Establishment of efficient ethanol production system from lignocellulosic biomass by using recombinants expressing *Aspergillus aculeatus* BGL I

TREEBUPACHATSAKUL Treesukon

June 2015

Contents

Chapter 1 General Introduction

1.1 Introduction

With increasing depletion of petroleum resources, bioethanol, has been considered a promising clean energy, which is widely used as a partial gasoline replacement. The second-generation of bioethanol becomes attractive alternative, which is gaining more attention worldwide because it can be produced from sustainable, renewable energy sources like lignocellulosic biomass (Lin et al. 2006, Mussatto et al. 2010, Sánchez et al. 2008). Since, lignocellulosic biomass is abundant, inexpensive and cannot be used as a food, suggesting its potential as a resource for the production of ethanol (Demain 2009, Gray et al. 2006). The utilization of bioethanol from lignocellulosic biomass can reduce greenhouse gas emission by 60-90% relative to conventional petroleum fuels by CO_2 released during the combustion of bioethanol is recycled through the photosynthesis process resulting in no net increase to CO_2 level thereby improving energy balance (Carlo et al. 2008) (Fig. 1.1).

Plants comprised primary and secondary cell wall. Primary cell walls typically contain cellulose, hemicellulose, pectin and proteins. Secondary cell wall composes of cellulose, hemicellulose and lignin and constitutes the majority of cell wall mass (Hayashi 2006, Jordan et al. 2012). Generally, lignocellulose is composed of three main fractions: cellulose (~45% of dry weight), hemicellulose (~30% of dry weight), and lignin (~25% of dry weight) (Zaldivar et al. 2001), however, the ratio of components varies on the type and source of biomass.

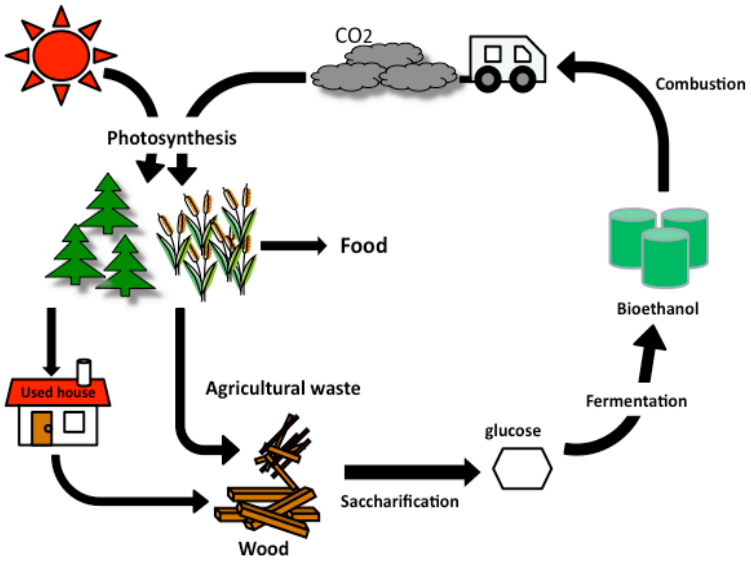


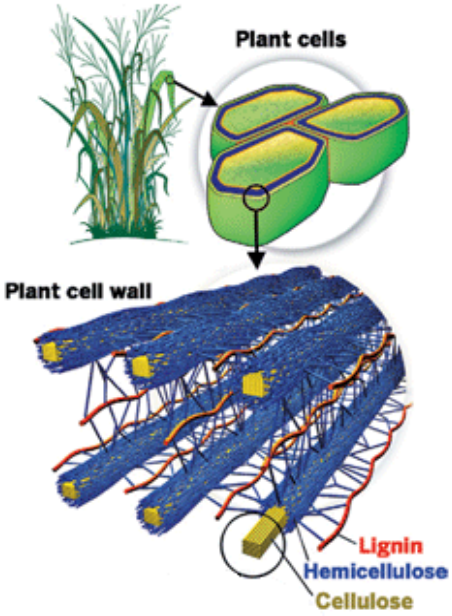
Fig. 1.1 Bioethanol production from biomass with carbon dioxide cycle

The conversion of lignocelluosic biomass into ethanol involves three main steps; (i) pretreatment of the biomass to alters the lignocellulose structure to increase the enzyme accessibility and enhance the digestibility of cellulose (Alvira et al. 2010, Hendriks et al. 2009) (ii) saccharification and (iii) fermentation of glucose and sugars to ethanol (Sun et al. 2002). The economic ethanol production from lignocellulosic biomass has been considered. The important factor for the cost effective production of ethanol from lignocellulosic materials is the high yield, high rate fermentation of biomass hydrolysates to ethanol (van Maris et al. 2006). The cost of enzyme involved in the conversion of the cellulose component into fermentable sugars or enzymatic hydrolysis is one of the major limitations of economic ethanol production from cellulosic biomass. It is necessary to improve yields and productivity of the enzyme to make to process economically attractive. Achieving rapid and complete enzymatic hydrolysis of lignocellulosic biomass at low protein loadings continues to be a major technical challenge in the commercialization of cellulose-based process converting biomass to ethanol (Acebal et al. 1986, Arantas et al. 2001, Gray et al. 2006, Wang et al. 2012).

1.2 Lignocellulosic materials

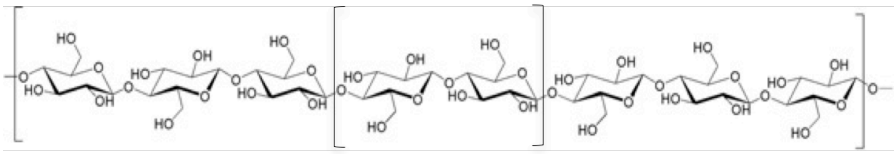
Lignocellulose is the most abundant organic material on earth, which mostly composed in plant cell wall. Lignocellulose constitutes of cellulose, hemicellulose and lignin (Fig. 1.2). Cellulose is the major constituent of lignocellulose Cellulose is a linear polymer of D-glucose molecule linked by β−(1,4)−glycosidic bond, where adjacent D-glucoses are flipped making cellobiose the fundamental repeating units (Fig. 1.3). The linear flat polymer allows for extensive hydrogen bonding within and between chains, as well as van der Waals stacking interactions between chain, making it has a strong tendency to form highly crystalline fibres (Hayashi 2006, Jordan et al. 2012, Kroon-Batenburg et al. 1997). The microfibrils of cellulose are insoluble cable-like structures that are typically composed of about 36 hydrogen-bonded glucan chains. Cellulose can exist in several crystalline polymorphs. However, it also has amorphous or soluble regions, in which the molecular are less compact and more easily hydrolyzed by cellulases (Zhao et al. 2012). Cellulose is the key substance in lignocellulose because glucose derived from cellulose by enzymatic degradation can be the substrate of microbial fermentation to produce ethanol.

Hemicellulose is the second most abundant polysaccharide in nature after cellulose. Hemicellulose is a diverse group of short-chain branched substituted polymer of sugars with a degree of polymerization ~70 to 200, and is usually characterized as the heterogeneous polysaccharides that bonds to the surface of cellulose microfibrils, and contributes to strengthening the cell wall by interaction with cellulose and, in some walls, with lignin. Hemicellulose polysaccharides are shorter than those of cellulose and they are often branched, with short chains containing other sugars, acetyl group and phenolic groups.



Stephen K. Ritter, 2008

Fig. 1.2 Lignocellulose



Jordan et al. 2012

Fig. 1.3 Cellulose chain

Hemicellulose structure and their compositions vary widely with plant species and tissue type and differ in type of glycosidic linkages, side-chain composition, and degree of polymerization (Scheller et al. 2010, Zhao et al. 2012). Classes of hemicellulose are named according to the main

sugar unit. Hemicelluloses mainly comprise xylans, which are degraded by xylanolytic enzymes (Polizeli et al. 2005). The principle hemicellulose in hardwoods is an *O*-acetyl-4-*O*-methylglucoronoxylans. A backbone of β-1,4-linked D-xylose that may include arabinose and glucuronic acid side chains. However, the main hemicellulose of softwoods is an *O*-acetyl-galactoglucomannan. A backbone of randomly arranged mannose and glucose units linked by β-1,4-glycosidic bond with α-1,6-glycosidic bond linked galactose branches attached to mannose backbone. In grasses, the main hemicellulose is glucuronoarabinoxylans, which are similar to hardwood but the amount of L-arabinose is higher (Fig. 1.4). Glucuronoxylans and glucuronoarabinoxylana are located mainly in the secondary wall and function as an adhesive by forming covalent and non-covalent bonds with lignin, cellulose and other polymers essential to the integrity of the cell wall (Hayashi 2006, Polizeli et al. 2005).

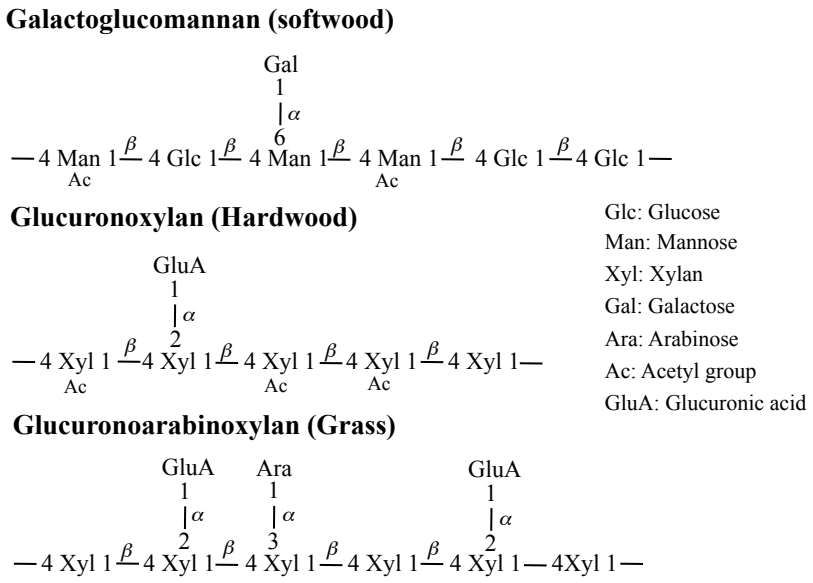


Fig. 1.4 Hemicellulose structures

Lignin is abundant substance composed of aromatic moieties in nature. As an organic substance binding the cells, fibers, and vessels, lignin has an important role in protecting the plants to impermeability and resistance against invasion by pathogens and insects. Lignin is an amorphous heteropolymer consisting of three different phenylpropane units; *p*-hydroxyphenyls-, gluaicyls-, and syringyl-phenylpropanoid, which vary between species and cell tissue type. The amorphous heteropolymer is also non-water soluble and optically inactive; all this makes the degradation of lignin very tough (Hendriks and Zeeman 2009, Jordan et al. 2012, Zhao et al. 2012).

Japanese cedar belongs to soft wood type, which presents in wood waste. It is regarded as one of the most promising substrates for the production of bioethanol. The hemicellulose contains in Japanese cedar is glucomannan and galactoglucomannan. In this wood, xylose units are scarce compared to hardwood or agricultural waste (Galbe and Zacchi 2002, Stenberg et al. 2000, Timell and Syracuse 1967).

The enzymatic hydrolysis of cellulose involves the adsorption of cellulases onto cellulose surface. Lignocellulose structural features are the main factors limiting the enzymatic hydrolysis of lignocellulose. Several structural factors have been found to affect the enzymatic digestibility of biomass, which include contents of lignin, hemicelluloses, acetyl group and cell wall proteins, cellulose crystallinity, degree of polymerization, porosity, accessible surface, particle size, cell wall thickness (Alvira et al. 2009, Sun et al, 2002, Zhao et al. 2012). Therefore, the pretreatment is a necessary step to alter some structural characteristics of lignocellulose, increasing glucan and xylan accessibility to the enzymatic attack.

1.3 Pretreatment of lignocellulosic materials

Woody is fairly resistant to enzymatic hydrolysis and different approaches have been tried to increase the efficiency of this step. Several pretreatment technologies for lignocellulosic materials such as physical pretreatment, chemical pretreatment, biological pretreatment, have been reported and widely studied to improve sugars conversion and ethanol productivity (Alvira et al. 2009, Hendriks and Zeeman 2009, Sun et al, 2002, Zheng et al. 2014). The difference pretreatment methods resulted in different lignocellulosic biomass compositions and structures after pretreatment and that these changed significantly influence the ethanol yield from lignocellulosic biomass (Liu et al 2010). Removal of lignin and hemicellulose, reduction of cellulose crystallinity, degree of polymerization and increase of porosity are the main substrate-related factors affecting the enzymatic hydrolysis. The pretreatment process can make the lignocellulose accessible to enzymatic reactions, which significantly improve the hydrolysis.

The presence of lignin and hemicellulose difficults the access of cellulase enzymes to cellulose, thus reducing the efficiency of hydrolysis (Manfield et al. 1999). Lignin has a great impact on the enzymatic hydrolysis but also, as one of the main components in lignocellulosic materials. Lignin limits the rate of enzymatic hydrolysis by acting as a physical barrier, preventing the digestible parts of the substrate. Besides, lignin appears to reduce cellulose hydrolysis by non-productively binding cellulolytic enzymes. Different strategies have been studied recently to overcome to non-productive adsorption of cellulase to lignin such as alkaline extraction and addition of protein (e.g. BSA) or other additive (e.g. polyethylene glycol, Tween) (Alvira et al. 2010, Börjesson et al. 2007, Pan et al. 2005).

Steam explosion is the most widely employed physico-chemical pretreatment for

lignocellulosic biomass. It is hydrothermal pretreatment, which uses high temperature and pressure, followed by sudden release to separate individual fibers with in wood. This pretreatment combines mechanical forces and chemical effects due to the hydrolysis of acetyl groups present in hemicellulose. Hydrolysis takes place when high temperatures promote the formation of acetic acid from acetyl groups; furthermore, water can also act as an acid at high temperature. The mechanical effects are caused because the pressure is suddenly reduced and fibers are separated owning to the explosive decompression. Most of hemicellulose is hydrolyzed and removed during steam explosion, contributing to explosion of cellulose surface and increases enzyme accessibility to the cellulose microfibrils. However, the steam explosion process does not result in net delignification (Alvira et al. 2010, Pan et al. 2005). Steam explosion is one of the most intensive studied pretreatment methods for bioconversion of softwood materials (Galbe and Zacchi 2002). However, steam pretreatment includes a risk on production of compounds, like furfural, HMF, and soluble phenolic compounds. These compounds are inhibiting the ethanol fermentation (Hendriks and Zeeman 2009).

Liquid hot water pretreatment is used instead of steam. The objective of the liquid hot water is to solubilize mainly the hemicellulose to make to cellulose better accessible and to avoid the formation of inhibitors. Liquid hot water has the major advantage that the solubilized hemicellulose and lignin products are present in lower concentrations compared to steam pretreatment, thus the risk on degradation products like furfural and the condensation and precipitation of lignin compound is reduced (Hendriks and Zeeman 2009).

Alkaline pretreatments increase cellulose digestibility and are more effective for lignin solubilization, exhibiting minor cellulose and hemicellulose solubilization than acid or hydrothermal processes. Sodium, potassium, calcium and ammonium hydroxides are suitable alkaline pretreatments NaOH causes swelling, increasing the internal surface of cellulose and decreasing the degree of

polymerization and crystallinity, which provokes lignin structure disruption. $Ca(OH)_2$ also known as lime, has been widely studied. Lime pretreatment removes amorphous substances such as lignin, which increases the crystallinity index. Lime also removes acetyl groups from hemicellulose reducing steric hindrance of enzymes and enhancing cellulose digestibility (Alvira et al. 2010). There is however an important aspect of alkali pretreatment is that the biomass on itself consumes some of the alkali. Alkal extraction can also cause solubilization, redistribution and condensation of lignin and modifying cellulose structure to a form that is denser and thermodynamically more stable than the native cellulose. However, there is often a loss of hemicellulose to degradation products and solubilized lignin components often have an inhibitory effect caused by the products forms from the lignin during akaline heat pretreatment. The loss of fermentable sugars and production of inhibitory compounds make the alkaline pretreatment less attractive for the ethanol production (Hendriks and Zeeman 2009).

Converge-milling is a novel technology for lignocellulosic biomass pretreatment that is mechano-chemically prepared using high intensive ball mill. Woody biomass is rapidly converted to a fine-particle low-crystalline form by treating using this pretreatment, thus enabling much higher efficiency of unground woody biomass (Fukumura et al. 2010; Nikaido et al. 2012). Takeda et al. (2009) reported that enzymatic digestion of rice straw enhanced by pretreatment with the high intensive ball milled. The digestion ratio of cellulose in converge-milled rice straw reached to more than 80% by 10 mg of *T. reesei* cellulase preparation caused by the destruction of the physical structure of cell walls, non-crystallization of cellulose and depolymerization another polysaccharides of plant cell wall. Therefore, the high intensive ball mill is an efficient pretreatment method for effective saccharification of plant cell wall. Therefore, converge-milling appears to be an efficient method for the pretreatment of cedar wood waste, used as a substrate in this study.

1.4 *Trichodema reesei*

Filamentous fungus mesophilic, *Trichoderma reesei* is an anamorph of the fungus *Hypocrea jecorina*. The discovery of this fungus dates back to World War II in the South Pacific and was identified as a microscopic fungus *T. viride* strain QM6a. Extensive studies of this fungus were conducted, resulting in the selection of hypercellulolytic fungal mutants, *T. viride* QM9414 and MCG77. In 1977, the fungus was renamed *T. reesei* in honor to Elwin Thomas Reese, who discovered it in the 1940s (Gusakov 2011, Shuster et al. 2010). *T. reesei* was realized that it had noteworthy cellulolytic capacity (Reese 1956, Mandels and Resse 1957). *T. reesei* is an extremely efficient producer of cellulose- and hemicellulose- degrading enzymes. It has long been considered the most productive of commercial cellulolytic enzyme that have received worldwide industrial interest. The cellulase and hemicellulase enzymes produced by *T. reesei* and the respective genes have been extensively studied (Polizeli et al. 2005, Shuster et al. 2010, van der Brink and de Vries 2011).

In Japan, *T. reesei* mutants have been improved as a part of National project. One mutant, PC-3-7, the sixth generation descendant of the standard strain QM9414 was a spontaneous derivative of KDG-12, which in turn was a descendant of the natural isolate QM6a (Nitta et al. 2012). It was found to exhibited enhance cellulase productivity during growth on variety of carbon sources, and high cellulase inducibility by L-sorbose relative to its parent (Kawamori et al. 1986, Nitta et al. 2012) (Fig. 1.5 and 1.6). Due to *T. reesei* PC-3-7 has high capable cellulase and hemicellulase production (Xu et al. 1998), PC-3-7 mutant has been used as a model strain in our laboratory and used for secreting enzyme protein for conversion of cellulosic biomass into ethanol in this study.

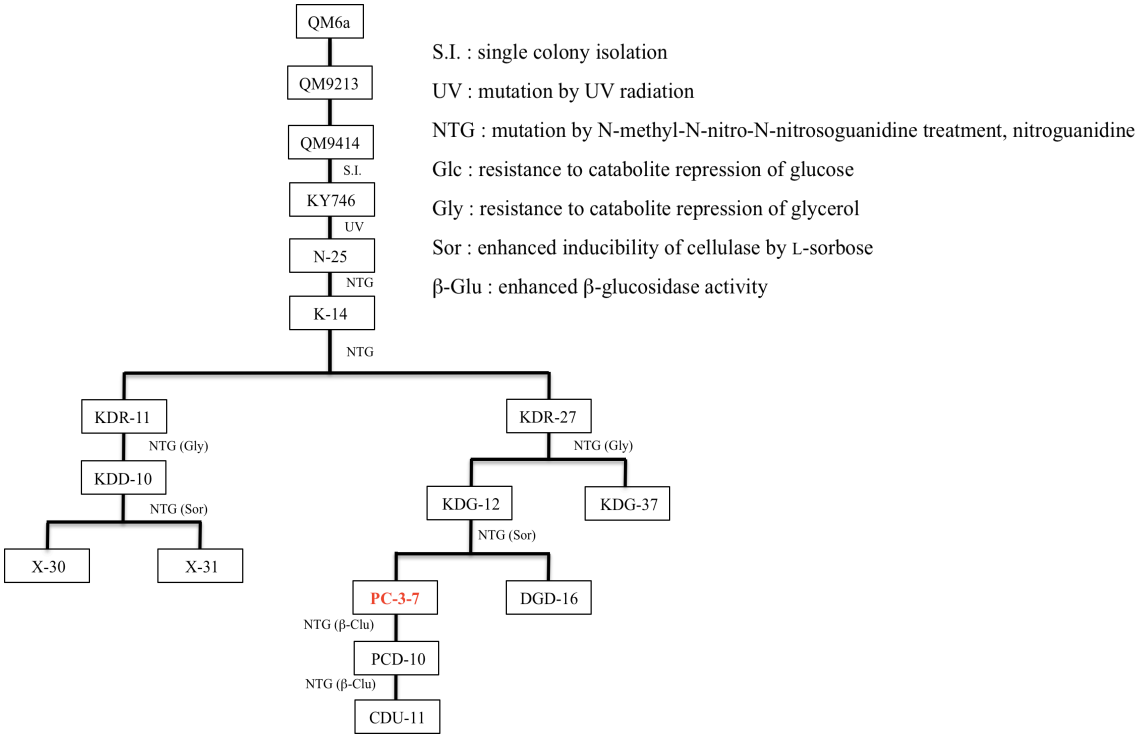


Fig. 1.5 Genealogy of *T. reesei* mutant strains developed in Japan.

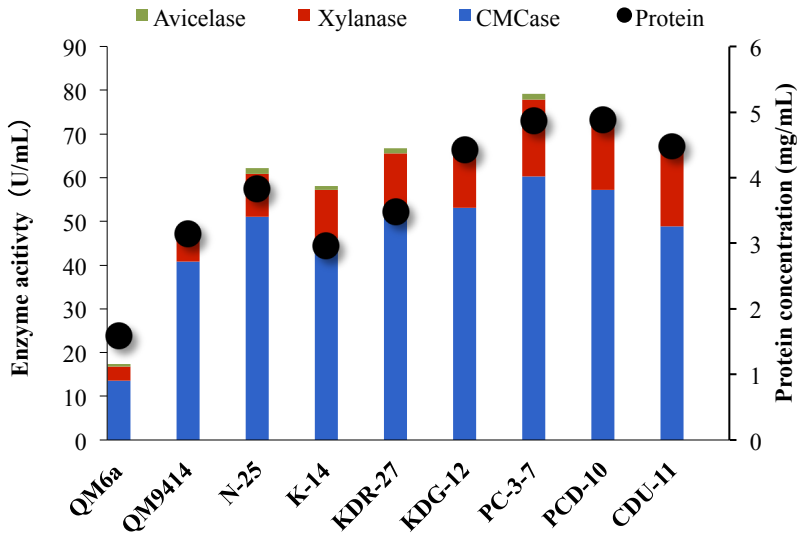


Fig. 1.6 Enzyme activity and protein production of *T. reesei* mutant strains

1.4.1 *Trichoderma reesei* cellulases

T. reesei is well known as a cellulolytic organism capable of secreting a large amount of cellulolytic enzymes (Shuster et al. 2010). *T. reesei* secreted cellulases include endo-β-1,4-glucanases (EG, EC 3.2.1.4), cellobiohydrolase (CBH, EC 3.2.1.91) and β-glucosidases (BGL, EC 3.2.1.21) (Gusakov 2011). Most of the fungal hydrolases are modular proteins, which contain a large catalytic domain (CD) and a small, distinct carbohydrate binding modules (CBM) joined by flexible peptide linker. CBM also form sequence based families and are numbered with arobic numerals (Bourne et al. 2001). Using extensive sequence comparison by amino acid sequence homology, CD of glycoside hydrolases (GHs) are classified in families as described in http://www.cazy.org/Glycoside-Hydrolases.html. CBM were previously classified as cellulose-binding domains (CBDs), based on the initial discovery of modules that bound cellulose (http://www.cazy.org/Carbohydrate-Binding-Modules.html). Although ten cellulases (two cellobiohydrolases and eight endoglucanases) belonging to different glycoside hydrolase families have been identified in the *T. reesei* genome (Table 1.1). Only four major cellulases are usually secreted in notable quantities, CBH I (Cel7A), CBH II (Cel6A), EG I (Cel7B) and EG II (Cel5A), in a proportion of 60:20:10:10, which can make up to 90-95% of the enzyme cocktail secreted by *T. reesei*. The major protein secretion capacity of *T. reesei* are CBH I making up to 50-60% and CBH II ~20% of the total secreted protein. The β-glucosidase typically makes up less than 1% (Gusakov 2011, Margeot et al 2009). Minor amounts of EG III (Cel12A) and EG V (Cel45A) are also detectable in enzyme preparations produced by some *T. reesei* strains. All the mentioned cellulases, except for EG III (Cel12A), have a two domains structure of catalytic domain (CD) and a cellulose-binding domain (CBD) connected with a flexible peptide linker. The CBD effects enzyme binding to the hydrophobic surface of crystalline cellulose by destabilizing the hydrogen bond structure of cellulose, and

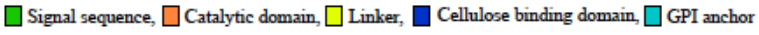
cellulases possessing the CBD display much higher activity on crystalline cellulose than do those lacking this module (Gusakov 2011, Medve et al. 1998, Mello et al. 2014, Merino et al. 2007, Sugimoto et al., Tomme et al. 1988). Although, EG IV (Cel61A) and EG VII (Cel61B) have been names endoglucanases, the question whether these proteins possess a hydrolytic is still unresolved. Nevertheless, it has been reported that glycoside hydrolase family 61 (GH61) proteins lack measurable hydrolytic activity by themselves but in some proteins of the GH61 family could act as enhancers of cellulase activity on lignocellulosic substrates (Gusakov 2011, Harris et al. 2010, Merino et al. 2007).

T. reesei has 10 putative *bgl* genes (*cel1a-b*, *cel3a-h*), of which the ones encoding BGL I (Cel3A) and II (Cel1A) have already been cloned, heterologously expressed and enzymatically characterized (Christopher et al. 1991, Chen et al. 1992, Cummings et al. 1996, Inglin et al. 1980, Saloheimo et al. 2002, Takashima et al. 1998). Of these, Cel3A (BGL I) is the main extracellular β-glucosidase induced by cellulose, with a specific activity of 23 U/mg toward cellobiose (Chen et al. 1992). The enzyme, when expressed in *S. cerevisiae*, undergoes N-glycosylation, resulting in a size-increase of 10-15 kDa (Cummings and Fowler 1996). Cel1A (BGL II) is an intracellular β-glucosidase (Inglin et al. 1980 and Saloheimo et al. 2002) and, compared to the other β-glucosidases of *T. reesei* QM6a, its gene is preferentially transcribed when sophorose is used as a cellulase inducer (Foreman et al. 2003). Five *bgl* genes (*cel1b*, *cel3b-e*) have been identified by EST analysis (Foreman et al. 2003) in the *T. reesei* QM6a genome by phylogenetic analysis (Martinez et al. 2008). Based on the deduced protein sequences, including predicted signal peptides, the genes *cel3a*, *b* and *e-g* encode secreted enzyme, while *cel1a*, *b* and *cel3c*, *d* and *h* code for intracellular proteins. Thus only two out of the ten *bgl* genes have been biochemically characterized. There is sufficient possibility to identify a β-glucosidase in *T. reesei*, that is comparable to any of the secreted

Aspergillus enzyme with a reported cellobiase specific activity of over 150-400 U/mg such as *Aspergillus aculeatus* BGL I (Dan et al. 2000, Langston et al. 2006, Riou et al. 1998, Sakamoto et al. 1985)

Table 1.1 Cellulases and β-glucosidases from *T. reesei*

Enzyme	GHF	Domain structure	Calculated M (kDa)	*T. reesei* M (kDa)
Cellobiohydrolases				
CBH I (Cel7A)	7		58	68
CBH II (Cel6A)	6		47	58
Endoglucanases				
EG I (Cel7B)	7		46	54
EG II (Cel5A)	5		42	50
EG III (Cel12A)	12		5	25
EG IV (Cel61A)	61		34	56
EG V (Cel45A)	45		23	36
EG VI (Cel74A)	74		85	85-105
EG VII (Cel61B)	61		25	
EG VIII (Cel5B)	5		45	
β-glucosidase				
BGL I (Cel3A)	3		75	71-76
BGL II (Cel1A)	1		52	
Cel1B	3		55	
Cel3B	3		92	
Cel3C	3		91	
Cel3D	3		77	
Cel3E	3		83	
Cel3F	3		95	
Cel3G	3		84	
Cel3H	3		125	



1.4.2 *Trichoderma reesei* hemicellulases

T. reesei produces high levels of both xylan- and cellulose-degrading enzymes when cultures with native substrates containing both cellulose and xylan or cellulose alone (Xu et al. 1998). Recently, the number of genes encoding enzyme hydrolysing hemicelluloses have been isolated from *T. reesei* and the enzymatic properties of the corresponding enzymes have been analyzed. The putative genes involve in hemicellulose degradation of *T. reesei* based on the CAZy database (http://www.cazy.org) was shown in Table 1.2 (van den Brink and Vries de Ronald 2011). The main enzymes degrading xylans are at least four characterized endo-β-1,4-D-xylanases. There are the *xyn1* and *xyn2* genes encoding xylanase, XYN I and II (EC 3.2.1.8) belong to the glycoside hydrolase (GH) families 11, *xyn3* encoding the third xylanase, XYN III has been classified in GH family 10 and the xyn4 gene encoding XYN IV was found not to belong to the two major xylanase families, but to GH family 30, which is a very diverse family including glucanases, mannanases and xylanases (Tenkanen et al. 2013). The two main xylanases, XYN I and XYN II have molecular masses of 19 and 20 kDa whereas the XYN III is larger with a molecular mass of 32 kDa (Tenkanen et al. 2013). However, XYN III was produced in quantity in *T. reesei* PC-3-7, whereas little or no XYN III was found in *T. reesei* QM9414 when Aicel or lactose was used as a carbon source (Herrmann et al. 1997, Margolles Clark et al. 1996abcd, Margolles Clark et al. 1997, Saarelainen et al. 1993, Tenkanen et al. 2013, Torronen and Rouvinen 1997, Xu et al. 1998). The *bxl1* gene encoding β-xylosidase (EC 3.2.1.37) mainly hydrolyses xylo-oligosaccharides. Most fungal β-xylosidases belong to GH family 3. The *man1* encoding β-mannanase (EC 3.2.1.78) hydrolyses the glucomannan backbone. Genes isolated encoding the side group cleaving enzymes include the acetyl xylan esterase (EC 3.1.1.72) (*axe1*) which cleaves acetyl groups from polymeric xylans and xylan oligomers, the α-glucuronidase (EC 3.2.1.139) which cleaves glucuronic acid mainly from arabinoglucuronoxylan oligomers, and the

α-L-arabinofuranosidase (EC 3.2.1.55) (*abf1*) which cleaves arabinose from arabinoglucuronoxylans. Also genes encoding three α-galactosidases have been characterized; *agl1* encodes an enzyme which release galactose side groups from polymeric galactoglucomannans and their oligomers, and *agl2* and *agl3* significantly only the action of β-mannanase (Polizeli et al. 2005, van den Brink and Vries de Ronald 2011).

Table 1.2 Number of putative genes involved hemicellulases of *T. reesei*

Total putative genes involved in hemicellulose degradation				***Trichoderma reesei***
Substrate	**Enzyme activity**	**Code**	**CAZy family**	
Xyloglucan	Xyloglucan b-1,4-endoglucanase	XEG	GH12,74	3
	α-arabinofuranosidase	ABF	GH51,54	2
	α-xylosidase	AXL	GH31	4
	α-fucosidase	AFC	GH29,95	4
	α-1,4-galactosidase	AGL	GH27,36	10
	β-1,4-galactosidase	LAC	GH2,35	8
Xylan	β-1,4-endoxylanase	XLN	GH10,11	5
	β-1,4-xylosidase	BXL	GH3,43	15
	α-arabinofuranosidase	ABF	GH51 54	2
	Arabinoxylan arabinofuranohydrolase	AXH	GH62	1
	α-glucuronidase	AGU	GH67,115	2
	α-1,4-galactosidase	AGL	GH27,36	10
	β-1,4-galactosidase	LAC	GH2,35	8
	Acetyl xylan esterase	AXE	CE1	3
Galactomannan	β-1,4-endomannanase	MAN	GH5,26	8
	β-1,4-mannosidase	MND	GH2	7
	β-1,4-galactosidase	LAC	GH2,35	10
	α-1,4-galactosidase	AGL	GH27,36	8

1.5 Enzymatic hydrolysis (Saccharification)

1.5.1 Cellulose degradation

T. reesei cellulases act synergistically to hydrolyze cellulose to glucose effectively (Fig. 1.7). CBHs have a higher reactivity on crystalline cellulose than EGs. Both CBHs and EGs digest long polysaccharide chains into smaller fragments, thereby reducing degree of polymerization of cellulose (Zhang et al. 2004). In the synergic model of cellulose degradation, multiple enzymes are involved, of which endoglucanases (EGs) fist randomly hydrolyze the internal bond of amorphous regions of cellulose, providing new chain ends preparation for cellobiohydrolases (CBHs) degradation. The cellobiohydrolases, in turn hydrolyze both the amorphous and crystalline cellulose progressive from the ends of cellulose chains to liberate mainly cellobiose. β-glucosidases (BGLs) hydrolyze cellooligosaccharides and cellobiose to produce glucose in the aqueous phase (Percival et al. 2006). CBH I cleaves cellobiose from reducing end and CBH II from non-reducing end of cellulose chains. The complementary activities of cellulases lead to synergy, i.e. enhancement of activity over the added activities of the individual enzymes (Benerjee et al. 2010, Miettinen-Oinonen et al. 2005, Wang et al. 2012). However, it has reported that the product in enzymatic hydrolysis of cellulose, cellobiose can strongly inhibit hydrolysis reaction of cellulases, especially CBH I while CBH II and EG I display less inhibition by cellobiose (Du et al. 2010). Nevertheless, cellobiose inhibitor has no effect on the adsorption of cellulase on cellulose surface. The inhibition mechanism was found that the binding of cellobiose to the active site of CBH I prevents further reactions, and that the binding of cellobiose induces conformational change in CBH I, thereby decreasing its activity (Yue et al. 2004). However, added β-glucosidase effectively decreased or even eliminates the cellobiose inhibition (Berlin et al. 2005). Thus, β-glucosidase activity controls the overall rate of cellulose hydrolysis, therefore, it is

considered as an important reaction for the efficient conversion of cellulose to glucose.

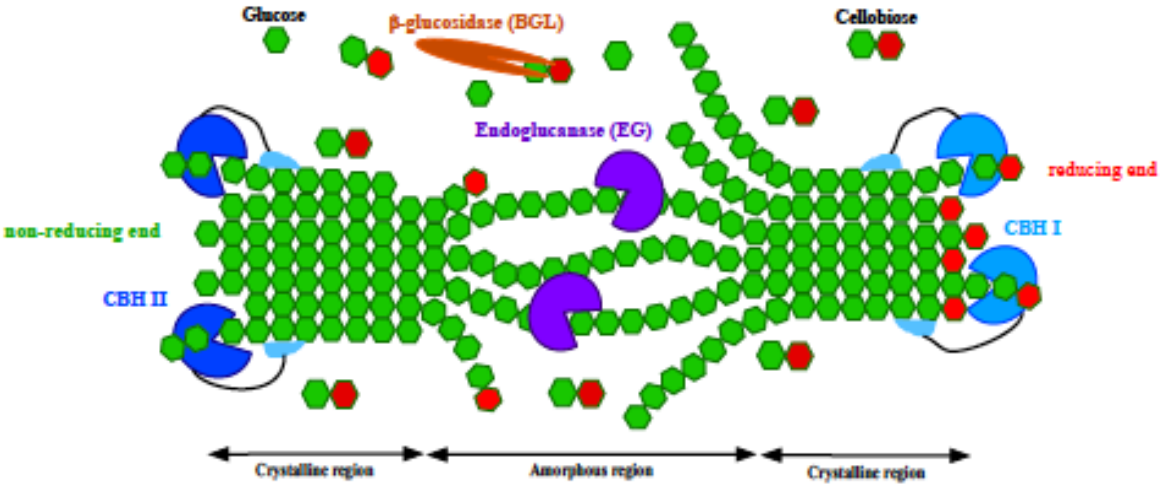


Fig. 1.7 Putative model of cellulose degradation by the synergistic action of cellulases

T. reesei has been reported to produce extracellular and intracellular β-glucosidase as mentioned above (1.4). The gene *bgl1*, encoding an extracellular β-glucosidase has been isolated from *T. reesei*. The BGL I enzyme is a major extracellular activity whereas BGL II is an intracellular β-glucosidase enzyme (Saloheimo et al. 2002). The endogenous extracellular β-glucosidases secreted by *T. reesei* have extremely low activity. Moreover, it is inhibited by its end-product glucose, leading to the retardation of cellulose hydrolysis with an insufficient conversion into ethanol as the result (Lee et al. 2013, Xiao et al. 2004).

1.5.2 Hemicellulose degradation

The complex structure of hemicellulose has led to a multiplicity of hemicellulases being found in *T. reesei* and other microorganisms (Polizeli et al. 2005, van den Brink and Vries de Ronald 2011). The enzymatic complete degradation of xylan is not requires only the action of xylanase but also the other hemicellulases that remove side-chains from the xylan backbone. Hydrolysis of the xylan backbone involves endoxylanases and β-xylosidase. Endoxylanases cleave the glycosidic bonds in the xylan backbone, bringing about a reduction in the degree of polymerization of the substrate (Fig. 1.8a). Xylan is not attacked randomly, but the bonds selected for hydrolysis depend on the nature of the substrate molecule, i.e. on the chain length, the degree of branching, and the presence of substituents. The released xylo-oligisaccharides are degraded by β-D-xylosidase. Acetylxylan esterase removes the *O*-acetyl groups from position 2 and/or 3 on the β-D-xylopyranosyl residues of acetyl xylan. Acetylxylan plays an important role in the hydrolysis of xylan, since the acetyl side-groups can interfere with the approach of enzymes that cleave the backbone, by steric hindrance, and their elimination thus facilitates the action of endoxylanases. α-glucuronidase hydrolyzed the α-1,2 bonds between the glucuronic acid residues and β-D-xylopyranosyl backbone units found in glucuronoxylan (Poliseli et al. 2005). L-arabinose is cleaved from arabinose-substituted xyloglucan and arabinoxlan by α-L-arabinofuranosidase and arabinoxylan arabinofuranohydrolases. Alpha-linked D-galactose residues are released from hemicellulose, xylan and galactoglucomannans by α-galactosidases. The major softwood hemicellulose is an *O*-acetyl-galactoglucomannan (15-20% of wood) with various structure variations (Timell and Syracuse 1967). Hydrolysis of manna-type polysaccharides by mannanases yield an array of oligomers, including the β-D-manno-oligosaccharides and a range of mixed oligosaccharides containing D-mannose, D-glucose and D-galactose. However, the patterns of enzyme action are differences. For example, the mannanase from *T. reesei* was able to hydrolyse

galactoglucomannan in pine kraft pulp whereas the mannanase from *Bacillus subtilis* was not (Tenkanen et al. 1997).

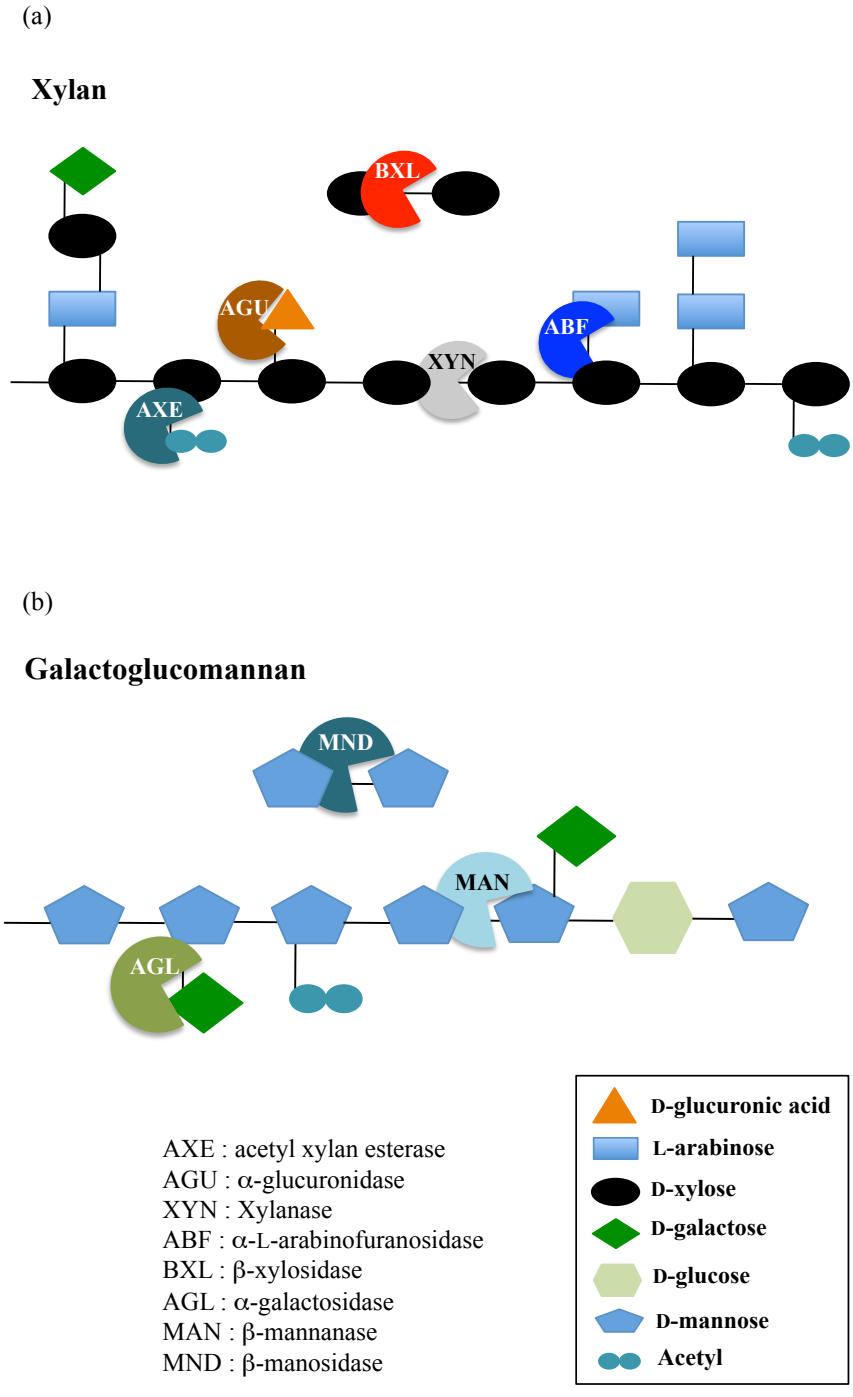


Fig. 1.8 Putative model of hemicellulose degradation by *T. reesei* hemicellulases of (a) xylan degradation and (b) galactoglucomannan degradation

1.6 Strategies to enhance the capability of cellulose conversion

The retardation of insufficient β-glucosidase activity secreted by *T. reesei* resulted in inefficient cellulose conversion thus the supplementation of β-glucosidase from other source is required. Addition of exogenous BGL is one of strategy to alleviate the limitation of cellulose hydrolysis and improve cellulose conversion yield. Berlin et al. (2007) has also reported that addition of further exogenous β-glucosidase of Novozyme 188 to *T. reesei* celluclase 1.5L could improve glucose yield. However, this approach result increased the cost of enzyme preparation. Therefore, the genetic gene engineering for increased β-glucosidase activity has been widely considered (Kawai et al. 2012, Lee et al. 2013, Nakazawa et al. 2011, Shen et al. 2008, Tang et al 2013, Wilde et al. 2012).

Recently, many microbial β-glucosidases have been heterologous expressed in *S. cerevisiae* since cellobiose is one of the major end products of *T. reesei* cellulase, acts as a strongly inhibitor, especially for cellobiohydrolase. Therefore, β-glucosidase is not only critical to catalyze glucose generation, but is also required to remove cellobiose inhibition of other enzymatic hydrolysis. Removal of the inhibitory cellobiose can be resolved by the addition of exogenous β-glucosidase or the construction of fermentation strain harboring high β-glucosidase activity conducted in simultaneous saccharification and fermentation (SSF) process (Lee et al. 2013, Tang et al. 2013). The β-glucosidases from *Aspergillus aculeatus* and *Aspergillus niger* have been often used for the supplementation of *T. reesei* cellulases (Chauve et al. 2010, Fujita et al. 2002, Yanase et al. 2010). *A. aculeatus* BGL I (AaBGL1) belongs to the glycoside hydrolase family 3 (GH3) has been reported that AaBGL1 was potently active not only on soluble cellooligosaccharides, such as cellotriose to cellohexose, but also on insoluble cellooligosaccharides, of which the average degree of polymerization was 20 (Sakamoto et al. 1985, Takada et al. 1998). AaBGL1 shows specific activity

against cellobiose, 3.3-9.5 times higher that that of *T. reesei* BGLs. Moreover, AaBGL1 was less sensitive to glucose inhibition than *T. reesei* BGL1. Consequently, AaBGL1 has significant advantages for used in biomass saccharification (Nakazawa et al. 2011).

A current goal in the biofuel industry is to generate microorganism that can degrade cellulose and convert the released sugars into alcohol without the need to add cellulase enzymes. The introducing cellulase gene in noncellulolytic organism that exhibits high product yield and enabling ethanol fermentability of *S. cerevisiae* is a favorable approach strategy (Fitzpatrick et al. 2014, Lynd et al. 2005, van Rooyen et al. 2005, Song et al. 2010). The advantages of *S. cerevisiae* include their high ethanol productivity and tolerance, a larger cells size simplifying their separation from the culture broth and resistance to viral infection. Although *S. cerevisiae* is a good choice for ethanol fermentation, it has also few shortcomings, such as inability to degrade polysaccharides since cellobiose and longer chain oligosaccharides is the major products of cellulose hydrolysis (van Rooyen et al. 2005). Various cellulase and β-glucosidase genes such as from *T. reesei*, *Saccharomycopsis fibuligera*, *Aspergillus aculeatus* or from other bacterial sources have been expressed in yeasts either individually and in various combinations with the aim of direct ethanol production from cellulose for low-cost ethanol production process of consolidated bioprocessing (CBP) (Cumming and Fowler 1996, Fitzpatrick et al. 2014, Fujita Y et al. 2002, Fujita et al. 2004, Tang et al. 2013). The genes have been expressed from different promoters and secretion of the proteins from the cell has been directed by secretory signals from heterologous genes (Fitzpatrick et al. 2014, Tang et al. 2013, van Rooyen et al. 2005). Fujita et al. (2004) has constructed the cellulose-degrading yeast strain by genetically codisplaying three cellulolytic enzymes, *T. reesei* endoglucanase II and cellobiohydrolase II and β-glucosidase 1 from *A. aculeatus* on the cell surface of *S. cereivisae* by using a cell surface engineering system. Their engineered yeast cells could directly

conversion of amorphous cellulose (phosphoric acid-swollen cellulose) to produce ethanol. Although, *S. cerevisiae* can be easily engineered by expressing heterologous β-glucosidase with the ability of utilizing cellobiose, but its effects are still limited from the lower utilizing rate of cellobiose as *S. cerevisiae* does not possess an active transporter for cellobiose. Therefore, the co-expressing cellodextrin transporter and β-glucosidase have been constructed and considered as an efficient direct ethanol production from cellulose (Guo et al. 2011, Yamada et al. 2013).

T. reesei has also been attempted to expressing many BGL targets but cellulase inducible promoter used for constructed recombinant *T. reesei* strain is restricted (Rahman et al. 2009). However, Nakazawa et al. (2011) has accomplished constructed the *T. reesei* expressing *Aspergillus aculeatus* β-glucosidase I (X3AB1) with high specific activity under the control of *xyn3* promoter. The enzyme preparation (JN11) obtained from the X3AB1 strain was evaluated for its activity and saccharification ability relative to the enzyme preparation of *T. reesei* PC-3-7. The yielded of enzyme preparation (JN11) exhibited high saccharification activity at a low enzyme dose. Moreover, Kawai et al. (2012) reported that JN11 effectively saccharified various types of pretreated biomass because it includes the best balance of cellulase and hemicellulase activities.

1.7 Ethanol fermentation by *Saccharomyces cerevisiae*

For the fermentation of sugars to ethanol on an industrial scale, *Saccharomyces cerevisiae* is preferably used, as, compared to other fermentative organism. *S. cerevisiae* wild type is a hexose-sugar utilizing fermentative organism that has high ethanol productivity and tolerance to ethanol and inhibitors produced during cellulose hydrolysis (Lau et al. 2010, Palmqvist et al. 2000, Stanley et al. 2010, van Maris et al. 2006). Fermentation is a metabolic process in which fermentative organism converts sugars to alcohol. The major process for oxidizing sugars is the sequence reactions known as glycolysis. Glycolysis produced ATP (Adenosine triphosphate) without the involvement of molecular oxygen (O_2). During glycolysis, a glucose molecule is converted into two molecules of pyruvate and two molecules of ATP are hydrolyzed to provide energy to drive the early steps, but four molecules of ATP are produced in the later step. Therefore, at the end of glycolysis, there is consequently a net gain of two molecules of ATP for each glucose molecule broken down. Two molecules of NADH are formed per molecule of glucose in the course of glycolysis. In aerobic condition, these NADH molecules donate their electrons to electron-transport chain, and NAD^+ formed from the NADH is used again for glycolysis. Then the pyruvate is rapidly transported into the mitochondria, where it is converted into CO_2 and acetyl CoA, which is then completely oxidized to CO_2 and H_2O via citric acid cycle or tricarboxylic acid cycle (TCA) (Fig. 1.9). In anaerobic state (Fermentation), yeast cells convert pyruvate to ethanol and CO_2 and oxidize NADH to NAD^+. Two reactions are required. First, pyruvate is carboxylated to acetaldehyde in a reaction catalyzed by pyruvate decarboxylase. Next, alcoholdehydrogenase catalyzes the reaction of acetaldehyde to ethanol by NADH. In this process, the NADH gives up its electrons and is converted back into NAD^+. This generation of NAD^+ is required to maintain the reactions of glycolysis (Alberts et al. 2008, Horton et

al. 2002).

1.7.1 Hexoses fermentation

Depending on the biomass feedstock, the resulting hydrolysates contain a large variety of sugars, which glucose is a notable sugar. *S. cerevisiae* contains an elaborate system for hexose transport. There are 32 members of hexose transporters (HXT) family in *S. cerevisiae*. However, they all transport glucose via facilitated diffusion, glucose uptake only requires a concentration gradient across the plasma membrane (Boles et al. 1997). After uptake, glucose assimilation proceeds via the glycolytic pathway, which oxidizes glucose to two pyruvates, resulting in the net formation of two ATP per glucose (Fig. 1.9). In anaerobic fermentation, the NADH formed by glyceraldehyde-3-phosphate dehydrogenase is reoxidized to NAD^+. This essential redox balancing involves the combined activity of pyruvate decarboxylase and alcohol dehydrogenase. However, glucose is not the only hexose sugar present in the hydrolysates but also mannose, galactose and fructose that can be fermented all by *S. cerevisiae*. Glucose, mannose and fructose are transported by all the different members of the HXT family, although K_m value of mannose and fructose were generally higher than that of glucose indicated that glucose is a preferable substrate (Nevado et al. 1993, van Maris et al. 2006). After phosphorylation by hexokinase, mannose 6-phophate is isomerized to fructose-6-phophate. Hexokinase also responsible for fructose to fructose-6-phophate, which is subsequently metabolized through glycolysis for producing pyruvate and ethanol afterward (Fig. 1.9). Galactose, another sugar that can fermented by *S. cerevisiae*, is fist taken up by dedicated member of the *HXT* family, the galactose permease Gal2p, and subsequent converted into glucose-6-phosphate by a pathway in which the galactose-1-phosphate formed exchanges with the glucose-1-phosphate

moiety of nucleotide sugar, uridine diphosphate glucose (UDP-glucose) by cleavage of pyrophosphate bond of UDP-glucose. This reaction is catalyzed by galactose-1-phosphate uridylyltransferase and produce glucose-1-phasphate and UDP-galactose. Glucose-1-phosphate can enter glycolysis after conversion to glucose-6-phosphate in a reaction catalyzed by phosphoglucomutase. UPD-galactose is recycled to UDP-glucose by the action of UPD-glucose-4-epimerase (Horton et al. 2002, Nevado et al. 1993, van Maris et al. 2006).

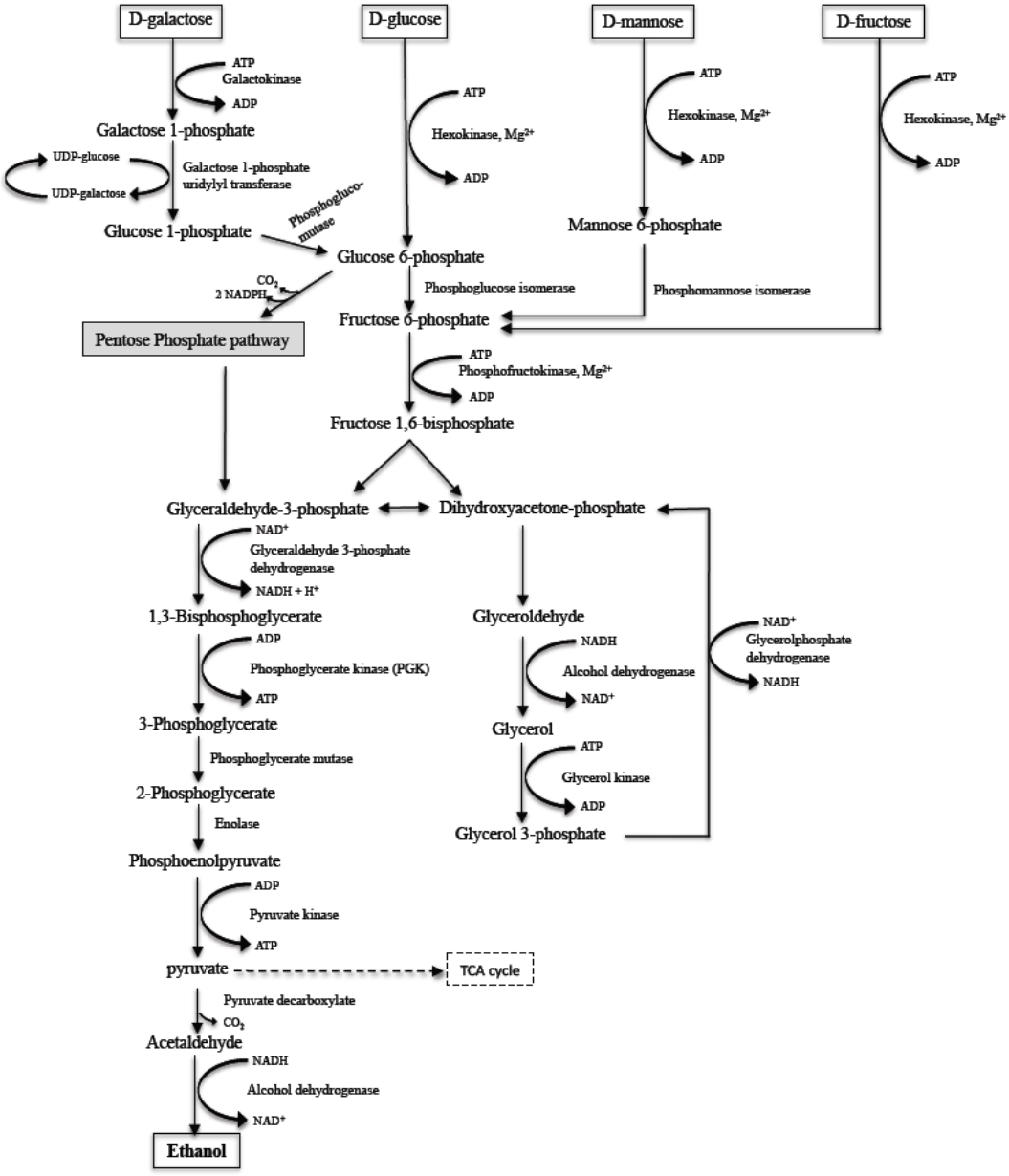


Fig. 1.9 Hexoses catabolism of *S. cerevisiae* into ethanol

Although *S. cerevisiae* has high ethanol productivity and ethanol tolerance, there are still some characteristics of *S. cerevisiae* that make problems of ethanol producing. Glucose repression is one of the main limitations in mixed lignocellulosic sugar fermentation for cost effective production of fuels and chemicals (Li et al. 2010). Glucose repression is a consequence of a complex regulatory phenomenon, when external glucose is sensed via a single transduction pathway (involving several kinases) the expression of several genes is repressed. Glucose repression influences several functions in yeast, both in the central metabolism (i.e. glyoxylatic cycle, gluconeogensis) and in the peripheral metabolism (i.e. sugar uptake and glycolysis) (Olsson and Nielsen 2000, Jan et al. 2009). The signal for glucose repression is related to the glucose concentration rather than the glucose flux, and evidence that cells have significant concentrations of intracellular glucose suggests that glucose itself could be a signaling molecule (Carlson 1999, Rolland et al. 2002).

During ethanol production (anaerobic condition), the energy for growth of yeast is provided by the glycolysis and fermentation pathway rather than the oxidative respiration pathway. However, without completely oxidizing glucose to CO_2, yeasts such as *S. cereivise* and *Schizosaccharoyces pombe* can accumulate ethanol even in the presence of oxygen, which are called Crabtree-positive yeasts, whereas those that degrade sugars to CO_2, such as *Kluyveromyces lactis* and *Candida albicans*, are designated as Crabtree-negative yeasts. Crabtree effect is alcoholic fermentation is a predominant pathway in the degradation of hexose sugars in the presence of oxygen, because of insufficient capacity or saturation or repression of the respiratory metabolism, leading to pyruvate overflow (De Deken 1966, Piskur et al. 2006). In *S. cerevisiae*, the Crabtree effect relies to a large extent, on a glucose represses respiration or under high glucose concentration (Postma et al. 1989). Indeed, after depletion of glucose and accumulation of ethanol, the metabolism in Crabtree-positive yeast changes. The fermentation product, ethanol becomes a substrate, which is

converted back to acetaldehyde if oxygen is presented (Fig. 1.10). This change in metabolism is known as the "diauxic shift". The acetaldehyde to ethanol conversion is catalyzed by alcohodehydrogenase (Adh), which can in principle catalyze the reaction in both directions. Adh activity is encoded by two genes, *ADH1* is expressed constitutively, whereas *ADH2* is expressed only when the internal sugar concentration drops. The enzyme Adh1 has an elevated K_m for ethanol, consistent with ethanol being a product of the reaction, whereas K_m of Adh2 is ten times lower, consistent with ethanol being its substrate (Piskur et al. 2006)

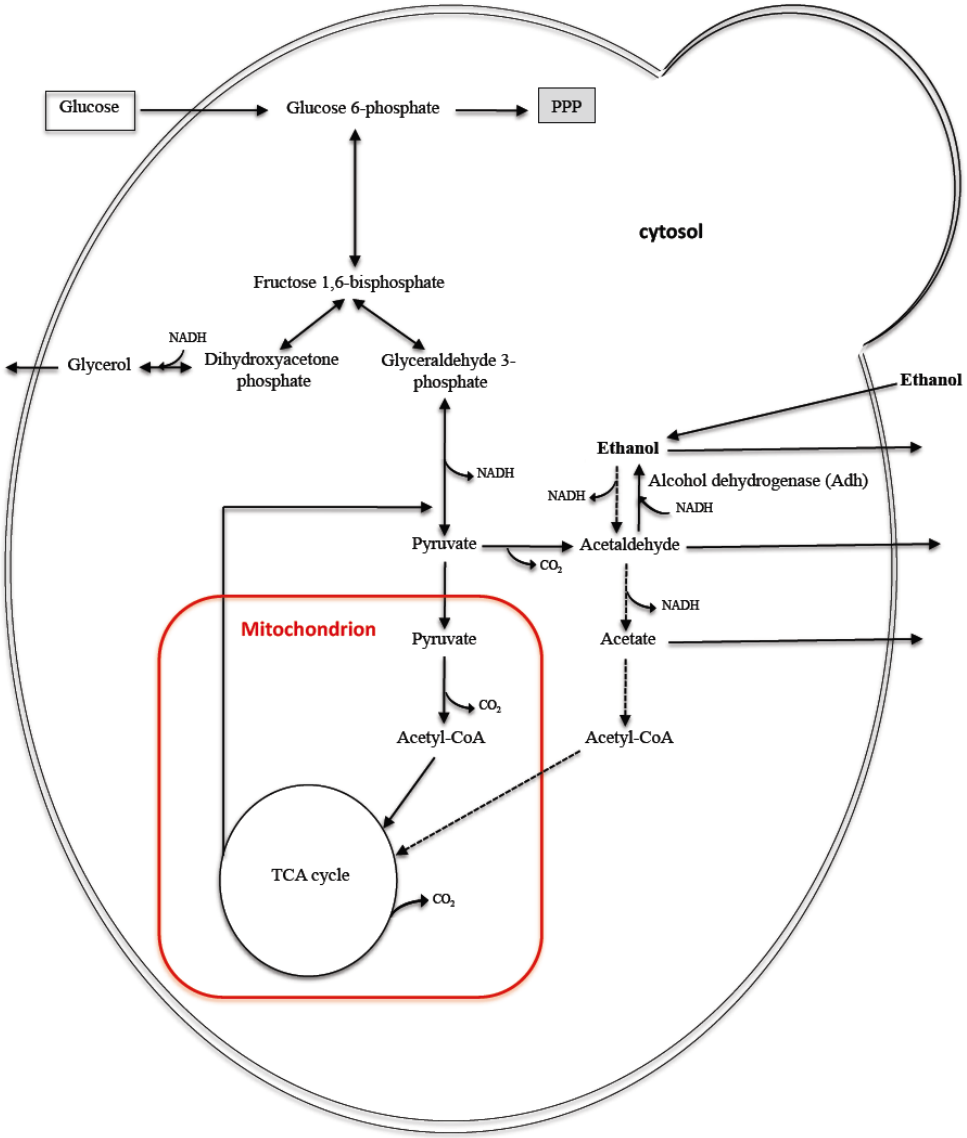


Fig. 1.10 A Schematic pathway of glucose and ethanol assimilation of *S. cerevisiae*

1.7.2 Xylose fermentation

In lignocellulose hydrolysates, the pentose sugars, especially xylose is the major monosaccharide that cannot be fermented by *S. cerevisiae* wild type. Although *S. cerevisiae* can neither ferment nor assimilate xylose, this is not a general characteristic of yeast. *S. cerevisiae* cells take up xylose with the same sugar permeases use for glucose (Lee et al. 2002). However, xylose uptake is very insufficient compared with that of glucose. Reported K_m values for xylose transport are at least 5-200 fold higher than that for glucose. *S. cerevisiae* can catabolize xylulose, but it cannot utilize xylose due to the inability to convert xylose to xylulose (Pitkänen et al. 2005). In naturally xylose utilizing fungi, D-xylose is first reduced to xylitol by D-xylose reductase (XR) that prefers NADPH over NADH. Xylitol is then oxidized to D-xylulose with NAD^+ by xylitol dehydrogenase (XDH) (Fig. 1.11). The different cofactor specificities, conversion of xylose into xylulose yields one $NADP^+$ and one NADH, lead to a serious cofactor imbalance during xylose consumption. NADPH and NAD^+ need to be regenerated in order to maintain redox balance. For NADPH can be accomplished by diverting part of the fructose 6-phosphate produced into the oxidative part of pentose phosphate pathway (PPP) (Fig. 1.12). Under aerobic conditions the NADH can be reoxidized via respiratory chain with molecular oxygen. However, under anaerobic conditions, another electron acceptor is required to reoxidize the NADH. If no electron acceptor is available, the yeast cell cannot maintain redox balance and will not ferment xylose under anaerobic condition (van Maris et al. 2006). Before entering the PPP, xylulose is phosphorylated to xylulose-5-phosphate by xylulokinase (XK). The non-oxidative reaction of PPP converts xyulose 5-phosphate to glyceraldehyde 3-phosphate and fructose 6-phosphate, which link the PPP to glycolysis (Fig. 1.10) (Pitkänen et al. 2005).

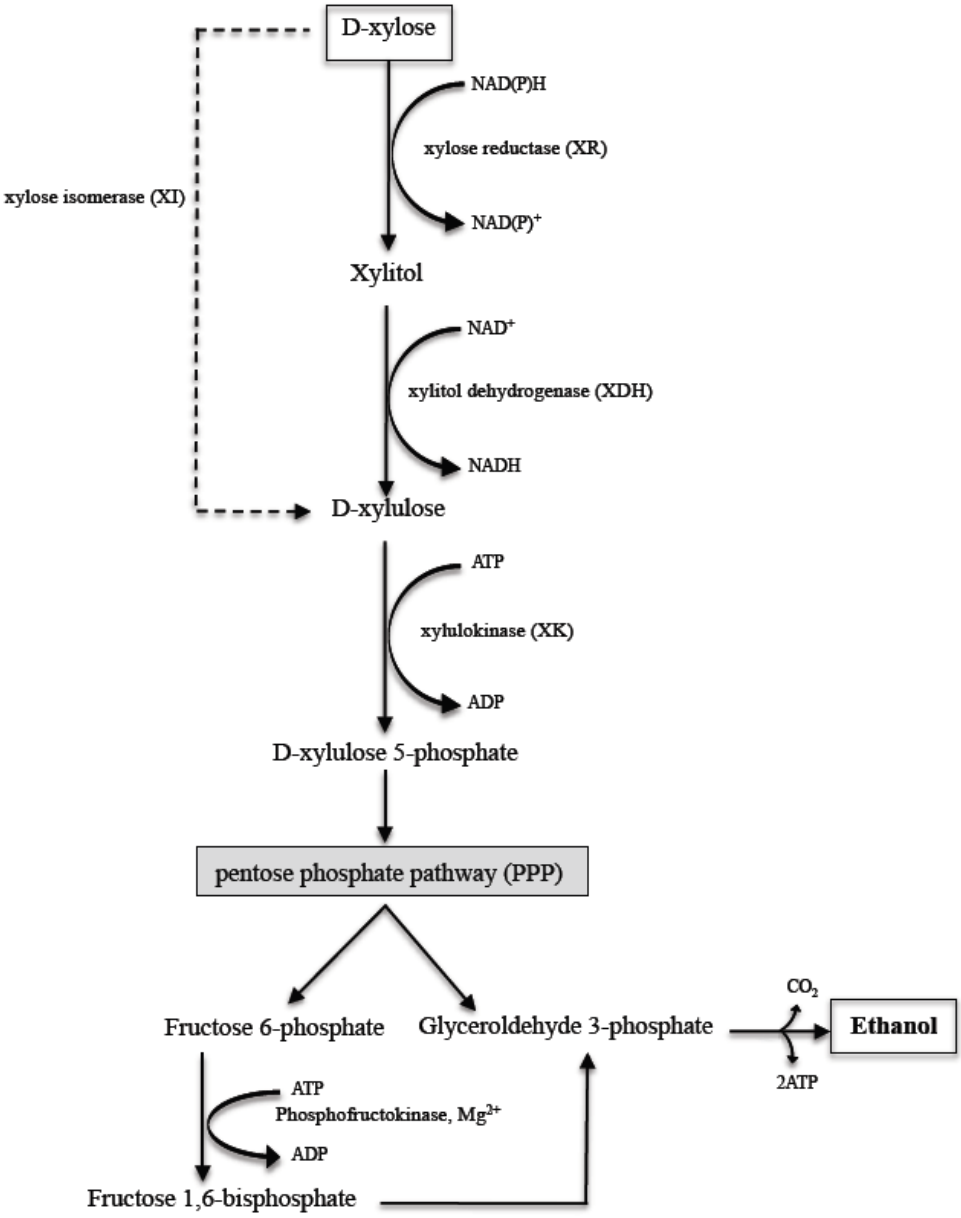


Fig. 1.11 Xylose catabolism of *S. cerevisiae* into ethanol

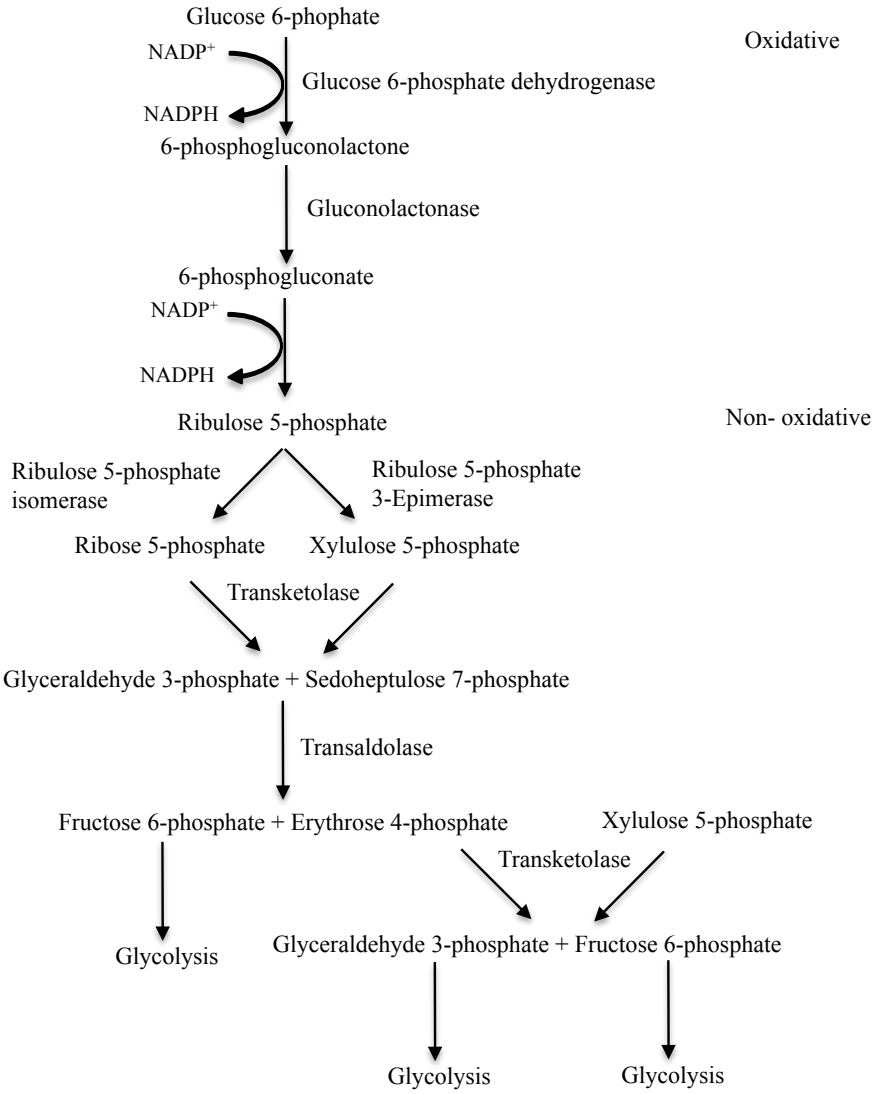


Fig. 1.12 Pentose phosphate pathway (PPP)

Insufficient xylose fermentation has been ascribed to the different cofactor preference of XR and XDH. Strategies to balance the cellular redox include efforts to increase intracellular NADPH availability by metabolic engineering of ammonium assimilate pathway. To circumvent the redox issue of xylose conversion by the oxidoreductive pathway, heterologous expression of xylose

isomerase (XI) has been tried (Fig. 1.11) (Kuyper et al. 2003). Another option would be select for yeast possessing a XR and XDH, which are linked to the same coenzyme system from NADPH towards NADH (Petschacher et al. 2005).

1.8 Ethanol production process

Ethanol production from lignocellulosic biomass comprised the following steps: degradation of the lignocellulosic materials to fermentable sugars followed by fermentation of fermentative organism and distillation of the fermentation broth to obtain 95% ethanol (Fig. 1.13) (Olsson et la. 1996). However, the problems encountered in the efficient conversion of the economic lignocellulosic hydrolysates to ethanol are biomass recalcitrance, inadequate of enzyme activity, and limitation of metabolic ability of fermentative organism. Highly encouraging progress and economically feasible ethanol production has been made with respect to decreasing the cost of enzymes, optimizing the method of pretreatment, and developing functional process organism and the development of process techniques of the fermentation step (Olofsson et al. 2008, Olsson et la. 1996).

1.8.1 Separate Hydrolysis and Fermentation (SHF)

The classic process employed for fermenting biomass hydrolysate involves a sequential process where the hydrolysis of cellulose and the fermentation are carried out in different units. This

configuration process is known as separate hydrolysis and fermentation (SHF). When solid fraction of pretreated lignocellulosic material is completely degraded by enzymatic hydrolysis (saccharification), the resulting cellulose hydrolysate is fermented and converted into ethanol by fermentative organism, *S. cerevisiae*. One of the main features of the SHF process is that each step can be performed at its optimal operating conditions. The most important factors to be taken into account for saccharification step are reaction time, temperature, pH, enzyme dosage and substrate loaded (Sánchez et al. 2008). However, the glucose produced during biomass saccharification strongly inhibits the cellulase activity, particularly the β-glucosidase component. Cellulases are also inhibited by cellobiose. These factors result in a large reduction in overall reaction rate (Alfani et al. 2000).

1.8.2 Simultaneous Saccharification and Fermentation (SSF)

The idea of performing the enzymatic hydrolysis together with the fermentation, instead of subsequent to the enzymatic hydrolysis is called simultaneous saccharification and fermentation (SSF). Since the glucose yield in traditional separate hydrolysis was low, probably due to end-produce, glucose and cellobiose inhibition of hydrolysis, SSF could relived this product inhibition of cellulase that the glucose formed during the enzymatic hydrolysis of cellulose be immediately consumed by the yeast cells converting it into ethanol. Thus, the inhibition effect caused by sugars over the cellulase is neutralized resulted in higher overall ethanol yield (Olofsson et al. 2008, Sánchez et al. 2008). The combination of hydrolysis and fermentation decreases the number of vessels needed and thereby investment costs. The decrease in ethanol production cost of SSF has been estimated to be larger than SHF (Wingren et al. 2003). The SSF process shows more attractive indexes than SHF as higher ethanol yield and less energetic consumption (Sánchez et al. 2008). Unavoidably, the optimum

temperature for enzymatic hydrolysis is typically higher than that of fermentation when using *S. cerevisiae* as the fermentative organism. Since the yeast *S. cerevisiae* has an optimal temperature around 30℃ and the cellulolytic enzyme around 55℃ (Olofsson et al. 2008). Therefore, a compromise temperature must be found in an SSF process. The running SSF temperature is regarded as a suitable compromise at the high end of what *S. cerevisiae* can tolerate. Furthermore, the yeast cannot be reused in an SSF process due to the problems of separating the yeast from the lignin after fermentation. One strategy to improve the total ethanol yield in SSF process is using prehydrolysis. Additional the initial condition of presaccharification in SSF is available for improving the ethanol yield and reducing the total reaction time of the process when compared to SHF process (de Souza et al. 2012).

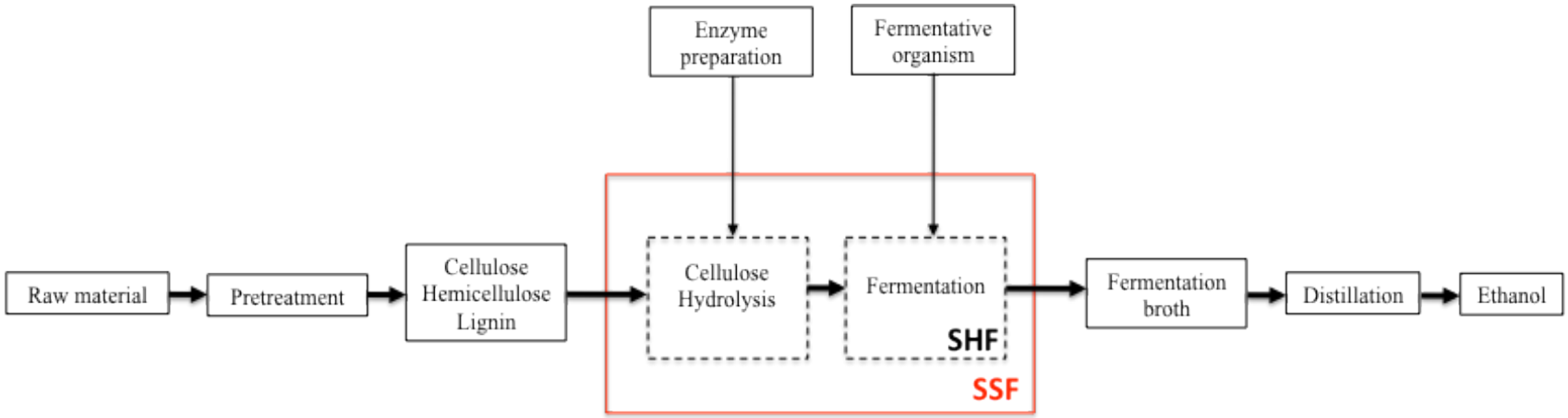


Fig. 1.13 Schematic representation of SHF and SSF processes

1.9 Gerneral objectives

Owing to the deficiency of homologous *T. reesei* β-glucosidase is the major retardation of efficient cellulose hydrolysis resulting to inefficient conversion of ethanol. This major problem can be overcome by using heterologous expression of β-glucosidase. In this study, we evaluated the efficient production of ethanol from lignocellulosic biomass of recombinant strains expressing *Aspergillus aculeatus* β-glucosidase I. The purposes of this study were as follows.

1. Evaluation and characterization of the appropriate recombinant *Saccharomyces cerevisiae* strain expressing either β-glucosidase of *T. reesei* Cel3B and *A. aculeatus* BGL I (AaBGL1) for ethanol production from lignocellulosic biomass. Determined the ethanol production efficiency of recombinant *S. cerevisiae* expressing *A. aculeatus* BGL I compared to the combination of *T. reesei* PC-3-7 culture supernatant with a commercially available BGL-preparation (β-glucosidase from *Aspergillus niger* or Novozyme 188) and wild type *S. cerevisiae* under the basis of simultaneous saccharification and fermentation (SSF) process.

2. Evaluation of the ethanol production efficiency of enzyme preparation produced by a recombinant *T. reesei* strain (X3AB1) expressing AaBGL1 (JN11) by comparing to the traditional enzyme mixture of *T. reesei* PC-3-7 culture supernatant reinforced by exogenous of commercial β-glucosidase from Novozyme 188 with *S. cereivisiae* wild type under the separate hydrolysis and fermentation (SHF) and SSF processes.

Chapter 2

Heterologously expressed *Aspergillus aculeatus* β-glucosidase in *Saccharomyces cerevisiae* is a cost-effective alternative to commercial supplementation of β-glucosidase in industrial ethanol production using *Trichoderma reesei* cellulases

2.1 Introduction

Since the β-glucosidase secreted by *T. reesei* is extremely low and the enzyme are inhibited by their end product, glucose, leading to the retardation of cellulose hydrolysis with an inefficient conversion into ethanol as the result. Supplementation of the β-glucosidase activity to overcome this problem is a good way to using *T. reesei* cellulase and taking advantage of its cellulolytic enzyme production capability. The insufficient β-glucosidase activity of the cellulases secreted by *T. reesei* can be supplemented by the heterologous expression of a β-glucosidase in the yeast, *Saccharomyces cerevisiae* (Lee et al. 2013, Tang et al. 2013). There are advantages to using *S. cerevisiae* for this purpose. *S. cerevisiae* is the fermentative organism of choice for the fermentation of sugars to ethanol on an industrial scale as it efficiently utilizes hexoses and is more tolerant to inhibitors produced during the hydrolysis of cellulose than most other organisms. Moreover, this yeast is also fairly resistant to ethanol (Lau et al. 2010, Olsson et al. 1996, Palmqvist et al. 2000, Stanley et al. 2010). Because *S. cerevisiae* lacks β-glucosidase and other enzymes necessary for the direct conversion of cellulose to glucose or for the degradation of cellobiose, the heterologous expression of β-glucosidase and the hydrolysis of cellulose with cellulases from *T. reesei* are essential steps before utilizing the yeast. Heterologous expression of a required but missing enzyme forms an attractive

alternative for reducing costs (Shen et al. 2008, Wilde et al. 2012), and can easily be integrated with the simultaneous saccharification and fermentation (SSF) process as an economical approach for efficient ethanol production. In this process, the sugars produced by hydrolysis of cellulose are immediately fermented so that product inhibition of the cellulases by glucose is diminished (Olofsson et al. 2008).

A BGL I from *A. aculeatus* has been reported that produced 180 U/mg of specific activity against cellobiose, 9.5 times higher than that of the known *T. reesei* BGL I. There is also a possibility of the existence of other yet to be identified BGL enzymes that are evolutionarily homologous to the *A. aculeatus* BGL I and are comparable to AaBGL1 in their cellobiase specific activity (Dan et al. 2000, Langston et al. 2006, Riou et al. 1998, Sakamoto et al. 1985).

In this work we identified a BGL from *T. reesei* (TrCel3B), which was found in a single cluster with all the highly active *Aspergillus* β-glucosidase, as the closet homolog of *A. aculeatus* BGL I and characterized this enzyme for the first time. We attempted to express it in *S. cerevisiae* to investigate its BGL activity. Recombinant *S. cerevisiae* strains expressing either *T. reesei* Cel3B or *A. aculeatus* BGL I (AaBGL1) were constructed and compared for their cellobiase activities. Our results demonstrated that the strain producing AaBGL1 was the most promising for the production of bioethanol under SSF conditions. We found that the recombinant *S. cerevisiae* expressing AaBGL1 was equally efficient in the conversion of Japanese cedar, a lignocellulosic biomass, as that of the wild type *T. reesei* PC-3-7 culture supernatant, supplemented with a commercially available BGL-preparation and wild-type baker's yeast.

2.2 Materials and Methods

2.2.1 Lignocellulosic raw material

As lignocellulosic substrate, Japanese cedar was prepared by converge-milling in Ichinoseki National College of Technology (Takeda et al. 2009) and its composition analyzed as described in Nakazawa et al. (2011). A high-energy ball mill for high speed and purity processing (converge mill) was used as the mechanochemical grinding machine. We have found that woody biomass can be rapidly converted to a fine-particle low-crystallinity form using a converge mill, thus enabling its glucose conversion by cellulose with a far higher efficiency than attainable with unground woody biomass (Fukumura et al. 2010, Nikaido et al. 2012). Converge mill (Type ET2 manufactured by Earthtechnica Co. Ltd.: pot (SUS) = 1L; media balls (SUJ-2) = ϕ 8mm; ball-filling ratio = 10% ; sample input = 30g; rotation speed = 800 rpm) was used in this study. Table 2.1 summarizes the ratio of cellulose, hemicellulose and lignin and the proportion of monosaccharides in converge-milled Japanese cedar.

Table 2.1 Composition of converge-milled Japanese cedar

Components	**(%wt)**	**Monosaccharides**	**(%wt)**
Cellulose	40	Glucose	44
Hemicellulose	13	Mannose	7.6
Lignin	36	Xylose	4.8
Ash	0.5	Galactose	1.2
No identify	10.5	Arabinose	1.0
		Fructose	0.06

2.2.2 *T. reesei* cultivation and cellulase preparation

The *T. reesei* PC-3-7 hypercellulase-producing strain (ATCC 66589) was used throughout this study. PC-3-7 is sixth generation descendant of the standard strain QM9414 (ATCC 26921). PC-3-7 was found to exhibited enhance cellulase productivity during growth on variety of carbon sources, and high cellulase inducibility by L-sorbose relative to its parent (Kawamori et al. 1986, Nitta et al. 2012). *T. reesei* PC-3-7 spores were inoculated into 300 mL of Erlenmeyer flask containing 50 mL of a basal medium described in Kawamori et al. (1986) with 1% (w/v) Avicel (Funacel; Funakoshi Co., Ltd., Tokyo, Japan) as a carbon source, and incubated at 28°C for 6 days on a rotary shaker at 220 rpm. The culture supernatant was obtained as an enzyme solution after filtration through Miracloth (Celbiochem).

2.2.3 Yeast strain, plasmids and media

S. cerevisiae INVSc1 (*MATα, his3*-D*1*, *leu2*, *trp1-289*, *ura3-52,* Invitrogen) was used as the host strain for BGL expression and fermentation. Full-length cDNAs for *T. reesei cel3a* and *cel3b* were amplified by PCR from a *T. reesei* QM9414 (ATCC 26921) cDNA library derived from Avicel-induced mycelium, while the cDNA for *A. aculeatus bgl1* (*aabgl1*) was obtained with pABG7 (Takada et al. 1998) as the template. Primers are listed in Table 2.2. Derived PCR products were placed under control of the *PGK1* promoter in pYEX-S1 (Clontech), a 2-micron based *S. cerevisiae* vector with an *URA3* gene as a selectable marker. Resultant plasmids were transformed using Yeastmaker System 2 (Clontech) and selected on SD medium (0.67% (w/v) YNB (Yeast Nitrogen Base w/o amino acid), 2% (w/v) glucose and 2% (w/v) agar) supplemented with 0.2 mg/mL L-histidine, 1 mg/mL L-leucine and 0.2 mg/mL L-tryptophan. To prepare cells for enzymatic and

fermentation studies, transformed yeast strains were cultivated in YPC medium containing 1% (w/v) yeast extract, 2% (w/v) peptone, and 1.9% (w/v) cellobiose as the sole carbon source in Erlenmeyer flasks and shaken at 30°C, 220 rpm. *S. cerevisiae* INVSc1 was cultivated in YPD (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose). The optical density of yeast cultures was determined by spectrophotometry at a wavelength of 600 nm and used for the estimation of dry cell weight (dcw) of cultures added in the fermentation experiments.

2.2.4 Sequence analysis

DNA sequences and deduced amino acid sequences of *T. reesei* Cel3A, Cel3B and *A. aculeatus* BGL1 were obtained from the Carbohydrate Active enZYme database (http://www.cazy.org/Home.html, Foreman et al. 2003). The DDBJ databases were searched for homologous proteins using BLAST (www.genome.jp/tools/blast/) and multiple sequence alignments were produced with ClustalW (www.genome.jp/tools/clustalw/) from which a phylogenetic tree was inferred using the neighbor-joining method (Saitou et al. 1987) and visualized with Figtree (http://tree.bio.ed.ac.uk/software/figtree).

2.2.5 RNA extraction, reverse transcription and first strand cDNA synthesis

Total RNA was extracted from frozen mycelia by a modified hot-phenol method using TRIzol (Invitrogen) for additional purification. The lysate was applied to an RNA Spin Mini column (GE Healthcare) to remove genomic DNA and was further purified following manufacturer's instructions. Total RNA (1 µg) was reverse-transcribed and complementary DNA was synthesized

using a Transcriptor First-Strand cDNA Synthesis Kit (Roche Applied Science).

2.2.6 Quantitative real-time PCR

Quantitative real-time PCR was performed using a LightCycler® 480 System (Roche Applied Science). Amplification reactions were performed in a final volume of 20 µL using a LightCycler® 480 SYBR Green I Master kit (Roche Applied Science) with 0.5 µM forward primer, 0.5 µM reverse primer, and 2 µl of cDNA (10-fold diluent of synthesized cDNA). Thermal cycling was conducted under the following conditions: 5min at 95°C followed by 45 cycles of 10s at 95°C, 10s at 60°C and 10s at 72°C. Assays were performed in triplicate with non-amplification controls. The specificity of the PCR amplification was documented by melting curve analysis. Relative expression levels were calculated as the $\log_2$ of ΔCt value by subtracting the Ct value of the house-keeping gene (*act1*, the gene encoding actin) from that of the target gene. qPCR primers used in this study are listed in Table 2.2.

Table 2.2 Primers used in this study

Genes	Primer name	Sequences (5'--->3')
For PCR		
cel3a	Caf	CTAAGCTTAAAATGCGTTACCGAACAGCAGC (HindIII)
	Car	AGAAGCTTTACGCTACCGACAGAGTGCTCG (HindIII)
cel3b	Cbf	CTTGGTCGCGGCCCCGAGATCTATGAAGACGT (BglII)
	Cbr	TCTTGTCAAGCTTATGGCAGGCGGGCGCT (HindIII)
aabgl1	Alf	CGTTTAAAATGAAGCTCAGTTGGCTTGAGGC (DraI)
	Alr	CGTTTAAATTATTGCACCTTCGGGAGCGC (DraI)
For qRT-PCR		
cel3a	cel3a F	AGTGACAGCTTCAGCGAG
	cel3a R	GGAGAGGCGTGAGTAGTTG
cel3b	cel3b F	AGACGTTGTCAGTGTTTGCT
	cel3b R	ATGGCGAAGGGTAGAAAG
cel3e	cel3e F	CAAACGCTCCATCATACATC
	cel3e R	CCAAGCAAGCATCTGAAG
cel3f	cel3f F	AGGACGCTGCATGAAGTA
	cel3f R	TGTTGAGAGCACGCAGT
cel3g	cel3g F	CCAACAGCAAGACTCTGAAT
	cel3g R	CATATCCATGCCAGCTTCT
cel1a	cel1a F	CGTGCTCTTCACCAACAA
	cel1a R	TCTTGCTGATCCACACCA
cel3c	cel3c F	GTTCATGACGGCGTACAAT
	cel3c R	GTACCAGTCGCTCATGATTAG
cel3d	cel3d F	GGCGAGGATGTTTACGTT
	cel3d R	GGTAGAATCTGGGAGCTTGA
cel3h	cel3h F	GAAGCATTGCGAAAGAAGT
	cel3h R	TCCGAGTTGATAAAGACCAT
cel1b	cel1b F	CGATTTCCAAGGCCATT
	cel1b R	TTGAGGGTGGTGTAGTCTGT
act1	actin F	TCCATCATGAAGTGCGAC
	actin R	GTAGAAGGAGCAAGAGCAGTG

Underlines are restriction enzyme site.

F: forward primers; R: reverse primers.

2.2.7 Purification of heterologously expressed enzymes

S. cerevisiae transformants expressing either *T. reesei* Cel3B or *A. aculeatus* BGL1 were cultivated in 100 mL YPC medium for 5 days during which growth and β-glucosidase activity in culture supernatant and cells were monitored. For further enzymatic characterization, heterologously expressed TrCel3B and AaBGL1 were purified from culture supernatants after 4 days incubation.

Recombinant *T. reesei* Cel3B in the culture supernatant was purified by addition of ammonium sulfate to 80% saturation, followed by desalting using a Bio-Gel P-6 gel filtration column (Bio-Rad laboratories, Inc.), and ion exchange chromatography with first a Hiprep Q XL 16/10 column and then a Hiprep SP FF 16/10 (GE Healthcare). The active fractions after Hiprep SP FF chromatography were collected and used as the purified enzyme. Recombinant *A. aculeatus* BGL1 was purified in the same manner, although the saturation with ammonium sulfate was from 80% to 100% and anion exchange column chromatography was done in one step with a Hiprep Q HP 16/10 (GE Healthcare) column. The resulting active fraction was used as the purified enzyme.

Purified proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970) and stained with Coomassie blue R250. The extent of N-glycosylation of TrCel3B and AaBGL1 expressed in *S. cerevisiae* was analyzed by SDS-PAGE after treating 0.5 μg of TrCel3B and 1.7 μg of AaBGL1 purified protein with Glycopeptidase F (TaKaRa) according to the manufacturer's instructions.

2.2.8 Enzyme assays and ethanol analysis

The concentrations of proteins used in the various enzyme assays were measured using the Lowry method with bovine serum albumin as a standard. Total cellulase activity was determined

using a standardized filter paper assay (Adney et al. 1996). Cellobiase activity (CBU) was determined by measuring the amount of glucose (using a Glucose C2 Test; Wako Pure Chemical Industries) released during incubation of 20 mM cellobiose (Sigma) in 200 mM acetate buffer pH 5.0 (in a total volume of 500 μL) at 50°C for 10 min. One unit of cellobiase activity (CBU) was defined as the amount of enzyme that produced 2 μmol of glucose per minute. The β-glucosidas activity was determined using *p*-nitrophenyl-β-D-glucopyranoside (*p*NPGlc, Sigma) as substrates at 50°C for 10 min. Reactions were stopped by addition of 1 M sodium carbonate and released *p*-nitrophenol was detected by spectrometry at 410 nm. One unit of enzyme activity was defined as the amount of enzyme that produced 1 μmol of *p*NP per minute.

For the determination of the optimal pH of the purified enzymes, the following buffers were used: pH 3-4, 100 mM citrate buffer; pH 4-6, 100 mM acetate buffer; pH 6-8, 100 mM phosphate buffer. For assessing the pH stability of enzymes their residual activity was measured after a 30 min pre-incubation in the absence of substrate at 50°C in 50 mM buffers of different pH. For measuring their thermostability, the enzymes were incubated in 100 mM acetate buffer (pH 5.0) at various temperatures for 60 min.

The amount of cellobiose, mannose, xylose and ethanol were determined by high performance liquid chromatography (HPLC) on a LC-20AD (Shimadzu, Japan) equipped with an HPX-87P Aminex column (Bio-Rad) and a RID-10A refractive index detector (Shimadzu, Japan). The temperature was 80°C, and water (Millipore) was used for the mobile phase at a flow rate of 0.6 mL/min. All samples were filtered with a 0.45 μm disposal hydrophobic filter (Dismic-13 HP, Advantec). The conversion of glucose or mannose was calculated as the concentration of glucose or mannose released in the total reaction volume compared to the initial glucose or mannose contents in Japanese cedar (Table 2.1). The efficiency of ethanol production was calculated as the ethanol

concentration in the total reaction volume compared to the theoretical ethanol yield based on the amount of glucose and mannose present in the initial substrate, i.e. 1 g of Japanese cedar (10% w/v). The theoretical yield was calculated from the stoichiometry of the conversion of glucose and mannose to ethanol, that is 1 mole of glucose or mannose yield 2 moles of ethanol. The data presented in the tables are the mean value of three independent experiments.

2.2.9 Fermentation of sugars by *S. cerevisiae*

Fermentation of the main sugars contained in converge-milled Japanese cedar was mimicked in 50 mL polyethylene tubes containing a 10 mL solution of 44 g/L glucose, 7.6 g/L mannose and 4.8 g/L xylose in 50 mM acetate buffer pH 5.0. Yeast cell mass prepared from exponentially growing *S. cerevisiae* INVSc1, equivalent to 8, 15 and 29 mg (38 OD_{600} units) dry cell weight, was added, after which the fermentation tubes were sealed to exclude exposure to oxygen in the air and incubated in a shaking incubator at 39°C, 100 spm (stroke per minute) for 12 - 72 h.

2.2.10 Simultaneous saccharification and fermentation (SSF)

To prepare the substrate for SSF, 1 g of converge-milled Japanese cedar was added to 50 mL polyethylene tubes and suspended in 10 mL of 50 mM acetate buffer pH 5.0. Presaccharification was done before SSF. *T. reesei* PC-3-7 culture supernatant containing 5 and 15 mg protein (corresponding to a cellobiase activity of 0.28 and 0.84 CBU, respectively) was added and incubated at 50°C with shaking at 200 spm for 24 h. SSF was started by addition of yeast cell culture preparations containing equal amounts of *S. cerevisiae* INVSc1 (cultivated in YPD medium at 30°C

for 24 h), either expressing AaBGL1 (cultivated in YPC medium at 30°C for 36 h) or supplemented with Novozyme 188 (Sigma), a commercial preparation of cellobiase from *Aspergillus niger*. Various amounts of yeast cell culture representing approximately 2, 3 or 5 CBU of total cellobiase activity were tested. The amount of dry cell weight added to tubes containing converge-milled Japanese cedar pretreated with 5 mg *T. reesei* culture supernatant protein was 10, 15 and 26 mg (26 OD_{600} units) and to tubes with 15 mg *T. reesei* culture supernatant protein, 8, 15 and 29 mg (38 OD_{600} units) dry cell weight was added. The tubes were sealed to create oxygen-limited conditions and incubated in a shaking incubator at 39°C at 100 spm, for 12 - 72 h.

2.3 Results

2.3.1 TrCel3B is the cellobiase in *T. reesei* with the highest sequence similarity to *Aspergillus* β-glucosidase

In this study, we aimed to improve the overall formation of ethanol from Japanese cedar by combining enzymatic hydrolysis using *T. reesei* cellulase with simultaneous fermentation of the produced sugars by *S. cerevisiae*. The cellobiase activity required for the conversion of cellobiose to glucose in the *T. reesei* extract, however, is poor and absent in baker's yeast. *S. cerevisiae* wild type cannot provide the cellobiase activity required for optimal ethanol production. Therefore we sought the appropriate BGL to complement cellobiase activity in cellulase of *T. reesei*.

The expression profiles of ten β-glucosidase genes of *T. reesei* under the cellulose

producing condition were analyzed and compared to the expression levels of the cellulase genes *cbh1* and *egl1* genes, encoding cellobiohydrolase I and endoglucanase I, respectively. Total mRNA derived from mycelia cultivated on Avicel cellulose as a sole carbon source was reverse-transcribed and quantitative real-time PCR analysis was performed using the cDNA as a template and a gene-specific primer set. In general, the expression level of all β-glucosidase genes was relatively lower compared to the *cbh1* and *egl1* genes (Fig. 2.1). The expression amount of *cel3b*, which encodes Cel3B, was extremely low compared to the cellulase genes, but a low level of expression could nevertheless be observed (Fig. 2.1). Therefore, it is possible that the Cel3B protein exists in the culture filtrate and contributes to cellulose saccharification together with Cel3A, the main β-glucosidase in *T. reesei*.

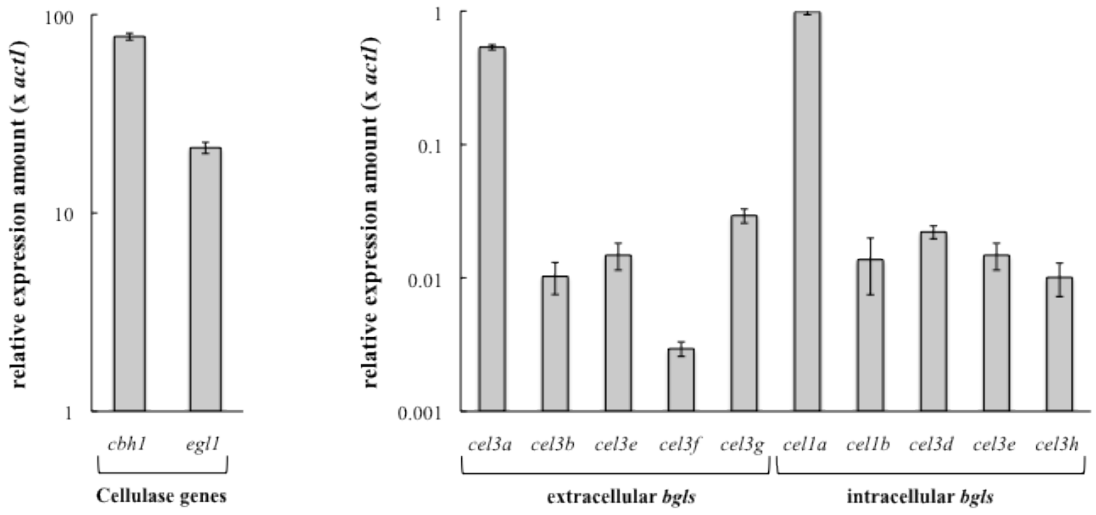


Fig. 2.1 Relative expression levels of cellulase genes *cbh1* and *egl1* (left) and β-glucosidase genes (right) are predicted. Values indicated are relative to expression levels of *act1* gene. Error bars represent standard derivation.

It has been know that many *Aspergillus* species synthesize β-glucosidases belonging to the Glycoside Hydrolase Family 3 (GH3), that have a very high cellobiase activity. Based on homology with enzymes of the GH3 family, ten putative *bgl* genes (*cel3a-h*) have been identified in the genome of *T. reesei* (Martinez et al. 2008). However, only two out of the *bgl* genes have been biochemically characterized. In this study, we performed a phylogenetic tree analysis based on an alignment generated by ClusterW from the predicted amino acid sequence of GH3 β-glucosidases from *T. reesei* and other fungi to identify the *T. reesei* homolog of *A. aculeatus* BGL I with a high cellobiase activity (Fig. 2.2). Cel3B from *T. reesei* (TrCel3B) was found in a single cluster with all the highly active *Aspergillus* β-glucosidases. TrCel3B has a predicted length of 874 amino acids and is encoded by a gene (*cel3b*) containing a putative coding region of 2622 bp that is interrupted by two nitrons of 514 and 55 bp. Sequence alignment showed that the protein shares 47% identity with its counterparts from *A. aculeatus* and *A. oryzae* (Fig. 2.3). The putative catalytic residues Asp and Glu as the catalytic nucleophile and acid/base residues are conserved between these proteins and at least four regions with a high degree of similarity appear to be unique for this group of enzymes. These regions were absent from *T. reesei* Cel3A, the main extracellular β-glucosidase induced after exposure to cellulose, which is only for 38% identical with the *Aspergillus* proteins.

Because of its identification as the closet homolog of *Aspergillus* β-glucosidases with a high cellobiase activity, we decided to test whether TrCel3B and *A. aculeatus* BGL I, heterologously expressed in *S. cerevisiae*, could compensate for the lack of cellobiase activity in the *T. reesei* extract used for hydrolysis of lignocellulosic biomass in the production of bioethanol from waste wood.

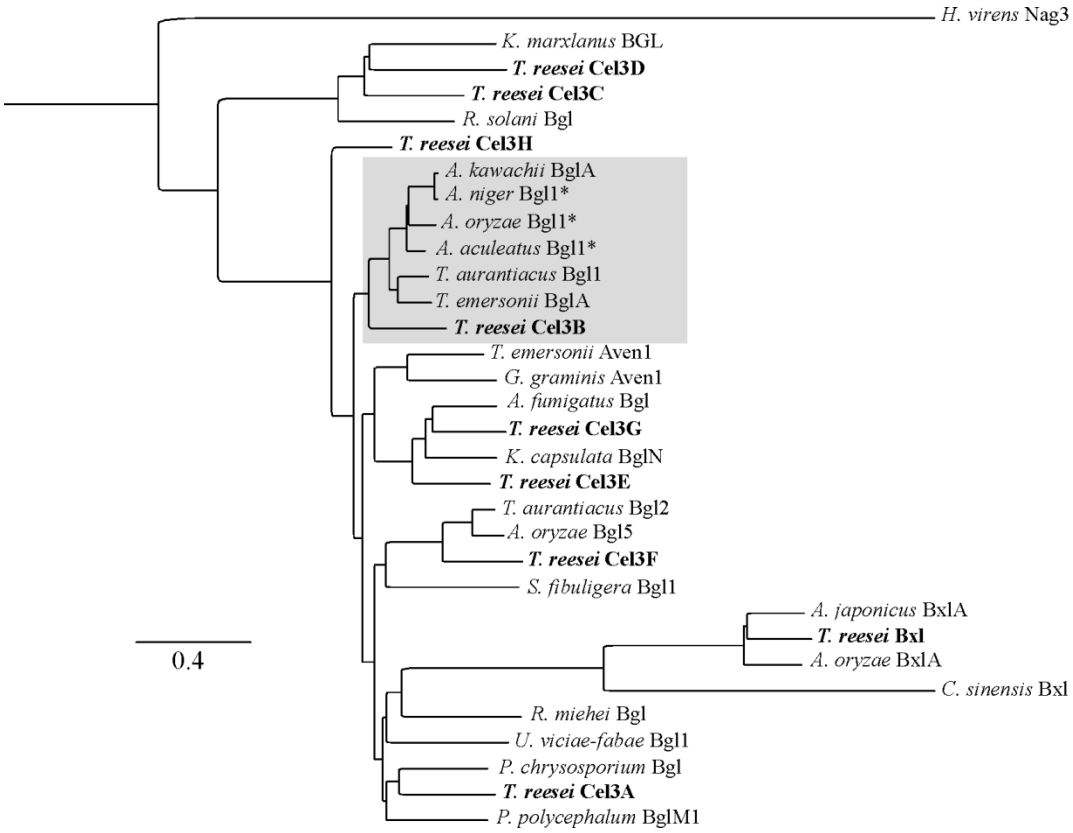


Fig. 2.2 Phylogenetic tree of fungal GH 3 enzymes. Scale corresponds to a genetic distance of 0.1 substitutions per position (10% difference). GenBank protein accession numbers are: *Trichoderma reesei* QM9414 β-glucosidase 1 (Bgl1, Cel3A): AAA18473.1, *Trichoderma reesei* QM6a Cel3B: JGI protein ID 121735, Cel3C: JGI protein ID 82227, Cel3D: JGI protein ID 46816, Cel3E: JGI protein ID 76227, Cel3F: JGI protein ID 47268, Cel3G: JGI protein ID 104797, Cel3H: JGI protein ID 66832, *Hypocrea virens* β-*N*-acetylglucosaminidase (Nag3): ACH72647.1, *Aspergillus kawachii* IFO4308 β-glucosidase A (BglA): BAA19913.1, *Aspergillus fumigatus* Af293 β-glucosidase (Bgl): EAL91070.1, *Physarum polycephalum* β-glucosidase 1 (BglM1): BAE43955.1, *Aspergillus aculeatus* F-50 β-glucosidase 1 (Bgl1): BAA10968.1, *Aspergillus japonicus* MU-2 β-1,4-xylosidase (XylA): BAG82824.1, *Aspergillus oryzae* KBN616 β-xylosidase (XylA): BAA28267.1, *Talaromyces emersonii* Avenacinase (Aven1): AAM94393.3, *Gaeumannomyces graminis* Avenacinase I (Aven1): AAB09777.1, *Uromyces viciae-fabae* β-glucosidase (Bgl1): CAE01320.1, *Thermoascus aurantiacus*

IFO 9748 β-glucosidase 1 (Bgl1): AAZ95587.1, *Thermoascus aurantiacus* IFO 9748 β-glucosidase 2 (Bgl2): AAY33982.1, *Aspergillus niger* NRRL 3135 β-glucosidase (Bgl): CBA02054.1, *Kluyveromyces marxianus* NBRC1777 β-glucosidase I (Bgl): ACY95404.1, *Rhizomucor miehei* NRRL 5282 β-glucosidase I (Bgl1): CAP58431.2, *Talaromyces emersonii* β-glucosidase (Cel3A): AAL69548.3, *Aspergillus oryzae* β-glucosidase I (Bgl1): CAD67686.1, *Saccharomycopsis fibuligera* ATCC 36309 β-glucosidase 1 (Bgl1): AAA34314.1, *Aspergillus oryzae* RIB40 β-glucosidase 1 (Bgl5): BAE57053.1, *Rhizoctonia solani* AG3 β-glucosidase: ABL67526.1, *Kuraishia capsulata* 35M5N β-glucosidase (BglN): AAA91297.1, *Camellia sinensis* β-xylosidase (Bxl): ACD93208.1, *Phanerochaete chrysosporium* K-3 glucan β-1,3-glucosidase (Bgl): BAB85988.1. The cluster containing BGLs with a very high cellobiase activity (*) is highlighted by a gray box.

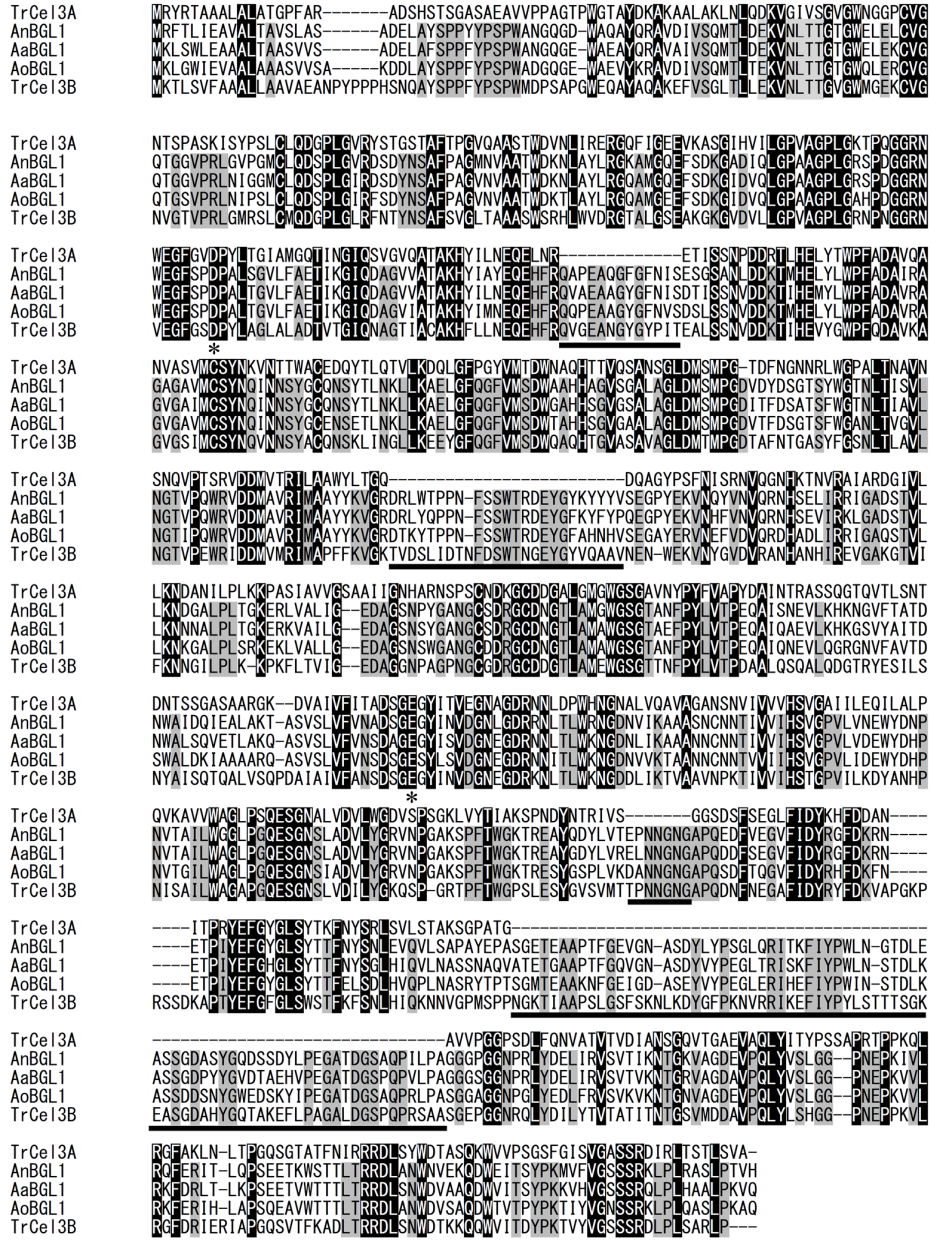


Fig. 2.3 Multiple alignment of *Aspergillus* β-glucosidases, *T. reesei* Cel3A and Cel3B. Identical amino acids are indicated by black boxes and amino acids common to *Aspergillus* BGL and Cel3B are denoted by gray boxes. Underlined bars represent areas absent in Cel3A. Putative catalytic residues (D and E) are indicated with asterisks. GenBank protein accession numbers: TrCel3A, *T. reesei* L27 BGL I (U09580); AnbglI, *A. nigar* BGL I (FN430671); AaBGL1, *A. aculeatus* BGL I (D64088); AobglI, *A. oryzae* BGL I (AX616738); TrCel3B, *T. reesei* Cel3B (AY281374).

2.3.2 Recombinant AaBGL1 shows higher activity and is more stable than TrCel3B

For the characterization of heterologous β-glucosidases expressed in *S. cerevisiae*, cDNAs encoding full-length *T. reesei cel3a* or *cel3b*, and *A. aculeatus bgl1* with their native signal sequences were cloned into the expression vector pYEX-S1 and transformed into *S. cerevisiae* INVSc1 under the control of *PGK* promoter as described in the Materials and Methods. After selection of transformants on media lacking uracil, cells were cultured in YPC medium at 30°C for 5 days during which their cell-density and enzymatic activity of β-glucosidase were monitored. For yeast strains to survive on YPC medium, the expression of a functional β-glucosidase is essential. Cell expressing AaBGL1 or TrCel3B reached a comparable cell-density during the stationary phase (Fig. 2.4a). Expression of TrCel3B resulted in a low level of β-glucosidase activity, both in the fraction containing the precipitated cells and in the culture supernatant (Fig. 2.4b). A significant increase in the conversion of *p*NPGlc was observed for expressing AaBGL1, although most of the activity remained in the cell-pellet, suggesting that the recombinants BGLs were localized the living yeast cell-bound and the culture supernatant. The activity level of TrCel3B was higher compared to TrCel3A, but was significantly lower than AaBGL1, which showed rebust *p*NPGlc activity, especially in the cell pellet fraction, which was suspended in acetate buffer (pH 5.0) with the same volume of culture medium. We decided to characterize TrCel3B and AaBGL1 and evaluate their suitability for the production of bioethanol.

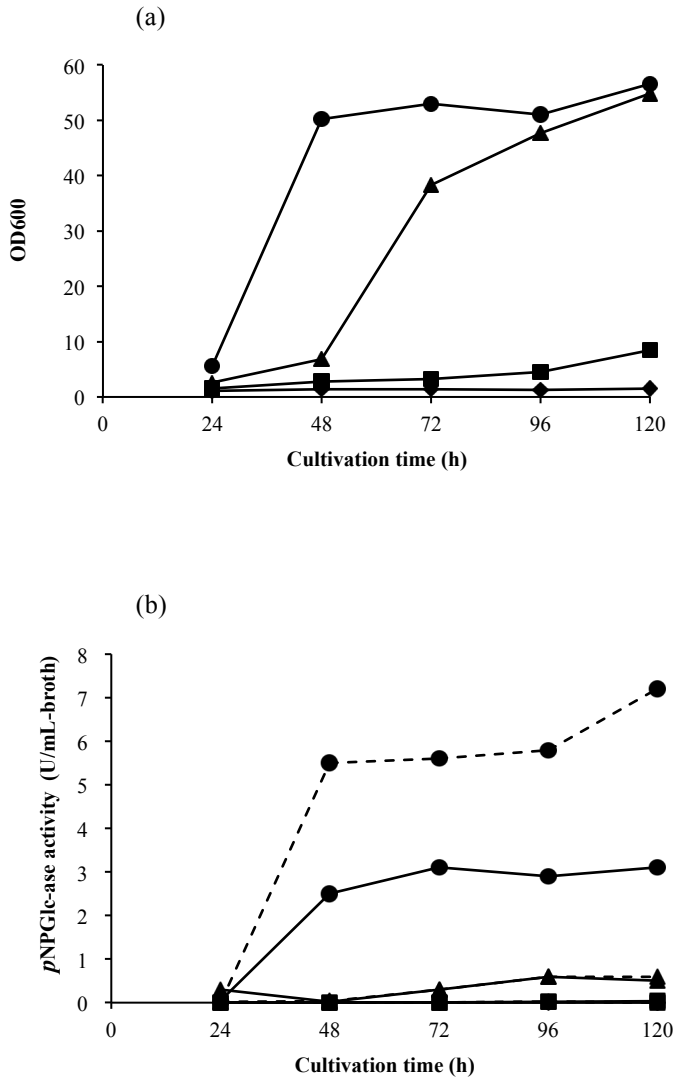


Fig. 2.4 Effect of heterologous expression of β-glucosidases on cell growth and cellobiase activity. Growth curves (a) of *S. cerevisiae* INVSc1 transformed with empty vector (diamond), or expressing TrCel3A (square), TrCel3B (triangle) and AaBGL1 (circle). Enzymatic activities (b) of culture supernatants (straight lines) and cell-precipitates (dotted lines) were determined by measuring the conversion of *p*-nitrophenyl-β-D-glucopyranoside (*p*NPGlc). Cultures were monitored over five days and cell densities were measured as OD600 as described in the Materials and Methods.

Recombinant TrCel3B and AaBGL1 were purified from the supernatant of cell-cultures that had been incubated for four days as described in Materials and Methods. Summary of TrCel3B and AaBGL1 purification steps with yields and protein on SDS-PAGE obtained at each stage were showed in Table 2.3 and Fig. 2.5 and Table 2.4 and Fig. 2.6, respectively. Enzymatic activities with *p*NPGlc as the substrate showed that the specific *p*NPGlc-ase activity of purified AaBGL1 was about 8.5 times higher than that of TrCel3B and about 9 times higher against cellobiose (Table 2.5). From these results, the amounts of secreted protein per mL of culture were estimated to be 8 μg AaBGL1 and 12 μg TrCel3B. The migration on SDS-PAGE indicated molecular masses of about 110 and 160-185 kDa for recombinant TrCel3B and AaBGL1, respectively (Fig. 2.7a and b lane 2), which exceeded their predicted molecular mass from the deduced amino acid sequence of 92 and 91 kDa. Proteins exported by *S. cerevisiae* are often glycosylated. In order to see whether recombinant TrCel3B and AaBGL1 had gained the observed increase in mass due to this modification, the purified proteins were treated with glycopeptidase F. This resulted in bands migration around 100 and 95 kDa, respectively (Fig. 2.7a and b lane 3), which corresponds well to the calculated molecular mass for TrCel3B and AaBGL1.

pH and temperature profiles of the purified TrCel3B were characterized and compared to that of AaBGL1. We found that the proteins showed slight difference in their stability (Table 2.5). AaBGL1 was more than 90% stable after an hour of exposure to a high temperature (60°C) whereas TrCel3B retained more than 80% of its activity after an hour of incubation at temperature up to 50°C (Table 2.5). In addition, the pH range at which the enzyme stable was also different. AaBGL1 could retain its activity at a broader range of pH compared to TrCel3B (Table 2.5). These results indicated that recombinant AaBGL1 is not only more active but also less sensitive to varying conditions than TrCel3B, which makes AaBGL1 was more suitable β-glucosidase to use for the supplementation of

hydrolytic enzymes in bioethanol production. Therefore, we used AaBGL1 expressed by S. *cerevisiae* in SSF for our subsequent fermentation studies.

Table 2.3 Purification step of recombinant *T. reesei* Cel3B from yeast culture supernatant

Steps	Total protein (mg)	Total activity[1] (U)	Yield (%)	Specific activity (U/mg)	Purification fold
Culture supernatant	18000	480	100	0.03	1
Ammonium sulfate precipitation	2800	600	100	0.21	7
Gel filtration	440	430	89	0.96	32
AKTA Hiprep Q XL	20	240	49	12	387
AKTA Hiprep SP FF	2	68	14	34	1133

[1] The enzyme activities were measured using *p*NPGlc as a substrate.

This data was obtained from Ms. Asami Nagaiwa.

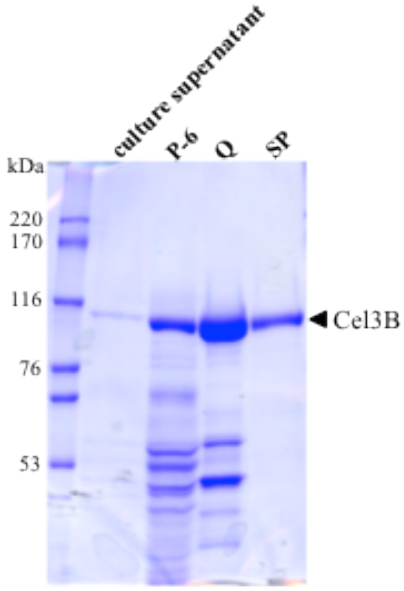


Fig. 2.5 SDS-PAGE of purification step of recombinant Cel3B from yeast culture supernatant. This data was obtained from Ms. Asami Nagaiwa.

Table 2.4 Purification step of recombinant *A. aculeatus* BGL I from yeast culture supernatant

Steps	Total protein (mg)	Total activity[1] (U)	Yield (%)	Specific activity (U/mg)	Purification fold
Culture supernatant	24000	5350	100	0.22	1
Ammonium sulfate precipitation	160	1650	31	10	46
Gel filtration	5.7	850	16	149	671
AKTA Hiprep Q HP	1.4	390	7	290	1304

[1] The enzyme activities were measured using *p*NPGlc as a substrate.

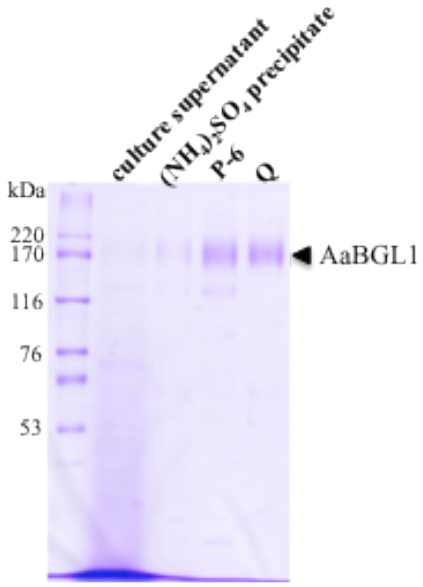


Fig. 2.6 SDS-PAGE of purification step of recombinant AaBGL1 from yeast culture supernatant

Table 2.5 Characteristics of the β-glucosidases expressed by *S. cerevisiae*

	TrCel3B	AaBGL1
Specific activity (U/mg-protein) for		
*p*NPGlc	34	290
Cellobiose	21	194
Optimal pH [1]	4.0-5.0	5.0
pH stability [1,2]	5.0-6.0	4.0-6.0
Temperature stability [2]	<50℃	<60℃

[1] Enzymatic activity was measured using *p*NPGlc in 50 mM citrate buffer (pH 3-4), 50 mM acetate buffer (pH 4-6) and 50 mM phosphate buffer (pH 6-7).

[2] pH and temperature stability were calculated as conditions when the enzyme retained more than 80% of activity**.**

TrCel3B data was obtained from Ms. Asami Nagaiwa.

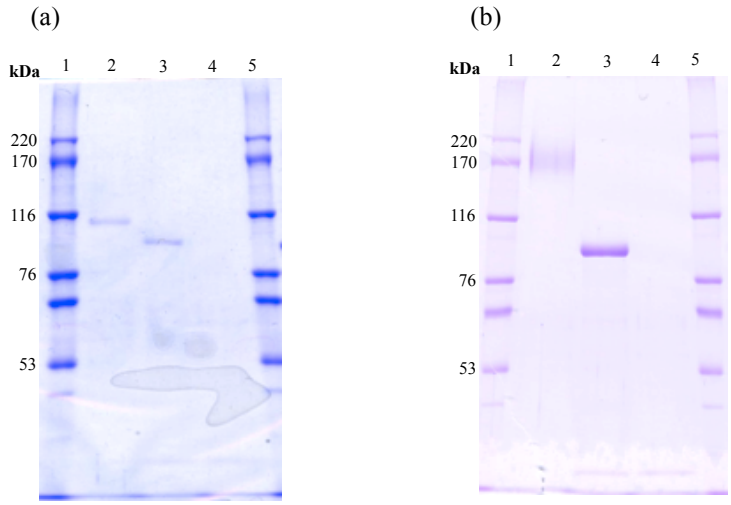


Fig. 2.7 SDS-PAGE of purified recombinant TrCel3B and deglycosylated TrCel3B (a) and purified AaBGL1 and deglycosylated AaBGL1 (b). Lanes 1 and 5, HMW SDS markers; lane 2, 0.5 μg of purified TrCel3B or 1.7 μg of purified AaBGL1; lane 3, deglycosylated TrCel3B or deglycosylated AaBGL1, lane 4, Glycopeptidase only as control. TrCel3B data was obtained from Ms. Asami Nagaiwa.

2.3.3 Yeast cell mass affects the fermentability of sugars contained in Japanese cedar

In the fermentation experiments, we attempted to use *S. cerevisiae* to convert glucose and other sugars like mannose and xylose produced by enzymatic degradation from converge-milled Japanese cedar to bioethanol. To determine the optimal amount of dry cell weight of the yeast needed for this conversion, we carried out ethanol fermentation using various amounts of yeast cells under oxygen-limited condition. A dose-dependent increase in the rate of glucose consumption was observed in the course of fermentation (Fig. 2.8a-c), with higher amount of addition of yeast yielding complete glucose consumption in as little as 12 hours (Fig. 2.8c). Similar dose-dependent response was also seen with mannose, but xylose on the other hand, was not fermented at all (Fig. 2.8a-c). This is because of the inability of *S. cerevisiae* to use pentose sugars (van Maris et al. 2006). Ethanol accumulation also showed some sort of dose dependence. The amount of ethanol produced increased in the first 12 hours with increase in dry weight from 8 to 15 mg and the time taken for producing maximum concentration of ethanol reduced at the same dosages (Fig. 2.8a and b). However, the efficiency of ethanol production declined to 74.6% when the amount of yeast added was almost doubled (to 29 mg dcw, Fig. 2.8c). These results suggest that the amount of yeast cell mass can itself become a limiting factor for ethanol production under our fermentation conditions.

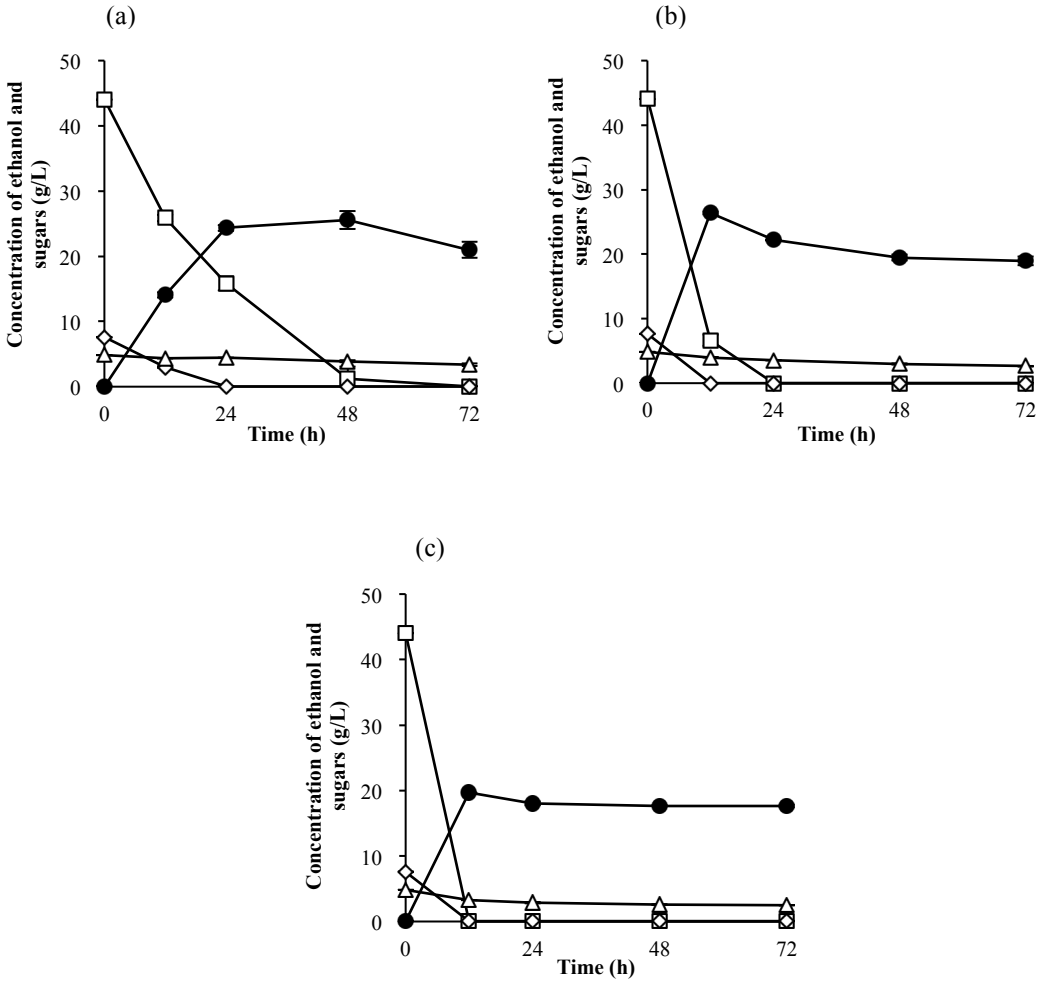


Fig. 2.8 Fermentation efficiency of *S. cerevisiae* INVSc1 on converge-milled Japanese cedar. Dry cell weight (dcw) of yeast added was: 8 (a), 15 (b) and 29 (c) mg. Ethanol concentration (filled circles) and those of residual sugars, such as glucose (open squares), mannose (open diamonds) and xylose (open triangles) are indicated. Data represent the averages of three independent experiments ± STD.

2.3.4 Simultaneous saccharification with *T. reesei* enzymes and fermentation with *S. cerevisiae* (SSF)

Synchronous saccharification with fermentation is based on the idea that the sugars produced by the extracellular *T. reesei* enzymes during hydrolysis of lignocellulosic biomass will directly be removed by *S. cerevisiae* for conversion to ethanol. In this manner, product inhibition of the hydrolytic enzymes of *T. reesei* would be bypassed. A bottleneck in this scheme is the poor efficiency conversion of cellobiose to glucose by the *T. reesei* enzymes, which *S. cerevisiae* wild type lacks the required β-glucosidase activity. Therefore, we tested the efficiency of SSF when cellobiase activity was added either exogenously in the form of a commercial preparation (Novozyme 188) or by heterologous expression of the enzyme in the yeast used for fermentation. For heterologous expression we chose AaBGL1 because of its high cellobiase activity and robustness to changes in temperature or pH. The substrate, 1 g converge-milled Japanese cedar, was presaccharified with *T. reesei* cellulase at 50°C for 24 h before SSF with the addition of yeast expressing recombinant AaBGL1 or supplementation with Novozyme 188. The concentration of glucose and mannose after presaccharification of 5 mg protein of *T. reesei* culture supernatant were 7.9 and 1.2 mg/mL, respectively.

Fig. 2.9 shows the concentrations of cellobiose, glucose and ethanol at varying yeast dry cell weight conditions of 10 (Fig. 2.9a), 15 (Fig. 2.9b) and 26 mg (Fig. 2.9c) using 5 mg protein of *T. reesei* culture supernatant. Both cellobiose and glucose were completely consumed with the addition of 10 mg yeast (corresponding to 2CBU) in 12 hours of fermentation (Fig. 2.9a). Mannose consumption followed the same pattern as well (data not shown). There was no significant difference observed with the addition of more yeast in sugar consumption (Fig. 2.9b and c). The sugar consumption pattern was similar irrespective of whether the supplementation of cellobiase activity

was achieved through the use of recombinant AaBGL1 or supplementation with the commercial Novozyme 188 (Fig. 2.9a-c). Ethanol concentration increased with time, achieving a peak at 72 hours of fermentation in all dry cell weight conditions, with no perceptible difference between the different cell weight conditions (Fig. 2.9a-c). Similar to sugar consumption, ethanol production efficiency was similar between recombinant AaBGL1 and Novozyme 188 with *S. cerevisiae* wild type (Table 2.6).

We found that cellobiose released during the saccharification of *T. reesei* enzyme was immediately converted by AaBGL1 or Novozyme 188. This was obvious when there was no supplementation of cellobiase activity (Fig. 2.10). Cellobiose concentration decreased to only about 50% even after 72 hours of fermentation (mainly due to the low CBU activity (- 0.28) of the added *T. reesei* enzymes) (Fig. 2.10). Ethanol concentration was also significantly lower (from about 12 g/L with supplementation to 7 g/L without supplementation) (compare Fig. 2.9a to Fig. 2.10).

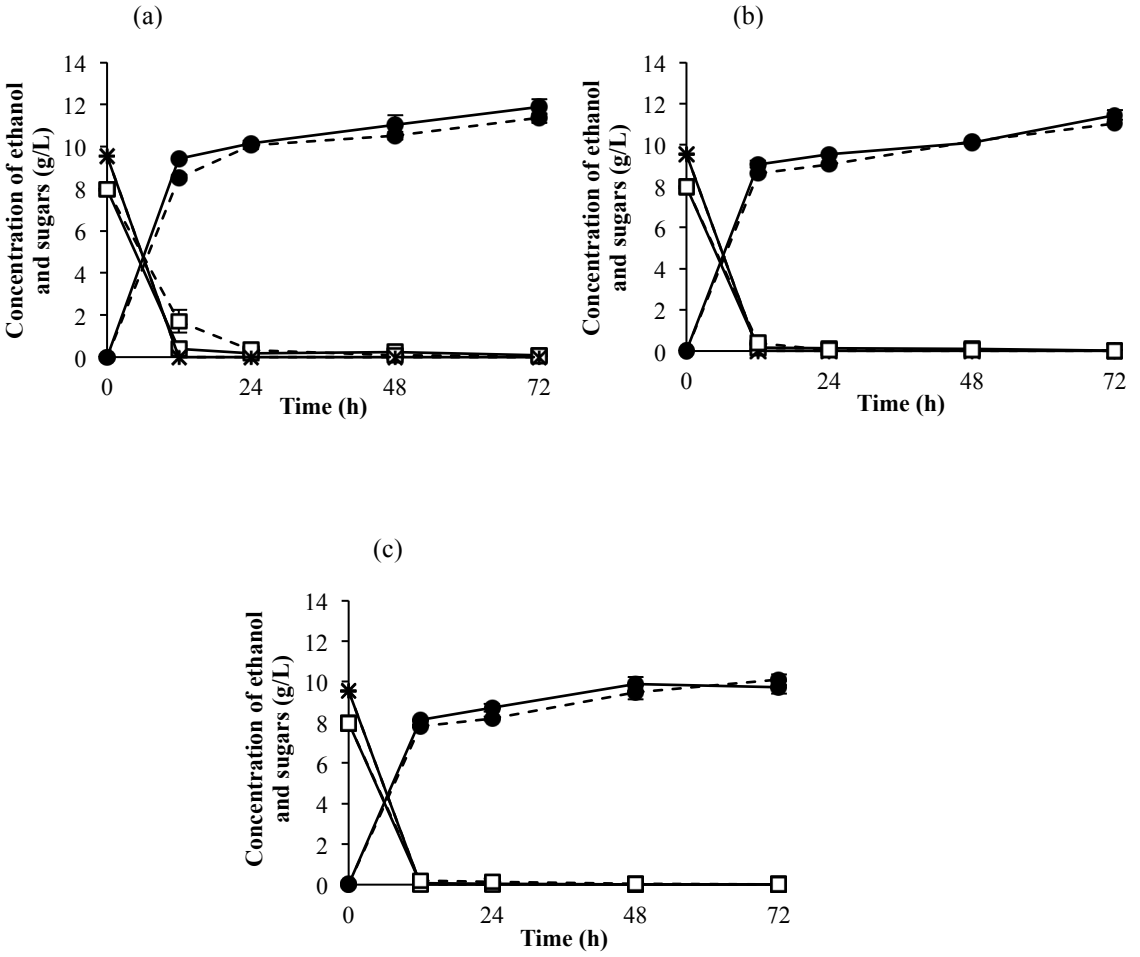


Fig. 2.9 SSF using 5 mg protein of *T. reesei* culture supernatant supplemented with varying cellobiase activities. Cellobiase activity was supplemented by heterologously expressed AaBGL1 (bold line) or with Novozyme 188 (dotted line) to an equivalent of 2 (a), 3 (b) and 5 (c) CBU equivalent to 10, 15 and 26 mg dry cell weight of added yeast, respectively. The concentration profiles of ethanol (filled circles), glucose (open square) and cellobiose (asterisks) are indicated. Data represent the averages of three independent experiments ±STD.

Table 2.6 Ethanol production from SSF of converge-milled Japanese cedar using 5 mg protein of *T. reesei* culture supernatant with *S. cerevisiae* INVSc1 expressing recombinant AaBGL1 or supplemented with 2, 3 and 5 CBU of Novozyme 188

cellobiase activity (CBU) / dcw (mg)	SSF Time (h)	Ethanol production efficiency (%)		
		INVSc1 (w/o added cellobiase activity)	INVSc1 +188	AaBGL1
2 / 10	12	22.3 ± 0.4	36.9 ± 0.5	40.9 ± 0.3
	24	24.9 ± 0.4	43.3 ± 0.9	43.9 ± 0.9
	48	28.9 ± 0.7	45.5 ± 0.8	47.7 ± 2.0
	72	31.9 ± 0.3	49.2 ± 1.0	51.5 ± 1.5
3 / 15	12	22.4 ± 0.3	39.9 ± 0.8	42.0 ± 0.7
	24	24.7 ± 0.5	41.9 ± 0.5	44.2 ± 0.1
	48	29.7 ± 0.7	47.2 ± 0.3	46.8 ± 0.5
	72	35.8 ± 1.6	51.1 ± 0.4	53.1 ± 1.1
5 / 26	12	22.8 ± 0.4	40.9 ± 0.3	42.5 ± 0.1
	24	25.0 ± 0.3	42.9 ± 0.4	45.7 ± 1.0
	48	33.7 ± 1.0	49.7 ± 1.8	51.9 ± 1.8
	72	38.4 ± 1.8	53.0 ± 1.4	51.0 ± 1.6

Data represent the averages of three independent experiments

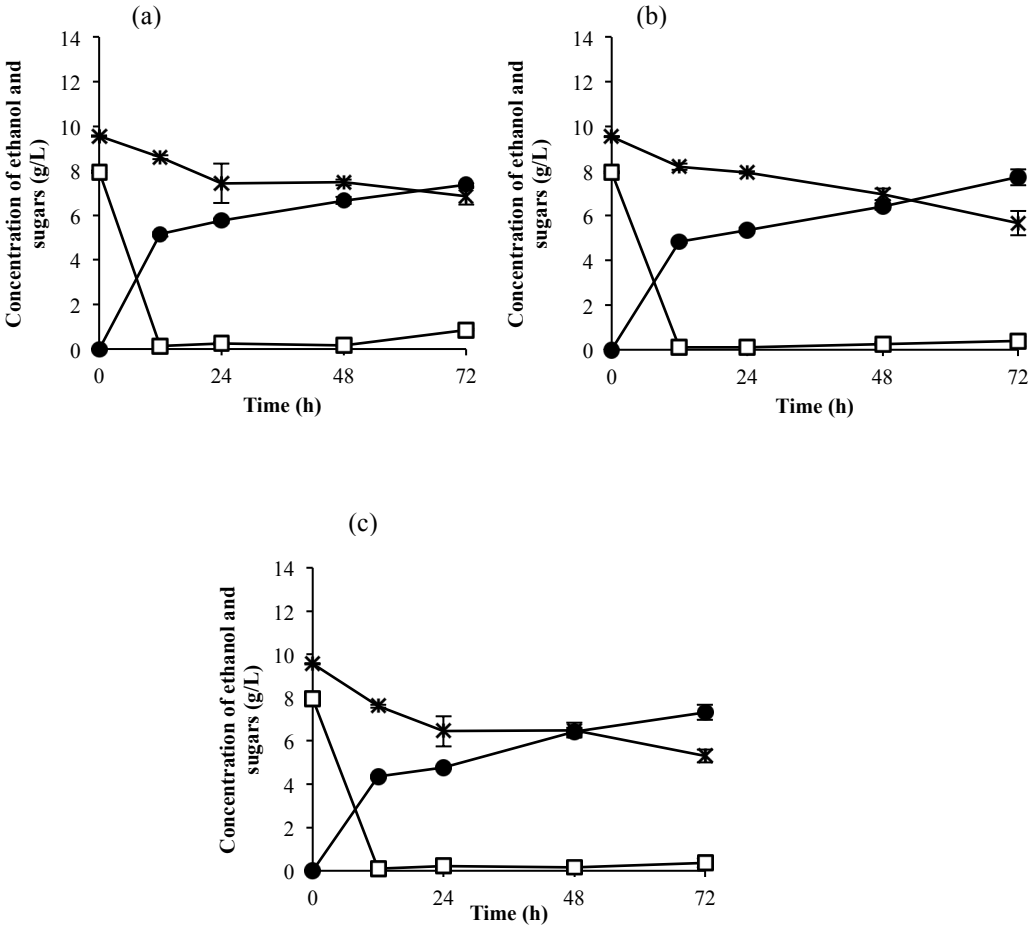


Fig. 2.10 SSF using 5 mg protein of *T. reesei* culture supernatant with 10 (a), 15 (b) and 26 (c) mg dry cell weight of *S. cerevisiae* INVSc1. The concentration profiles of ethanol (filled circles), glucose (open square) and cellobiose (asterisks) are indicated. Data represent the averages of three independent experiments ± STD

To analyze whether increased amount of cellulase affects glucose conversion and subsequent ethanol productivity, we increased the load of *T. reesei* culture supernatant to 15 mg and carried out SSF with varying amounts of yeast, supplemented with heterologous expression using AaBGL1 or with Novozyme 188. Interestingly, we observed a marked difference in the manner in which the sugars were metabolized between AaBGL1 and Novozyme 188 at this *T. reesei* culture supernatant protein load. The pattern was very different with immediate glucose consumption in the presence of Novozyme 188 (Fig. 2.11a). Ethanol production of recombinant AaBGL1 was accumulated to 13.3 g/L corresponding to 54.6% of ethanol production efficiency at 72 h of SSF and 10.5 g/L of glucose remained in supernatant for 72 h of SSF. With Novozyme 188 and *S. cerevisiae* INVSc1, the highest ethanol concentration obtained was 17.5 g/L corresponding to 72% of ethanol production efficiency at 24 h of SSF with almost complete glucose consumption of yeast (Fig. 2.11a and Table 2.7). However, the ethanol produced by Novozyme 188 and *S. cerevisiae* INVSc1 decreased after 24 h of SSF. On the other hand, complete glucose consumption of recombinant AaBGL1 was observed at 48 h of SSF when higher dcw of yeast (15 mg) was used (Fig. 2.11b). The highest ethanol production efficiency of 79.1% was observed by recombinant AaBGL1 under this 15 mg dcw containing 3 CBU at 48 h of SSF. The glucose consumption rate of recombinant AaBGL1 was significantly lower than Novozyme 188 with *S. cerevisiae* INVSc1 for the initial 24 hours, but the ethanol accumulation was almost similar at 24 h of SSF (Fig. 2.11b). Similar pattern of glucose metabolism and ethanol accumulation were also observed when the amount of yeast was double (Fig. 2.11c). However, this difference in the metabolism of glucose and the resultant accumulation of ethanol between the AaBGL1 and Novozyme 188 diminished with extended hours of fermentation. The concentration of ethanol and production efficiency at the end of the fermentation period (72 hours) was comparable between AaBGL1 and Novozyme 188 in all three yeast concentrations (Fig.

2.11 and Table 2.7). The final ethanol concentration was slightly higher with supplementation with AaBGL1 or Novozyme 188 (~15 g/L) compared to SSF without any supplementation (~12 g/L) (Fig. 2.12). This could be due to the high initial concentration of the sugars and also due to the addition of 0.84 CBU of *T. reesei* cellulase that could have contributed to the decrease in sugars by more than 50% at 72 hours of fermentation.

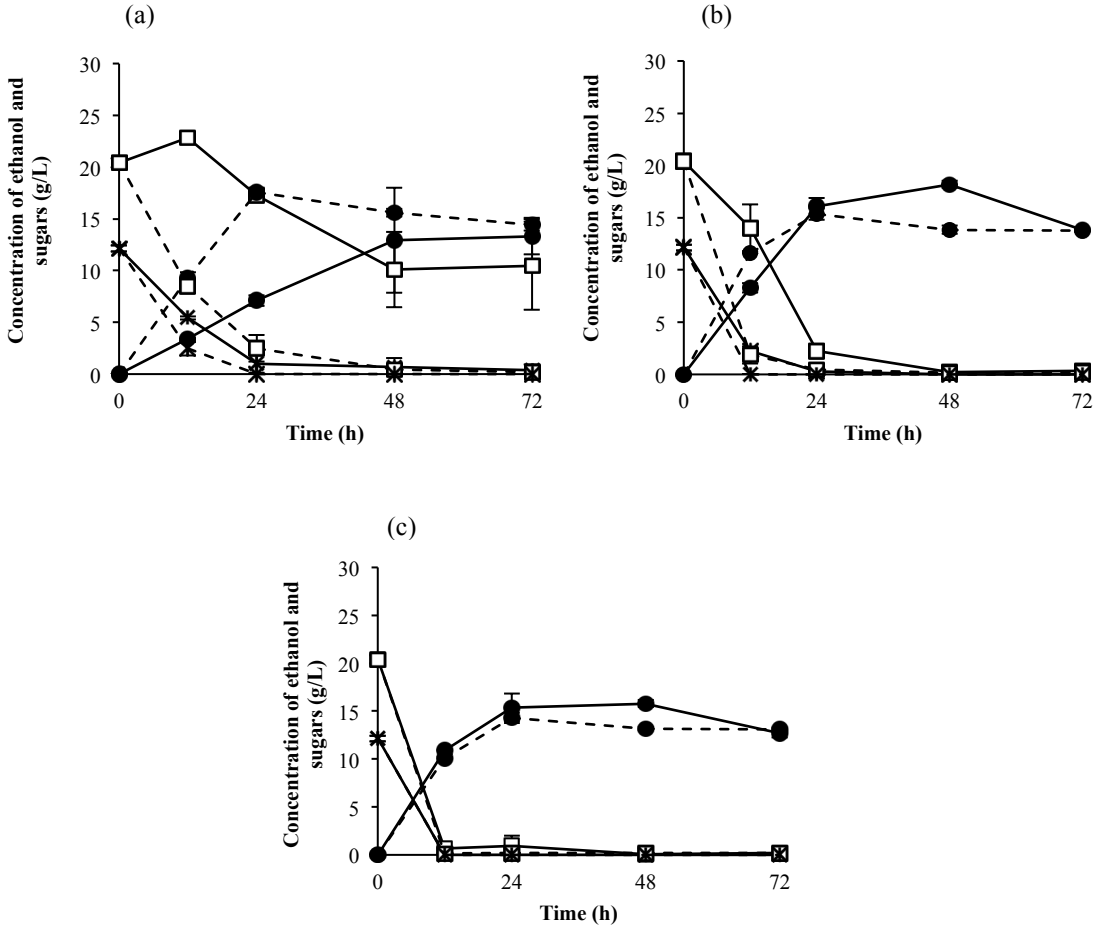


Fig. 2.11 SSF using 15 mg protein of *T. reesei* culture supernatant supplemented with varying cellobiase activities. Cellobiase activity was supplemented by heterologously expressed AaBGL1 (bold line) or with Novozyme 188 (dotted line) to an equivalent of 2 (a), 3 (b) and 5 (c) CBU equivalent to 8, 15 and 29 mg dry cell weight of added yeast, respectively. The concentration profiles of ethanol (filled circles), glucose (open square) and cellobiose (asterisks) are indicated. Data represent the averages of three independent experiments ±STD.

Table 2.7 Ethanol production from SSF of converge-milled Japanese cedar using 15 mg protein of *T. reesei* culture supernatant with *S. cerevisiae* INVSc1 expressing recombinant AaBGL1 or supplemented with 2, 3 and 5 CBU of Novozyme 188

cellobiase activity (CBU) / dcw (mg)	SSF Time (h)	Ethanol production efficiency (%)		
		INVSc1 (w/o added cellobiase activity)	INVSc1 +188	AaBGL1
2 / 8	12	37.3 ± 0.8	38.1 ± 2.2	13.9 ± 0.3
	24	37.9 ± 1.0	72.0 ± 0.8	29.2 ± 2.1
	48	59.3 ± 1.1	63.9 ± 1.5	53.0 ± 21.0
	72	54.1 ± 3.3	59.1 ± 2.3	54.6 ± 7.3
3 / 15	12	41.7 ± 0.9	50.5 ± 0.1	36.0 ± 1.9
	24	33.4 ± 3.2	66.7 ± 2.4	70.1 ± 3.3
	48	58.1 ± 0.8	60.3 ± 1.7	79.1 ± 1.5
	72	52.6 ± 0.9	59.8 ± 0.4	60.2 ± 0.7
5 / 29	12	34.3 ± 0.5	49.2 ± 0.6	53.1 ± 0.2
	24	35.8 ± 0.8	69.5 ± 2.6	74.9 ± 7.0
	48	63.0 ± 0.2	64.1 ± 0.9	76.8 ± 1.7
	72	62.1 ± 1.0	63.8 ± 0.9	61.8 ± 2.2

Data represent the averages of three independent experiments.

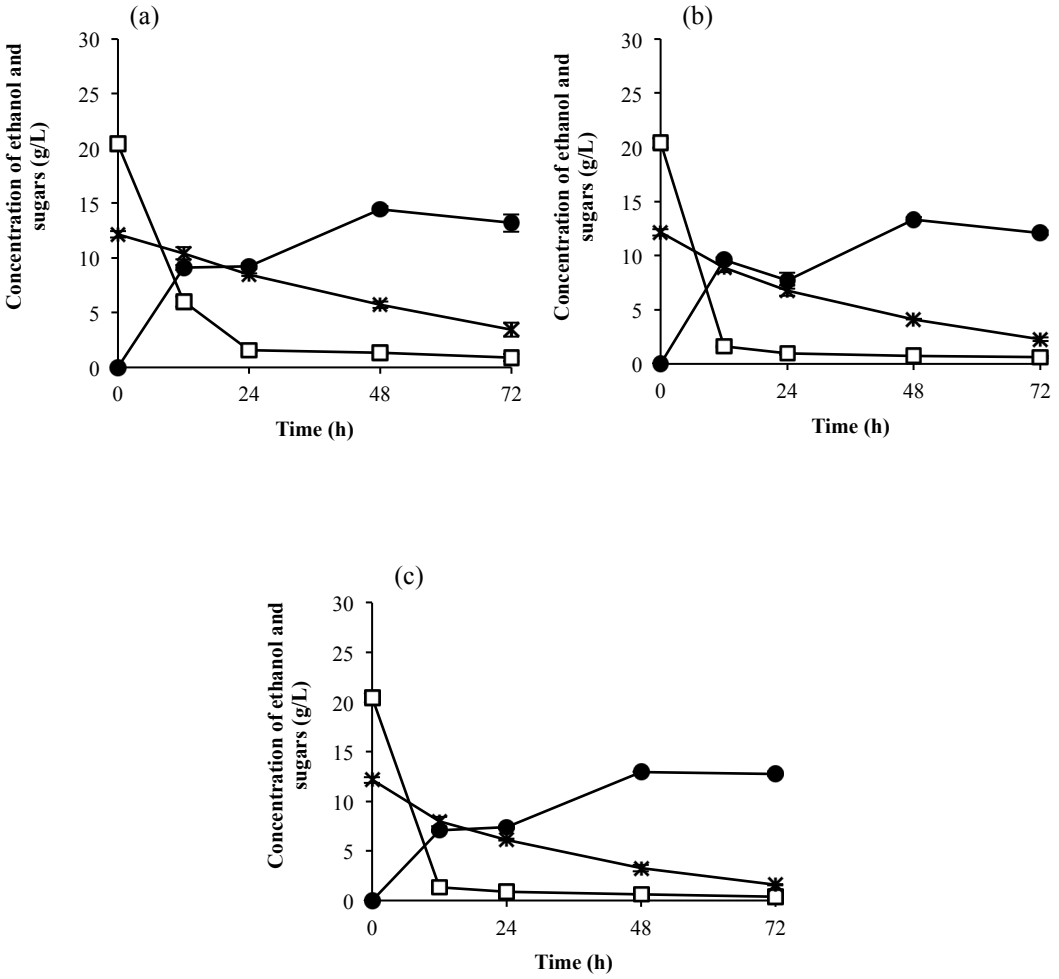


Fig. 2.12 SSF using 15 mg protein of *T. reesei* culture supernatant with 8 (a), 15 (b) and 29 (c) mg dry cell weight of *S. cerevisiae* INVSc1. The concentration profiles of ethanol (filled circle), glucose (open square) and cellobiose (asterisk) are indicated. Data represent the averages of three independent experiments ± STD.

2.4 Discussion

The lack of sufficient β-glucosidase activity in *T. reesei* cellulase preparation retards efficient conversion of cellulose to fermentable glucose resulting in low level of ethanol conversion. The construction of recombinant *T. reesei* to express β-glucosidase has been attempted to overcome this retardation in cellobiose conversion. Nakazawa et al. 2011 successfully constructed a recombinant *T. reesei* strain, X3AB1 expressing *A. aculeatus* BGL I under the control of *xyn3* promoter that displayed high specific activity. The enzyme produced from this X3AB1 called JN11 showed higher cellobiase specific activity than that of parental strain, PC-3-7. However, the efforts to express BGL targets in *T. reesei* are limited by the availability of cellulase-inducible promoters (Rahman et al. 2009). Therefore, a better strategy is to introduce the cellulase genes into a noncellulolytic organism that exhibits high product yield such as *Saccharomyces* (Fitzpatrick et al. 2014, Lynd et al. 2005, Song et al. 2010, van Rooyen et al. 2005). A previous study reported the expression of *A. aculeatus* BGL I in *S. cerevisiae*, but these studies lacked information on the specific cellobiase and *p*NPGase activity of recombinant protein (Takada et al. 1998). So, we set out to characterize this recombinant *A. aculeatus* BGL I in terms of its specific activity as well as ethanol production ability.

Our phylogenetic analysis showed a new *T. reesei* β-glucosidase, TrCel3B belonging to the same cluster as homologous proteins with *Aspergillus* β-glucosidases that exhibit high cellobiase activity (Fig. 2.2 and 2.3). Therefore, *cel3b* of *T. reesei* seemed like a good β-glucosidase candidate for testing expression in *S. cerevisiae*.

Examination of cellobiase activity of the recombinant strain using either TrCel3B or AaBGL1 indicated that the recombinant AaBGL1 showed higher *p*NPGase activity than that of

TrCel3B, revealing that *S. cerevisiae* INVSc1 expressing AaBGL1 had higher cellobiose metabolizing capacity than that of TrCel3B (Fig. 2.4b). Although the amount of secreted protein of AaBGL1 culture was lower than that in TrCel3B, the specific activity of AaBGL1 against *p*NPGlc and cellobiose were evidently higher than that of TrCel3B (Table 2.3 and Table 2.4). Additionally, the specific activity of native AaBGL1 has been characterized by National project. AaBGL1 was found to 180 and 210 U/mg against cellobiose and *p*NPG, respectively whereas the recombinant AaBGL1 expressed by *S. cerevisiae* showed 290 U/mg against *p*NPG. Further characterization also revealed post-translational modifications, such as glycosylation, in these recombinant proteins, suggesting structural modifications that could potentially enhance enzyme activities. Suzuki et al. (2013) have reported the AaBGL1 monomer consists of three domains; a catalytic TIM (triosephosphateisomerase) barrel-like domain, an α/β sandwich domain and a FnIII (fibronectin type III) domain. The AaBGL1 crystal structure contains many large *N*-glycan chains. The subsite +1 of AaBGL1, is formed by three aromatic residues (Tyr^{511}, Trp^{68}, Phe^{305}) and Trp^{358} is presented at subsite +4, exhibits high activity against cellooligosaccharides with DP (degree of polymerization) greater than 4. This subsite +1 is uniquely present in AaBGL1. At present, the protein structure of TrCel3B has not been characterized. However, based on protein sequence alignment of TrCel3B was different compared to AaBGL1 at this high active site (Fig. 2.3) resulted in in the difference substrate specificity between TrCel3B and AaBGL1. Stability of AaBGL1 was also superior to TrCel3B under various pH and thermal conditions tested, prompting us to select recombinant yeast expressing AaBGL1 for use in the subsequent SSF process.

SSF has been considered an effective process for bioethanol production from lignocellulosic biomass because it increases the yield of ethanol by minimizing product inhibition, eliminating the need to separate saccharification and fermentation (SHF) process (Deshpande et al.

1983, Olofsson et al. 2008, Sun et al. 2002). SSF has been shown to circumvent glucose inhibition of the hydrolytic enzymes due to rapid assimilation of sugars by the yeast used for fermentation (Hari et al. 2001). The conversion of cellobiose to glucose has been indentified as the limiting step in cellulosic SSF. Tang et al. (2013) has studied using a recombinant strain of *S. cerevisiae*, which expressed the β-glucosidase of *S. fibuligera*. They concluded that the capability of the yeast strains to ferment cellobiose could enhance the efficiency of cellulose hydrolysis. In our hands, the use of *S. cerevisiae* INVSc1 expressing heterologous β-glucosidase of *A. aculeatus* was as effective for ethanol production as the addition of equivalent amounts of exogenous cellobiase Novozyme 188 with the fermenting yeast when SSF was initiated.

Examination of various amounts of *T. reesei* culture supernatant (5 vs 15 mg protein) indicated that increases in the amount of saccharifying protein, which significantly increased the dosage of cellulases in the process, enhanced the yield and rate of cellulose hydrolysis and consequently in increased ethanol conversion. Interestingly, an unusually high concentration of glucose (10.5 g/L) was observed at the higher protein concentration (8 mg dcw) with scarce ethanol accumulation at 72 h of SSF (Fig. 2.11a), but the final ethanol productivity was almost similar between recombinant AaBGL1 and Novozyme 188 with *S. cerevisiae* INVSc1 (Fig. 2.11a). The cause of this phenomenon is not very clear, but glucose repression could be attributed as one of probable reasons. Glucose repression influences several functions in yeast, both in the central metabolism (i.e. glyoxylatic cycle, gluconeogensis) and in the peripheral metabolism (i.e. sugar uptake and glycolysis), therefore, in the presence of glucose the family-specific inducer as well as the individual gene and their expressions are subject to repression (Olsson and Nielsen 2000, Piskur et al. 2006, Rolland et al. 2002). Nevertheless, another probable reasons were considered. Assuming that the remaining glucose at 72 h of SSF was metabolized and converted into ethanol according to theoretical calculations, the

predicted final ethanol that would have a accumulated using recombinant AaBGL1 would amount to 18.6 g/L, corresponding to 76.6% of ethanol production efficiency, which is almost similar to 72.0% of the highest ethanol production efficiency from Novozyme 188 with *S. cerevisiae* INVSc1 at 24 h of SSF. This indicates that the amount of recombinant yeast cells and the conditions used for characterization were probably suboptimal for the conversion of glucose into ethanol compared to the exogenous BGL of Nocozyme 188 and *S. cerevisiae* INVSc1. The differences in glucose conversion and metabolism and ethanol productivity could also be attributed to the differences in BGL characterization arising from secreted AaBGL1 and β-glucosidase from *A. niger* or from yeast characterizations of wild type and recombinant strains. However, the suboptimal efficiency of ethanol conversion by the recombinant AaBGL1 could be relieved by increased yeast cell mass. Highest ethanol productivity was observed when 15 mg dcw of recombinant AaBGL1 was used for 48 h of SSF (Fig. 2.11b). Increasing the amount of cellobiase unit to attributed to rapid cellobiose degradation rate but did not significantly affect the production of ethanol probably because it resulted in increased yeast cell mass of recombinant AaBGL1 thereby limiting the ethanol production (Fig. 2.11c).

The cost of cellulase production is considered as an important factor in the commercialization of lignocellulosic biomass to ethanol. The addition of β-glucosidase requiring the cultivation of microorganism or supplementation of commercial sources will increase the cost significantly (Lynd et al. 2005, Merino et al. 2007). Our results show that the ethanol production by using a recombinant strain expressing AaBGL1 can be as effective as supplementing the process with commercial cellobiase activity, providing a means of reducing the cost of industrial conversion of lignocellulosic biomass into ethanol.

2.5 Conclusion

Cel3B from *T. reesei* (TrCel3B) was found in a single cluster with all the highly active *Aspergillus* β-glucosidases. In this report, we show the characterization of Cel3B from *T. reesei* for the first time. Comparison of AaBGL1 and TrCel3B showed that AaBGL1 was better suited for downstream use in the SSF process. The specific activity of AaBGL1 against *p*NPGlc was about 8.5 times higher than that of TrCel3B and against cellobiose about 9 times higher. Other enzyme characteristics of AaBGL1 such as pH and thermal stability indicated that this enzyme would be more suitable than TrCel3B for use in the process of SSF. Therefore, we tested *S. cerevisiae* expressing AaBGL1 for investigation of ethanol production efficiency in an SSF process. We have demonstrated efficiency ethanol production from converge-milled Japanese cedar by means of SSF in which culture supernatant of Avicel-grown *T. reesei* provided cellulase and hemicellulase activities and an engineered *S. cerevisiae* strain expressing the β-glucosidase gene of *A. aculeatus* aided in the conversion of cellobiose and in the fermentation of the generated sugars. The results of ethanol production from converge-milled Japanese cedar by using 5 and 15 mg protein of *T. reesei* culture supernatant during simultaneous saccharification and fermentation indicated that 15 mg protein of *T. reesei* culture supernatant created the optimal balance for efficient conversion of cellulosic material to ethanol. Our results show that the ethanol production by using a recombinant strain expressing AaBGL1 can be as effective as supplementing the process with commercial cellobiase activity, providing a means of reducing the cost of industrial conversion of lignocellulosic biomass to ethanol.

Chapter 3

Utilization of recombinant *Trichoderma reesei* expressing *Aspergillus aculeatus* β-glucosidase I (JN11) for a more economical of bioethanol from lignocellulosic biomass

3.1 Introduction

Conversion of cellulosic materials to glucose is the key step of lignocellulosic ethanol production, which mainly depende on the degradation capacity of cellulases secreted by cellulolytic microorganisms and the accessibility of these enzymes to cellulose (Arantes et al. 2011, de Souza et al. 2012, Zhang et al. 2004). Highly efficient enzymatic hydrolysis is required for efficient ethanol production. The filamentous fungus *Trichoderma reesei* is well known as a source of cellulolytic enzymes, however, its secreting β-glucosidase activity is relatively low compared to other cellulases and end-product inhibition by glucose limits the activity and cellulose hydrolysis results in inefficient ethanol production. Another approach to overcoming insufficient BGL activity and improving glucose yield is the addition of extracellular β-glucosidase, such as Novozyme 188 to *T. reesei* culture supernatant. However, the addition of further exogenous β-glucosidase (Novozyme 188) to *T. reesei* culture supernatant result in increased cost of ethanol production. Utilization of recombinant technology for increased BGL activity has been very successful in recent years. Nakazawa et al. (2011) constructed a recombinant *T. reesei* strain (X3AB1) expressing AaBGL1 under the control of *xyn3* promoter and the enzyme preparation produced by the recombinant X3AB1 grown on 1% Avicel called JN11 The BGL activity of JN11 showed over 60 times more than the parental strain PC-3-7 when grown on Avicel cellulose. Subsequently, Kawai et al. (2012) showed that JN11 exhibited the

best balance of cellulase and hemicellulase activities and is very effective for saccharification and is preferred for enzymatic hydrolysis of various cellulosic biomasses. These characteristics make JN11 worthy of further investigation of its potential in the conversion of lignocellulose into ethanol. However, the X3AB1 strain had AaBGL1 expressed under the control of the *T. reesei xyn3* promoter but this strain expressed reduced xylanase (XYN) activity due to a loss of native *xyn3*. So, they added xylan to the basal medium to induce XYN and β-xylosidase (BXL) genes in order to recover the reduced XYN activity. The enzyme produced by X3AB1 grown on a Avicel supplement of 0.5% xylan called JN11H. In addition, JN11H showed increased xylanase activity over JN11 (Nakazawa et al. 2011). Therefore, the lignocellulosic ethanol by using JN11H is further tested in this study.

In this chapter, we determined the optimal protein concentration required to produce a comparable yield of glucose and ethanol for recombinant *T. reesei* expressing AaBGL1 and the cocktail enzyme, PC-3-7 culture supermatant reinforced by commercial BGL from *Aspergillus niger* (Novozyme 188), for both SHF and SSF processes. In addition, we have also demonstrated in this study, the result of JN11 adsorption to biomass during the saccharification step.

3.2 Materials and Methods

3.2.1 Lignocellulosic raw material

Lignocellulosic biomass substrate, Japanese cedar was pretreated by converge-milled pretreatment, which similar to chapter 2. The components of converge-milled Japanese cedar were 40% (wt.) of cellulose, 13% (wt.) of hemicellulose, which constitutes to 44% (wt.) of glucose, 7.6% (wt.) of mannose, 1.2% (wt.) of galactose and 4.8% (wt.) of xylose, and 36% (wt.) of lignin.

3.2.2 *T. reesei* cultivation and cellulase preparation

T. reesei PC-3-7 cellulase hyper-producing stain (ATCC 66589) obtained from Kyowa Hakko Kogyo Co. Ltd. was used in this research. The cultivation of *T. reesei* PC-3-7 was carried out as described previously (Kawamori et al. 1986) with 1% (w/v) Avicel (Funacel; Funakoshi Co., Ltd., Tokyo, Japan) as a carbon source. 1 x 10^7 conidia were inoculated in 50 mL culture medium and incubated at 28°C for 6 days on a rotary shaker at 220 rpm. The culture supernatant as enzyme preparation was obtained as filtrate Miracloth (Calbiochem). Novozyme 188 was purchased from SIGMA was used to reinforce the BGL activity in *T. reesei* PC-3-7 culture supernatant (PC-3-7). The culture supernatant from recombinant *T. reesei* expressing *A. aculeatus* β-glucosidase I, X3A1 strain was prepared as using methods described previously (Nakazawa et al. 2011).

3.2.3 Yeast strain and culturing conditions

S. cerevisiae INVSc1 (*MATa, his3*-D*1*, *leu2*, *trp1-289*, *ura3-52,* Invitrogen) was used as the fermentative organism. A single colony of the subculured *S. cerevisiae* INVSc1 on YPD agar plate was aerobically cultivated in a sterilized 50 mL of YPD (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose) medium, its pH wadjusted to 5.2 by HCl (Nacalai Tesque, inc., Kyoto, Japan) and inoculated at 30°C, 130 spm (stroke per minute) for 24 h (exponential growth phase). The optical density of yeast cell in cultured was determined by UV-VIS spectrophotometer with OD 600 nm and used for the estimation of dry cell weight (dcw) of cultures added in the fermentation experiments.

3.2.4 Enzyme assay and ethanol analysis

The protein concentration of PC-3-7, Novozyme 188, JN11 and JN11H were measured by Lowery method using bovine serum albumin as a standard. Total cellulase activity reported in filter paper unit (FPU), was determined according to the Laboratory Analytical Procedure published by the National Renewable Energy Laboratory (LAP, Adney et al. 1996). The cellobiase activity was determined in 200 mM of acetate buffer (pH 5.0) at 50°C with final concentration of 20 mM of cellobiose (SIGMA) as a substrate in total volume of 500 μL. The amount of glucose released by enzyme reaction was determined by Glucose C2 test Wako (Wako pure chemical). One unit of cellobiase activity (CBU) was defined as the amount of enzyme that produced 2 μmol of glucose per minute. β-glucosidase, β-mannosidase and β-xylosidase activities were determined using the *p*-nitrophenyl-β-D-glucopyranoside (*p*NPGlc, SIGMA), *p*-nitrophenyl-β-D-mannopyranoside (*p*NPMan, SIGMA) and *p*-nitrophenyl-β-D-xylopyranoside (*p*NPXyl, SIGMA), respectively, as

substrates at 50°C for 10 min (pH 5.0). The released *p*-nitrophenol was detected at 410 nm after adding 1 M sodium carbonate to stop the reaction. One unit of enzyme activity was defined as the amount of enzyme that produced 1 μmol of *p*NP per minute. Avicelase, carboxymethylcellulase (CMCase), xylanase and mannanase activities were measured as the amount of reducing sugar produced using the Somogyi-Nelson method (Somogyi 1952). The final concentration of Avicel, carboxymethylcellulose or birch wood xylan (SIGMA) was 1%, and that of glucomannan (low viscosity) was 0.5% in 50 mM acetate buffer (pH 5.0) at 50°C for 10 min of CMCase activity, 15 min of mannanase activity and 30 min of Avicelase and xylanase activities. One unit of enzyme activity was defined as the amount of enzyme that produced reducing sugars corresponding to 1 μmol of glucose or xylose equivalent.

The amount of cellobiose, glucose, mannose and ethanol derived from SSF or SHF experiment was determined using a high performance liquid chromatography (HPLC), LC-20AD (Shimadzu, Japan) system equipped with HPX-87P Aminex column (BIO-RAD) and refractive index detector of RID-10A (Shimadzu, Japan). The temperature was set at 80°C, and water (Millipore) was used for the mobile phase at a flow rate of 0.6 mL/min. All samples were filtered with 0.45 μm disposable hydrophobic filter before being subjected to HPLC (Dismic-13 HP, ADVANTEC). The data presented in the tables are the mean value of three independent experiments.

3.2.5 Saccharification

Saccharification was conducted in a 50 mL polyethylene bottle (PLK, Japan). 10%(w/v) of converge-milled Japanese cedar was used as substrate. JN11 was loaded at 5 and 15 mg protein corresponding to 17.25 and 51.75 CBU, respectively/g-converge-milled Japanese cedar. The protein

loading amount of cocktail enzyme was determined under 5 and 15 mg protein of PC-3-7 corresponding to 0.28 and 0.84 CBU, respectively with various supplementing protein amounts of Novozyme 188 to reinforce cellobiase activity in PC-3-7. The amounts of protein supplements with Novozyme 188 increased the CBU up to 0.5, 1, 2, 3, 5 and 10 for 5 mg protein loading and 1, 2, 3, 5, 10 and 20 for 15 mg protein loading of PC-3-7/g-converge milled Japanese cedar. 50 mM final concentration of acetate buffer (pH 5.0) and distilled water were added to set the total volume to 10 mL. The saccharification bottles were incubated in a shaking incubator (Bioshaker BR-43FL, TAITEC) at 50°C, 200 spm for 24 h.

For the SHF process, the saccharification step was performed under similar cellobiase activity of JN11 and cocktail enzyme. JN11 was loaded at 5, 10 and 15 mg protein corresponding to 17.25, 34.50 and 51.75 CBU of JN11/g-converge-milled Japanese cedar. The cocktail enzyme was loaded under 5, 10 and 15 mg protein of PC-3-7 and reinforced by 5.7, 11.4 and 17.1 mg protein of Novozyme 188, which made the total protein loading amount of cocktail enzyme to 10.7, 21.4 and 32.1 mg for corresponding to 17.25, 34.50 and 51.75 CBU, respectively. After incubation at 50°C, 200 spm for 72 h, the saccharification samples were boiled for 10 min to denature the enzyme and centrifuge at 3000 rpm for 10 min. The amount of glucose in the saccharified supernatant was measured by Glucose C2 test solution. The conversion of glucose was calculated as the concentration of glucose released in the total reaction volume compared to the initial glucose contained in converge-milled Japanese cedar.

3.2.6 Fermentation

Fermentation was performed by adding *S. cerevisiae* INVSc1 culture at 1 OD_{600} unit (exponential growth phase), which was equivalent to 9 mg dry cell weight (dcw), into the boiled saccharified liquid supernatant. The fermentation bottles were sealed to prevent air transfer into the bottle (limiting oxygen) and incubated in a shaking incubator at 30°C, 100 spm for 12-24 h. The amount of glucose in the supernatant was measured by Glucose C2 test solution.

3.2.7 Enzyme characterization

For examining cellulase adsorption of JN11 and cocktail enzyme ~~on~~ to the biomass precipitate, saccharification was tested under similar cellobiase activity of JN11 and cocktail enzyme. JN11 was loaded at 5.2 CBU, corresponding to 1.5 mg protein/0.1 g-converge-milled Japanese cedar and the cocktail enzyme at 0.1 CBU, corresponding to 1.5 mg protein of PC-3-7 with 5.1 CBU of Novozyme 188. 50 mM final concentration of acetate buffer (pH 5.0) and distilled water were added for a total volume of 500 μL, dispensed into a 96 deep-well plate, and incubated in a shaking incubator (Bio-shake M BR-024, TAITEC) at 50°C, 1200 spm for 2-72 h. Saccharified supernatant and biomass precipitate were separated by centrifugation and the biomass precipitate was washed three times by Millipore water. The free proteins in the saccharified supernatant and proteins adsorbed to the saccharified biomass precipitate were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% polyacrylamide gel slabs as described by Laemmli 1970. Gels were stained with Coomassie blue R250. The molecular mass marker used was the Precision Plus Dual Standard Marker kit for SDS-PAGE (Bio-Rad Laboratories, Hercules, CA). *p*NPGlc-ase activity of all the samples was evaluated in total sample volume.

3.2.8 Simultaneous saccharification and fermentation (SSF)

Pre-saccharification was performed before SSF start. 10%(w/v) of converge-milled Japanese cedar was added to a polyethylene bottle. JN11 and cocktail enzyme were loaded as in the saccharification step of SHF described in section 3.2.5. JN11H was loaded at 5, 10 and 15 mg protein corresponded to 25, 50 and 75 CBU, respectively. Pre-saccharification bottles were placed in a shaking incubator at 50°C and the shaking speed was set at 200 spm for 24 h. SSF was carried out by adding *S. cerevisiae* INVSc1 culture at 1 OD_{600} unit (exponential growth phase), which was equivalent to 9 mg dcw into pre-saccharified solution. The SSF bottle was sealed to prevent air transfer (limiting oxygen) and incubated at 39°C with a shaking speed of 100 spm for 6-24 h. The amount of glucose in the presaccharified and SSF supernatant were measured by Glucose C2test solution. The efficiency of ethanol production was calculated as the actual ethanol concentration in the total reaction volume compared to the theoretical ethanol yield based on the amount of glucose and mannose present in the initial biomass substrate.

3.3 Results

3.3.1 JN11, JN11H and Novozyme 188 show high BGL activity compared to PC-3-7

We first measured the cellulase and hemicellulase activities of JN11, JN11H, PC-3-7 and Novozyme 188 preparations used in ethanol production to confirm their enzyme activity (Table 3.1). Both recombinant X3AB1 and *T. reesei* PC-3-7 were cultivated in medium containing 1% Avicel as a cellulase inducer to obtain JN11 and PC-3-7 culture supernatant. The JN11H was produced by recombinant X3AB1 cultivated in medium containing 1% Avicel supplement of 0.5% xylan. JN11 and JN11H showed remarkably higher BGL activity of cellobiase and *p*NPGlc-ase activities than those of PC-3-7. The endo- and exo- cellulases activities, as represented by CMCase and Avicelase activities were comparable between JN11 and PC-3-7. The total cellulase activity represented by filter paper unit (FPU) of JN11 and JN11H were higher than that of PC-3-7, indicating that the high BGL activity has a strong impact on cellulase activity of JN11. Supplementation with Novozyme 188 increased the BGL activity compared to PC-3-7, but had no effect on CMCase and Avicelase activities. The specific cellobiase activity of JN11 and Novozyme 188 was similar (around 3 CBU), but *p*NPGlc-ase of JN11 was about two fold higher than that of Novozyme 188. Interestingly Novozyme 188 showed higher mannanase activity, exhibited about 9-fold higher compared to JN11, 6-fold higher compared to JN11H and 14 fold higher compared to that of PC-3-7. Moreover, JN11H showed higher xylanase activity than that of JN11 and Novozyme 188.

Table 3.1 Specific activity of enzyme preparations

	Specific activity (U/mg-protein)			
	PC-3-7	Novozyme 188	JN11	JN11H
FPU	0.41±0.02	N.D.	0.71±0.11	0.96±0.08
Cellobiase	0.06±0.00	2.98±0.06	3.45±0.24	4.98±0.19
*p*NPGlc-ase	0.15±0.00	1.96±0.03	4.01±0.04	6.77±0.19
CMCase	7.96±0.64	0.06±0.00	6.81±0.40	11.52±0.28
Avicelase	0.68±0.21	0.01±0.00	0.81±0.10	0.76±0.02
Mannanase	0.32±0.02	4.43±0.15	0.49±0.03	0.71±0.06
*p*NPMan-ase	0.008±0.000	0.03±0.00	0.008±0.000	0.006±0.000
Xylanase	1.30±0.04	3.07±0.06	2.18±0.12	3.83±0.54
*p*NPXyl-ase	0.20±0.00	0.02±0.00	0.26±0.00	0.23±0.01

N.D.: not determined

Data represent one batch of enzyme preparation.

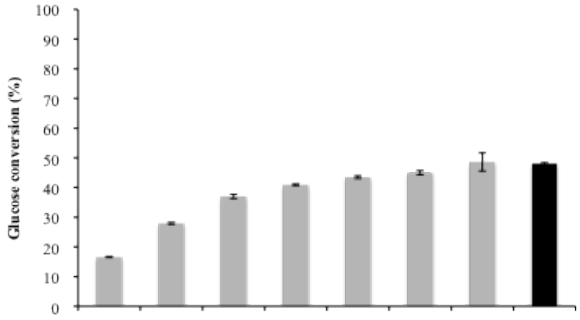
Values represent the averages of three independent measured activity ± STD.

3.3.2 More Novozyme 188 and protein loading is required to attain the same level of CBU attained with JN11

PC-3-7, in enough itself, possesses inadequate BGL activity for efficient glucose conversion. Therefore, next we determined the amount of Novozyme 188 enzyme required to complement the cellulase reaction to attain the same level of activity as that of JN11. We test this with

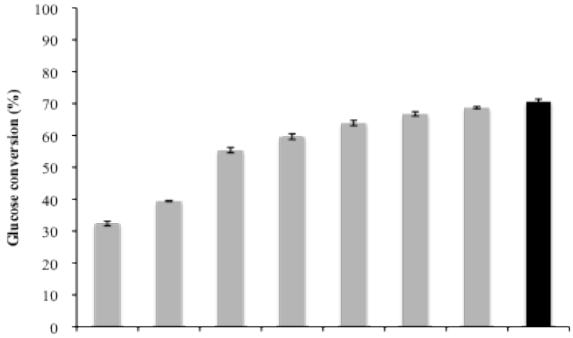
two different amounts of protein loading (5 and 15 mg) corresponding to 17.25 and 51.75 CBU of JN11. Glucose conversion of JN11 increased from 48% at 5 mg to 70% at 15 mg protein loading (Fig. 3.1a and b). For the increased amount of protein loaded, PC-3-7 glucose conversion was at 17 and 32% (Fig. 3.1a and b). This data revealed that PC-3-7 required more β-glucosidase activity for glucose conversion comparable to that of JN11. We added varying amounts of Novozyme 188 to supplement for the β-glucosidase activity required to reach the threshold attained by JN11 (see Table beneath Fig. 3.1a and b). The percentage of glucose conversion increased corresponding to the increase in the amount of enzyme supplemented for the same protein loading PC-3-7 (Fig. 3.1a and b). This data suggested that more protein amount of the cocktail enzyme is needed to attain the same CBU as that of JN11 (8.26 and 21.43 mg protein for the cocktail mixture compared to 5 and 15 mg protein of JN11). From these results, we evaluated protein concentrations to be used for further SSF and SHF processes. Results of this experiment indicated that JN11 has inherent cellobiase activity and that the cocktail enzyme needed supplementation of enzyme and more protein loading to bring the activity comparable to that of JN11.

(a)



mg protein	PC-3-7	5.00	5.00	5.00	5.00	5.00	5.00	5.00	JN11
	Nov. 188	0.00	0.07	0.24	0.58	0.91	1.58	3.26	
	Total	5.00	5.07	5.24	5.58	5.91	6.58	8.26	5.00
Total cellobiase unit		0.28	0.50	1.00	2.00	3.00	5.00	10.00	17.25

(b)



mg protein	PC-3-7	15.00	15.00	15.00	15.00	15.00	15.00	15.00	JN11
	Nov. 188	0.00	0.05	0.39	0.72	1.40	3.07	6.43	
	Total	15.00	15.05	15.39	15.72	16.40	18.07	21.43	15.00
Total cellobiase unit		0.84	1.00	2.00	3.00	5.00	10.00	20.00	51.75

Fig. 3.1 Glucose conversion profile of 5 (a) and 15 (b) mg protein loading of JN11 (Black bar) and PC-3-7 in cocktail enzyme (Gray bar). Various cellobiase activities relative to protein amounts of added Novozyme 188 are showed in the Table. Data represent the averages of three independent experiments ±STD

3.3.3 JN11 and cocktail enzyme showed comparable performance during saccharification and ethanol fermentation

We used the data from the previous experiment to compare the saccharification efficiency of JN11 with that of the cocktail enzyme. Three different protein amounts (5, 10 and 15 mg of JN11 or 10.7, 21.4 and 32.1 mg of cocktail enzyme) were used for the SHF process. No cellobiose remained throughout the saccharification process, indicating that β-glucosidase activity under this cellobiase unit was sufficient to complement the cellulase activity of cocktail enzyme and JN11. The amount of glucose and mannose converted using JN11 and the cocktail enzyme was comparable with all protein loads, with the cocktail enzyme producing slightly higher conversion of both sugars compared to JN11 (Fig. 3.2a-c). Glucose concentration was higher than that of mannose and both showed same level of dose- and time-dependent increases.

We compared the ethanol produced from saccharified sugars released by JN11 and the cocktail enzyme. Both JN11 and the cocktail enzyme saccharification produced comparable amounts of ethanol upon fermentation, with JN11 showing slightly higher efficiency than the cocktail enzyme, especially at longer incubation time (24 hours) (Table 3.2). We also observed that glucose and mannose in the saccharified supernatant incubated for 72 h were completely consumed by yeast at 12 h of fermentation time of all enzyme dosage conditions (data not shown). Ethanol production efficiency under SHF increased with increase in protein loading.

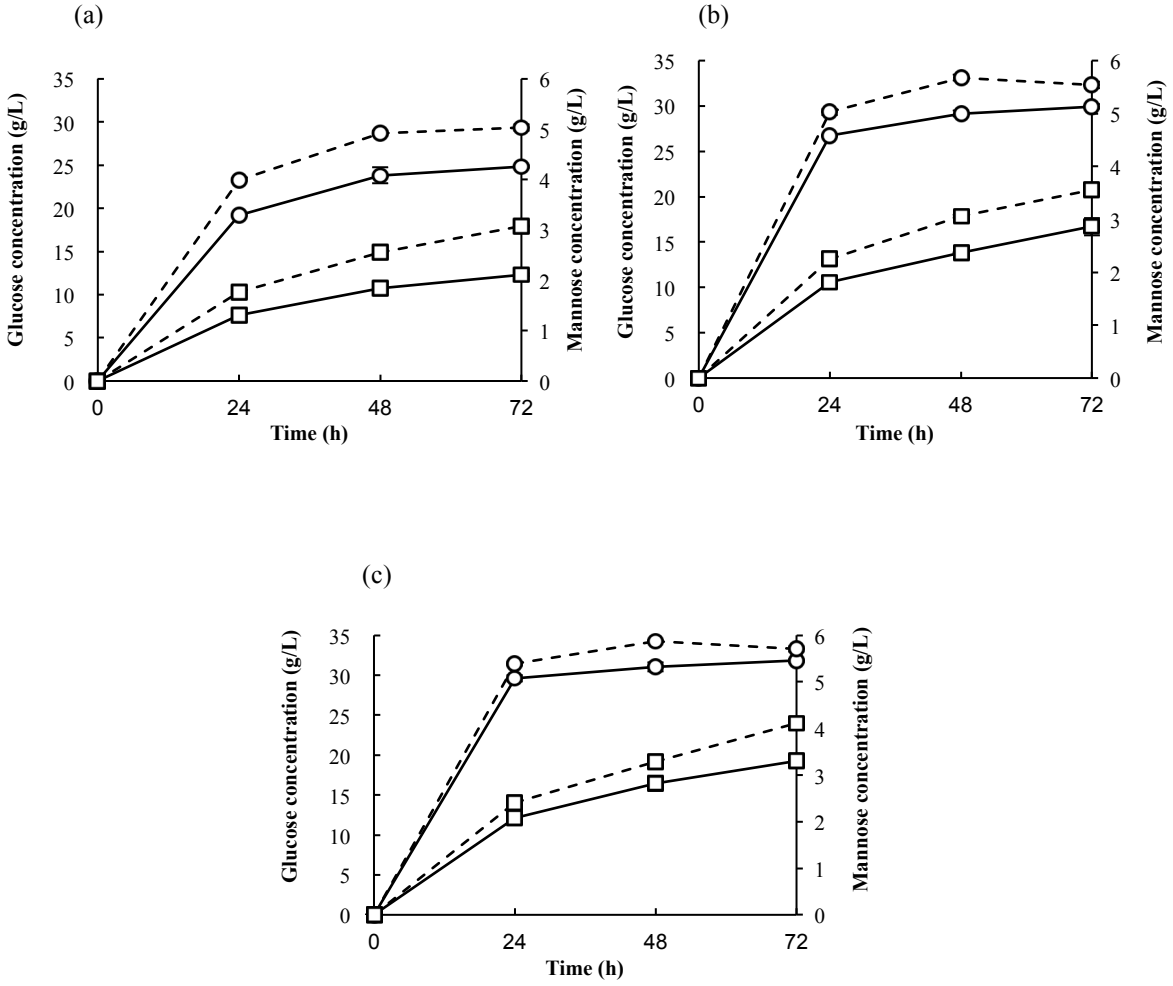


Fig. 3.2 SHF process of converge-milling Japanese cedar by JN11 (bold line) and cocktail enzyme (dotted line) of 17.25 CBU (5 mg protein of JN11 or 10.7 mg protein of cocktail enzyme) (a), 34.50 CBU (10 mg protein of JN11 or 21.4 mg protein of cocktail enzyme) (b) and 51.75 CBU (15 mg protein of JN11 or 32.1 mg protein of cocktail enzyme) (c). The concentration of glucose (circle) and mannose (square) were indicated. Data represent the averages of three independent experiments ±STD.

Table 3.2 Ethanol production from fermentation of SHF process

Cellobiase activity (CBU) of enzyme loading	Time (h)	g/L (% production efficiency)	
		JN11[1]	cocktail enzyme[2]
17.25	12	9.8±0.1 (33.4±0.4)	10.2±0.2 (34.9±0.7)
	24	10.4±0.3 (35.7±1.0)	10.0±0.1 (34.1±0.3)
34.50	12	11.6±0.1 (39.6±0.2)	11.3±0.0 (38.8±0.1)
	24	12.5±0.2 (42.8±0.8)	10.6±0.1 (36.4±0.5)
51.75	12	12.3±0.1 (42.1±0.5)	12.0±0.1 (41.2±0.2)
	24	12.8±0.1 (43.9±0.4)	10.9±0.2 (37.3±0.7)

[1] Amounts of protein loaded for JN11 were 5, 10 and 15 mg, which corresponded to 17.25, 34.50 and 51.75 CBU, respectively.

[2] Amounts of protein loaded for PC3-7 were 5, 10, and 15 mg with supplementation of Novozyme 188 of 5.7, 11.4 and 17.1 mg. The amounts of total protein of the cocktail enzyme loaded were 10.7, 21.4, 32.1 mg protein, which corresponded to 17.25, 34.50 and 51.75 CBU, respectively.

Data represent the averages of three independent experiments ±STD.

3.3.4 AaBGL1 rapidly adsorbed to biomass

We were intrigued by the reduction in the amount of glucose released using JN11 during the saccharification process compared to the cocktail enzyme, even though we had used appropriate amount of protein with appropriate CBU, using values determined from the previous experiment (shown in Fig. 3.2). Therefore, we decided to check *p*NPGlc-ase activity in the reactant during the saccharification process. To our surprise, we found that there was no *p*NPGlc-ase activity in

saccharified supernatant of JN11. We hypothesized that the recombinant protein AaBGL1 adsorbed to the biomass during enzymatic hydrolysis and as a result no endogenous *p*NPGlc-ase activity could take place. To test this hypothesis, we looked at the *p*NPGlc-ase activity of JN11 throughout the saccharification process and compared it to the cocktail enzyme. We carried out smaller-scale saccharification based on 1.5 mg protein (equivalent to 5.2 CBU of JN11/0.1 g-converge-milled Japanese cedar) and measured BGL activity both in the precipitate biomass as well as in the supernatant and biomass wash solutions. Results showed that *p*NPGlc-ase activity of JN11 was seen only in the biomass precipitate but not in the supernatant or wash solutions during the course of saccharification, even through it starts with a high *p*NPGlc-ase activity at the time 0 (Fig. 3.3a). The cocktail enzyme on the other hand showed amount of *p*NPGlc-ase activity both in the precipitate as well as in the supernatant and wash solutions (Fig. 3.3b). The cocktail enzyme showed time-dependent increase in BGL activity in the biomass precipitate and a decrease in the supernatant and wash solutions, whereas JN11 showed steady BGL activity in biomass precipitate fraction (Fig.3.3a and b).

The adsorption of AaBGL1 to the biomass was confirmed by SDS-PAGE analysis, which showed the absence of a band of recombinant AaBGL1, estimated at 95 kDa, in the saccharified supernatant, but presence in the saccharified biomass precipitate (Fig. 3.3c). This explained the *p*NPGlc-ase activity results we observed in Fig. 3.3a. Several distinct bands with molecular mass greater than 75 kDa, which almost belong to Novozyme 188 (Fig. 3.4) were also visible in the saccharified supernatant of cocktail enzyme and some faint bands were observed in biomass precipitate (Fig. 3.3d). CBH I, II and EG I were also identified in JN11 or cocktail enzyme in the saccharified biomass throughout the saccharification period (Fig. 3.3c and d).

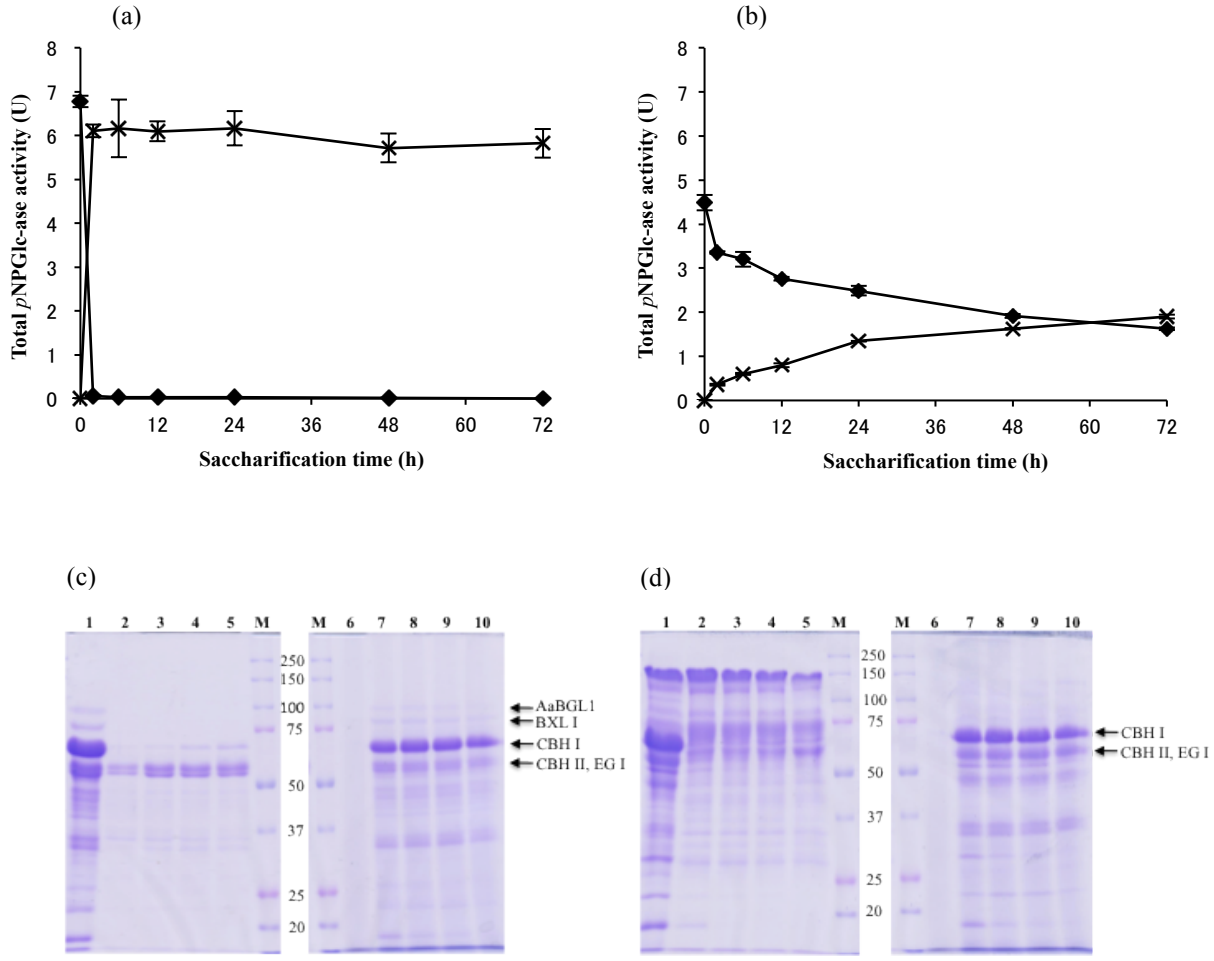


Fig. 3.3 Adsorption characterization of JN11 and cocktail enzyme. *p*NPGlc-ase activity of JN11 (a) and cocktail enzyme (b). The activity of saccharified biomass precipitated (cross) and the sum of activity in supernatant and saccharified biomass wash solution (diamond) are indicated. Data represent averages of three independent experiments ± STD. SDS-PAGE results from JN11 (c) and the cocktail enzyme (d) are shown. Lanes M, molecular weight marker; lane 1, biomass blank; lanes 2-5, saccharified supernatants at 2, 12, 24 and 72 h; lane 6, enzyme blank; lanes 7-10, saccharified biomass precipitated at 2, 12, 24 and 72 h. Note the presence of the 95 kDa band of AaBGL 1 in the JN11 saccharified biomass.

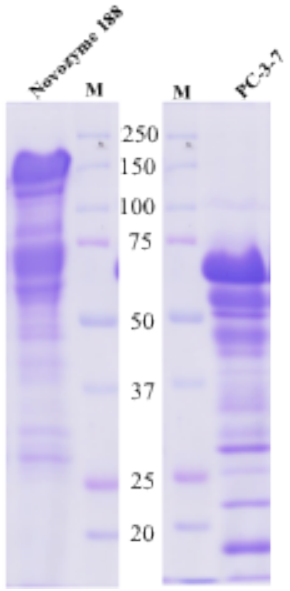


Fig. 3.4 SDS-PAGE of Novozyme 188 and PC-3-7 used in this study. These data were analyzed by SDS-PAGE using a 10% polyacrylamide gel.

3.3.5 Both JN11 and the cocktail enzyme exhibited similar ethanol production efficiency but JN11H exhibited the highest under SSF

We evaluated the conversion efficient of cellulosic material into ethanol under concomitant enzymatic hydrolysis of JN11 and cocktail enzyme under the SSF process. We determined that 39°C was the optimal temperature at which *S. cerevisiae* can function well and the SSF process can take place efficiently. We found that ethanol production efficiency of cocktail enzyme was comparable to that of JN11 under similar cellobiase activity (Fig. 3.5a-c). We also observed a dose-dependent increase in ethanol productivity, indicating that ethanol productivity depended on the dosage of the

saccharifying enzyme. Approximately 50% glucose conversion had been achieved at 17.25 CBU at time 0 or pre-saccharification, and with the increased cellobiase activity at 51.75 CBU, the conversion increased to approximately 70% at the same time (Fig. 3.5a and c and Table 3.3.). Mannose was detected in the pre-saccharified supernatant, and its percentage conversion by JN11 was slightly lower than that of cocktail enzyme. However, mannose was entirely consumed at 6 h of SSF of all enzyme dosage conditions. Interestingly, glucose consumption rate of *S. cereivisiae* under JN11 saccharification was higher than that of cocktail enzyme at 6 h of SSF and glucose was almost entirely consumed at 12 h of SSF (Fig. 3.5a-c).

When 15 mg protein of PC-3-7 was used without the supplementation of exogenous β-glucosidase of Novozyme 188, 11.8 g/L of cellobiose was detected at time 0 and decreased to just of 5.4 g/L cellobiose at 24 h of SSF. Ethanol concentration also decreased with only 7.5 g/L corresponding to 38% ethanol production efficiency at 24 h of SSF, indicating that the β-glucosidase activity of PC-3-7 was inadequate (Fig. 3.6).

Ethanol conversion and accumulation occurred throughout the SSF process and the conversion efficiency between JN11 and the cocktail enzyme was similar throughout. A dose-dependent increase in ethanol productivity was apparent. The highest ethanol production efficiency of 70% was achieved at 51.75 CBU by both JN11 and the cocktail enzyme at 12 h of SSF, while with the lower CBU of 17.25, only 50% ethanol production was achieved for the same amount of SSF time (Table 3.3).

We also further evaluated the efficiency of ethanol production by using JN11H. The glucose from JN11H presaccharification (0 time) showed similar conversion to JN11 and cocktail enzyme (Fig. 3.5). The glucose consumption rate of *S. cerevisiae* during JN11H saccharification was almost similar to cocktail enzyme, which was slower than JN11 (Fig. 3.5a-c). Ethanol conversion and

accumulation of JN11H saccharification showed higher than that of JN11 and cocktail enzyme at 12 h when glucose was depleted. Reached 75% of ethanol production efficiency of JN11H saccharification was obtained at 12 h of SSF. However, the dose-dependent increase in ethanol production was also observed (Fig. 3.5 and Table 3.3).

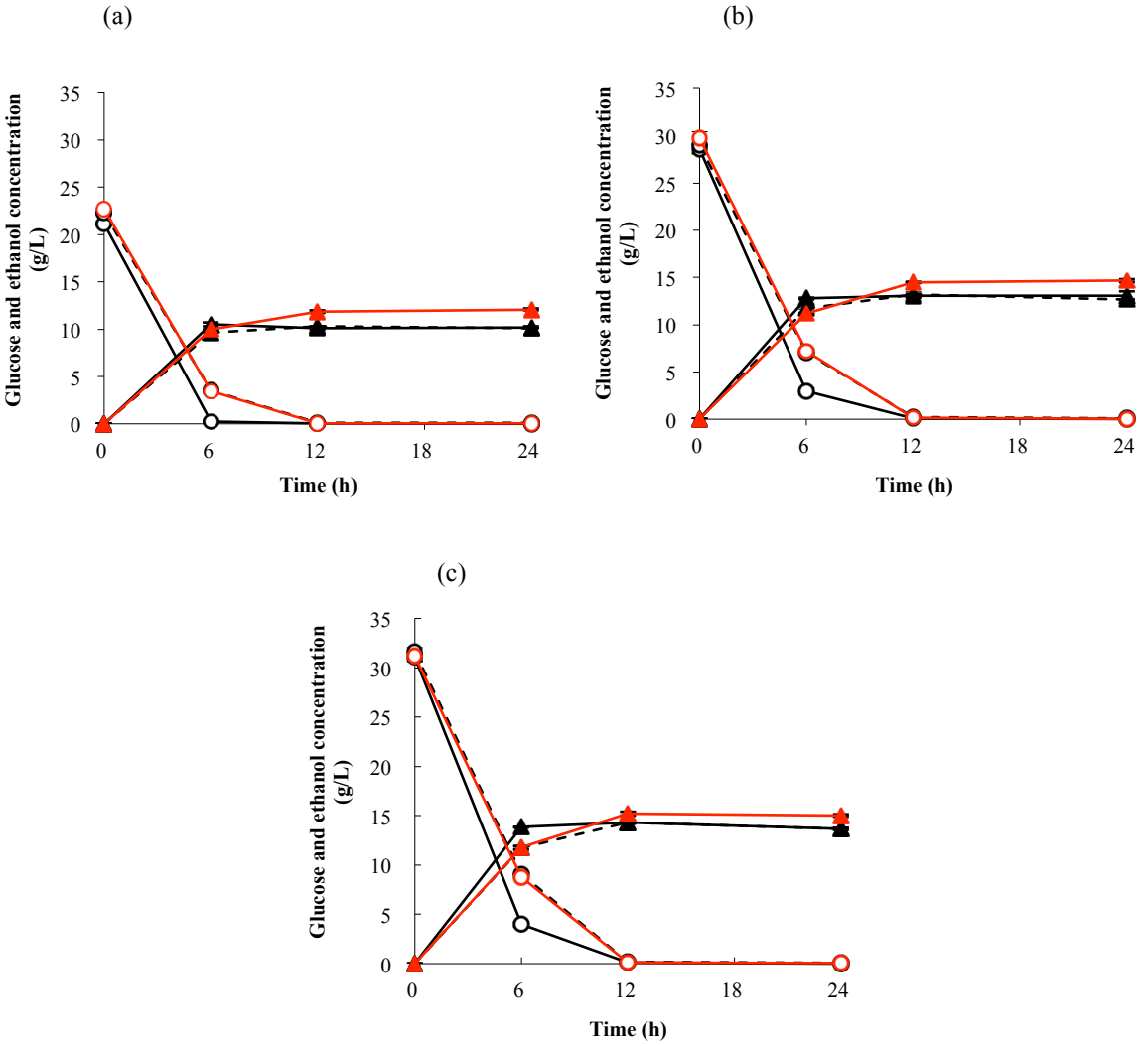


Fig. 3.5 The SSF of converge-milling Japanese cedar by JN11 (black bold line), cocktail enzyme (black dotted line) and JN11H (red bold line) with 17.25 CBU (5 mg protein of JN11 or 10.7 mg protein of cocktail enzyme) or 25 CBU of 5 mg protein of JN11H (a), 34.50 CBU (10 mg protein of JN11 or 21.4 mg protein of cocktail

enzyme) or 50 CBU of 10 mg protein of JN11H (b) and51.75 CBU (15 mg protein of JN11 or 32.1 mg protein of cocktail enzyme) or 75 CBU of 15 mg protein of JN11H (c). The concentrations of glucose (open circle) and ethanol (filled triangle) throughout the SSF process are indicated. Data represent-averages of three independent experiments ± STD.

Table 3.3 Glucose conversion and ethanol production efficiency of SSF

Cellobiase activity (CBU) of enzyme loading	SSF Time (h)	(%)					
		Glucose conversion			Ethanol production efficiency		
		JN11[1]	cocktail enzyme[2]	JN11H[3]	JN11[1]	cocktail enzyme[2]	JN11H[3]
17.25 (JN11 and cocktail enzyme) 25 (JN11H)	0	48.1±0.3	50.7±.0.3	51.6±0.2	0.0±.00	0.0±.00	0.0±0.0
	6	0.8±0.2	10.5±0.2	10.3±0.1	52.0±1.0	47.7±0.2	49.2±0.2
	12	0.2±0.1	0.4±0.0	0.2±0.1	50.1±0.9	50.9±1.6	58.6±0.6
	24	0.0±0.0	0.2±0.0	0.1±0.1	50.5±0.5	50.1±0.0	59.8±0.4
34.50 (JN11 and cocktail enzyme) 50 (JN11H)	0	65.0±1.0	65.9±.0.3	67.6±1.6	0.0±.00	0.0±.00	0.0±0.0
	6	8.7±0.9	20.9±0.1	21.4±0.1	63.2±0.5	58.1±0.5	55.4±0.2
	12	0.4±0.4	0.7±.0.2	0.5±0.1	64.6±1.1	65.2±1.3	71.7±0.4
	24	0.0±0.0	0.3±.0.1	0.1±0.1	64.7±2.1	62.6±0.3	72.6±0.8
51.75 (JN11 and cocktail enzyme) 75 (JN11H)	0	70.6±0.9	72.0±.0.9	70.9±0.6	0.0±.00	0.0±.00	0.0±0.0
	6	11.7±0.6	26.8±0.2	25.9±0.3	68.4±0.2	57.8±1.3	58.1±0.8
	12	0.3±0.1	0.6±.0.0	0.4±0.1	70.8±0.7	70.6±0.3	75.2±1.0
	24	0.0±0.0	0.2±.0.0	0.1±0.0	67.4±0.2	67.5±0.9	74.2±0.6

[1] Amounts of protein loaded for JN11 were 5, 10 and 15 mg, which corresponded to 17.25, 34.50 and 51.75 CBU, respectively.

[2] Amounts of protein loaded for PC-3-7 were 5, 10, and 15 mg with supplementation of Novozyme 188 of 5.7, 11.4 and 17.1 mg. The amounts of total protein of the cocktail enzyme loaded were 10.7, 21.4, 32.1 mg protein, which corresponded to 17.25, 34.50 and 51.75 CBU, respectively.

[3] Amounts of protein loaded for JN11H 5, 10 and 15 mg protein corresponding to 25, 50 and 75 CBU, respectively.

Data represent the averages of three independent experiments ±STD

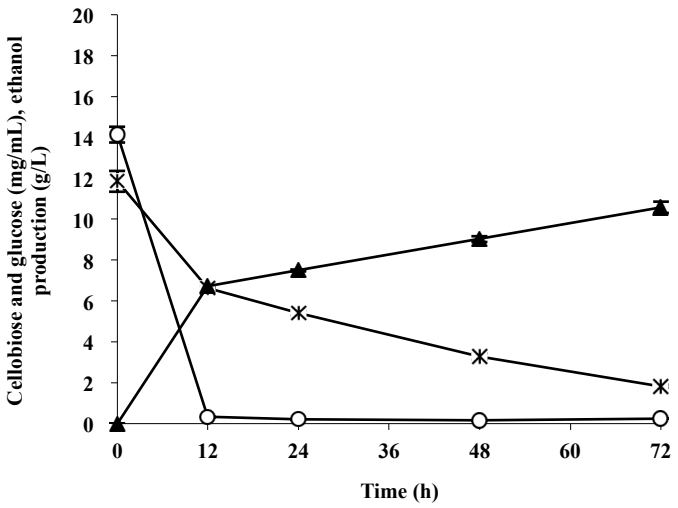


Fig. 3.6 The SSF of converge-milling Japanese cedar by PC-3-7 15 mg protein (1 CBU). The concentrations of cellobiose (asterisk), glucose (open circle) and ethanol (filled triangle) throughout the SSF process are indicated. Data represent-averages of three independent experiments ± STD.

3.4 Discussion

Saccharification is the key step of ethanol production from lignocellulosic materials. The low level of endogenous BGL activity of PC-3-7 limits cellulose hydrolysis, resulting in low level of saccharification. From the specific data of the prepared enzyme used in this study, we found that JN11 has s great BGL activity while the endo- and exo- cellulase activities were maintained and were comparable to the enzyme from its parental strain, PC-3-7 (Table 3.1). This is accordance with the finding of Kawai et al. 2012 and Nakazawa et al. 2011, who reported that JN11 has higher saccharification capability for various pretreated biomass compared to that of PC-3-7 or other commercial enzymes. JN11 has since been considered as a promising enzyme for efficient ethanol production from lignocellulosic material.

In this study, we also examined the specific activities of Novozyme 188, the commercially available BGL from *A. niger*. We found that the BGL activity of this enzyme was high and comparable to that of JN11, but its endo- and exocellulase activities were limited. Novozyme 188 showed high hemicellulose-specific (mannanase and xylanase) activities, which also contributed towards its saccharification ability. Our data show that Novozyme 188 is indeed a suitable supplementary enzyme for reinforcing *T. reesei* cellulase, which remains the traditionally favorable enzyme mixture for lignocellulosic materials degradation (Berlin et al. 2007, Rana et al. 2014). In addition, the culture supernatant from *T. reesei* X3AB1 grown on a supplement of 0.5% xylan, JN11H showed increased BGL and xylanase activities over that of JN11. Increasing of xylanase activities suggested that xylanase activity was induced by xylan counterbalanced the XYNII loss caused by the deletion of *xyn3* (Nakazawa et al. 2011).

The addition of exogenous β-glucosidase with Novozyme 188 supplementation to PC-3-7

could improve not only the efficiency of cellulose hydrolysis, but also the rate of glucose production (Fig. 3.1). To achieve the glucose conversion comparable to that by JN11, PC-3-7 required more supplemental protein, with the total protein load required to achieve the same CBU was significantly different between the two. This difference can be attributed to the insufficient native BGL activity in PC-3-7. Moreover, increased glucose production depended on the enzyme dosage because increasing amount accrued cellulase activities (Fig. 3.1).

The hemicellulose contained in Japanese cedar is glucomannan and galactoglucomannan. Mannose is the second most abundant sugar contained in converge-milled Japanese cedar. Therefore, it was not surprising that mannose was detected during the saccharification process (Fig. 3.2). The presence of mannose revealed the additional advantage for Japanese cedar as a suitable substrate for ethanol production because yeast can also utilize mannose for conversion to ethanol (Nevado et al. 1993, Olofsson et al. 2008, van Maris et al. 2006).

Considering that the cellobiose activity was similar for both JN11 and the cocktail enzyme, it was surprising to see a reduction in glucose conversion by JN11 compared to Novozyme 188. The significant difference in *p*NPGlc-ase activity between the two led us to suspect that perhaps the recombinant protein was adsorbed to the biomass. Measurement of *p*NPGlc-ase activity during the entire process of saccharification showed that during initial stages (at t=0) the *p*NPGlc-ase activity of JN11 was about 1.5 fold higher than that of cocktail enzyme (Fig. 3.3a and b), whereas their measured cellobiase activity was almost similar. Similar results were obtained when specific *p*NPGlc-ase and cellobiase activities were measured (Table 3.1). However, SDS-PAGE analysis of the different fractions showed that there was no free AaBGL1 present in the saccharified supernatant or wash solutions since 2 h of after saccharification, suggesting that the recombinant protein strongly adsorbed to biomass throughout the saccharification process (Fig. 3.3a and c). The cocktail enzyme showed an

increased *p*NPGlc-ase activity in the biomass precipitate, but decrease in the free BGL activity during the saccharification process, suggesting that the enzyme also adsorbed to the biomass, but the binding affinity in this case was weaker than it was for the recombinant AaBGL1 (Fig. 3.3b and d). In addition, to clarify the question whether of protein amount effect to JN11, we incubated JN11 with BSA (Bovine serum albumin) under the similar protein amount to cocktail enzyme. We found that recombinant AaBGL1 rapidly adsorbed to biomass precipitate although protein amount was increased (Fig. 3.7), indicating that the increasing protein amount to those of cocktail enzyme did not affect cellulose adsorption of JN11. To investigate if a possible activity could have an influence on the glucose conversion results, we incubated JN11 and the cocktail enzyme without the biomass substrate for 2-72 h and found that the cellulase proteins and BGL activity were stable throughout the incubation time, indicating that the protease did not affect the glucose conversion of JN11 and cocktail enzyme (Fig. 3.8 and 3.9). SDS-PAGE analysis of the saccharified biomass precipitate showed not only BGL but also almost all cellulases, indicating that these cellulases were also adsorbed to the saccharified biomass (Fig. 3.3c and d). CBH I, for example, is a major cellulase produced by *T. reesei*, and is an important enzyme for crystalline cellulose degradation (Boer et al. 2000, Rosgaard et al. 2007). CBH I, seen as a distinct band at 68 kDa in Fig. 3.3a and b, preferentially adsorbs and promotes degradation on the crystalline cellulose surface because of its cellulose-binding domain (CBD) in the saccharified debris as reported by Mansfield et al. 1999, Sugimoto et al. 2012, Tomme et al. 1987. However, there was some CBH I released into the supernatant during saccharification of JN11, suggesting that cellulases would bind to and hydrolyze cellulose and then redissolve into the supernatant when the polymeric substrate is depolymerized, as suggested in previous reports (Xu et al. 2008). Palonen et al. 2003 reported nonspecific adsorption of CBH I and EG II to lignin of steam-pretreated softwood. This report supported our results that PC-3-7 cellulase

may bind to lignin-particle surface in converge-milled Japanese cedar. It has also been shown that cellulases may be irreversibly adsorbed by the substrate when lignin was present, resulting in decrease in efficiency of cellulose hydrolysis (Sun et al. 2002, Xu et al. 2008). Therefore, it will be reasonable to suppose that differences in adsorption characteristics between JN11 and the cocktail enzyme may influence the conversion of the lignocellulosic materials to glucose. The adsorption of AaBGL1 has also been considered as an interesting point of efficient and economical cellulase enzyme. The further evidences concerning adsorption characterization of AaBGL1 have been expected for clarifying this adsorption phenomenon. Another possible cause of difference in glucose conversion between JN11 and cocktail enzyme has been considered. Hemicelluose contained in secondary cell wall of softwood are glucomannan and galactoglucomannan, which consists of a backbone randomly β-1,4- linked D-glucose and D-mannose residues, carrying single α-1,6-D-galactose units attached to D-glucose or D-mannose residues. Considering the mannannase activity of Novozyme 188 showed 9 fold higher than that of JN11 (Table 3.1), therefore, hemicellulose contained in Japanese cedar was more hydrolyzed to glucose and mannose than by JN11 hydrolysis as a resulted in higher released glucose and mannose concentration from cocktail enzyme hydrolysis.

SSF has been identified as more effective a process for lignocellulosic ethanol conversion than SHF since it combines cellulose hydrolysis and ethanol production in a single process and increases the yield of ethanol by minimizing product inhibition of cellulase (Olofsson et al. 2008, Sánchez et al. 2008, Vásquez et al. 2007). Increasing the enzyme dosage in the SHF and SSF processes can enhance the yield of ethanol because the enzymatic step determines the availability of glucose to ethanologenic fermentation (Philippidis et al. 1993, Sun et al. 2002, Wang et al. 2012). Addition of exogenous β-glucosidase or heterologous expression of β-glucosidase could improve ethanol productivity because of the compensation of BGL activity by the complementation of

cellulase reaction. Without the concomitant enzymatic activity in the fermentation of SHF process, the ethanol production efficiency of both these approaches was similar in our experiments. The cocktail enzyme showed similar production efficiency to that of JN11 under same cellobiase activity, probably because the amount of glucose released from the JN11 and cocktail enzyme saccharification for 72 h were not significantly different to affect the ethanol production level. We did not observe any remarkable difference in ethanol produced using JN11 and cocktail enzyme in the SSF process, especially at 12 h of SSF incubation time. With the same cellobiase activity of both the cocktail enzyme and JN11, the protein amount needed by the cocktail enzyme for ethanol conversion was about 2 fold higher than for JN11, indicating that JN11 is an intrinsic economical enzyme for bioethanol production from lignocellulosic biomass. Results of our experiments show that the SSF process with ethanol fermentation and concomitant enzyme activity is highly efficient than SHF process for ethanol production from Japanese cedar.

Considering the conversion of converge-milled Japanese cedar by JN11H saccharification, the glucose conversion of JN11H showed higher than that from JN11 and cocktail enzyme (Fig. 3.5), indicating that increased β-glucosidase and xylanase activities of JN11H improved the capability of cellulose hydrolysis resulted in subsequently enhancing ethanol conversion efficiency.

The cost of cellulases used for biomass degradation is an important consideration in commercial ethanol production from lignocellulosic biomass. The addition of β-glucosidase from microbial resources increases the cost significantly (Lynd et al. 2005, Merino et al. 2007). Our results demonstrate that using the saccharifying enzyme of recombinant AaBGL1 expressed in *T. reesei* for ethanol production can be as effective as supplementing the process with commercial cellobiase activity. Therefore, JN11 or JN11H could eliminate the cost of supplementing BGL, thereby significant reducing the cost of industrial conversion of lignocellulosic biomass into ethanol.

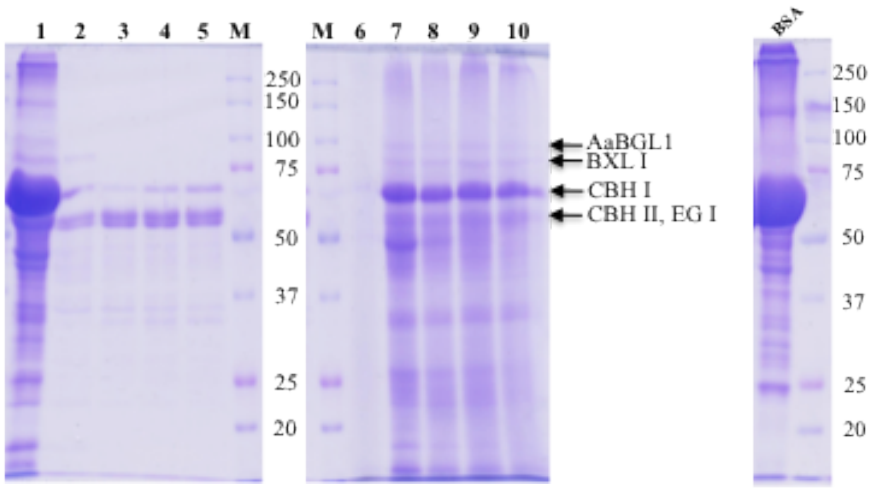


Fig. 3.7 The increased protein amount effect to JN11 adsorption. JN11 was loaded at 5.2 CBU, corresponding to 1.5 mg protein/0.1 g-converge-milled Japanese cedar with BSA protein amount was similar to that of Novozyme 188 in cocktail enzyme. Saccharification incubated at 50°C, 1200 spm for 0-72 h. Lanes M, molecular weight marker; lane 1, biomass blank; lanes 2-5, saccharified supernatants at 2, 12, 24 and 72 h; lane 6, enzyme blank; lanes 7-10, saccharified biomass precipitated at 2, 12, 24 and 72 h. Note the presence of the 95 kDa band of AaBGL1 in the JN11 saccharified biomass. Right panel is SDS-PAGE of BSA protein. These data were analyzed by SDS-PAGE using a 10% polyacrylamide gel.

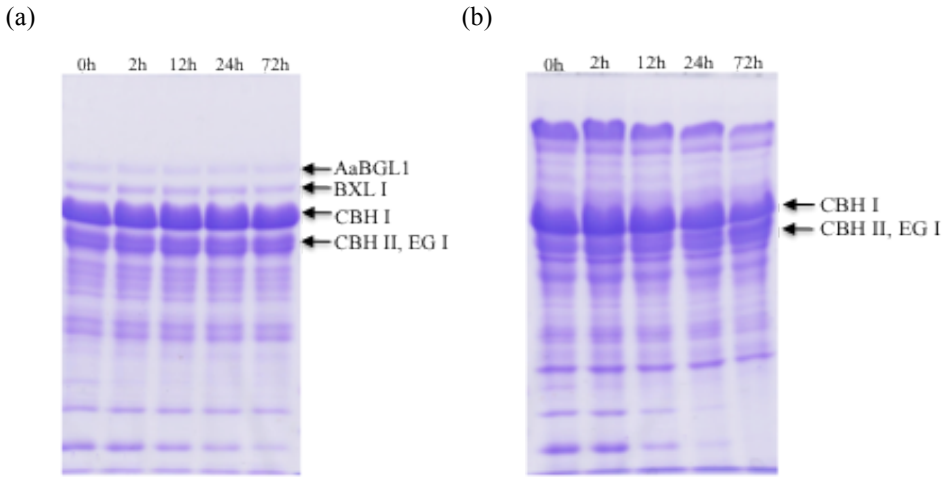


Fig. 3.8 Protein stability of 5.2 CBU of JN11 (a) and cocktail enzyme (PC-3-7 with Novozyme 188) (b) incubated without the biomass substrate at 50°C, 1200 spm for 0-72 h. These data were analyzed by SDS-PAGE using a 10% polyacrylamide gel.

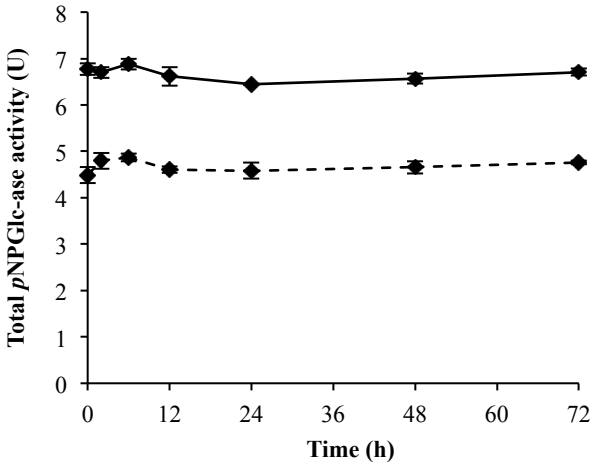


Fig. 3.9 The activity profile of β-glucosidase stability. *p*NPGlc-ase activity of JN11 (bold line) and cocktail enzyme (dotted line) incubated without the biomass substrate at 50°C, 1200 spm for 0-72 h. Data represent averages of three independent experiments ± STD.

3.5 Conclusion

In this study, we investigated the ethanol production ability of the recombinant *T. reesei* mutant expressing *A. aculeatus* β-glucosidase I protein (JN11) and compared it with Novozyme 188, the commercial β-glucosidase supplementary source of PC-3-7, for the degradation of lignocellulosic material into ethanol. Our results suggest that JN11 exhibited similar lignocellulosic ethanol conversion as that of cocktail enzyme Novozyme 188, but needed less protein loading to attain similar efficiency. We also found that JN11 adsorbed to biomass with high affinity compared to the cocktail enzyme, but still was more effective in lignocellulosic ethanol production. The ethanol production efficiency from converge-milled Japanese cedar depended on the amount of enzyme loading and that the concomitant occurrence of saccharification and fermentation during the SSF process enhanced the efficiency of ethanol production compared to SHF. We concluded that the use of JN11 in an SSF process for lignocellulosic ethanol production is very cost effective compared to supplementation using commercial enzymes and results in similar ethanol production efficiency.

Chapter 4 General conclusions

Many problems encounter the efficient lignocellulosic biomass into ethanol such as inadequate of β-glucosidase activity of *T. reesei* cellulase, end products inhibition effect to cellulase reactions and the limitation of fermentability of *S. cerevisiae* to utilize cellobiose. Our research on the ethanol production from lignocellulosic biomass using *T. reesei* culture supernatant under SSF process with both recombinant strains expressing *Aspergillus aculeatus* β-glucosidase I (AaBGL1) could relieve these obstacle. Enzyme characteristic of recombinant AaBGL1 such as high specific activity, pH and thermal stability indicated that AaBGL1 would be suitable for downstream used in SSF process. The ethanol production of recombinant *S. cerevisiae* expressing AaBGL1 and using enzyme preparation produced by recombinant *T. reesei* expressing AaBGL1 showed a comparable ethanol productivity to commercial exogenous Novozyme 188 with *S. cerevisiae* wild type. Consequently, recombinant AaBGL1 efficiently aided in the conversion of cellobiose and resulted in improved the fermentation of generated sugars. Ethanol production from lignocellulosic biomass on SSF with both recombinant strains, *S. cerevisiae* and *T. reesei* expressing AaBGL1 are cost-effective strategy because of the potential to eliminate the cost of supplementing BGL, thereby significant reducing the cost of industrial conversion of lignocellulosic biomass into ethanol. Although the ethanol production efficiency of recombinant *S. cerevisiae* expressing AaBGL1 was observed to be higher than using enzyme preparation (JN11) from recombinant *T. reesei* expressing AaBGL1 but the reaction time needed for accumulating ethanol production of recombinant *S. cerevisiae* expressing AaBGL1 was longer than using enzyme preparation. Consequently, JN11 was an intrinsic economic enzyme preparation for bioethanol production from lignocellulosic biomass.

References

Acebal C, Castillon MP, Estrada P, Mata I, Costa E, Aguado J, Romero D, Jimenez F (1986) Enhanced cellulase production from *Trichoderma reesei* QM9414 on physically treated wheat straw. Appl Microbiol Biotechnol 24:218-223. doi: 10.1007/BF00261540

Adney B, Baker J (1996) Measurement of cellulase activities. NREL Laboratory Analytical Procedure, National Renewable Energy Laboratory, Technical Report NREL/TP-510-42628, Golden, Colorado.

Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P (2008) Molecular biology of the cell, 5th edn. Garland science, Taylor and Francis group, New York. pp. 88-91.

Alfani F, Gallifuoco A, Saporosi A, Spera A, Cantarella M (2000) Comparisonof SHF and SSF processes for the bioconversion of steam-exploded wheat straw. J Ind Microbiol Biotechnol 25:184-192. doi: 10.1038/sj.jim.7000054

Alvira P, Tomás-Pejó E, Ballesteros M, Negro MJ (2010) Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review. Bioresour Technol 101:4851-4861. doi: 10.1016/j.biortech.2009.11.093

Arantes V, Saddler JN (2011) Cellulose accessibility limits the effectiveness of minimum cellulase loading on the efficient hydrolysis of pretreated lignocellulosic substrates. Biotechnol Biofuels 4:3. doi: 10.1186/1754-6834-4-3

Berlin A, Gilkes N, Kilburn D, Bura R, Markov A, Skomarovsky A, Okunev O, Gusakov A, Maximenko V, Gregg D, Sinitsyn A, Saddler J (2005) Evaluation of novel fungal cellulase preparations for ability to hydrolyze softwood substrates - evidence for the role of accessory enzymes. Enz Microbial Technol 37:175-184. doi: 10.1016/j.enzmictec.2005.01.039

Berlin A, Maximenko V, Gilkes N, Saddler J (2007) Optimization of enzyme complexes for lignocellulosic hydrolysis. Biotechnol Bioeng 97:287-296. doi: 10.1002/bit.21238

Boer H, Teeri TT, Koivula A (2000) Characterization of *Trichoderma reesei* cellobiohydrolase Cel7A secreted from *Pichia pastoris* using two different promoters. Biotechnol Bioeng 69:486-494. doi: 10.1002/1097-0290(20000905)69:53.0.CO;2-N

Boles E, Hollenberg CP (1997) The molecular genetics of hexose transport in yeasts. FEMS Microbiol Rev 21:85-111. doi: 10.1111/j.1574-6976.1997.tb00346.x

Börjesson J, Engqvist M, Sipos B, Tjerneld F (2007) Effect of poly(ethylene glycol) on enzymatic hydrolysis and adsorption of cellulase enzymes to pretreated lignocellulose. Enz Microbiol Technol 41:186-195. doi: 10.1016/j.enzmictec.2007.01.003

Bourne Y, Henrissat B (2001) Glycoside hydrolases and glycosyltransferases: families and functional modules. Curr Opin Struct Biol 11:593-600. doi: 10.1016/S0959-440X(00)00253-0

Carlo RC, Richard S, Nazim C, David BL (2008) Third generation biofuels via direct cellulose fermentation. Int J Mol Sci 9:1342-1360. doi: 10.3390/ijms9071342

Carlson M (1999) Glucose repression in yeast. Curr Opin Microbiol 2:202-207. doi:10.1016/S1369-5274(99)80035-6

Chauve M, Mathis H, Huc D, Casanave D, Monot F, Lopes Ferreira N (2010) Comparative kinetic analysis of two fungal β-glucosidase. Biotechnol Biofuels 3:3. doi: 10.1186/1754-6834-3-3

Chen H, Hayn M, Esterbauer H (1992) Purification and characterization of two extracellular β-glucosidases from *Trichoderma reesei*. Biochim Biophys Acta 1121:54-60. doi: 10.1016/0167-4838(92)90336-C

Christopher CB, Randy MB, Tim F (1991) Cloning and amplification of the gene encoding an

extracellular β-glucosidase from *Trichoderma reesei:* Evidence for improved rates of saccharification of cellulosic substrates. Nature Biotechnol 9:562 - 567. doi: 10.1038/nbt0691-562

Cummings C, Fowler T (1996) Secretion of *Trichoderma reesei* β-glucosidase by *Saccharomyces cerevisiae*. Curr Genet 29:227-233. doi: 10.1007/s002940050040

Dan S, Marton I, Dekel M, Bravdo BA, He S, Withers SG, Shoseyov O (2000) Cloning, expression, characterization, and nucleophile identification of family 3, *Aspergillus niger* β-glucosidase. J Biol Chem 275:4973-4980. doi: 10.1074/jbc.275.7.4973

De Deken RH (1966) The crabtree effect: a regulatory system in yeast. J Gen Microbiol 44:149-156. doi: 10.1099/00221287-44-2-149

Demain AL (2009) Biosolutions to the energy problem. J Ind Microbiol Biotechnol 36:319-332. doi: 10.1007/s10295-008-0521-8

Deshpande V, Raman HS, Rao M (1983) Simultaneous saccharification and fermentation of cellulose to ethanol using *Penicillium funiculosum* cellulase and free or immobilized *Saccharomyces uvarum* cells. Biotechnol Bioeng 25:1679-1684. doi: 10.1002/bit.260250623

de Souza CJ, Costa DA, Rodrigues MQ, dos Santos AF, Lopes MR, Abrantes AB, dos Santos Costa P, Silveira WB, Passos FM, Fietto LG (2012) The influence of presaccharification, fermentation and yeast strain on ethanol production from sugarcane bagasse. Bioresour Technol 109:63-69. doi: 10.1016/j.biortech.2012.01.024

Du F, Wolger E, Wallace L, Liu A, Kaper T, Kelemen B (2010) Determination of product inhibition of CBH1, CBH2, and EG1 using a novel cellulase activity assay. Appl Biochem Biotechnol 161:313-317. doi: 10.1007/s12010-009-8796-4

Fitzpatrick J, Kricka W, James TC, Bond U (2014) Expression of three *Trichoderma reesei* cellulase genes in *Saccharomyces pastorianus* for the development of a two-step process of hydrolysis and fermentation of cellulose. J Appl Microbiol 117:96-108. doi: 10.1111/jam.12494

Foreman PK, Brown D, Dankmeyer L, Dean R, Diener S, Dunn-Coleman NS, Goedegebuur F, Houfek TD, England GJ, Kelley AS, Meerman HJ, Mitchell T, Mitchinson C, Olivares HA, Teunissen PJM, Yao J, and Ward M (2003) Transcriptional regulation of biomass-degrading enzymes in the filamentous fungus *Trichoderma reesei*. J Biol Chem 278:31988-31997. doi: 10.1074/jbc.M304750200

Fujita Y, Ito J, Ueda M, Fukuda H, Kondo A (2004) Synergistic saccharification, and direct fermentation to ethanol, of amorphous cellulose by use of an engineered yeast strain codisplaying three types of cellulolytic enzyme. Appl Environ Microbiol 71:1207-1212. doi: 10.1128/AEM.70.2.1207-1212.2004

Fujita Y, Takahashi S, Ueda M, Tanaka A, Okada H, Morikawa Y, Kawaguchi T, Arai M, Fukuda H, Kondo A (2002) Direct and efficient production of ethanol from cellulosic material with a yeast strain displaying cellulolytic enzymes. Appl Environ Microbiol 68:5136-5141. doi: 10.1128/AEM.68.10.5136–5141.2002

Fukumura T, Osada M, Totani K, Nikaido M (2010) Effect of converge mill grinding on the enzymatic saccharification characteristics of woody materials. J Jpn Inst Energy 89:968-974

Galbe M, Zacchi G (2002) A review of the production of ethanol from softwood. Appl Microbiol Biotechnol 59:618-628. doi: 10.1007/s00253-002-1058-9

Gray KA, Zhao L, Emptage M (2006) Bioethanol. Curr Opin Chem Biol 10:141-146. doi: 10.1016/j.cbpa.2006.02.035

Guo ZP, Zhang L, Ding ZY, Gu ZH, Shi GY (2011) Development of an industrial

ethanol-producing strain for efficient utilization of cellobiose, Enzyme Microb Technol 49:105-112. doi: 10.1016/j.enzmictec.2011.02.008

Gusakov AV (2011) Alternatives to *Trichoderma reesei* in biofuel production. Trends Biotechnol 29:419-425. doi: 10.1016/j.tibtech.2011.04.004

Hari Krishna S, Janardhan Reddy T, and Chowdary GV (2001) Simultaneous saccharification and fermentation of lignocellulosic wastes to ethanol using a thermotolerant yeast. Bioresour Technol 77:193-196. doi: 10.1016/S0960-8524(00)00151-6

Harris PV, Welner D, McFarland KC, Re E, Navarro Poulsen JC, Brown K, Salbo R, Ding H, Vlasenko E, Merino S, Xu F, Cherry J, Larsen S, Lo Leggio L (2010) Stimulation of lignocellulosic biomass hydrolysis by proteins of glycoside hydrolase family 61: structure and function of a large, enigmatic family. Biochemistry 49:3305-3316. doi: 10.1021/bi100009p

Hayashi T (2006) The science and lore of the plant cell wall: Biosynthesis, structure, and function, Boca Raton, Florida, pp. 1-7

Hendriks AT, Zeeman G (2009) Pretreatments to enhance the digestibility of lignocellulosic biomass. Bioresour Technol 100:10-18. doi: 10.1016/j.biortech.2008.05.027

Herrmann MC, Vrsanska M, Jurickova M, Hirsch J, Biely P, Kubickey CP (1997) The β-D-xylosidase of *Trichoderma reesei* is a multifunctional β-D-xylan xylohydrolase. Biochem J 321:375-381

Horton HR, Moran LA, Ochs RS, Rawn JD, Scrimgeour KG (2002) Principles of biochemistry, 3rd edn, Pearson Education, United state

Inglin M, Feinberg BA, and Loewenberg JR (1980) Partial purification and characterization of a new intracellular β-glucosidase of *Trichoderma reesei*. Biochem J 185:515–519

Jan H, Jianan F, Lars MB (2009) Correlation between TCA cycle flux and glucose uptake rate during respiro-fermentative growth of *Saccharomyces cerevisiae*. Microbiology 115:3827-3837. doi: 10.1099/mic.0.030213-0

Jordan DB, Bowman MJ, Braker JD, Dien BS, Hector RE, Lee CC, Mertens JA, Wagschal K (2012) Plants cell walls to ethanol. Biochem J 442:241-252. doi: 10.1042/BJ20111922

Kawai T, Nakazawa H, Ida N, Okada H, Tani S, Sumitani J, Kawaguchi T, Ogasawara W, Morikawa Y, Kobayashi Y (2012) Analysis of the saccahrification capability of high-functional cellulase JN11 for various pretreated biomasses through a comparison with commercial available counterparts. J Ind Microbiol Biotechnol 39:1741-1749. doi: 10.1007/s10295-012-1195-9

Kawamori M, Morikawa, Y, Takasawa S (1986) Induction and production of cellulases by L-sorbose in *Trichoderma reesei*. Appl Microbiol Biotechnol 24:449-453. doi: 10.1007/BF00250321

Kroon-Batenburg LMJ, Kroon J (1997) The crystal and molecular structure of cellulose I and II. Glycoconjugate J 14:677-690. doi: 10.1023/A:1018509231331

Kuyper M, Harhangi HR, Stave AK, Winkler AA, Jetten MS, de Laat WT, den Ridder JJ, Op den Camp HJ, van Dijken JP, Pronk JT (2003) High-level functional expression of a fungal xylose isomerase: the key to efficient ethanolic fermentation of xylose by *Saccharomyces cerevisiae*?. FEMS Yeast Res 4:69-78. doi: 0.1016/S1567-1356(03)00141-7

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685. doi: 10.1038/227680a0

Langston J, Sheehy N, Xu F (2006) Substrate specificity of *Aspergillus oryzae* family 3 β-glucosidase. Biochim Biophys Acta 1764:972-978. doi: 10.1016/j.bbapap.2006.03.009

Lau MW, Gunawan C, Balan V, Dale BE (2010) Comparing the fermentation performance of

Escherichia coli K011, *Saccharomyces cerevisiae* 424A(LNH-ST) and *Zymomonas mobilis* AX101 for cellulosic ethanol production. Biotechnol Biofuels 3:11. doi: 10.1186/1754-6834-3-11

Lee WH, Nan H, Kim HJ, Jin YS (2013) Simultaneous saccharification and fermentation by engineered *Saccharomyces cerevisiae* without supplementing extracellular β-glucosidase. J Biotechnol 167:316-22. doi: 10.1016/j.jbiotec.2013.06.016

Lee WJ, Kim MD, Ryu YW, Bisson LF, Seo JH (2002) Kinetic studies on glucose and xylose transport in *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 60:186-191. doi: 10.1007/s00253-002-1085-6

Li S, Du J, Sun J, Galazka JM, Glass NL, Cate JH, Yang X, Zhao H (2010) Overcoming glucose repression in mixed sugar fermentation by co-expressing a cellobiose transporter and a β-glucosidase in *Saccharomyces cerevisiae*. Mol Biosyst 6:2129-2132. doi: 10.1039/c0mb00063a

Lin Y, Tanaka S (2006) Ethanol fermentation from biomass resources: current state and prospects. Appl Microbiol Biotechnol 69:627-642. doi: 10.1007/s00253-005-0229-x

Liu K, Lin X, Yue J, Li X, Fang X, Zhu M, Lin J, Qu Y, Xiao L (2010) High concentration ethanol production from corncob residues by fed-batch strategy. Bioresour Technol 101:4952-4958. doi: 10.1016/j.biortech.2009.11.013

Lynd LR, van Zyl WH, MaBride JE, Laser M (2005) Consolidated bioprocessing of cellulosic biomass: an update. Curr Opin Biotechnol 16:577-583. doi: 10.1016/j.copbio.2005.08.009

Mandels M and Reese ET (1957) Induction of cellulase in *Trichoderma viride* as influenced by carbon sources and metals. J Bacteriol 73:269-278.

Mansfield SD, Mooney D, Saddler JN (1999) Substrate and enzyme characteristics that limit cellulose hydrolysis. Biotechnol Prog 15:804-816. doi: 10.1021/bp9900864

Margeot A, Hahn-Hagerdal B, Edlund M, Slade R, Monot F (2009) New improvements for

lignocellulosic ethanol. Curr Opin Biotechnol 20:372-380. doi: 10.1016/j.copbio.2009.05.009

Margolles Clark E, Tenkanen M, Nakari-Setälä T, Penttilä M (1996a) Cloning of genes encoding α-L-arabinofuranosidase and β-xylosidase from *Trichoderma reesei* by expression in *Saccharomyces cerevisiae*. Appl Environ Microbiol 62:3840-3846

Margolles Clark E, Tenkanen M, Söderlund H, Penttilä M (1996b) Acetyl xylan esterase from *Trichoderma reesei* contains an active site serine residue and a cellulose binding domain. Eur J Biochem 237:553-560

Margolles Clark E, Saloheimo M, Siika-aho M, Penttilä M (1996c) The α-glucuronidase-encoding gene of *Trichoderma reesei*. Gene 172:171-172

Margolles Clark E, Tenkanen M, Luonteri E, Penttilä M (1996d) Three α-galactosidase genes of *Trichoderma reesei* cloned by expression in yeast. Eur J Biochem 240:104-111

Margolles Clark E, Ilmen M, Penttila M (1997) Expression patterns of ten hemicellulase genes of the filamentous fungus *Trichoderma reesei* on various carbon sources. J Biotechnol 57:167-179

Martinez D, Berka RM, Henrissat B, Saloheimo M, Arvas M, Baker SE, Chapman J, Chertkov O, Coutinho PM, Cullen D, Danchin EG, Grigoriev IV, Harris P, Jackson M, Kubicek CP, Han CS, Ho I, Larrondo LF, de Leon AL, Magnuson JK, Merino S, Misra M, Nelson B, Putnam N, Robbertse B, Salamov AA, Schmoll M, Terry A, Thayer N, Westerholm-Parvinen A, Schoch CL, Yao J, Barabote R, Nelson MA, Detter C, Bruce D, Kuske CR, Xie G, Richardson P, Rokhsar DS, Lucas SM, Rubin EM, Dunn-Coleman N, Ward M, Brettin TS (2008) Genome sequencing and analysis of the biomass-degrading fungus *Trichoderma reesei* (syn. *Hypocrea jecorina*). Nat Biotechnol 26:553-560. doi: 10.1038/nbt1403

Medve J, Karlsson J, Lee D, Tjerneld F (1998) Hydrolysis of microcrystalline cellulase by cellobiohydrolase I and endoglucanase I and endoglucanase II from *Trichoderma reesei*:

adsorption, sugar production pattern and synergism of the enzymes. Biotechnol Bioeng 59:621-634. doi: 10.1002/(SICI)1097-0290(19980905)59:5<621::AID-BIT13>3.0.CO;2-C

Mello BL, Polikarpov I (2014) Family 1 carbohydrate binding-modules enhance saccharification rates. AMB Express 4:36. doi: 10.1186/s13568-014-0036-9

Merino ST, Cherry J (2007) Progress and challenges in enzyme development for biomass utilization. Adv Biochem Engin/Biotechnol 108:95-120. doi: 10.1007/10_2007_066

Miettinen-Oinonen A, Paloheimo M, Lantto R, Suominen P (2005) Enhanced production of cellobiohydrolases in *Trichoderma reesei* and evaluation of the new preparations in biofinishing of cotton. J Biotechnol 116: 305-317. doi: 10.1016/j.jbiotec.2004.10.017

Mussatto SI, Dragone G, Guimarã PM, Silva JP, Carneiro LM, Roberto IC, Vicente A, Domingues L, Teixeira JA (2010) Technological trends, global market, and challenges of bio-ethanol production. Biotechnol Adv 28:817-830. doi: 10.1016/j.biotechadv.2010.07.001

Nakazawa H, Kawai T, Ida N, Shida Y, Kobayashi Y, Okada H, Tani S, Sumitani J, Kawaguchi T, Morikawa Y, Ogasawara W (2011) Construction of a recombinant *Trichoderma reesei* strain expressing *Aspergillus aculeatus* β-glucosidase 1 for efficient biomass conversion. Biotechnol Bioeng 109:92-99. doi: 10.1002/bit.23296

Nevado J, Navarro MA, Heredia CF (1993) Galactose inhibition of the constitutive transport of hexoses in *Saccharomyces cerevisiae*. Yeast 9:111-119, doi: 10.1002/yea.320090202

Nikaido M, Totani K, Fukumura T, Osada M, Osada M, Yanagawa K, KOgawa J (2012) Effective utilization of woody biomass using converge mill and enzymatic saccharification characteristics. J Soc Powder Technol Japan 49:675-682

Nitta M, Furukawa T, Shida Y, Mori K, Kuhara S, Morikawa Y, Ogasawara W (2012) A new Zn(II)(2)Cys(6)-type transcription factor BglR regulates β-glucosidase expression in *Trichoderma*

reesei. Fungal Genet Biol 49:388-397. doi: 10.1016/j.fgb.2012.02.009

Olofsson K, Bertilsson M, Lidén G (2008) A short review on SSF- an interesting process option for ethanol production from lignocellulosic feedstocks. Biotechnol Biofuels 1:7. doi: 10.1186/1754-6834-1-7

Olsson L, Hahn-Hägerdal B (1996) Fermentation of lignocellulosic hydrolysates for ethanol production. Enz and Microbiol Technol 18:312-331. doi: 10.1016/0141-0229(95)00157-3

Olsson L, Nielsen J (2000) The role of metabolic engineering in the improvement of *Saccharomyces cerevisiae*: utilization of industrial media. Enzyme Microb. Technol. 26, 785-792. doi: 10.1016/S0141-0229(00)00172-1

Palmqvist E, Hahn-Hägerdal B (2000) Fermentation of lignocellulosic hydrolysates. I: inhibition and detoxification. Bioresour Technol 74:17-24. doi: 10.1016/S0960-8524(99)00160-1

Palonen H, Tjerneld F, Zacchi G, Tenkanen M (2003) Adsorption of *Trichoderma reesei* CBH I and EG II and their catalytic domains on steam pretreated softwood and isolated lignin. J Biotechnol 107:65-72. doi: 10.1016/j.jbiotec.2003.09.011

Pan X, Xie D, Gilkes N, Gregg DJ, Saddler JN (2005) Strategies to enhance the enzymatic hydrolysis of pretreated softwood with high residual lignin content. Appl Biochem Biotechnol 121-124:1069-1080

Petschacher B, Leitgerb S, Kavanagh KL, Wilson DK, Nidetzxy B (2005) The coenzyme specificity of Candida tenuis xylose reductase (AKR2B5) explored by site-directed mutagenesis and X-ray crystallography. Biochem J 385:75-83. doi: 10.1042/BJ20040363

Percival Zhang YH, Himmel ME, Mielenz JR (2006) Outlook for cellulase improvement: screening and selection strategies. Biotechnol Adv 24: 452-481. doi: 10.1016/j.biotechadv.2006.03.003

Piskur J, Rozpedowska E, Polakova S, Merico A, Compagna C (2006) How did *Saccharomyces*

evolve to become a good brewer?. Trends Genet 22:183-186. doi: 10.1016/j.tig.2006.02.002

Pitkänen JP, Rintala E, Aristidou A, Ruohonen L, Penttilä M (2005) Xylose chemostat isolates of *Saccharomyces cerevisiae* show altered metabolite and enzyme levels compared with xylose, glucose, and ethanol metabolism of the original strain. Appl Microbiol Biotechnol 67:827-837. doi: 10.1007/s00253-004-1798-9

Polizeli ML, Rizzatti AC, Monti R, Terenzi HF, Jorge JA, Amorim DS (2005) Xylanases from fungi: properties and industrial apllications. Appl Microbiol Biotechnol 67:577-591. doi: 10.1007/s00253-005-1904-7

Postma E, Verduyn C, Scheffers WA, Van Dijken JP (1989) Enzymic analysis of the crabtree effect in glucose-limited chemostat cultures of *Saccharomyces cerevisiae*. Appl Environ Microbiol 55:468-477

Rahman Z, Shida Y, Furukawa T, Suzuki Y, Okada H, Ogasawara W, Morikawa (2009) Evaluation and characterization of *Trichoderma reesei* cellulase and xylanase promoters. Appl Microbiol Biotechnol 82:899–908. doi: 10.1007/s00253-008-1841-3

Rana V, Eckard AD, Ahring BK (2014) Comparison of SHF and SSF of wet exploded corn stover and loblolly pine using in-house enzymes produced from *T. reesei* RUT C30 and *A. saccharolyticus*. Springerplus 3:516. doi: 10.1186/2193-1801-3-516

Reese ET (1956) A microbiological process report. Enzymatic hydrolysis of cellulose. Appl Microbiol 4:39-45.

Riou C, Salmon JM, Vallier MJ, Günata Z, Barre P (1998) Purification, characterization, and substrate specificity of a novel highly glucose-tolerant β-glucosidase from *Aspergillus oryzae*. Appl Environ Microbiol 64:3607-3614

Rosgaard J, Pedersen S, Langston J, Akerhielm D, Cherry JR, Meyer AS (2007) Evaluation of

minimal *Trichoderma reesei* cellulase mixtures on different pretreated barley straw substrates. Biotechnol Prog 23:1270-1276. doi: 10.1021/bp070329p

Rolland F, Winderickx J, Thevelein JM (2002) Glucose-sensing and -signalling mechanisms in yeast. FEMS Yeast Res. 2:183-201. doi: 10.1111/j.1567-1364.2002.tb00084.x

Saarelainen R, Paloheimo M, Richard F, Suominen PL, Nevalainen Helena KM (1993) Cloning, sequencing and enhanced expression of the *Trichoderma reesei* endoxylanase II (pI 9) gene *xln2*. Mol Gen Genet 241:497-503.

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425

Sakamoto R, Arai M, Sawano M (1985) Enzymatic properties of Three β-glucosidases from *Aspergillus aculeatus* No. F-50. Agric Biol Chem 49:1283-1290. doi: 10.1271/bbb1961.49.1283

Saloheimo M, Kuja-Panula J, Ylösmäki E, Ward M, Penttilä M (2002) Enzymatic properties and intracellular localization of the novel *Trichoderma reesei* β-glucosidase BGLII (Cel1A). Appl Environ Microbiol 68:4546–4553. doi: 10.1128/AEM.68.9.4546-4553.2002

Sánchez ÓJ, Cardona CA (2008). Trends in biotechnological production of fuel ethanol from different feedstocks. Bioresour Technol 99:5270-5295. doi: 10.1016/j.biortech.2007.11.013

Scheller HV, Ulvskov P (2010) Hemicelluloses. Annu Rev Plant Biol 61:263-289. doi: 10.1146/annurev-arplant-042809-112315

Shen Y, Zhang Y, Ma T, Bao X, Du F, Zhuang G, Qu Y (2008) Simultaneous saccharification and fermentation of acid-pretreated corncobs with a recombinant *Saccharomyces cerevisiae* expressing β-glucosidase. Bioresour Technol 99:5099-5103. doi: 10.1016/j.biortech.2007.09.046

Shuster A, Schmoll M (2010) Biology and biotechnology of *Trichoderma*. Appl Microbiol Biotechnol 87:787-799. doi: 10.1007/s00253-010-2632-1

Somogyi M (1952) Notes on sugar determination. J Biol Chem 195:19-23

Song J, Liu B, Liu S, Yang Q (2010) Cloning of two cellobiohydrolase genes from *Trichoderma viride* and heterogeneous expression in yeast *Saccharomyces cerevisiae*. Mol Biol Rep 37:2135-2140. doi: 10.1007/s11033-009-9683-3

Stanley D, Bandara A, Fraser S, Chambers PJ, Stanley GA (2010) The ethanol stress response and ethanol tolerance of *Saccharomyces cerevisiae*. J Appl Microbiol 109:13-24. doi: 10.1111/j.1365-2672.2009.04657.x

Stenberg K, Bolló M, Réczey K, Galbe M, Zacchi G (2000) Effect of substrate and cellulase concentration on simultaneous saccharification and fermentation of stream-pretreated softwood for ethanol production. Biotechnol Bioeng 68:204-210. doi: 10.1002/(SICI)1097-0290(20000420)68:2<204::AID-BIT9>3.0.CO;2-4

Stephen K. Ritter (2008) Lignocellulose : A complex Biomaterial. Chemical and Engineering News 86:15.

Sugimoto N, Igarashi K, Wada M, Samejima M (2012) Adsorption characteristics of fungal family 1 cellulose-binding domain from *Trichoderma reesei* cellobiohydrolase I on crystalline cellulose: negative cooperative adsorption via a steric exclusion effect. Langmuir 28:14323-14329. doi: 10.1021/la302352k

Sun Y, Chang J (2002) Hydrolysis of lignocellulosic materials for ethanol production: a review. Bioresour Technol 83:1-11. doi: 10.1016/S0960-8524(01)00212-7

Suzuki K, Sumitani J, Nam YW, Nishimaki T, Tani S, Wakagi T, Kawaguchi T, Fushinobu S (2013) Crystal structures of glycoside hydrolase family 3 β-glucosidase 1 from *Aspergillus aculeatus*. Biochem J 452: 211-221. doi: 10.1042/BJ20130054

Takada G, Kawaguchi T, Sumitani J, Arai M (1998) Expression of *Aspergillus aculeatus* No. F-50

cellobiohydrolase I (*cbhI*) and β-glucosidase 1 (*bgl1*) genes by *Saccharomyces cerevisiae*. Biosci Biotechnol Biochem 62:1615-1618. doi: 10.1271/bbb.62.1615

Takashima S, Iikura H, Nakamura A, Hidaka M, Masaki H, Uozumi T (1998) Overproduction of recombinant *Trichoderma reesei* cellulases by *Aspergillus oryzae* and their enzymatic properties. J Biotechnol 65:163-171. doi: 10.1016/S0168-1656(98)00084-4

Takeda T, Nikaido M, Totani K, Obara M, Nakano Y, Uchimiya H (2009) Evaluation of high intensive ball mill method for effective saccharification of plant cell wall materials. J Appl Glycosci 56:71-76

Tang H, Hou J, Shen Y, Xu L, Yang H, Fang X, Bao X (2013) High β-glucosidase secretion in *Saccharomyces cerevisiae* improves the efficiency of cellulase hydrolysis and ethanol production in simultaneous saccharification and fermentation. J Microbiol Biotechnol 23:1577-1585. doi: 10.4014/jmb.1305.05011

Tenkanen M, Makkonen M, Perttula M, Viikari L, Teleman A (1997) Action of *Trichoderma reesei* mannanase on galactoglucomannan in pine kraft pulp. J Biotechnol 57:191-204. doi: 10.1016/S0168-1656(97)00099-0

Tenkanen M, Vrsanská M, Siika-aho M, Dominic WW, Vladimir P, Penttilä M, Saloheimo M, Biely P (2012) Xylanase XYN IV from *Trichoderama reesei* showing exo- and endo-xylanase activity. FEBS 280:285-301. doi: 10.1111/febs.12069

Timell TE, Syracuse NY (1967) Recent progress in the chemistry of wood hemicelluloses. 1:45-70. Wood Science and Technology 1:45-70. doi: 10.1007/BF00592255

Tomme P, Van Tibeurgh H, Pettersson G, Van Damme J, Vandekerckhove J, Knowles J, Teeri T, Claeyssens M (1987) Studies of the cellulolytic system of *Trichoderma reesei* QM9414. Analysis of domain function in two cellobiohydrolases by limited proteolysis. Eur J Biochem

170:575-581. doi: 10.1111/j.1432-1033.1988.tb13736.x

Torronen A and Rouvinen J (1997) Structure and functional properties of low molecular weight endo-1,4- β-xylanases. J Biotechnol 57:137-149.

van den Brink J, de Vries RP (2011) Fungal enzyme sets for plant polysaccharide degradation. Appl Microbiol Biotechnol 91:1477-1492. doi: 10.1007/s00253-011-3473-2.

van Maris AJ, Abbott DA, Bellissimi E, van den Brink J, Kuyper M, Luttik MA, Wisselink HW, Scheffers WA, van Dijken JP, Pronk JT (2006) Alcoholic fermentation of carbon sources in biomass hydrolysates by *Saccharomyces cerevisiae*: current status. Anton van Leeuw 90:391-418. doi: 10.1007/s10482-006-9085-7

van Rooyen R, Hahn-Hägerdal B, La Grange DC, van Zyl WH (2005) Construction of cellobiose-growing and fermenting *Saccharomyces cerevisiae* strains. J Biotechnol 120:284-295. doi: 10.1016/j.jbiotech.2005.06.013

Vásquez MP, da Silva JN, de Souza MB Jr, Pereira N Jr (2007) Enzymatic hydrolysis optimization to ethanol production by simultaneous saccharification and fermentation. Appl Biochem Biotechnol 137-140:141-153. doi: 10.1007/s12010-007-9046-2

Wang M, Li Z, Fang X, Wang L, Qu Y (2012) Cellulolytic enzyme production and enzymatic hydrolysis for second-generation bioethanol production. Adv Biochem Eng Biotechnol 128:1-24. doi: 10.1007/10_2011_131. doi: 10.1007/10_2011_131

Wilde C, Gold ND, Bawa N, Tambor JH, Mougharbel L, Storms R, Martin VJ (2012) Expression of a library of fungal β-glucosidases in *Saccharomyces cerevisiae* for the development of a biomass fermenting strain. Appl Microbiol Biotechnol 95:647-659. doi: 10.1007/s00253-011-3788-z

Wingren A, Galbe M, Zacchi G (2003) Techno-economic evaluation of producing ethanol from softwood: comparison of SSF and SHF and identification of bottlenecks. Biotechnol Prog 19:1109-1117. doi: 10.1021/bp0340180

Xiao Z, Zhang X, Gregg DJ, Saddler JN (2004) Effects of sugars inhibition on cellulases and β-glucosidase during enzymatic hydrolysis of softwood substrates. Appl Biochem Biotechnol 113-116:1115-1126. doi: 10.1385/ABAB:115:1-3:1115

Xu J, Takakuwa N, Nogawa M, Okada H, Morikawa Y (1998) A third xylanase from *Trichoderma reesei* PC-3-7. Appl Microbiol and Biotechnol 49:718-724.

Xu F, Ding H, Osborn D, Tejitian A, Brown K, Albano W, Sheehy N (2008) Partition of enzymes between the solvent and insoluble substrate during the hydrolysis of lignocellulose by cellulases. J Mol Catal B-Enzym 51:42-48. doi: 10.1016/j.molcatb.2007.10.004

Yamada R, Nakatani Y, Ogino C, Kondo A (2013) Efficient direct ethanol production from cellulose by cellulase- and cellodextrin transporter-co-expressing *Saccharomyces cerevisiae*. AMB express 3:34. doi: 10.1186/2191-0855-3-34

Yanase S, Yamada R, Kaneko S, Noda H, Hasunuma T, Tanaka T, Ogino C, Fukuda H, Kondo A (2010) Ethanol production from cellulosic materials using cellulase-expressing yeast. Biotechnol J 5:449-455. doi: 10.1002/biot.200900291

Yue Z, Bin W, Baixu Y, Peiji G (2004) Mechanism of cellobiose inhibition in cellulose hydrolysis by cellobiohydrolase. Sci Chian C Life Sci 47:18-24. doi: 10.1360/02yc0163

Zaldivar J, Nielsen J, Olsson L (2001) Fuel ethanol production from lignocellulose: a challenge for metabolic engineering and process integration. Appl Microbio Biotechnol 56:17-34. doi 10.1007/s002530100624

Zhang YH, Lynd LR (2004) Toward an aggregated understanding of enzymatic hydrolysis of cellulose: noncomplexed cellulase systems. Biotechnol Bioeng 88:797-824. doi: 10.1002/bit.20282

Zheng Y, Zhao J, Xu F, Li Y (2014) Pretreatment of lignocellulosic biomass for enhance biogas production. Progress in Energy and Combustion Science 42:35-53. doi: 10.1016/j.pecs.2014.01.001

Zhoa X, Zhang L, Liu D (2012) Biomass recalcitrance. Part I: the chemical compositions and physical structures affecting the enzymatic hydrolysis of lignocellulose. Biofpr 6:465-482. doi: 10.1002/bbb.1331

Zhou Q, Xu J, Kou Y, Lv X, Zhang X, Zhao G, Zhang W, Cheng G, Liu W (2012) Differential involvement of β-glucosidases from *Hypocrea jecorina* in rapid induction of cellulase genes by cellulose and cellobioase. Eukaryot Cell 11:1371-1381. doi: 10.1128/EC.00170-12

Acknowledgements

Firstly, I own my deepest gratitude to my supervisor, Professor Hirofumi Okada who always guided, encouraged and supported me to understanding in this research. He is in my mind and my memory forever.

I greatly appreciate my supervisor, Associate professor Wataru Ogasawara for greatly helped, encouraged and supported me to set up the both manuscripts and this thesis with his patience and knowledge.

I would like to thank Professor Mitsuru Nikaido, Department of Chemical Engineering of Ichinoseki Colledge of Technology for pretreatment of converge-milled Japanese cedar.

I am heartily thank Dr. Yosuke Shida and Dr. Koki Shioya for great helping and supporting me to set up manuscripts and this thesis.

I would like to thank Mr. Nobuhiro Ochiai and Ms. Asami Nagaiwa for *T. reesei* Cel3B expressed in *S. cerevisiae* strain and its characteristic data.

I deeply impress all members of Okada and Ogasawara laboratory who always gave me an encouragement. All of impression experiences and great deal from all of you would be engraved on my mind forever.

Finally, I would like to thank my family for love, encouragement and supporting me throughout my life.

List of publications

Treesukon Treebupachatsakul, Hikaru Nakazawa, Hideaki Shinbo, Hiroki Fujikawa, Asami Nagaiwa, Nobuhiro Ochiai, Takashi Kawaguchi, Mitsuru Nikaido, Kazuhide Totani, Koki Shioya, Yosuke Shida, Yasushi Morikawa, Wataru Ogasawara, Hirofumi Okada. Heterologously expressed *Aspergillus aculeatus* β-glucosidase in *Saccharomyces cerevisiae* is a cost-effective alternative to commercial supplementation of β-glucosidase in industrial ethanol production using *Trichoderma reesei* cellulases. J Biosci Bioeng. Received 18 December 2014, accepted 8 May 2015.

Treesukon Treebupachatsakul, Koki Shioya, Hikaru Nakazawa, Takashi Kawaguchi, Yasushi Morikawa, Yosuke Shida, Wataru Ogasawara, Hirofumi Okada. Utilization of recombinant *Trichoderma reesei* expressing *Aspergillus aculeatus* β-glucosidase I (JN11) for a more economical production of ethanol from lignocellulosic biomass. J Biosci Bioeng. Received 13 February 2015, accepted 22 April 2015.