as a waste product from the paper-making plant was focused on as an unused cellulosic material for lactic acid production. To remove the unnecessary inclusion such as inorganic materials and deinked reagents used for paper manufacturing which inhibit cellulases activity and/or fermentation, pretreatment of raw PS was investigated. PS treatment of soaking in NaOH and subsequently HCl was very efficient in the exposure of cellulosic fiber from PS. Ca from $CaCO_3$ in the loading material and Al from $Al_2Si_2O_5(OH)_4$ in kaolin were mainly decreased by the treatment. Considering the tolerance to high temperature and the ability of lactic acid

production into consideration, *R. oryzae* NBRC 5384 was selected as the fungus for the production of lactic acid by SSF of PS at 40°C. The strain was able to produce lactic acid from glucose in high yield under the aerobic condition at 40°C. Moreover, the fungus was able to directly bioconvert various sugars to lactic acid. The optimization of cellulase reagent by the DOE method resulted in construction of a cellulase cocktail for PS hydrolysis. Lactic acid was able to be produced by SSF of the treated PS at 40°C with *R. oryzae* NBRC 5384 and the cocktail. The SSF process from PS will be able to achieve the production of various biomaterials such as not only lactic acid, but also other organic acids or ethanol in the near future.

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General conclusion

In this study, effective conversion system of biomass into useful substances, ethanol or lactic acid, was developed by simultaneous saccharification and fermentation (SSF) with desirable cellulases and novel fermenting fungi in order to prepare for exhaustion of petroleum resources in the future and reduce generation of carbon dioxide gas and wastes. Bioprocess engineering approach for efficient ethanol or lactic acid production requires selection of fermenting microorganism, comparison of pretreatment effect for biomass, optimization of cellulases for hydrolysis of biomass, and construction of SSF.

In chapter 1, high performing fungus for ethanol production was first selected from 90 strains of zygomycota *Mucor* spp. by cultivation with 100 g/L of glucose or 50 g/L of xylose under aerobic and anaerobic conditions. The results described that strains of the genus was able to grow all conditions. In glucose culture, anaerobic condition resulted in high ethanol production with NBRC 4554, 4569, 4572, 8092, and 30470 up to 40 g/L ethanol. When the fungi were cultured under aerobic condition, by increase of dry cell weight the amount of produced ethanol about 25% of anaerobic culture was reduced, and among these fungi 27 g/L ethanol was maximum production by NBRC 4572 and 8092. In xylose cultivation under aerobic condition, ethanol was able to produce from xylose by NBRC 4570, 4572, and 5774 in higher yield than that of the other strains. From these results, *M.ciecinelloides* NBRC 4572 was identified as high performing fungus on ethanol production by evaluating the fermentation ability in the aspect of substrate and aeration. Moreover, substrate specificity of the strain for fermentation was validated by using monosaccharides, disaccharides, and polysaccharides. It was able to convert all hexoses and xylose (main pentose contained in plants) to ethanol and some disaccharides and starch was also fermented. This result suggested that the selected fungus can secrete hydrolytic enzymes such as α- and β-glucosidase, glucoamylase *etc.* The strain could be characterized as a novel fungus that has unique capability of not only ethanol production at any conditions but also secretion of various hydrolysis enzymes.

Next in chapter 2, pretreatment methods and hydrolysis cellulases for rice straw degradation, and its application to SSF were investigated from the effective and economical view point. By comparing five kinds of pretreatment methods different effect of degradation for

components ratio of rice straw was able to explain. All types of treatments resulted in reduce of lignin and increase of sugar content. Steam explosion and hydrothermal treatment was able to increase cellulase content. Though alkali treatment was most effective method to obtain fermentable-sugars, steam exploded straw was used in this chapter from the point of relatively low energy demand. In characterization of commercial cellulase reagents, it was revealed respective enzyme possess various activities for hydrolysis of cellulosic biomass. However, decision of the most appropriate cellulase reagent alone for hydrolysis was difficult. Therefore, two cellulase reagents were selected by using multivariate analysis since combination of some reagents is considered as the best approach for effective reaction. Moreover, estimation of mixing ratio of these enzymes achieved progress of fermentable-sugar production from steam exploded rice straw. When SSF of the rice straw 100 g/L was performed with fermenting fungus selected in chapter 1 and an optimized cellulase cocktail, glucose was produced efficiently and subsequently was converted to ethanol of 13.2 g/L for 96 h. While by-products derived by steam explosion treatment was harmful to growth and fermentation of microorganism, the strain could produce ethanol effectively with tolerability of these derivatives.

In chapter 3, bioethanol production from rice straw by SSF with statistical optimized cellulase cocktail and fermenting-fungus was estimated. Since rice straw that was pretreated by alkali contained higher amount of fermentable sugars than that of treated by other methods, it was used as substrate for ethanol production by SSF. On the process, selection and optimization of cellulase reagents suitable for the straw were performed. Comparison of 15 kinds of cellulase reagents described that these reagents possessed various enzymatic activities and hydrolysis activities of the straw by them showed multiple results. These values were used as variables for multivariate analysis and regression expression was obtained for selection of suitable reagents to the treated straw. From weight of the coefficient in the expression, cellobiohydrolase, *endo*-β-glucanase, filter paper degradation unit, and BEMCOT degradation unit were required for the hydrolysis of the straw. From these viewpoints, Cellulase T Amano 4, Cellulase Onozuka 3S, and Pectinase G were chosen as candidates for preparing a cocktail. Additionally, an optimum ratio of mixture containing the three reagents was calculated by Design of Experiment (DOE) method with response surface graph. When hydrolysis reactions were performed with

the constructed cellulase cocktail or these component reagents only, the cocktail induced production of the highest amount of fermentable sugars in high hydrolysis efficiency of 83.3%. Decision of cellulase by statistical approach suggested appropriate condition for desirable performance. Combination of three cellulase reagents induced synergy each other for effective hydrolysis of cellulose because they possess different activities. SSF of the treated rice straw with the cocktail and fermenting fungus, *M. circinelloides* J, produced ethanol efficiently. Concentration of ethanol and the fermentation efficiency achieved 30.6 g/L and 90% of hydrolyzed fermentable sugar basis, respectively. Since fermentable sugar production was progressed by not only synergy activities of three reagents but also suppression of product inhibition by accumulation of them by the fungus, subsequent ethanol production was increased from that of SSF with single reagent.

In order to utilize the cellulase secreting ability of *Mucor* spp. for direct ethanol production, co-culture of two high performing fungi was challenged in chapter 4. Screening for ethanol producing fungus was carried out from 11 strains in our library of *M.circinelloides* because *M.circinelloides* NBRC 4572 was the best fermenting fungus as described in Chapter 1. Meanwhile, cellulase secretions from these fungi were estimated by comparing the activities of *endo*-β-glucanase (EBG) and β-glucosidase (BG). The highest activities of these hydrolase were recorded by NBRC 5398 and the strain was selected as the best secreting fungus. When cellulase secreting performance of 5398 was confirmed by cultivation with rice straw, EBG activity increased from the beginning of cultivation and then BG secretion started belatedly about 36 h later. SSF was carried out with these fungi for direct ethanol production without cellulase reagent. To balance the ratio of fermentable-sugar production and conversion to ethanol, inoculum ratio of 5398 and 4572 was investigated. The blending fungi prepared by the ratio of 9 : 1 could achieve ethanol production directly and the amount was 1.28 g/L for 96 h. However, only EBG activity was observed in this SSF. Improvement of cellulase secretion by fungi would make the coculture system advance and increase ethanol production.

Chapter 5 described the details of β-glucosidase (BGL) secreted by NBRC 4572 by purification, characterization, and molecular cloning of the gene. First, condition for efficient production of BGL was investigated. The strain secreted the highest activity of BGL at static

cultivation on rice straw with limited volume of medium. The activity was 293-fold higher than shaking culture under aerobic condition. Purification of the BGL resulted in detection of two kinds of enzymes (BGL 1 and 2). The molecular masses (Ms) of these BGLs were determined around 78 kDa. Two BGLs showed similar enzyme characteristics in optimal temperature and pH of 50-55°C and 3.5-5.5, respectively. When effect of various chemicals and metal ions to the activities were investigated, Ag^{+} and Hg^{2+} inhibited BGLs 1 and 2 activities while other metal ions did not inhibit them. Glucose and gluconolactone also inhibited them. Cellobiose addition decreased BGL 2 while did not inhibit BGL 1. Moreover, the kinetic parameters of them were calculated and K_m and V_{max} values were determined. Furthermore, cloning of the genes encoding the BGLs was performed. Their amino acids sequences were determined from purified BGLs. The result of cloning of these complete genes showed 81% identify and exhibited less than 60% identify with the known family-3 BGLs. Considering the properties, BGL1 is likely to be more suitable for bioethanol production than BGL2 via simultaneous saccharification and fermentation (SSF) of rice straw with the fungus as an application of ethanol producing fungi.

Ethanol production from chitin as one of the abundant resources was successfully demonstrated in Chapter 6. First, evaluation of fermenting strain for *N*-acetyl-D-glucosamine (GlcNAc), constituent of chitin, and colloidal chitin was carried out selected from *Mucor* and *Rhisomucor* strains. Most strains were able to produce ethanol from GlucNAc and some strains were also able to produce a little ethanol from colloidal chitin. Among these strains, NBRC 4572, 6746, and 8092 achieved effective ethanol production in high yields of up to 80% to theoretical amount. This was unprecedented report on high ethanol production from GlcNAc by native microorganism. Moreover, cultivation of the three strains with colloidal chitin or chitin powder enabled direct ethanol production and also induced extracellular chitinolytic enzymes, *endo*-chitinase and GlcNAcase, activities and further enabled direct ethanol production. These enzymes were more activated or secreted at cultivation under aerobic condition than microaerobic with both substrates while no significant ethanol accumulation was unfortunately observed. Especially, the highest *endo*-chitinase activity of 22.7 U/L was detected from culture broth of NBRC 8092 with chitin powder. In order to improve ethanol production from chitin substrate by NBRC 4572, SSF was performed with increase of inoculum volume and addition of

concentrated chitinolytic enzyme from NBRC 8092. As a result, ethanol production from colloidal chitin was able to increase 1.5 times higher than the cultivation without enzyme addition. These results suggest that *Mucor* strains have potential for the SSF of chitin biomass.

As application of SSF, chapter 7 examined high temperature SSF of paper sludge (PS) with thermotolerant fungus for lactic acid production. Removal of inorganic materials and various compounds in PS, which were paper filler substances, was successfully carried out by NaOH and subsequent HCl treatment. The washed material contained higher cellulose content than that of raw PS. In order to dissolve inefficient process on SSF at low temperature caused by difference of optimum temperature between cellulase and microorganism, thermotolerant strain was screened among *Rhizopus* sp. that can produce lactic acid. *R.oryzae* NBRC 5384 was selected as the best strain for the purpose, which was able to grow up to 50°C and produce lactic acid of about 80 g/L from 120 g/L glucose. Cellulase cocktail for SSF of the treated PS was constructed by DOE method as described in chapter 3 and the cocktail was consisted by Cellulase Onozuka, Accellerase, and Pectinase G. When hydrolysis of the PS by the cocktail was performed at 28 and 40°C, yield of fermentable sugar was increased with temperature rising because cellulases were activated at high temperature near optimum condition. Finally, high temperature SSF of the PS with the cocktail and the selected strain produced lactic acid of 9.3 g/L for 96 h. Although PS was complicated material which containing various reagents and difficult to convert useful substances, treatment of PS for removal of unwanted parts and thermotolerant strain induced achievement of lactic acid production by high temperature SSF.

Abundant waste biomass having a possibility of substrate for bioconversion of not only lignocellulose but also other organic carbon materials exists abundantly on the earth. Many researches have been carried out approaches to bioconversion of them. However, complex structures of biomasses make pretreatment and scale up difficult. One bioconversion system with a pretreatment and hydrolase suitable for a biomass could not generally apply another. Respective biomasses require the own system and optimization every process. Therefore, rather than developing a bioprocess for individual biomass conversion, it is necessary to develop a highly applicable system. This research demonstrates a performance of selection process to apply the main rice straw into ethanol production.

Among many kinds of microorganism used for ethanol production, *Mucor* spp. was demonstrated that it possesses superior fermentation and enzyme secretion ability compare with other species. General microbes have dedicated ability such as enzyme secretion or fermentation, whereas *Mucor* can both secrete hydrolysis enzyme and produce ethanol. It can be expected to be applied to consolidated bioprocessing, which is the next generation-type fermentation bioprocess, by breeding strains that have improved enzyme secretion ability by utilizing various mutagenesis methods based on this fungus.

On the bioconversion from lignocellulosic biomass, which is applied not only to ethanol but also to the other fermentation products, SSF is a more valid process than separate hydrolysis and fermentation (SHF) because the process can be carried out in one tank batch. SHF can achieve higher yields of fermentation products than SSF since it is started with high initial fermentable sugars of concentrated hydrolysate. However, SSF require only one tank and can progress production of ethanol and lactic acid effectively because it can avoid product inhibition against hydrolases by fermentable sugars as fermentation microorganisms consume sugars formed by hydrolases at once. Moreover, the application of thermotolerant fermenting microorganisms for high temperature SSF is effectual. Enhancement of cellulases activity by increasing the temperature can lead to a reduction of cellulase usage in SSF and therefore reduction of production cost will be accomplished. The high temperature SSF also has an advantage that the contamination from biomass can be avoided easily though lignocellulosic biomasses include a large amount of environmental microbes such as bacteria and fungi. Therefore, SSF is great potential process for bioconversion of lignocellulosic feedstock to fermentation products by thermotolerant zymogenic microorganisms.

Many researches have been examined about bioconversion of lignocellulosic biomasses to various substances for development of next-generation energy and its derivatives. They will be evolved according as increasing needs for various biomass conversions in every field. The processes described in this study about biomass pretreatment, cellulase optimization, coculture, cellulase secretion, and fermentation of complicated substrate have application possibility to any other bioconversion processes. I desire that this research will help development of sustainable biofuel and the derivatives production system.