Effects of xylan on production of cellulolytic enzymes by the basidiomycete *Phanerochaete chrysosporium*

担子菌 *Phanerochaete chrysosporium* の

セルロース分解関連酵素生産に及ぼすキシランの影響に関する研究

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堀　千明

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1.1 Composition and structure of plant cell wall

On the earth, lignocellulosic biomass in the form of plant cell wall is the most abundant organic resource and has drawn considerable attention as a promising resource for conversion to useful products such as bioethanol and bioplastic (Himmel*, et al.*, 2007). Plant cell wall is made largely of polysaccharides such as cellulose and hemicellulose as well as the recalcitrant phenolic compound lignin. The ratio of those components varies dependent on plant species, cell type or development stage. Generally the composition of plant cell wall is about 50 % cellulose, subsequently 20-25 % hemicellulose and 30-25 % lignin (Eriksson*, et al.*, 1990). Those components are configured in the form of a structurally complex framework not a simple mixture, which provide mechanical strength and resistance to microbial attack, as shown in Fig. 1-1. The microstructure of plant cell wall consists of thin primary wall, thick secondary wall (the outer, middle and inner layers) and middle lamella, which is located between the adjacent cells (Sjostrom, 1981), as shown in Fig. 1-1A. The ultrastructure of primary wall is extensively studied because this layer is related to cell growth (Scheller & Ulvskov, 2010). This simplified model is presented and it displays that cellulose fibrils are cross-linked with tethering hemicellulose, i.e. xyloglucans (McNeil*, et al.*, 1984). On the other hand, the ultrastructure of secondary wall is known to consist of several concentric lamellae by many microscopic studies and chemical analyses and they exhibit the presence of fibril aggregates, composed of cellulose and hemicellulose, and hemicellulose-lignin matrix, which is located between the fibril aggregates (Salmen, 2004), as showing in Fig. 1-1B. The model of secondary wall for arrangement of cellulose, hemicellulose and lignin is proposed by Kerr and Goring in 1975 (Kerr & Goring, 1975), as shown in Fig. 1-1C. In the complex,

moreover, cellulose is adhesively connected with hemicellulose possibly via hydrogen bounds while hemicellulose forms the covalent cross-linkages with lignin (Ebringerová*, et al.*, 2005).

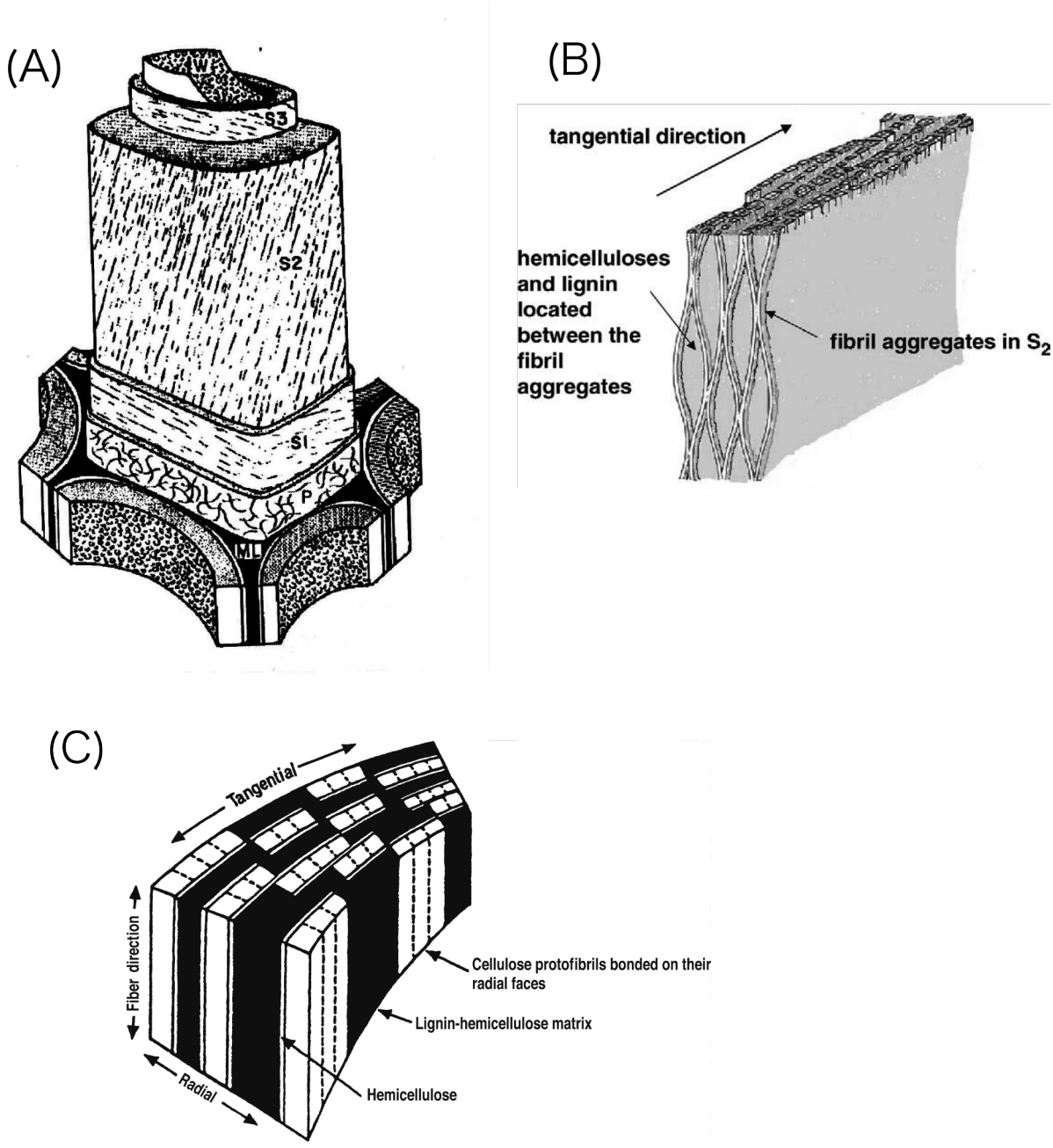


Fig. 1-1. (A) Microstructure of plant cell wall. ML, middle lamella; P, primary cell wall; S, secondary cell wall (S1, outer layer; S2, middle layer; S3, inner layer). (Based on 1981 Sjostrom) (B) Illustration of fibril aggregates in secondary wall (C) Model of ultrastructure in secondary wall proposed by Kerr (1975)).

1.1.1 Cellulose in plant cell wall

Cellulose is the major component of plant cell wall and the most abundant organic polymer on earth, whose total amount has been estimated around 7×10^{11} tons (Coughlan, 1985). The structure of cellulose is a linear homo-polymer of beta-1,4-linked glucose residues and the basic repeating structure unit of the molecular chain of cellulose is cellobiose rather than glucose, as shown Fig. 1-2. The end of cellulose chain with an anomeric carbon is referred to as the reducing end and the other end of the polymer is the non-reducing end. Cellulose chains are firmly hold together by intra- and inter-molecular bonds via hydrogen bonds, hydrophobic interaction and van der Waals forces, forming highly crystalline structure, called cellulose microfibril (Gardner & Blackwell, 1974). The size of the cellulose microfibril is dependent on the origin source but the microfibril in plant consist of some elementary fibrils packing together tightly and the width of elementary fibril is approximately 3.5 nm, which contains 36 cellulose chains (Hon, 1994, Persson*, et al.*, 2004). The length of cellulose chains is defined by degree of polymerization (DP) and DP in secondary wall is high and rather well defined than primary wall, DP 2,000 or more (Hon, 1994). In addition to crystalline region, cellulose contains less ordered region, called amorphous region. Amorphous cellulose is generally considered to occur as a nick between crystalline regions in the microfibril (Hon, 1994). However, some studies suggested that amorphous cellulose forms the surface of the microfibrils (Verlhac*, et al.*, 1990, O'sullivan, 1997). In nature, moreover, cellulose is embedded with hemicellulose in plant cell wall as described above.

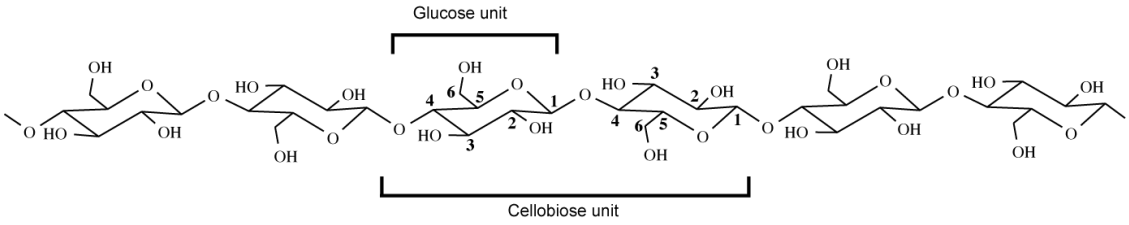


Fig. 1-2. Structure of cellulose chain

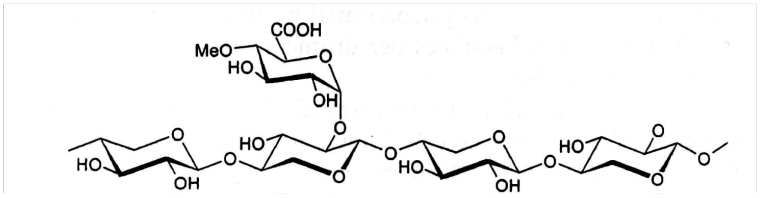
1.1.2 Xylan in plant cell wall

Hemicellulose is the structural polysaccharides of plant cell wall except for cellulose and pectin. It is composed of different polymers, usually divided into four general groups of structurally different polysaccharide type; xyloglycans (xylans), mannoglycans (mannans), xyloglucans and mixed-linkage beta-glucan (Ebringerová*, et al.*, 2005, Ebringerova, 2006). For most species of land plants, xylan is the most abundant component of the hemicellulose fraction, representing more than 30% of the dry weight (Joseleau*, et al.*, 1992). Xylan is heteropolymer consisting of main chain of 1,4-linked β-D-xylopyranose unit substituted with mainly acetyl, arabinofuranosyl and glucuronosyl residue. The type and frequency of substitution are dependent on source. However, based on the origin from hardwood, softwood and grass, xylans are mainly divided into three groups, glucuronoxylan, arabinoglucuronoxylan and arabinoxylan, respectively, as summarized in Fig. 1-3. The frequency of substitution determines its ability to bind cellulose, meaning that xylan with high frequency of substitutions are more water-soluble and bind less tightly to cellulose (McNeil*, et al.*, 1975). Main woody xylan, mostly distributed in hardwood, has side chain of (4-*O*-methyl-)β-D-glucuronic acid (GlcA) attached at position 2 of xylose unit (Xyl), called glucuronoxylan, and the mole ratio of GlcA:Xyl varied between 1:4 and 1:16; on average 1:10 with heavy acetylation at position 3 or 2 of more than 50 % xylose unit (Ebringerova & Heinze, 2000). Arabinoglucuronoxylan, glucuronoxylan attached by single α-L-arabinose (Ara) at position 3 of xylose unit, is also distributed as a minor hemicellulose in softwood and the ratio of Xyl:GlcA:Ara is 100:20:13 ((Sunna & Antranikian, 1997) and references therein). In contrast, main grass xylan has side chain of α-L-arabinose attached at position 2 and/or 3 of xylose unit, called arabinoxylan, and the mole ratio of Ara/Xyl extremely varied in the range about 0.5-1 (Izydorczyk & Biliaderis, 1995). In addition, beta-D-galactose residue is found to attach to side chains at position 5 of arabinose unit or at position 4 of xylose unit attached at

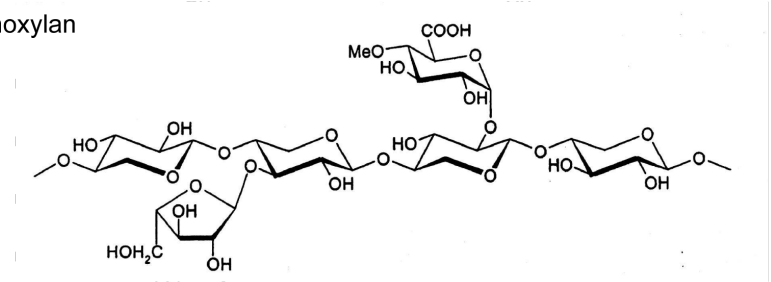
position 2 of arabinose unit. Moreover, xylan also contains ester linkages with phenolic compound, mainly ferulic and *p*-coumaric acids, attached at position 5 of arabinose unit (Ishii, 1997) and thus primary linkage between hemicellulose and lignin are thought to be associated with xylan. The length of xylan is much shorter than cellulose and average DP is between 150-200 in hardwood and between 70-130 in softwood ((Sunna & Antranikian, 1997) and references therein).

	Glucronoxylan	Arabinoglucronoxylan	Arabinoxylan
Sourse	Hardwood	Softwood	Grass
Main chain	β-1,4-linked β-D-xylopyranose		
Side chain	Singly substituted α-1,2-linked α-D-4-*O*-methyl grucronic acid	α-1,2-linked α-D-4-*O*-methyl grucronic acid α-1,3-linked α-L-arabinofuranose	Singly or doubly substituted α-1,2-linked α-1,3-linked α-L-arabinofuranose
Ratio	Xyl: GlcA= 10: 1	Xyl: GlcA: Ara= 100: 20: 13	Xyl: Ara= 1: 0.5-1

Glucronoxylan



Arabinoglucronoxylan



Arabinoxylan

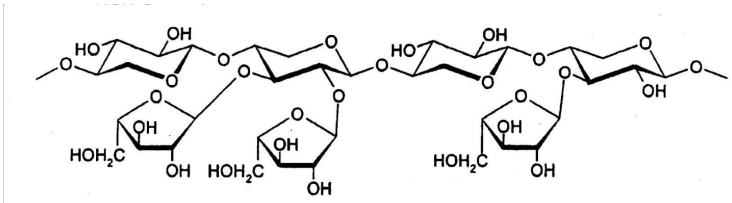


Fig. 1-3. Properties and structures of xylan

1.2 Degradation of plant cell wall by fungi

In nature, plant cell wall is degraded by numerous microorganisms and among them filamentous fungi, belonging to Basidiomycota and Ascomycota, are omnipotent degraders of plant cell wall components such as cellulose, hemicellulose and lignin (Eriksson*, et al.*, 1990). Wood-rot fungi have been separated into three groups, white-rot fungi, brown-rot fungi and soft-rot fungi based on different microscopic characteristics of decayed wood. White-rot fungi degrade all components of plant cell wall while brown-rot fungi and soft-rot fungi degrade only the polysaccharides, especially the decay of soft-rot fungi occurs at the wood surface. White-rot and brown-rot fungi belong to Basidiomycota and soft-rot fungi belong to Ascomycota. Fungi are known to secret a number of enzymes to convert polymers into oligo- or monomers, capable of uptake into the cell as nutrients. Generally, many types of extracellular glycoside hydrolases (GHs) are secreted by fungi to degrade polysaccharides such as cellulose and hemicellulose. In addition to GHs, fungi produce various extracellular carbohydrate esterases (CEs) and oxidative enzymes to degrade plant cell wall components. Those GHs and CEs have been classified into GH families and CE families and the classification is based on the hydrophobic cluster analysis of amino acid sequences to reflect on the feature of three dimensional structure (Henrissat*, et al.*, 1989, Henrissat, 1991, Henrissat & Bairoch, 1993, Henrissat & Bairoch, 1996, Henrissat, 1998, Cantarel*, et al.*, 2009). Those enzymes have been deposited into the Carbohydrate-Active enZymes (CAZy) database (http://www.cazy.org/). Meanwhile, oxidative enzymes classified into lignin oxidase (LO) and lignin degrading auxiliary enzyme (LDA) families based on the amino acid sequence similarities (Levasseur*, et al.*, 2008), integrated into the Fungal Oxidative Lignin enzymes (FOLy) database (http://foly.esil.univ-mrs.fr/). These two categories, LO and LDA, are reflected by their direct or indirect action on lignin degradation, respectively. LOs contain laccases, peroxidases such as lignin peroxidase and manganese peroxidase, and cellobiose

dehydrogenase (CDH). Most LDAs such as aryl-alcohol oxidase and glyoxal oxidase produce H_2O_2, which is supplied to the peroxidase. However, lignin degradation is not still completely understood and thus contribution of several FOLys such as CDH to lignin degradation remains controversial (Henriksson*, et al.*, 2000).

1.2.1 Enzymatic degradation of cellulose by fungi

1.2.1.1 Hydrolytic enzymes in cellulose degradation

Cellulose contains only beta-1,4-glucosidic linkages although it is so complicated morphology, as described above, that cellulolytic enzymes related to degradation of cellulose contain various glycoside hydrolases which cleave beta-1,4-glucosidic linkage, called cellulases. In addition, several oxidative enzymes, which utilize cellulose or cellooligosaccharides as a substrate, were also included in cellulolytic enzymes. First, C_1-C_x hypothesis was proposed as mechanism of cellulose degradation that C_1-cellulase acts for cellulose in the non-hydrolysis manner like a hydrogen bondase prior to hydrolysis of cellulose chains by C_x-cellulase, followed by beta-glucosidase (Reese*, et al.*, 1950, Reese & Levinson, 1952, Mandels & Reese, 1964). However, endo-exo hypothesis is newly proposed since a purified C_1-cellulase produced cellobiose from cellulose, named exo-glucanase, which was called cellobiohydrolase later, and it showed synergistic activity with C_x-cellulase, defined as enzymes reacting mainly for carboxymethyl-cellulose, which was named endo-glucanase (Wood & McCrae, 1972). Enzymatic hydrolysis of cellulose is now thought as shown in Fig. 1-4. In the first step, endo-glucanase acts at the middle of cellulose chains especially on amorphous region of cellulose and subsequently exo-glucanase acts at the generated ends of cellulose chains and then exo-endo enzymes act on cellulose synergistically. The resulting cellobiose or cellooligosaccharides are usually processed by extracellular or intracellular beta-glucosidase into glucose.

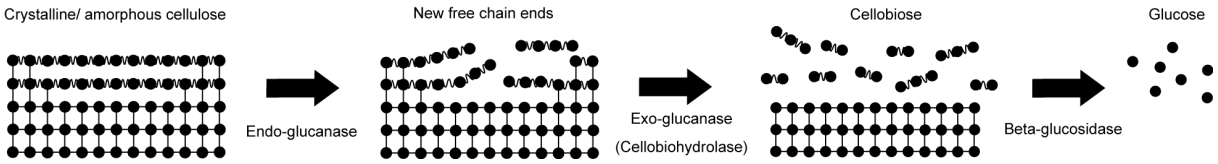


Fig. 1-4. Enzymatic hydrolysis of cellulose

The architecture of cellulase generally consists of two domains, catalytic domain (CD) and non-catalytic cellulose-binding domain, which is renamed as carbohydrate binding module (CBM) as shown in Fig. 1-5. The two domains are linked together by a linker region (Knowles*, et al.*, 1987). Function of CBM is mainly considered as concentrating cellulases on insoluble substrate, cellulose (Tomme*, et al.*, 1988). Fungal cellobohydrolases belong to GH family 7 and 6, which have been named CBHI or CBHII, respectively. CBHI has a CBM at N-terminal and hydrolyzes cellulose chain from the reducing end while CBHII has a CBM at C-terminal and hydrolyzes reversely from the non-reducing end (Knowles*, et al.*, 1987). In addition, electroscopic analyses revealed that both CBHs are also different in the fashion of degrading cellulose microfibril (Chanzy & Henrissat, 1983, Chanzy*, et al.*, 1983, Chanzy*, et al.*, 1984, Chanzy & Henrissat, 1985, Igarashi*, et al.*, 2011). To date, fungal EGs have been reported to belong to GH family 5, 7, 9, 12, 45, 61 and 74 while fungal BGLs assigned to GH family 3 and 1 (referred to the complete set of cellulase in the best-studied cellulase-producer *Trichoderma reesei* (Jia Ouyang, 2006)).

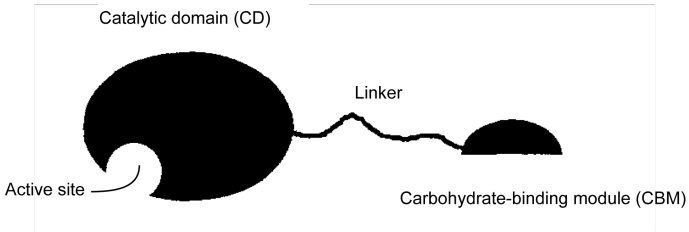


Fig. 1-5. The architecture of common cellulase (based on Henrissat (1994))

1.2.1.2 Oxidative enzymes in cellulose degradation

In addition to hydrolytic degradation of cellulose, oxidative reaction was demonstrated to be important for cellulose degradation (Eriksson*, et al.*, 1974) and cellobiose dehydrogenase (CDH) was isolated in cellulose degrading culture (Westermark & Eriksson, 1974, Ayers*, et al.*, 1978, Eriksson, 1978). CDH consists of two domains, b-type heme domain and flavin adenine nucleotide (FAD) domain, and oxidizes cellobiose and cellooligosaccharides into the corresponding 1,5-δ-lactones (1985 Morpeth, 1991 Henriksson) as shown in Fig. 1-6. CDH utilizes metal-containing compounds, for example Fe(III)-binding protein, ferricytochrome-*c*, as much more effective electron acceptors than molecular oxygen (1990 Wilson, 1992 Kremer, 1992 Samejima). The FAD domain derived from proteolysis product of CDH exhibited cellobiose-oxidizing ability in the existence of quinone but does not utilize Fe(III)-containing compounds as electron acceptors (1991 Henriksson, 1992 Wood, 1993 Habu). Therefore, heme domain is required for reduction of metal-containing compound and natural acceptor has not been determined yet but might be metal-binding protein like cytochrome. In the case of CDH from basidiomycete *Trametes velsicolor*, the heme domain is required for reduction of metal-containing compound and it revealed that *K*m value of Cu(II) used as an electron acceptor is much lower than that of Fe(III) (Roy*, et al.*, 1996), suggesting that CDH can utilize a wide variety of metal as an electron acceptor. On the other hand, in the metabolism of cellooligosaccharides, oxidization by CDH is much more likely to occur than hydrolysis by extracellular beta-glucosidase (Igarashi*, et al.*, 2003, Kawai*, et al.*, 2004, Yoshida*, et al.*, 2004). CDH was reported to enhance cellobiohydrolase activities by relieving product inhibition (Igarashi*, et al.*, 1998). CDH is also aborbed on cellulose well as cellulases, but amorphous region of cellulose is preferable site for location and it might be related to the function of FAD domain (1991 Henriksson, 1990 Renganathan, 1997 Samejima, 1997 Igarashi). Those results strongly indicated that CDH is associated with cellulose degradation

although biological role of CDH in cellulose degradation remains uncertain. In addition, it was reported that a CDH-deficient mutant of *T. versicolor* grows poorly not only on crystalline cellulose, but also on wood, implying that CDH may have role in invasion of the plant cell wall (Dumonceaux*, et al.*, 2001).

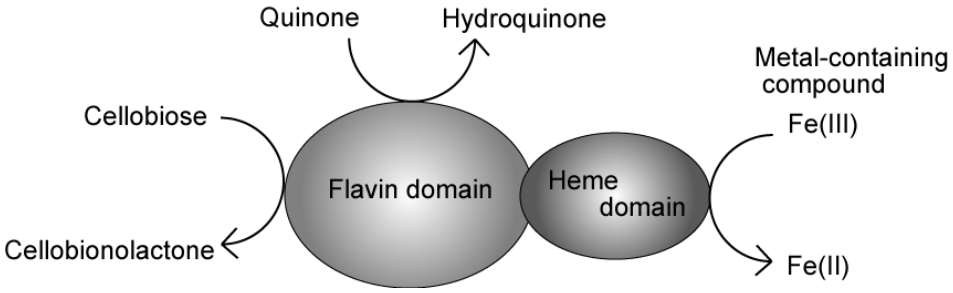


Fig. 1-6. Catalytic reaction of CDH

1.2.2 Enzymatic degradation of xylan by fungi

The enzymes related to degradation of xylan into monomeric sugar are called as xylanolytic enzymes and they have various catalytic properties as shown in Fig. 1-7, owing to heterogeneous of xylan. Xylanolytic enzymes include generally endo-beta-1,4-xylanase and beta-D-xylosidase for degrading main chain and alpha-D-glucuronidase, alpha-L-arabinofuranosidase, beta-D-galactosidase, acetyl xylan esterase, feruloyl esterase and *p*-coumaroyl esterase for acting on glycosidically-bound side-chains and ester-linked substitutions (Biely, 1985, Sunna & Antranikian, 1997, Subramaniyan & Prema, 2002, Polizeli*, et al.*, 2005). In addition, 4-*O*-methyl-glucuronyl methylesterase (called Cip2 before clarifying the function) belonging to CE family 15 (Spániková & Biely, 2006, Li*, et al.*, 2007, Spániková*, et al.*, 2007, Ďuranová*, et al.*, 2009, Vafiadi*, et al.*, 2009), which assigned to CAZymes recently, could be a member in xylanolytic system since this protein has been postulated to hydrolyze ester linkages between glucuronic acid residue in xylan and phenyl propane residue in lignin. Some xylanolytic enzymes are the modular enzymes with a CBM

like other carbohydrate active enzymes such as cellulase. Xylanolytic system is extremely investigated in *Aspergillus spp.* and see the review and references therein (de Vries & Visser, 2001). Main fungal endo-xylanases, cleaving beta-1,4 xyloside bonds, belong to GH family 10 and 11 and those enzymes have some different features (Biely*, et al.*, 1997, Collins*, et al.*, 2005, Kolenová*, et al.*, 2006). One subject is about physicochemical property since GH10 xylanases are higher molecular weight and acidic proteins while GH11 xylanases are lower molecular weight and basic proteins. Another subject is about substrate specificity in recognition of side chain since GH10 xylanasesc can recognize side chain of xylose residue in +1 subsite and thus act in the highly branched region of xylan while GH11 xylanase can act only in low branched region, resulting in non-reducing end of products with side chain in the case of GH10 and without side chain in the case of GH11. Main fungal xylosidases belong to GH family 3 (Poutanen & Puls, 1988, Margolles-Clark*, et al.*, 1996) and this enzyme releases xylose from non-reducing end of xylooligosaccharides after hydrolysis of xylan by endo-xylanase. Arabinofuranosidases, liberating arabinose from xylose residue, also contain two type enzymes and one enzyme belonging to GH family 3, 51, 54 and 62 cleaves both alpha-1,2 and alpha-1,3 linkages of singly arabinose-substituted xylose residue whereas the other enzyme belonging to GH family 43 cleaves only alpha-1,3 linkages of doubly arabinose-substituted xylose residue (Sørensen*, et al.*, 2006). Alpha-glucuronidase liberates glucuronic acid from xylan in the presence of xylanase, suggesting that it could act only on short oligomer, especially side chain attached to non-reducing end, and it belongs to GH family 67 (Puls*, et al.*, 1987, Margolles-Clark*, et al.*, 1996, Ruile*, et al.*, 1997, De Vries*, et al.*, 1998) and 115, which is a novel family and it has the ability of liberating glucuronic acid from not only non-reducing xylose residue but also internal xylose residue (Ryabova*, et al.*, 2009, Kolenová*, et al.*, 2010). Acetyl xylan esterase belongs to CE family 1, 2, 3, 4, 5, 6, 7, 12 and it removes the *O*-acetyl group attached to xylose residue at positions 2 and 3 since

acetylation inhibits endo-xylanase activity (Koseki*, et al.*, 1997). Feruroyl and *p*-coumaroyl esterase liberate ferulic acid and *p*-coumaric acid, respectively, from arabinose residue and only feruroyl esterase is assigned to CE family 1 (Kroon*, et al.*, 2000). Those xylanolytic enzymes cooperate in degradation of xylan in the synergistic manner.

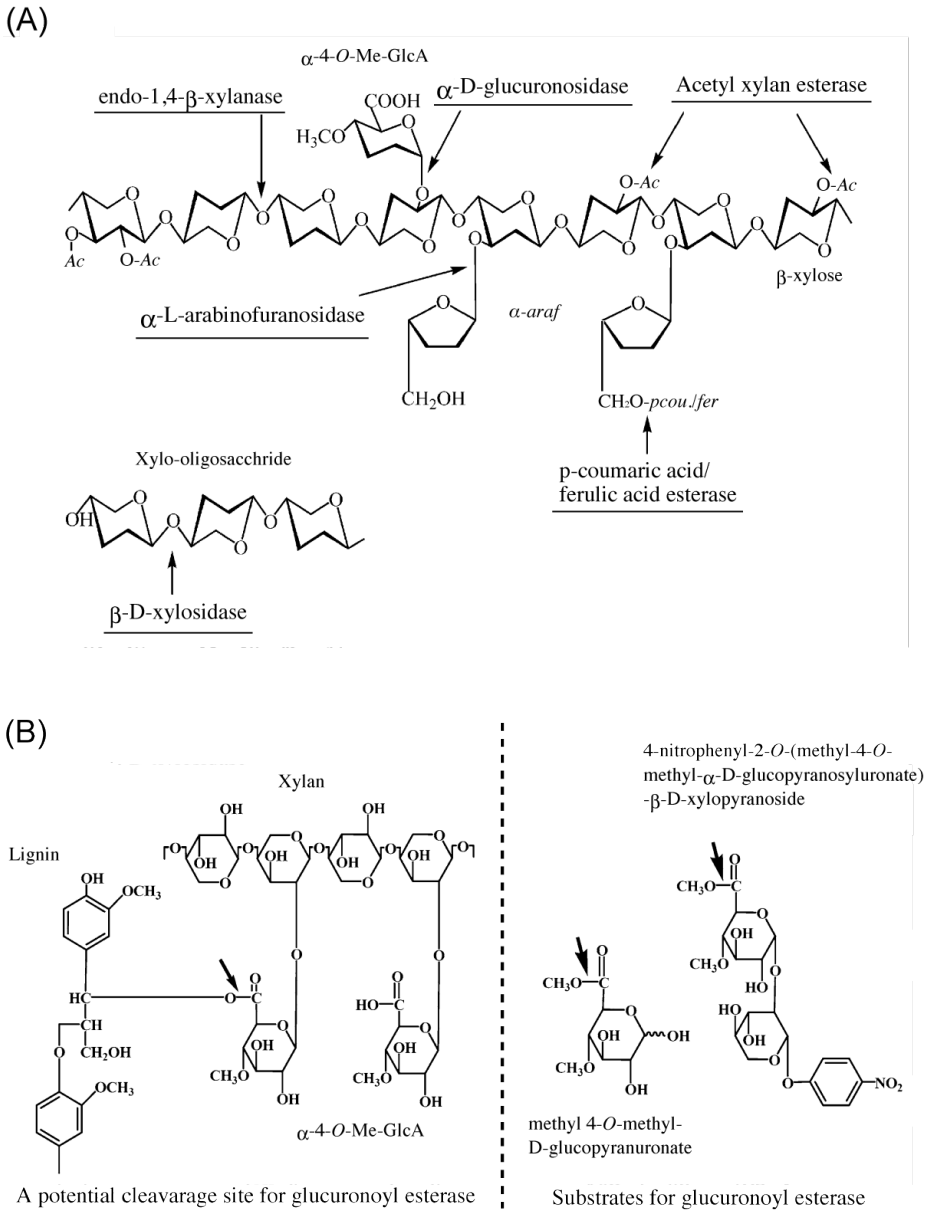


Fig. 1-7. Enzymatic hydrolysis of xylan (A) the cleavage sites for xylanolytic enzymes. *Ac*, acetyl group; alpha-*araf*, alpha-arabinofuranose; alpha-4-O-Me-GlcA, alpha-4-O-methyl-glucuronic acid; p-cou., *p*-coumaric acid; fer, ferulic acid. (Based on Collins (2005)) (B) the cleavage sites for glucuronoyl esterase (Based on Duranova (2009))

1.3 Regulation of the gene transcription encoding cellulolytic enzymes of filamentous fungi

Fungi produce a subset of extracellular cellulolytic enzymes for utilizing cellulose as nutrients (Vinzant*, et al.*, 2001). Accordingly, cellulolytic enzymes is generally produced in the culture containing cellulose whereas production of cellulase is reduced or not detected in the presence of glucose, which is a preferable nutrient. The latter phenomenon by a preferable nutrient as glucose is explained in the term of "carbon catabolite repression" of cellulase synthesis, which is widely observed in various enzymes of many organisms (Gancedo, 1998, Deutscher, 2008). In addition, repression of cellulase synthesis is provoked by monomeric sugar such as a large amount of xylose (Margolles-Clark*, et al.*, 1997). On the other hand, the former phenomenon is explained that soluble signal molecules, exhibiting the existence of insoluble cellulose, act as inducers of cellulase synthesis. Among them, the enzymatic products or derivatives converted from cellulose outside the cell are considered to act as natural inducers. Therefore, soluble carbon source-dependent induction of cellulase has been extensively investigated using various sugars in ascomycete *Hypocrea jecorina*, since early times of 1950s (Mandels & Reese, 1957). Several studies provided lactose and sorbose as efficient inducers of cellulase whereas cellobiose and longer cellooligosaccharides or cellobiono-1,5-lactone as natural inducers (Mandels & Reese, 1960, Vaheri*, et al.*, 1979, Iyayi*, et al.*, 1989, Szakmary*, et al.*, 1991, Margolles-Clark*, et al.*, 1997, Nogawa*, et al.*, 2001, Foreman*, et al.*, 2003). In addition, sophorose (2-O-beta-D-glucopyranosyl-D-glucose) was found as the strongest inducer of cellulase (Mandels*, et al.*, 1962) but it is also not a natural inducer since there is no report that purified enzymes can transform cellooligosaccharides into sophorose. The other study, however, demonstrated that potent sophorose is formed after incubation of cellobiose with cell extract (Crook & Stone, 1957), suggesting that its natural association with cellulase synthesis is still controversial. On the other hand, it is proposed that constitutive extracellular cellulase at low basal level is required for cellulase synthesis due to

formation of soluble inducers from cellulose as shown in Fig. 1-8. Based on this concept, antibodies to block activities of the major extracellular cellulases (Cel7A, Cel7B, Cel6A, Cel5A and Cel3A; the corresponding enzymes CBHI, EGI, CBHII, EGII and BGLI respectively) were used for induction experiment of *cel7A* gene. As a result, mRNA of Cel7A was detectable during growth on sophorose whereas not during growth on cellulose, which was added in the culture after supplement with antibodies (El-Gogary*, et al.*, 1989). This result strongly indicated that basal cellulase is responsible for cellulase synthesis at transcript level. Subsequent studies revealed that basal expression of Cel6A, Cel5A and Cel7B at transcript level are indispensable to form the inducers of CBHs from cellulose (Messner & Kubicek, 1991, Seiboth*, et al.*, 1992, Carle-Urioste*, et al.*, 1997, Seiboth*, et al.*, 1997). Among those cellulases, Cel6A plays a critical role for forming inducer since cellooligosaccharides are mainly produced from cellulose by CBH. As previously suggested, the induction and repression of cellulase synthesis are regulated at transcript level and thus several transcriptional regulators acting on the promoter region of the genes encoding cellulolytic enzymes were found in *H. jecorina*. XYR1, ACE2 and HAP2/3/5 complex were demonstrated as potent transcriptional activators while CRE1 and ACE1 proved to be repressors (Ilmen*, et al.*, 1996, Zeilinger*, et al.*, 1998, Saloheimo*, et al.*, 2000, Aro*, et al.*, 2001, Zeilinger*, et al.*, 2001, Aro*, et al.*, 2003, Aro*, et al.*, 2005, Stricker*, et al.*, 2006). Some cellulases are co-regulated via each transcriptional regulator since cellulases cooperate in degradation of cellulose in synergistic manner.

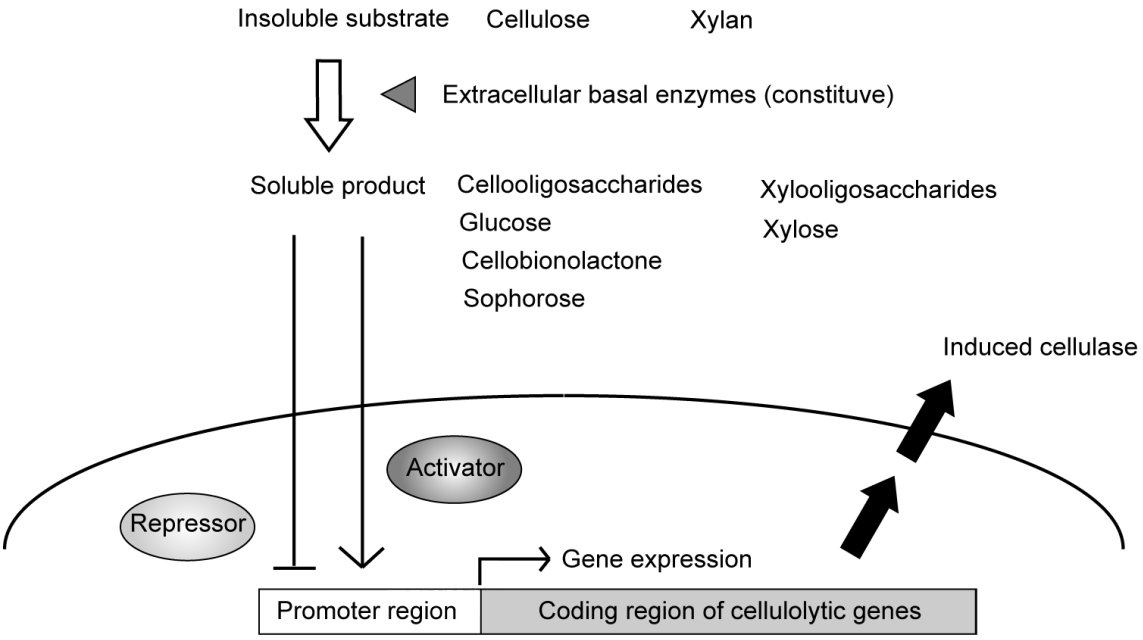


Fig. 1-8. Model of cellulase regulatory system

In addition to induction of cellulase by cellulose derivatives and related compounds, some reports revealed that cellulase production was coordinately regulated with xylanolytic enzymes by xylan and xylan derivatives. It might be reflected by the natural circumstances where cellulose is closely connected with xylan in plant cell wall. The most famous regulatory system for production of both cellulolytic and xylanolytic enzymes is the XlnR-mediated regulation in Ascomycete *Aspergillus* spp.. Originally, the gene encoding transcriptional activator XlnR was isolated in *Aspergillus niger* (Van Peij*, et al.*, 1998), based on the hypothesis that carbon catabolite repression by carbon catabolite repressor (CreA) (Dowzer & Kelly, 1991) acts directly on the *xlnA* gene encoding endo-xylanase, which is cloned in *Aspergillus tubingensis* but its homologue is lacking in *A. niger*, and indirectly by repressing transcription of the route-specific transcriptional activator (Graaff*, et al.*, 1994). This activator was reported to regulate not only major xylanolytic genes but also some cellulolytic genes, encoding two endoglucanases and two cellobiohydrolases, and a xylose metabolism gene via inducer of xylan and xylose in *A. niger* (Van Peij*, et al.*, 1998, de Vries*,*

et al., 1999, Gielkens*, et al.*, 1999, Hasper*, et al.*, 2000). All the genes are not induced by xylose but specific sugars, indicating that in addition to XlnR-mediated regulation other regulations could exist, and the overall mechanism of induction of plant polysaccharide-degrading enzymes was proposed as follows; xylose induces XlnR and subsequently induces the xylanolytic and cellulolytic genes, which are mediated by XlnR. The corresponding enzymes are secreted and the enzymatic products from natural substrate such as cellobiose induced the other cellulolytic genes (de Vries & Visser, 2001). On the other hand, in the analysis of major extracellular beta-xylosidase-deficient mutant, transcript of the gene encoding endo-xylanase increased with the accumulation of xylobiose and xylotriose (Van Peij*, et al.*, 1997). This result indicated that not only xylose but also xylobiose and/or xylotriose was inducing compound for the plant polysaccharides-degrading system. In the case of *Trichoderma* spp., moreover, two genes encoding endo-xylanases was induced differentially by xylose or xylobiose and it also indicated that xylobiose is responsible for production of plant-polysaccharide degrading enzymes, which is conducted separately from xylose-induction (Zeilinger*, et al.*, 1996, Würleitner*, et al.*, 2003). Meanwhile, it is also reported that xylobiose is not an effective inducer alone for cellulase activities whereas xylobiose in the presence of cellotetraose, which is also not an effective inducer alone, induced cellulase activities. This finding indicated that xylobiose is not direct inducer of cellulase but xylobiose induced a slight amount of cellulase and/or the related enzymes and then the enzymatic product from cellotetraose, hydrolysate such as shorter cellooligosaccharides or derivatives, is predominant inducer of cellulase activities (Royer & Nakas, 1990). Therefore, induction of cellulolytic enzymes production seems to be affected by xylan and xylan derivatives.

1.4 Cellulolytic and xylanolytic enzymes produced from *Phanerochaete chrysosporium*

1.4.1 Cellulolytic enzymes produced from *P. chrysosporium*

The basidiomycete *Phanerochaete chrysosporium* (Anamorph; *Sporotricum pulverulentum*) is one of the best-studied fungus from the viewpoint of wood degradation since this fungus is categorized into white-rot fungi which degrade all the components of wood, cellulose, hemicellulose and lignin. Cellulolytic system as well as lignolytic system has been extensively investigated in this fungus as shown in Fig. 1-9. In 1970s, Eriksson and co-workers revealed that the extracellular cellulolytic enzyme system included multiple endo-glucanases (EGs), an exo-beta-glucanase (CBH) and one or several beta-glucosidase (BGLs) (Almin*, et al.*, 1975, Eriksson & Pettersson, 1975, Eriksson & Pettersson, 1975, Streamer*, et al.*, 1975, Deshpande*, et al.*, 1978, Eriksson, 1978). In 1990s, those glucanases were reassessed and thus major cellulases in cellulolytic culture compose of two CBHI and one CBHII (CBH62, CBH58 and CBH50). They were also assigned to Cel7C, Cel7D and Cel6A respectively). Multiple endoglucanases composed of EG44, EG38 and EG28 and they were also assigned to Cel5B, Cel5A and Cel12A respectively. In addition, strong synergistic activities between cellobiohydrolases and endo-glucanases were demonstrated (Uzcategui*, et al.*, 1991, Uzcategui*, et al.*, 1991, Henriksson*, et al.*, 1999). Recently, the endo-glucanase assigned to Cel45A was discovered in cellulolytic culture and the recombinant protein showed synergistic activity with Cel6A as well as other cellulases (Igarashi*, et al.*, 2008). In addition, cDNAs encoding Cel61A and Cel9A were isolated (Wymelenberg*, et al.*, 2002) and the recombinant GH61D protein produced gluconic acid from amorphous cellulose (Westereng*, et al.*, 2011), suggesting that this enzyme is involved in oxidative degradation of cellulose. In the case of *P. chrysosporium*, a GH family 74 protein with CBM (Xgh74B) showed no cellulase activity but xyloglucanase activity (Ishida*, et al.*, 2007).

In 1970s, Eriksson and co-workers also pointed out that oxidative reaction is important for degradation of cellulose in this fungus (Eriksson*, et al.*, 1974). Cellobiose dehydrogenase (CDH) was purified and characterized and this enzyme is thought to play an important role in oxidative degradation of cellulose since cellulase activity was enhanced in the presence of CDH (Eriksson, 1978). Originally cellobiose oxidase (CBO) and cellobiose:quinone oxidoreductase (CBQ) were isolated but later CBO was renamed CDH since this enzyme utilizes other compounds as electron acceptor more effectively than molecular oxygen and CBQ became a partial product of CDH. Recent evidence of its relationship in cellulose degradation was referred to 1.2.1.2 section. In addition, the gene coding carbohydrate-binding cytochrome b_{562} (CBCyt.b_{562}) was isolated and the recombinant protein showed similar redox characteristics to CDH and adsorption with high affinity on both cellulose and chitin, suggesting that it might have an electron transfer function in the localization of the surface of cellulose or fungus (Yoshida*, et al.*, 2005).

Recently, the metabolism pathway of cellobiose and longer cellooligosaccharides after hydrolysis of EGs and CBHs is extensively investigated. For a long time, cellobiose and longer cellooligosaccharides or the corresponding lactones were thought to be hydrolyzed into glucose and gluconolactone by extracellular BGL (BGL3A) (Deshpande*, et al.*, 1978, Eriksson, 1978, Smith & Gold, 1979). However, it was shown that K_m value of cellobiose for BGL3A is considerably higher than that for CDH, and BGL3A does not hydrolyze cellobionolactone, suggesting that a kinetic disadvantage of BGL3A under cellulolytic conditions (Igarashi*, et al.*, 2003). Moreover, BGL3A is reported to be functionally a glucan 1,3-beta-glucosidase rather than 1,4-beta-glucosidase, suggesting that this enzyme plays an important role in fungal cell wall metabolism rather than cellulose degradation (Kawai*, et al.*, 2004). Transcription of *bgl3A* was repressed by cellobiose while that of *cdh* was promoted and thus it also indicated that CDH is more important than BGL3A in the extracellular

cellulose degradation (Yoshida, *et al.*, 2004). On the other hand, the genes encoding intracellular BGL belonging to GH family 1 (*bgl1A* and *bgl1B*) were isolated and kinetic parameter for cellobiose of the recombinant enzymes (BGL1A and BGL1B) were characterized and compared with that of BGL3A. As a result, BGL1B showed the best kinetic advantage for cellobiose (Tsukada, *et al.*, 2006). Moreover, the transcription of both *bgl1A* and *bgl1B* were observed in cellobiose culture while only that of *bgl1B* was repressed by glucose as well as other cellulases, suggesting that intracellular BGL1B may be responsible for cellobiose and longer cellooligosaccharide metabolism (Tsukada, *et al.*, 2006). However, the transport system of cellooligosaccharides has not been investigated in *P. chrysosporium*.

In cellulose degradation of this fungus, multiplicity of cel7s has drawn considerable attention since six genes encoding CBH1 (*cel7A-F*) were identified in the genome whereas only one gene for CBH2 (*cel6A*) was identified in about 1990 (Sims, *et al.*, 1988, Covert, *et al.*, 1992, Covert, *et al.*, 1992) and there is a speculation that multiple genes own different biological functions. Function of Cel7 isozymes have not been characterized except for two major isozymes (Cel7C and Cel7D), exhibiting almost the same enzymatic characteristic (Eriksson & Pettersson, 1975, Uzcategui, *et al.*, 1991) Structure of Cel7D was determined and homology modeling of other Cel7s based on its structure revealed that Cel7A and Cel7B possibly have endoglucanase-like characters compared to Cel7D while other isozymes, Cel7C-F, didn't show the structurally differences (Muñoz, *et al.*, 2001). On the other hand, several transcriptional analyses were performed and the genes were expressed differentially on the different substrates (Wymelenberg, *et al.*, 1993, Tempelaars, *et al.*, 1994, Birch, *et al.*, 1995, Broda, *et al.*, 1995, Lamar, *et al.*, 1995, Broda, *et al.*, 1996, Vallim, *et al.*, 1998). Among *cel7s*, highly expressed *cel7C* and *cel7D* were coordinately regulated in the substrates such as avicel, ball-milled straw and glucose (Tempelaars, *et al.*, 1994, Birch, *et al.*, 1995, Broda, *et al.*, 1995, Broda, *et al.*, 1996) although in culture containing the aspen wood chips

cel7C were abundant while *cel7D* was hardly observed (Vallim*, et al.*, 1998). In addition, *cel7C* was highly expressed under glucose repression condition and after consuming glucose transcript of *cel7C* didn't increase so much, compared to *cel7D*, suggesting that *cel7C* is a constitutive gene rather than *cel7D* (Suzuki*, et al.*, 2009). Moreover, transcripts of *cel7s* in glucose and cellooligosaccharides culture revealed that *cel7C* responded to cellooligosaccharides more quickly and highly than *cel7D* (Suzuki*, et al.*, 2010), indicating that Cel7C seems to play a key role as a basal expressed enzyme to form cellooligosaccharides and those compounds might act as inducer for production of cellulases in *P. chrysosporium*.

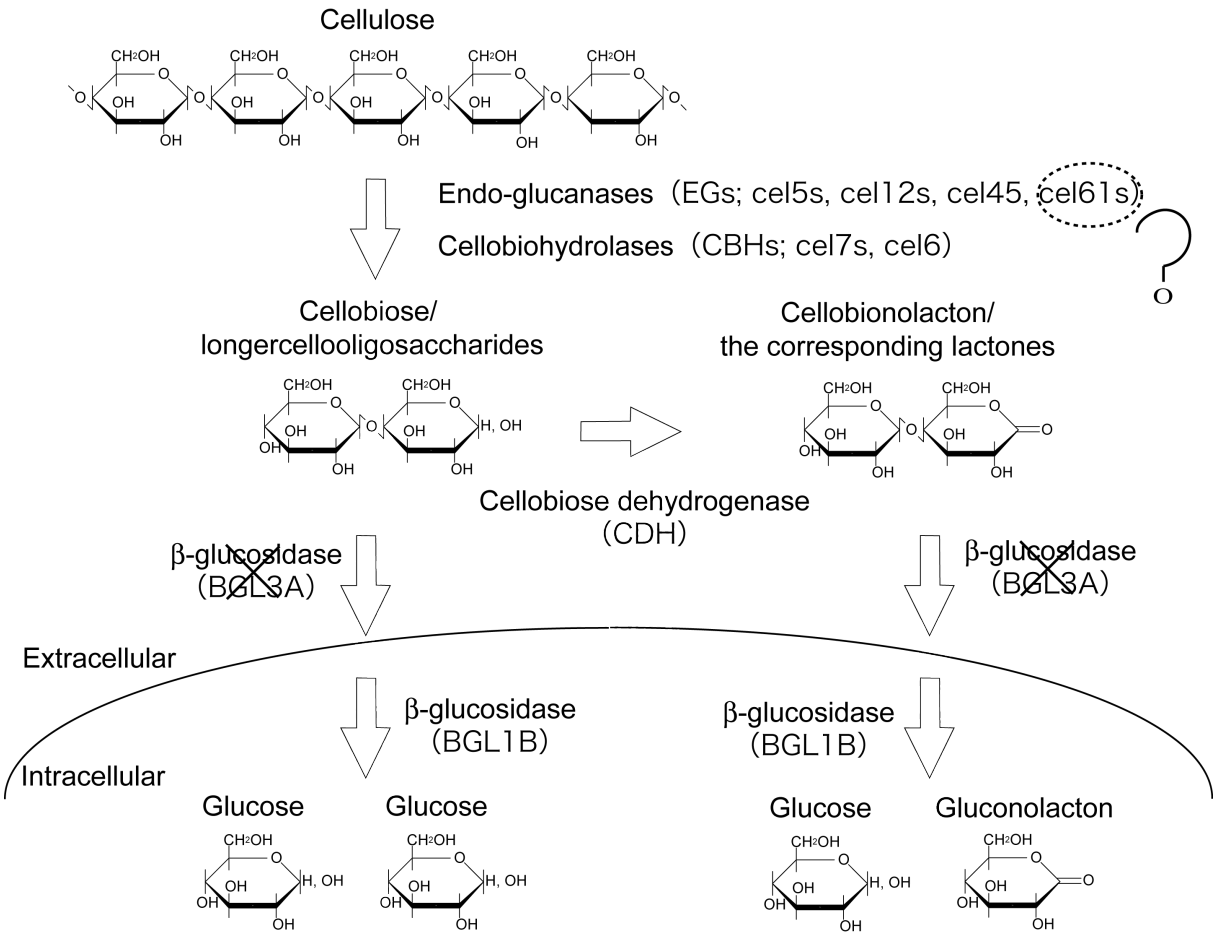


Fig. 1-9. Enzymatic degradation of cellulose in *P. chrysosporium*

1.4.2 Xylanolytic enzymes produced from *P. chrysosporium*

In contrast to cellulolytic enzymes, xylanolytic enzymes were little understood in *P. chrysosporium*. First endo-xylanase activities were detected with lignin degrading enzymes in the course of degrading pulp (Datta*, et al.*, 1991). Later, initial investigation of extracellular xylanolytic enzymes revealed that multiple endo-xylanases, one or several beta-xylosidases and arabinofuranosidases were produced in culture containing not only xylan but also cellulose, which is preferable substrate for growth (Dobozi*, et al.*, 1992, Copa-Patino*, et al.*, 1993). Moreover, alpha-glucuronidase was purified in the culture containing lignocellulose and characterized (Castanares*, et al.*, 1995). One major xylosidase was purified and characterized and, as a result, this enzyme has specificity for beta-1,3-glucan linkage rather than xylobiose (Copa-Patiño & Broda, 1994). Recently, the genes encoding several xylanases (XynA, XynB and XynC; the corresponding genes, *xyn10A*, *xyn11B* and *xyn10C*) were isolated and the corresponding enzymes were heterologously expressed and characterized (Decelle*, et al.*, 2004, Huy*, et al.*, 2011). In addition, glucuronoyl esterase activities were detected during growth on sugar beet pulp and two genes encoding glucuronoyl esterase (*ge1* and *ge2*) were isolated (Duranová*, et al.*, 2009). After its genome sequence was disclosed, several secretome analyses revealed that a putative arabinofuranosidase (GH family 51), a putative acetyl xylan esterase (CE family 1) and a putative glucuronyl esterase (CE family 15) were produced coincidentally with the expression of several endo-xylanases, suggesting that almost complete extracellular xylanolytic enzymes were produced by *P. chrysosporium* (Wymelenberg*, et al.*, 2005, Wymelenberg*, et al.*, 2009).

1.5 Genome-wide analyses of *P. chrysosporium*

In filamentous fungi, the subject of proteomic analysis is mainly secreted proteins because filamentous fungi secrete numerous useful enzymes as described above and the study of

secretome is called secretomic analysis (Kim, *et al.*, 2007). The genome-wide analyses have been exponentially performed after the genome sequence was disclosed in many species because of availability of second-generation sequencing technologies. In addition, advances in mass-spectrometry and protein detection methodology also contribute to comprehensive proteomic analysis. DNA array technology also made a breakthrough for exhibiting expression patterns of the genes in the genome and, moreover, recently it began to analyze transcriptome directly using second-generation sequencer. Those highly throughput genome-wide analyses, determining expression patterns of the genes at both transcript and protein level, provide an overall understanding of biological process. Since the potential of *P. chrysosporium* for efficient degradation of lignocellulose, its total genome was sequenced as the first filamentous fungi in 2004 (Martinez*, et al.*, 2004), showing that the genes encoding not only several lignin-degrading enzymes but also a vast number of carbohydrate active enzymes were predicted. In the next year, an initial secretome analysis of this fungus in the cellulose culture was reported (Wymelenberg*, et al.*, 2005) and it showed that not only an array of common cellulolytic enzymes but also several hemicellulolytic enzymes were expressed coincidently, suggesting that a subset of enzymes involved in lignocellulose is required for efficient conversion. Subsequently, several secretomic analyses from viewpoint of conversion of lignocellulose have been conducted under a variety of conditions (Abbas*, et al.*, 2005, Vanden Wymelenberg*, et al.*, 2006, Sato*, et al.*, 2007, Ravalason*, et al.*, 2008, Shary*, et al.*, 2008, Wymelenberg*, et al.*, 2009, Mahajan & Master, 2010, Vanden Wymelenberg*, et al.*, 2010, Adav & Ravindra, 2011, Vanden Wymelenberg*, et al.*, 2011). In addition, at transcriptomic level the expressed sequence tag analysis and microarray analysis were also performed (Sato*, et al.*, 2009, Wymelenberg*, et al.*, 2009, Vanden Wymelenberg*, et al.*, 2010, Vanden Wymelenberg*, et al.*, 2011). On the other hand, proteome and metabolome analysis concerning intracellular metabolism were also performed (Shimizu*, et al.*, 2005,

Matsuzaki, *et al.*, 2007, Ozcan, *et al.*, 2007). Those powerful analyses elucidated complex expression patterns of many enzymes but it is so complicated to compare since those analyses were carried out under completely different conditions, only in the culture containing glucose, cellulose and natural lignocellulose substrates as carbon sources. To resolve those problems that important information was difficult to extract from integrated massive data, future analysis will be conducted using comprehensive technique after an interesting phenotype is assayed in detail.

1.6 Aim of this research

Lignocellulose is promising the most abundant natural resource. Thus, effective conversion of it into fermentable monomeric sugars such as glucose and xylose is a key step to obtain useful products. In nature, among micoorganisms, the wood rotting basidiomycetes have distinct feature on degradation abilities of all components of lignocellulose. The basidiomycete *Phanerochaete chrysosporium* has a complete subset of extracellular enzymes related to cellulose and xylan degradation. In addition, this fungus might also have effective regulatory strategies on production of those enzymes. Cellulose is closely connected with xylan in plant cell wall. Therefore, production of cellulolytic enzymes might be controlled not only by soluble compounds of cellulolysis, but also by those of xylanolysis. In the cellulolytic system in *P. chrysosporium*, it has been already known that production of cellulolytic enzymes is regulated by cellulose and cellooligosaccharides. However, the effects of xylan on production of cellulolytic enzymes has not been investigated yet. Therefore, the principal aim of this research is to reveal this point.

First of all in Chapter 2, to get the initial clue for the above target, the effects of xylan on fangal growth and total amount of extracellular protein in the culture of *P. chrysosporium* grown on cellulose were investigated. Moreover, in the same culture, the

effects of xylan on the activities of cellulase, xylanase and cellobiose dehydrogenase were also assayed.

P. chrysosporium is the first filamentous fungus for disclosing total genome sequences and the genome information enables to do proteomic analysis. By using these informations, in Chapter 3, two-dimensional electrophoresis of extracellular proteins and successive liquid chromatography-tandem mass spectrometry (LC-MS/MS) performed to identify and quantify each protein secreted. Based on the results obtained, to elucidate the effects of xylan on cellulolytic enzymes produced by this fungus, the profile of the proteins up-regulated or down-regulated in the presence of xylan.

In Chapter 4, to clarify the effects of xylooligosaccharides from xylan on expression pattern of genes encoding cellulolytic enzymes, transcript levels of the related genes were quantified by real-time PCR. The results obtained were also compared with the cases by addition of cellooligosaccharides.

Finally, I attempted to discuss the effects of xylan on the regulatory system of cellulolytic enzyme system of *P. chrysosporium*.

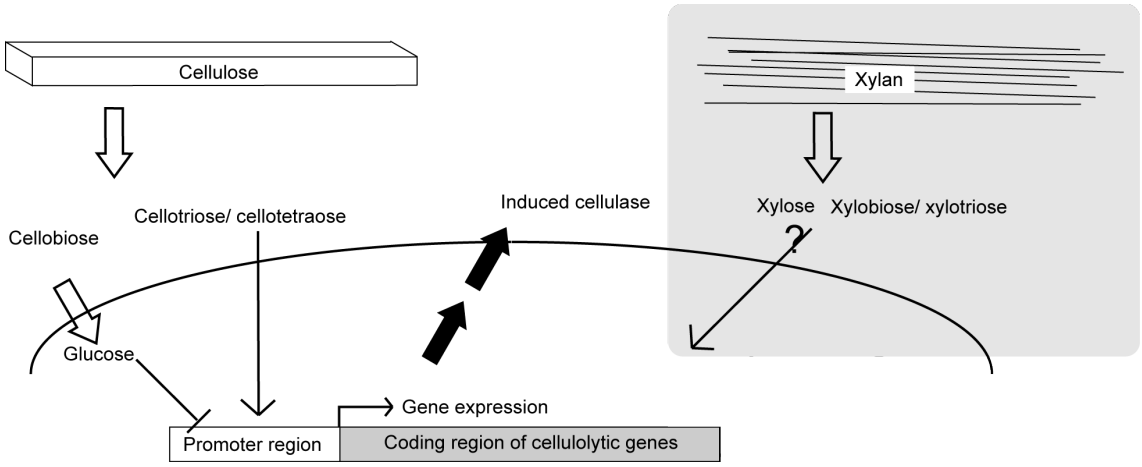


Fig. 1-10. Regulation system of cellulolytic enzymes in *P.chrysosporium* and the relation of xylan to cellulolytic enzymes production subjected in this study.

References

Abbas A, Koc H, Liu F & Tien M (2005) Fungal degradation of wood: initial proteomic analysis of extracellular proteins of Phanerochaete chrysosporium grown on oak substrate. *Curr Genet* **47**: 49-56.

Adav S & Ravindran A (2011) Quantitative proteomic analysis of lignocellulolytic enzymes by *Phanerochaete chrysosporium* on different lignocellulosic biomass. *J proteomics*.

Almin KE, Eriksson K-E & Pettersson B (1975) Extracellular enzyme system utilized by the fungus *Sporotrichum pulverulentum* (*Chrysosporium lignorum*) for the breakdown of cellulose. 2. Activities of the five endo-1,4-beta-glucanases towards carboxymethylcellulose. *Eur J Biochem* **51**: 207-211.

Aro N, Pakula T & Penttila M (2005) Transcriptional regulation of plant cell wall degradation by filamentous fungi. *FEMS Microbiol Rev* **29**: 719-739.

Aro N, Saloheimo A, Ilmén M & Penttilä M (2001) ACEII, a novel transcriptional activator involved in regulation of cellulase and xylanase genes of *Trichoderma reesei*. *J Biol Chem* **276**: 24309-24314.

Aro N, Ilmén M, Saloheimo A & Penttila M (2003) ACEI of *Trichoderma reesei* is a repressor of cellulase and xylanase expression. *Appl Environ Microbiol* **69**: 56.

Ayers AR, Ayers SB & Eriksson KE (1978) Cellobiose oxidase, purification and partial characterization of a hemoprotein from *Sporotrichum pulverulentum*. *Eur J Biochem* **90**: 171-181.

Biely P (1985) Microbial xylanolytic system. *Trends Biotechnol* **3**: 286-290.

Biely P, VrSanskh M, Tenkanen M & Kluepfel D (1997) Endo-[beta]-1,4-xylanase families: differences in catalytic properties. *J Biotechnol* **57**: 151-166.

Birch PR, Sims PF & Broda P (1995) Substrate-dependent differential splicing of introns in the regions encoding the cellulose binding domains of two exocellobiohydrolase I-like genes in *Phanerochaete chrysosporium. Appl Environ Microbiol* **61**: 3741-3744.

Brandstrom J (2001) Micro-and ultrastructural aspects of Norway spruce tracheids: a review. *Iawa Journal* **22**: 333-354.

Broda P, Birch P, Brooks P & Sims P (1995) PCR-mediated analysis of lignocellulolytic gene transcription by *Phanerochaete chrysosporium*: substrate-dependent differential expression within gene families. *Appl Environ Microbiol* **61**: 2358.

Broda P, Birch PR, Brooks PR & Sims PF (1996) Lignocellulose degradation by *Phanerochaete chrysosporium*: gene families and gene expression for a complex process. *Mol Microbiol* **19**: 923-932.

Cantarel BL, Coutinho PM, Rancurel C, Bernard T, Lombard V & Henrissat B (2009) The Carbohydrate-Active EnZymes database (CAZy): an expert resource for Glycogenomics. *Nucleic Acids Res* **37**: D233-238.

Carle-Urioste JC, Escobar-Vera J, El-Gogary S*, et al.* (1997) Cellulase induction in *Trichoderma reesei* by cellulose requires its own basal expression. *J Biol Chem* **272**: 10169-10174.

Castanares A, Hay AJ, Gordon AH, McCrae SI & Wood TM (1995) D-xylan-degrading enzyme system from the fungus *Phanerochaete chrysosporium*: isolation and partial characterisation of an alpha-(4-O-methyl)-D-glucuronidase. *J Biotechnol* **43**: 183-194.

Chanzy H & Henrissat B (1983) Electron microscopy study of the enzymic hydrolysis of *Valonia* cellulose. *Carbohydrate Polymers* **3**: 161-173.

Chanzy H & Henrissat B (1985) Undirectional degradation of *Valonia* cellulose microcrystals subjected to cellulase action. *FEBS Lett* **184**: 285-288.

Chanzy H, Henrissat B & Vuong R (1984) Colloidal gold labelling of l, 4-beta-D-glucan

cellobiohydrolase adsorbed on cellulose substrates. *FEBS Lett* **172**: 193-197.
Chanzy H, Henrissat B, Vuong R & Schulein M (1983) The action of 1, 4-[beta]-D-glucan cellobiohydrolase on *Valonia* cellulose microcrystals: An electron microscopic study. *FEBS Lett* **153**: 113-118.
Collins T, Gerday C & Feller G (2005) Xylanases, xylanase families and extremophilic xylanases. *FEMS microbiol reviews* **29**: 3-23.
Copa-Patino J, Kim Y & Broda P (1993) Production and initial characterization of the xylan-degrading system of *Phanerochaete chrysosporium*. *Appl Microbiol Biot* **40**: 69-76.
Copa-Patiño JL & Broda P (1994) A *Phanerochaete chrysosporium* beta-D-glucosidase/beta-D-xylosidase with specificity for 1,3-beta-D-glucan linkages. *Carbohydr Res* **253**: 265-275.
Coughlan M (1985) The properties of fungal and bacterial cellulases with comment on their production and application. *Biotechnol gen eng rev* **3**: 39-109.
Covert SF, Bolduc J & Cullen D (1992) Genomic organization of a cellulase gene family in *Phanerochaete chrysosporium*. *Curr Genet* **22**: 407-413.
Covert SF, vanden Wymelenberg A & Cullen D (1992) Structure, organization, and transcription of a cellobiohydrolase gene cluster from *Phanerochaete chrysosporium*. *Appl Environ Microbiol* **58**: 2168-2175.
Crook E & Stone B (1957) The enzymic hydrolysis of β-glucosides. *Biochem J* **65**: 1.
Datta A, Bettermann A & Kirk TK (1991) Identification of a specific manganese peroxidase among ligninolytic enzymes secreted by *Phanerochaete chrysosporium* during wood decay. *Appl Environ Microbiol* **57**: 1453-1460.
Davies G & Henrissat B (1995) Structures and mechanisms of glycosyl hydrolases. *Structure* **3**: 853-859.
de Vries RP & Visser J (2001) *Aspergillus* enzymes involved in degradation of plant cell wall polysaccharides. *Microbiol Mol Biol Rev* **65**: 497-522, table of contents.
de Vries RP, Poulsen CH, Madrid S & Visser J (1998) *aguA*, the gene encoding an extracellular alpha-glucuronidase from *Aspergillus tubingensis*, is specifically induced on xylose and not on glucuronic acid. *J Bacteriol* **180**: 243.
de Vries RP, van den Broeck HC, Dekkers E, Manzanares P, de Graaff LH & Visser J (1999) Differential expression of three alpha-galactosidase genes and a single beta-galactosidase gene from *Aspergillus niger*. *Appl Environ Microbiol* **65**: 2453-2460.
Decelle B, Tsang A & Storms RK (2004) Cloning, functional expression and characterization of three *Phanerochaete chrysosporium* endo-1,4-beta-xylanases. *Curr Genet* **46**: 166-175.
Deshpande V, Eriksson KE & Pettersson B (1978) Production, purification and partial characterization of 1, 4− β −glucosidase enzymes from *Sporotrichum pulverulentum*. *Eur J Biochem* **90**: 191-198.
Deutscher J (2008) The mechanisms of carbon catabolite repression in bacteria. *Curr Opin Microbiol* **11**: 87-93.
Divne C, Stahlberg J, Reinikainen T*, et al.* (1994) The three-dimensional crystal structure of the catalytic core of cellobiohydrolase I from *Trichoderma reesei*. *Science* **265**: 524.
Dobozi MS, Szakács G & Bruschi CV (1992) Xylanase activity of *Phanerochaete chrysosporium*. *Appl Environ Microbiol* **58**: 3466-3471.
Dowzer C & Kelly JM (1991) Analysis of the *creA* gene, a regulator of carbon catabolite repression in *Aspergillus nidulans*. *Mol Cell Biol* **11**: 5701.
Dumonceaux T, Bartholomew K, Valeanu L, Charles T & Archibald F (2001) Cellobiose dehydrogenase is essential for wood invasion and nonessential for kraft pulp delignification by *Trametes versicolor*. *Enzyme Microb Technol* **29**: 478-489.

Duranová M, Spániková S, Wösten HAB, Biely P & de Vries RP (2009) Two glucuronoyl esterases of *Phanerochaete chrysosporium*. *Arch Microbiol* **191**: 133-140.

Ďuranová M, Hirsch J, Kolenová K & Biely P (2009) Fungal glucuronoyl esterases and substrate uronic acid recognition. *Biosci Biotechnol Biochem* **73**: 2483-2487.

Ebringerova A (2006) Structural diversity and application potential of hemicelluloses. *Macromol Symp* **232**: 1-12.

Ebringerova A & Heinze T (2000) Xylan and xylan derivatives–biopolymers with valuable properties, 1. Naturally occurring xylans structures, isolation procedures and properties. *Macromol rap com* **21**: 542-556.

Ebringerová A, Hromádková Z & Heinze T (2005) Hemicellulose. *Adv in Poly Sci***186**: 1-67.

El-Gogary S, Leite A, Crivellaro O, Eveleigh DE & el-Dorry H (1989) Mechanism by which cellulose triggers cellobiohydrolase I gene expression in *Trichoderma reesei*. *P Natl Acad Sci Usa* **86**: 6138-6141.

Eriksson K (1978) Enzyme Mechanisms Involved in Cellulose Hydrolysis by the Rot Fungus *Sporotrichum pulverulentum*. *Biotechnol Bioeng* **20**: 317-332.

Eriksson K-E, Blanchette RA & Ander P (1990) *Microbial and Enzymatic Degradation Of Wood and Wood Components*. Springer-Verlag, Berlin.

Eriksson KE & Pettersson B (1975) Extracellular enzyme system utilized by the fungus *Sporotrichum pulverulentum* (*Chrysosporium lignorum*) for the breakdown of cellulose. 1. Separation, purification and physico-chemical characterization of five endo-1,4-beta-glucanases. *Eur J Biochem* **51**: 193-206.

Eriksson KE & Pettersson B (1975) Extracellular enzyme system utilized by the fungus *Sporotrichum pulverulentum* (*Chrysosporium lignorum*) for the breakdown of cellulose. 3. Purification and physico-chemical characterization of an exo-1,4-beta-glucanase. *Eur J Biochem* **51**: 213-218.

Eriksson KE, Pettersson B & Westermark U (1974) Oxidation: an important enzyme reaction in fungal degradation of cellulose. *FEBS Lett* **49**: 282-285.

Foreman PK, Brown D, Dankmeyer L*, et al.* (2003) Transcriptional regulation of biomass-degrading enzymes in the filamentous fungus *Trichoderma reesei*. *J Biol Chem* **278**: 31988.

Forsberg Z, Vaaje-Kolstad G, Westereng B*, et al.* (2011) Cleavage of cellulose by a CBM33 protein. *Protein Sci* **20**: 1479-1483.

Gancedo JM (1998) Yeast carbon catabolite repression. *Microbiol Mol Biol Rev* **62**: 334-361.

Gardner K & Blackwell J (1974) The structure of native cellulose. *Biopolymers* **13**: 1975-2001.

Gielkens MM, Dekkers E, Visser J & de Graaff LH (1999) Two cellobiohydrolase-encoding genes from *Aspergillus niger* require D-xylose and the xylanolytic transcriptional activator XlnR for their expression. *Appl Environ Microbiol* **65**: 4340-4345.

Graaff LK, Broeck HC, Ooijen AJJ & Visser J (1994) Regulation of the xylanase ‐ encoding xlnA gene of *Aspergilius tubigensis*. *Mol Microbiol* **12**: 479-490.

Harris PV, Welner D, Mcfarland KC*, et al.* (2010) Stimulation of lignocellulosic biomass hydrolysis by proteins of glycoside hydrolase family 61: structure and function of a large, enigmatic family. *Biochem* **49**: 3305–3316.

Hasper AA, Visser J & de Graaff LH (2000) The *Aspergillus niger* transcriptional activator XlnR, which is involved in the degradation of the polysaccharides xylan and cellulose, also regulates D-xylose reductase gene expression. *Mol Microbiol* **36**: 193-200.

Henriksson G, Johansson G & Pettersson G (2000) A critical review of cellobiose dehydrogenases. *J Biotechnol* **78**: 93-113.

Henriksson G, Nutt A, Henriksson H, Pettersson B, Ståhlberg J, Johansson G & Pettersson G (1999) Endoglucanase 28 (Cel12A), a new Phanerochaete chrysosporium cellulase. *Eur J*

Biochem **259**: 88-95.
Henrissat B (1991) A classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochemical Journal* **280**: 309.
Henrissat B (1994) Cellulases and their interaction with cellulose. *Cellulose* **1**: 169-196.
Henrissat B (1998) Glycosidase families. *Biochemical Society Transactions* **26**: 153.
Henrissat B & Bairoch A (1993) New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochem J* **293**: 781-788.
Henrissat B & Bairoch A (1996) Updating the sequence-based classification of glycosyl hydrolases. *Biochem J* **316 (Pt 2)**: 695-696.
Henrissat B, Driguez H, Viet C & Schülein M (1985) Synergism of Cellulases from Trichoderma reesei in the Degradation of Cellulose. *Nature Biotechnology* **3**: 722 - 726.
Henrissat B, Claeyssens M, Tomme P, Lemesle L & Mornon JP (1989) Cellulase families revealed by hydrophobic cluster analysis. *Gene* **81**: 83-95.
Himmel ME, Ding S-Y, Johnson DK, Adney WS, Nimlos MR, Brady JW & Foust TD (2007) Biomass recalcitrance: engineering plants and enzymes for biofuels production. *Science* **315**: 804-807.
Hon DNS (1994) Cellulose: a random walk along its historical path. *Cellulose* **1**: 1-25.
Huy ND, Kim S-W & Park S-M (2011) Heterologous expression of endo-1,4-beta-xylanase C from *Phanerochaete chrysosporium* in *Pichia pastoris*. *J Biosci Bioeng*
Igarashi K, Samejima M & Eriksson K (1998) Cellobiose dehydrogenase enhances *Phanerochaete chrysosporium* cellobiohydrolase I activity by relieving product inhibition. *Eur J Biochem* **253**: 101-106.
Igarashi K, Tani T, Rie K & Masahiro S (2003) Family 3 beta-glucosidase from cellulose-degrading culture of the white-rot fungus *Phanerochaete chrysosporium* is a glucan 1,3-beta-glucosidase. *J Biosci Bioeng.* **95**: 572-576.
Igarashi K, Ishida T, Hori C & Samejima M (2008) Characterization of an endoglucanase belonging to a new subfamily of glycoside hydrolase family 45 of the basidiomycete *Phanerochaete chrysosporium*. *Appl Environ Microbiol* **74**: 5628-5634.
Igarashi K, Koivula A, Wada M, Kimura S, Penttila M & Samejima M (2009) High speed atomic force microscopy visualizes processive movement of *Trichoderma reesei* cellobiohydrolase I on crystalline cellulose. *J Biol Chem* **284**: 36186-36190.
Igarashi K, Uchihashi T, Koivula A*, et al.* (2011) Traffic jams reduce hydrolytic efficiency of cellulase on cellulose surface. *Science* **333**: 1279-1282.
Ilmen M, Thrane C & Penttilä M (1996) The glucose repressor genecre1 of *Trichoderma*: Isolation and expression of a full-length and a truncated mutant form. *Mol Gener Genet MGG* **251**: 451-460.
Ishida T, Yaoi K, Hiyoshi A, Igarashi K & Samejima M (2007) Substrate recognition by glycoside hydrolase family 74 xyloglucanase from the basidiomycete *Phanerochaete chrysosporium*. *FEBS J* **274**: 5727-5736.
Ishii T (1997) Structure and functions of feruloylated polysaccharides. *Plant Science* **127**: 111-127.
Iyayi C, Bruchmann EE & Kubicek C (1989) Induction of cellulase formation in *Trichoderma reesei* by cellobiono-1, 5-lacton. *Arch Microbiol* **151**: 326-330.
Izydorczyk M & Biliaderis C (1995) Cereal arabinoxylans: Advances in structure and physicochemical properties. *Carbohyd Polym* **28**: 33-48.
Jia Ouyang M (2006) A complete protein pattern of cellulase and hemicellulase genes in the filamentous fungus *Trichoderma reesei*. *Biotechnol J* **1**: 1266-1274.
Joseleau JP, Comtat J & Ruel K (1992) Chemical structure of xylans and their interaction in the plant cell walls. *Xylans and xylanases,*(Visser J, Beldman G, Kusters-van Someren MA & Voragen AGJ, ed.^eds.), p.^pp. 1-15. Elsevier, Amsterdam.

Karkehabadi S, Hansson H, Kim S, Piens K, Mitchinson C & Sandgren M (2008) The first structure of a glycoside hydrolase family 61 member, Cel61B from *Hypocrea jecorina*, at 1.6 A resolution. *J Mol Biol* **383**: 144-154.

Karlsson J, Saloheimo M, Siika-Aho M, Tenkanen M, Penttilä M & Tjerneld F (2001) Homologous expression and characterization of Cel61A (EG IV) of *Trichoderma reesei*. *Eur J Biochem* **268**: 6498-6507.

Kawai R, Igarashi K, Kitaoka M, Ishii T & Samejima M (2004) Kinetics of substrate transglycosylation by glycoside hydrolase family 3 glucan 1,3-beta-glucosidase from the white-rot fungus *Phanerochaete chrysosporium*. *Carbohydr Res* **339**: 2851-2857.

Kerr AJ & Goring DAI (1975) The ultrastructural arrangement of the wood cell wall. *Cellul. Chem. Technol.* **9**: 563-573.

Kleywegt GJ, Zou JY, Divne C*, et al.* (1997) The crystal structure of the catalytic core domain of endoglucanase I from *Trichoderma reesei* at 3.6 Å resolution, and a comparison with related enzymes1. *J Mol Biol* **272**: 383-397.

Knowles J, Lehtovaara P & Teeri T (1987) Cellulase families and their genes. *Tre Biotechnol* **5**: 255-261.

Kolenová K, Vrsanská M & Biely P (2006) Mode of action of endo-beta-1,4-xylanases of families 10 and 11 on acidic xylooligosaccharides. *J Biotechnol* **121**: 338-345.

Kolenová K, Ryabova O, Vrsanská M & Biely P (2010) Inverting character of family GH115 α-glucuronidases. *FEBS Lett* **584**: 4063-4068.

Koseki T, Furuse S, Iwano K, Sakai H & Matsuzawa H (1997) An *Aspergillus awamori* acetylesterase: purification of the enzyme, and cloning and sequencing of the gene. *Biochem J* **326**: 485.

Koseki T, Mese Y, Fushinobu S*, et al.* (2008) Biochemical characterization of a glycoside hydrolase family 61 endoglucanase from *Aspergillus kawachii*. *Appl Microbiol Biot* **77**: 1279-1285.

Kroon PA, Williamson G, Fish NM, Archer DB & Belshaw NJ (2000) A modular esterase from *Penicillium funiculosum* which releases ferulic acid from plant cell walls and binds crystalline cellulose contains a carbohydrate binding module. *Eur J Biochem* **267**: 6740-6752.

Lamar RT, Schoenike B, vanden Wymelenberg A, Stewart P, Dietrich DM & Cullen D (1995) Quantitation of fungal mRNAs in complex substrates by reverse transcription PCR and its application to *Phanerochaete chrysosporium*-colonized soil. *Appl Environ Microbiol* **61**: 2122-2126.

Langston J, Shaghasi T, Abbate E, Xu F, Vlasenko E & Sweeney M (2011) Oxidoreductive cellulose depolymerization by the enzymes cellobiose dehydrogenase and glycoside hydrolase 61. *Appl Environ Microbiol* **77**: 7007.

Larsen MJ, Winandy JE & Green F (1995) A proposed model of the tracheid cell wall of southern yellow pine having an inherent radial structure in the S2 layer. *Material und Organismen* **29**: 197-210.

Levasseur A, Piumi F, Coutinho PM*, et al.* (2008) FOLy: an integrated database for the classification and functional annotation of fungal oxidoreductases potentially involved in the degradation of lignin and related aromatic compounds. *Fungal Genet Biol* **45**: 638-645.

Li X-L, Spániková S, de Vries RP & Biely P (2007) Identification of genes encoding microbial glucuronoyl esterases. *FEBS Lett* **581**: 4029-4035.

Mahajan S & Master ER (2010) Proteomic characterization of lignocellulose-degrading enzymes secreted by *Phanerochaete carnosa* grown on spruce and microcrystalline cellulose. *Appl Microbiol Biot*.

Mandels M & Reese ET (1957) Induction of cellulase in *Trichoderma viride* as influenced by

carbon sources and metals. *J Bacteriol* **73**: 269.
Mandels M & Reese ET (1960) Induction of cellulase in fungi by cellobiose. *J Bacteriol* **79**: 816.
Mandels M & Reese ET (1964) Fungal cellulases and the microbial decomposition of cellulosic fabric. *Develop. Ind. Microbiol.* **5**: 5-20.
Mandels M, Parrish FW & Reese ET (1962) Sophorose as an inducer of cellulase in *Trichoderma viride*. *J Bacteriol* **83**: 400.
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.
Margolles-Clark E, Saloheimo M, Siika-Aho M & Penttilä M (1996) The α-glucuronidase-encoding gene of *Trichoderma reesei*. *Gene* **172**: 171-172.
Margolles-Clark E, Margolles-Clark E, Tenkanen M*, et al.* (1996) Cloning of genes encoding alpha-L-arabinofuranosidase and beta-xylosidase from *Trichoderma reesei* by expression in *Saccharomyces cerevisiae*. *Appl Environ Microbiol* **62**: 3840.
Martinez D, Larrondo LF, Putnam N*, et al.* (2004) Genome sequence of the lignocellulose degrading fungus *Phanerochaete chrysosporium* strain RP78. *Nat Biotechnol* **22**: 695-700.
Matsuzaki F, Matsuzaki F, Shimizu M, Shimizu M, Wariishi H & Wariishi H (2007) Proteomic and metabolomic analyses of the white-rot fungus *Phanerochaete chrysosporium* exposed to exogenous benzoic acid. *J Proteome Res.*
McNeil M, Albersheim P, Taiz L & Jones RL (1975) The Structure of plant cell walls: VII. Barley Aleurone Cells 1. *Plant Physiology* **55**: 64.
McNeil M, Darvill A, Fry S & Albercheim P (1984) Structure and Function of the Primary Cell Walls of plants. *Ann Rev Biochem* **53**: 625-663.
Messner R & Kubicek CP (1991) Carbon source control of cellobiohydrolase I and II formation by *Trichoderma reesei*. *Appl Environ Microbiol* **57**: 630-635.
Muñoz IG, Ubhayasekera W, Henriksson H*, et al.* (2001) Family 7 cellobiohydrolases from *Phanerochaete chrysosporium*: crystal structure of the catalytic module of Cel7D (CBH58) at 1.32 A resolution and homology models of the isozymes. *J Mol Biol* **314**: 1097-1111.
Nidetzky B, Steiner W, Hayn M & Claeyssens M (1994) Cellulose hydrolysis by the cellulases from *Trichoderma reesei*: a new model for synergistic interaction. *Biochem J* **298**: 705.
Nogawa M, Goto M, Okada H & Morikawa Y (2001) L-Sorbose induces cellulase gene transcription in the cellulolytic fungus *Trichoderma reesei*. *Curr Genet* **38**: 329-334.
O'sullivan AC (1997) Cellulose: the structure slowly unravels. *Cellulose* **4**: 173-207.
Ozcan S, Yildirim V, Kaya L, Albrecht D, Becher D, Hecker M & Ozcengiz G (2007) *Phanerochaete chrysosporium* soluble proteome as a prelude for the analysis of heavy metal stress response. *Proteomics* **7**: 1249-1260.
Persson PV, Hafrén J, Fogden A, Daniel G & Iversen T (2004) Silica nanocasts of wood fibers: A study of cell-wall accessibility and structure. *Biomacromol* **5**: 1097-1101.
Phillips CM, Beeson IV WT, Cate JH & Marletta MA (2011) Cellobiose dehydrogenase and a copper-dependent polysaccharide monooxygenase potentiate cellulose degradation by *Neurospora crassa*. *ACS Chem Biol*.
Polizeli M, Rizzatti A, Monti R, Terenzi H, Jorge J & Amorim D (2005) Xylanases from fungi: properties and industrial applications. *Appl Microbiol Biotechnol* **67**: 577-591.
Poutanen K & Puls J (1988) Characteristics of *Trichoderma reesei* β-xylosidase and its use in the hydrolysis of solubilized xylans. *Appl Microbiol Biotechnol* **28**: 425-432.
Puls J, Schmidt O & Granzow C (1987) Alpha-glucuronidase in two microbial xylanolytic

systems. *Enzyme Microb Tech* **9**: 83-88.
Quinlan RJ, Sweeney MD, Lo Leggio L*, et al.* (2011) Insights into the oxidative degradation of cellulose by a copper metalloenzyme that exploits biomass components. *Pro Nat Acad Sci USA*.
Raguz S, Yagüe E, Wood DA & Thurston CF (1992) Isolation and characterization of a cellulose-growth-specific gene from *Agaricus bisporus*. *Gene* **119**: 183-190.
Ravalason H, Jan G, Mollé D*, et al.* (2008) Secretome analysis of *Phanerochaete chrysosporium* strain CIRM-BRFM41 grown on softwood. *Appl Microbiol Biot* **80**: 719-733.
Reese ET & Levinson HS (1952) A comparative study of the breakdown of cellulose by microorganisms. *Physiologia Plantarum* **5**: 345-366.
Reese ET, Siu RGH & Levinson HS (1950) The biological degradation of soluble cellulose derivatives and its relationship to the mechanism of cellulose hydrolysis. *J Bacteriol* **59**: 485.
Roy B, Dumonceaux T, Koukoulas A & Archibald F (1996) Purification and characterization of cellobiose dehydrogenases from the white rot fungus *Trametes versicolor*. *Appl Environ Microbiol* **62**: 4417.
Royer JC & Nakas J (1990) Interrelationship of xylanase induction and cellulase induction of *Trichoderma longibrachiatum*. *Appl Environ Microbiol* **56**: 2535.
Ruile P, Winterhalter C & Liebl W (1997) Isolation and analysis of a gene encoding α-glucuronidase, an enzyme with a novel primary structure involved in the breakdown of xylan. *Mol Microbiol* **23**: 267-279.
Ryabova O, Vrsanská M, Kaneko S, van Zyl WH & Biely P (2009) A novel family of hemicellulolytic alpha-glucuronidase. *FEBS Lett* **583**: 1457-1462.
Salmen L (2004) Micromechanical understanding of the cell-wall structure. *Comptes Rendus Biologies* **327**: 873-880.
Saloheimo A, Aro N, Ilmén M & Penttilä M (2000) Isolation of the ace1 gene encoding a Cys2-His2 transcription factor involved in regulation of activity of the cellulase promoter cbh1of *Trichoderma reesei*. *J Biol Chem* **275**: 5817.
Sato S, Liu F, Koc H & Tien M (2007) Expression analysis of extracellular proteins from *Phanerochaete chrysosporium* grown on different liquid and solid substrates. *Microbiol (Reading, Engl)* **153**: 3023-3033.
Sato S, Feltus FA, Iyer P & Tien M (2009) The first genome-level transcriptome of the wood-degrading fungus *Phanerochaete chrysosporium* grown on red oak. *Curr Genet* **55**: 273-286.
Scheller HV & Ulvskov P (2010) Hemicelluloses. *Annu Rev Plant Biol* **61**: 263-289.
Seiboth B, Messner R, Gruber F & Kubicek CP (1992) Disruption of the *Trichoderma reesei cbh2* gene coding for cellobiohydrolase II leads to a delay in the triggering of cellulase formation by cellulose. *Microbiol* **138**: 1259-1264.
Seiboth B, Hakola S, Mach RL, Suominen PL & Kubicek CP (1997) Role of four major cellulases in triggering of cellulase gene expression by cellulose in *Trichoderma reesei*. *J Bacteriol* **179**: 5318-5320.
Shary S, Kapich AN, Panisko EA, Magnuson JK, Cullen D & Hammel KE (2008) Differential expression in *Phanerochaete chrysosporium* of membrane-associated proteins relevant to lignin degradation. *Appl Environ Microbiol* **74**: 7252-7257.
Shimizu M, Yuda N, Nakamura T, Tanaka H & Wariishi H (2005) Metabolic regulation at the tricarboxylic acid and glyoxylate cycles of the lignin-degrading basidiomycete *Phanerochaete chrysosporium* against exogenous addition of vanillin. *Proteomics* **5**: 3919-3931.
Sims P, James C & Broda P (1988) The identification, molecular cloning and characterisation

of a gene from *Phanerochaete chrysosporium* that shows strong homology to the exo-cellobiohydrolase I gene from *Trichoderma reesei*. *Gene.* **74**: 4 I l-422

Sjostrom E (1993) *Wood Chemistry Fundamentals and applications.* Academic Press Inc., London.

Smith MH & Gold MH (1979) *Phanerochaete chrysosporium* beta-glucosidases: induction, cellular localization, and physical characterization. *Appl Environ Microbiol* **37**: 938-942.

Sørensen HR, Jørgensen CT, Hansen CH, Jørgensen CI, Pedersen S & Meyer AS (2006) A novel GH43 alpha-L-arabinofuranosidase from *Humicola insolens*: mode of action and synergy with GH51 alpha-L-arabinofuranosidases on wheat arabinoxylan. *Appl Microbiol Biot* **73**: 850-861.

Spániková S & Biely P (2006) Glucuronoyl esterase: novel carbohydrate esterase produced by *Schizophyllum commune*. *FEBS Lett* **580**: 4597-4601.

Spániková S, Poláková M, Joniak D, Hirsch J & Biely P (2007) Synthetic esters recognized by glucuronoyl esterase from *Schizophyllum commune*. *Arch Microbiol* **188**: 185-189.

Streamer M, Eriksson KE & Pettersson B (1975) Extracellular enzyme system utilized by the fungus *Sporotrichum pulverulentum* (*Chrysosporium lignorum*) for the breakdown of cullulose. Functional characterization of five endo-1,4-beta-glucanases and one exo-1,4-beta-glucanase. *Eur J Biochem* **59**: 607-613.

Stricker AR, Grosstessner-Hain K, Würleitner E & Mach RL (2006) Xyr1 (xylanase regulator 1) regulates both the hydrolytic enzyme system and D-xylose metabolism in *Hypocrea jecorina*. *Eukaryotic Cell* **5**: 2128-2137.

Subramaniyan S & Prema P (2002) Biotechnology of microbial xylanases: enzymology, molecular biology, and application. *Crit Rev Biotechnol* **22**: 33-64.

Sunna A & Antranikian G (1997) Xylanolytic Enzymes from Fungi and Bacteria. *Critical Rev Biotechnol* **17**: 39-67.

Suzuki H, Igarashi K & Samejima M (2009) Quantitative transcriptional analysis of the genes encoding glycoside hydrolase family 7 cellulase isozymes in the basidiomycete *Phanerochaete chrysosporium*. *FEMS Microbiol Lett* **299**: 159-165.

Suzuki H, Igarashi K & Samejima M (2010) Cellotriose and Cellotetraose as Inducers of the Genes Encoding Cellobiohydrolases in the Basidiomycete *Phanerochaete chrysosporium*. *Appl Environ Microbiol* **76**: 6164.

Szakmary K, Wotawa A & Kubicek CP (1991) Origin of oxidized cellulose degradation products and mechanism of their promotion of cellobiohydrolase I biosynthesis in *Trichoderma reesei*. *Microbiology* **137**: 2873-2878.

Tempelaars CA, Birch PR, Sims PF & Broda P (1994) Isolation, characterization, and analysis of the expression of the cbhII gene of *Phanerochaete chrysosporium*. *Appl Environ Microbiol* **60**: 4387-4393.

Tomme P, Tilbeurgh H, Pettersson G*, et al.* (1988) Studies of the cellulolytic system of *Trichoderma reesei* QM 9414. Analysis of domain function in two cellobiohydrolases by limited proteolysis. *Eur J Biochem* **170**: 575-581.

Tsukada T, Igarashi K, Yoshida M & Samejima M (2006) Molecular cloning and characterization of two intracellular beta-glucosidases belonging to glycoside hydrolase family 1 from the basidiomycete *Phanerochaete chrysosporium*. *Appl Microbiol Biot* **73**: 807-814.

Uzcategui E, Johansson G, Ek B & Information GPAC (1991) The 1,4-β-glucan glucanohydrolases from *Phanerochaete chrysosporium*. Re-assessment of their significance in cellulose degradation mechanisms *J Biotechnol* **21**: 143-159.

Uzcategui E, Ruiz A, Montesino R, Johansson G & Pettersson G (1991) The 1,4-beta-D-glucan cellobiohydrolases from *Phanerochaete chrysosporium*. I. A system of synergistically acting enzymes homologous to *Trichoderma reesei*. *J Biotechnol* **19**:

271-285.
Vaaje-Kolstad G, Westereng B, Horn SJ, Liu Z, Zhai H, Sørlie M & Eijsink VGH (2010) An oxidative enzyme boosting the enzymatic conversion of recalcitrant polysaccharides. *Science* **330**: 219-222.
Vafiadi C, Topakas E, Biely P & Christakopoulos P (2009) Purification, characterization and mass spectrometric sequencing of a thermophilic glucuronoyl esterase from *Sporotrichum thermophile*. *FEMS Microbiol Lett* **296**: 178-184.
Vaheri MP, Vaheri MEO & Kauppinen VS (1979) Formation and release of cellulolytic enzymes during growth of *Trichoderma reesei* on cellobiose and glycerol. *Appl Microbiol Biot* **8**: 73-80.
Vallim M, Janse B, Gaskell J, Pizzirani-Kleiner A & Cullen D (1998) *Phanerochaete chrysosporium* cellobiohydrolase and cellobiose dehydrogenase transcripts in wood. *Appl Environ Microbiol* **64**: 1924.
Van Peij NN, Brinkmann J, Vrsanská M, Visser J & de Graaff LH (1997) beta-Xylosidase activity, encoded by *xlnD*, is essential for complete hydrolysis of xylan by *Aspergillus niger* but not for induction of the xylanolytic enzyme spectrum. *Eur J Biochem* **245**: 164-173.
Van Peij NN, Gielkens MM, de Vries RP, Visser J & de Graaff LH (1998) The transcriptional activator XlnR regulates both xylanolytic and endoglucanase gene expression in *Aspergillus niger*. *Appl Environ Microbiol* **64**: 3615-3619.
Van Peij NNME, Visser J & De Graaff LH (1998) Isolation and analysis of xlnR, encoding a transcriptional activator co‐ordinating xylanolytic expression in *Aspergillus niger*. *Molecular microbiology* **27**: 131-142.
Vanden Wymelenberg A, Gaskell J, Mozuch M*, et al.* (2010) Comparative transcriptome and secretome analysis of wood decay fungi *Postia placenta* and *Phanerochaete chrysosporium*. *Appl Environ Microbiol* **76**: 3599-3610.
Vanden Wymelenberg A, Gaskell J, Mozuch M*, et al.* (2011) Gene expression of wood decay fungi *Postia placenta* and *Phanerochaete chrysosporium* is significantly altered by plant species. *Appl Environ Microbiol* AEM.00508-00511v00501.
Vanden Wymelenberg A, Minges P, Sabat G*, et al.* (2006) Computational analysis of the *Phanerochaete chrysosporium* v2.0 genome database and mass spectrometry identification of peptides in ligninolytic cultures reveal complex mixtures of secreted proteins. *Fungal Genet Biol* **43**: 343-356.
Verlhac C, Dedier J & Chanzy H (1990) Availability of surface hydroxyl groups in Valonia and bacterial cellulose. *J Polym Sci Part A: Polymer Chem* **28**: 1171-1177.
Vinzant T, Adney W, Decker S*, et al.* (2001) Fingerprinting *Trichoderma reesei* hydrolases in a commercial cellulase preparation. *Appl biochem and biotechnol* **91**: 99-107.
Westereng B, Ishida T, Vaaje-Kolstad G & Wu… M (2011) The putative endoglucanase PcGH61D from *Phanerochaete chrysosporium* is a metal-dependent oxidative enzyme that Cleaves Cellulose. *PLoS ONE*.
Westermark U & Eriksson K (1974) Cellobiose: quinone oxidoreductase, a new wood-degrading enzyme from white-rot fungi. *Acta chem. scand. B* **28**: 209-214.
Wood TM & McCrae SI (1972) The purification and properties of the C1 component of *Trichoderma koningii* cellulase. *Biochem J* **128**: 1183.
Würleitner E, Pera L, Wacenovsky C, Cziferszky A, Zeilinger S, Kubicek CP & Mach RL (2003) Transcriptional regulation of xyn2 in *Hypocrea jecorina*. *Eukaryotic Cell* **2**: 150-158.
Wymelenberg AV, Covert S & Cullen D (1993) Identification of the gene encoding the major cellobiohydrolase of the white rot fungus *Phanerochaete chrysosporium*. *Appl Environ Microbiol* **59**: 3492-3494.

Wymelenberg AV, Gaskell J, Mozuch M, Kersten P, Sabat G, Martinez D & Cullen D (2009) Transcriptome and secretome analyses of *Phanerochaete chrysosporium* reveal complex patterns of gene expression. *Appl Environ Microbiol* **75**: 4058-4068.

Wymelenberg AV, Sabat G, Martinez D*, et al.* (2005) The *Phanerochaete chrysosporium* secretome: database predictions and initial mass spectrometry peptide identifications in cellulose-grown medium. *J Biotechnol* **118**: 17-34.

Wymelenberg AV, Denman S, Dietrich D*, et al.* (2002) Transcript analysis of genes encoding a family 61 endoglucanase and a putative membrane-anchored family 9 glycosyl hydrolase from *Phanerochaete chrysosporium*. *Appl Environ Microbiol* **68**: 5765-5768.

Yoshida M, Igarashi K, Kawai R, Aida K & Samejima M (2004) Differential transcription of beta-glucosidase and cellobiose dehydrogenase genes in cellulose degradation by the basidiomycete *Phanerochaete chrysosporium*. *FEMS Microbiol Lett* **235**: 177-182.

Yoshida M, Igarashi K, Wada M*, et al.* (2005) Characterization of carbohydrate-binding cytochrome b562 from the white-rot fungus *Phanerochaete chrysosporium*. *Appl Environ Microbiol* **71**: 4548-4555.

Zeilinger S, Mach RL & Kubicek CP (1998) Two adjacent protein binding motifs in the cbh2 (cellobiohydrolase II-encoding) promoter of the fungus *Hypocrea jecorina* (*Trichoderma reesei*) cooperate in the induction by cellulose. *J Biol Chem* **273**: 34463.

Zeilinger S, Mach RL, Schindler M, Herzog P & Kubicek CP (1996) Different inducibility of expression of the two xylanase genes xyn1 and xyn2 in *Trichoderma reesei*. *J Biol Chem* **271**: 25624-25629.

Zeilinger S, Ebner A, Marosits T, Mach R & Kubicek C (2001) The *Hypocrea jecorina* HAP 2/3/5 protein complex binds to the inverted CCAAT-box (ATTGG) within the cbh2 (cellobiohydrolase II-gene) activating element. *Mol Genet Genom* **266**: 56-63.

Chapter 2

Effects of xylan on growth and cellulolytic enzymes production of *P. chrysosporium*

Chapter 2

Effects of xylan on growth and cellulolytic enzymes production of *P. chrysosporium*

2.1 Introduction

The most potent degrader of wood, basidiomycete *Phanerochaete chrysosporium* could have an efficient subset of extracellular polysaccharide-degrading enzymes and effective strategies of regulating those enzymes because of its distinct feature of degrading all components of plant cell wall by itself. On the other hand, cellulose is closely connected with xylan in plant cell wall in nature so that cellulolytic enzyme production is controlled in the existence of not only cellulose and those soluble products but also xylan and those soluble products in other filamentous fungi. In *P. chrysosporium*, it is well known that cellulolytic enzymes production is regulated by cellulose and its hydrolysis although the effect of xylan on extracellular cellulolytic enzymes production has not been investigated so much. Through this research the principal aim is, therefore, to reveal the effect of xylan on cellulolytic enzymes production.

In this chapter, to get an initial clue, we examined the effects of xylan on fungal growth and total amount of extracellular proteins of *P. chrysosporium.* In addition, enzymatic activities, both cellulase and xylanase activities, of culture filtrates were measured by PHBAH method. PHBAH method can determine amount of reducing sugar released by enzymatic reaction using the *p*-hydroxybenzoic acid hydrazide with high sensitivity and accuracy (Lever, 1972). Moreover, different xylans derived from glass or wood are comparatively examined since structure of xylan is diverse dependent on species. Xylan is a heteropolymer consisting of main chain of 1,4-linked β-D-xylose unit substituted with mainly acetyl, arabinofuranosyl and glucuronosyl residue. The type and frequency of substitution are dependent on source and main woody (hardwood) xylan has side chain of 4-O-methyl-β-D-glucuronic acid while main grass xylan has side chain of α-L-arabinose.

2.2 Materials and Methods

Fungal strain and cultivation conditions

Phanerochaete chrysosporium strain K-3 (Johnsrud & ERIKSSON, 1985) was cultivated in Kremer and Wood medium (Kremer & Wood, 1992) containing 2.0 % (w/v) cellulose (CF11; Whatman, Fairfield, NJ), 2.0 % (w/v) cellulose + 0.2 % (w/v) xylan from oat-spelt (Nakarai Chemicals Ltd., Kyoto, Japan) and 2.0 % (w/v) cellulose + 0.2 % xylan from beech (SIGMA-ALDRICH, St. Louis, MO) as carbon sources. The culture medium (400 mL) was inoculated with 10^9 spores $liter^{-1}$ in 1 L Erlenmeyer flasks, incubated at 37 ˚C and shaken at 150 rpm for 2 days. In order to evaluate fungal growth, 5 ml aliquots were collected, left to stand for 30 min, and the volume of fungal mycelia was taken as representing the growth. After cultivation, culture filtrates were separated from mycelia and insoluble substrate using a glass filter membrane (ADVANTEC® GA-100; Tokyo Roshi Kaisya, Tokyo, Japan). Protein concentration of the culture filtrate was determined by means of the Bradford assay (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions.

Enzyme assays

For enzyme assays, the culture filtrates of cellulose culture and cellulose + oat-spelt xylan media were used. Reducing sugar released by enzymatic reaction was measured using the *p*-hydroxybenzoic acid hydrazide (PHBAH; Wako) method (Lever, 1972), with some modifications.

For Avicelase activity, 100 µl of culture filtrate and 0.1 % (w/v) Avicel (Funakoshi Co. Ltd., Tokyo, Japan) in 250 µl (final volume) of 50 mM sodium acetate, pH 5.0, were incubated for 300 min at 30 ˚C. The reaction was stopped by the addition of 250 µl of 1.0 M NaOH. The solution was mixed with 500 µl PHBAH solution (0.1 M PHBAH, 0.2 M

NaK-tartrate and 0.5 M NaOH) and incubated at 96 ˚C for 5 min followed by measuring the absorbance at 405 nm of the reaction mixture. One unit of Avicelase was defined as the amount of enzyme required to release 1 µmol of reducing sugar per minute under the assay conditions using predetermined standard curve obtained with glucose (ε_{405} = 4.03 mM^{-1} cm^{-1}).

For xylanase activity, 100 µl of culture filtrate and 0.1 % (w/v) xylan from oat-spelt in 250 µl (final volume) of 50 mM sodium acetate, pH 5.0, were incubated for 10 min at 30 ˚C. The reaction was stopped by addition of 250 µl 1.0 M NaOH and incubation was continued at 96 ˚C for 5 min, then the absorbance at 405 nm of the reaction mixture was measured. One unit of xylanase was defined as the amount of enzyme required to release 1 µmol of reducing sugar per minute under the assay conditions; xylose was used as a standard (ε_{405} = 2.81 $mM^{-1}cm^{-1}$).

For cellobiose dehydrogenase (CDH) activities of the supernatants of the cultures, cytochrome *c*-reducing activities were assayed using cellobiose as a substrate as described previously (Samejima & Eriksson, 1992).

2.3 Results

P. chrysosporium was cultivated for 4 days in synthetic media containing cellulose (C), cellulose + oat-spelt oxylan (COX), cellulose + beech xylan (CBX) as carbon sources. As shown in Fig. 2-1A, the fungal volumes in COX and CBX cultures were apparently larger than in C culture during cultivation. The differences of the fungal growth between COX or CBX and C culture exhibited best record at day 2. The amount of extracellular proteins produced in COX and CBX cultures were also larger than this in C culture during cultivation, and finally reached more than 2-fold increase 0.37 g/L and 0.34 g/L in COX and CBX cultures respectively, compared to 0.14 g/L in C culture, as shown in Fig. 2-1B. These results suggested that addition of xylans substituted with different side-chains appeared to be almost the same effects on the fungal growth and extracellular protein production in cellulose-degrading culture.

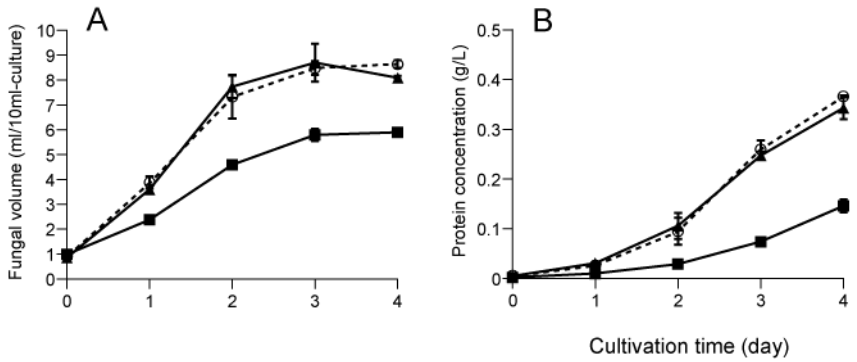


Fig. 2-1. Time courses of (A) fungal volumes and (B) concentrations of extracellular protein of *P. chrysosporium* grown in the medium containing cellulose (filled square), cellulose + oat-spelt xylan (open circle) and cellulose + beech xylan (filled triangle) during four days. Error bars show the standard deviation in triplicate tests.

Cellulase (Avicelase) and xylanase activities in culture filtrates in cellulose (C) and cellulose + oat-spelt xylan (CX) media after 2 days of cultivation were measured and the results are shown in Fig. 2-2. In the cellulose culture without addition of xylan, not only cellulase activity (0.66 U/L), but also xylanase activity (77 U/L) was detected. Addition of

xylan to cellulose culture resulted a significant increase of xylanase activity, and also increased cellulase activity.

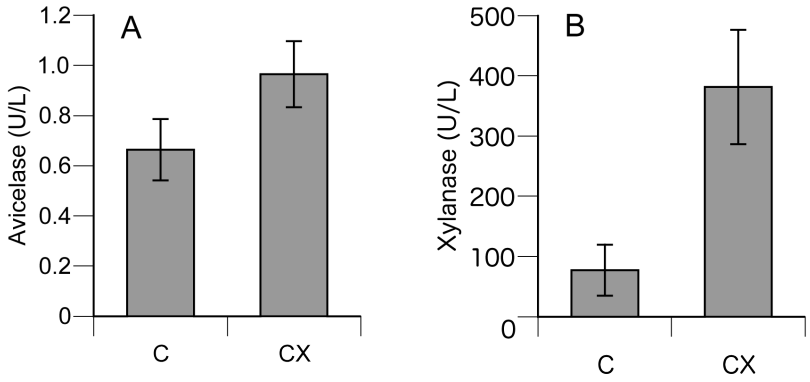


Fig. 2-2. Cellulase (A) and xylanase (B) activities of 2-day cultures of *P. chrysosporium* in C and CX media. All the enzyme activities were measured in sodium acetate, pH 5.0 at 30˚C. 0.1 % (w/v) Avicel and oat-spelt xylan were used as substrates, respectively, and the newly formed reducing ends were estimated by means of the *p*-hydroxybenzoic acid hydrazide (PHBAH) method as described in Materials and Methods.

CDH activities in culture filtrates were also measured and the results are shown in Fig. 2-3. In the case of C culture, CDH activities were hardly observed during cultivation, while in CX, CDH activities were clearly observed on day 2 (8.4 U/L) and final values reached 42 U/L (data not shown).

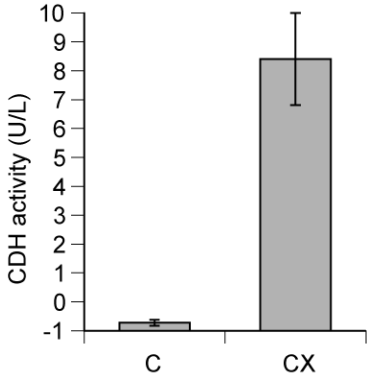


Fig. 2-3. CDH activities of 2-day cultures of *P. chrysosporium* in C and CX media.

2.4 Discussion

In filamentous fungi, various plant cell wall-degrading enzymes were produced during growth on polysaccharides. Generally polysaccharides induced production of those enzymes; e.g. cellulose induces cellulose-degrading enzymes production while xylan induces xylan-degrading enzymes production. To examine the effect of xylan on production of extracellular enzymes of *P. chrysosporium*, therefore, we cultivated the fungus in the synthetic culture containing cellulose and cellulose + xylan and compared its growth and enzymatic activities for extracellular proteins.

Surprisingly, as shown in Fig. 2-1A, addition of small amount of xylan to cellulose-degrading culture enhanced fungal growth of this fungus although xylan itself could not be a preferable nutrient because *P. chrysosporium* hardly grew when xylan was used as a sole carbon source in our experiments and some previous reports (Dobozi*, et al.*, 1992, Copa-Patino*, et al.*, 1993). Accordingly, we also investigated extracellular protein production and it revealed that the addition of xylan promoted extracellular protein production, as shown in Fig 2-1B. It suggested that enhancement of production of secreted proteins is related to initial fungal growth. Moreover, xylans with different side-chains similarly enhanced initial fungal growth and extracellular protein production, indicating that those effects could be caused by main chain rather than side chain. Furthermore, the differences between xylans derived from wood and glass are not only kind of side-chains, glucuronic acid or arabinose residue, but also frequency of substitution and length of polymer, although the difference caused the almost same effects on fungi, suggesting that small amount of fragments degraded from main chain, xylose or xylooligosaccharides, could cause those effects like signal molecule.

Cellulase and xylanase activities were measured in C and CX (cellulose + oat-spelt

xylan) culture filtrates, as shown in Fig. 2-2. In this time, extracellular protein concentrations in C and CX cultures are about 0.1 g/L and 0.15 g/L, respectively. Cellulase activity was enhanced by xylan at the same level as enhancement of extracellular protein concentration while xylanase activity was enhanced three-hold lager than enhancement of extracellular protein concentration. Xylanase activity was enhanced by xylan and this phenomenon is broadly observed in other filamentous fungi. This xylanase activity was reflected by endo-xylanase or other xylanolytic enzymes. Cellulase production also increased in the existence of xylan so that initial fungal growth might be promoted since cellulose is a preferable nutrient to xylan. Since cellulase activities represented the cooperation and synergism among many kinds of cellulolytic enzymes acting on cellulose, it is needed that the effect of xylan on cellulolytic enzyme production should be revealed at each component protein level.

CDH activities were also enhanced by xylan and this enhancement level is much more than the enhancement of extracellular protein production level, as shown in Fig. 2-3. It is very interesting phenomena since CDH cannot utilize xylose as a substrate at all.

References

Copa-Patino J, Kim Y & Broda P (1993) Production and initial characterization of the xylan-degrading system of *Phanerochaete chrysosporium*. *Appl Microbiol Biot* **40**: 69-76.

Dobozi MS, Szakács G & Bruschi CV (1992) Xylanase activity of *Phanerochaete chrysosporium*. *Appl Environ Microbiol* **58**: 3466-3471.

Habu N, Samejima M, Dean JF & Eriksson K (1993) Release of the FAD domain from cellobiose oxidase by proteases from cellulolytic cultures of *Phanerochaete chrysosporium*. *FEBS Lett* **327**: 161-164.

Johnsrud SC & Eriksson K-E (1985) Cross-breeding of selected and mutated homokaryotic strains of *Phanerochaete chrysosporium* K-3 - New cellulase deficient strains with increased ability to degrade lignin. *Appl Microbiol Biot* **21**: 320-327.

Kremer SM & Wood PM (1992) Evidence that cellobiose oxidase from *Phanerochaete chrysosporium* is primarily an Fe(III) reductase. Kinetic comparison with neutrophil NADPH oxidase and yeast flavocytochrome b_2. *Eur J Biochem* **205**: 133-138.

Lever M (1972) New reaction for colorimetric determination of carbohydrates. *Anal Biochem* **47**: 273-&.

Samejima M & Eriksson KEL (1992) A comparison of the catalytic properties of cellobiose: quinone oxidoreductase and cellobiose oxidase from *Phanerochaete chrysosporium*. *Eur J Biochem* **207**: 103-107.

Chapter 3

Effects of xylan on profile of extracellular enzymes produced from *P. chrysosporium* in cellulose-degrading culture

Chapter 3

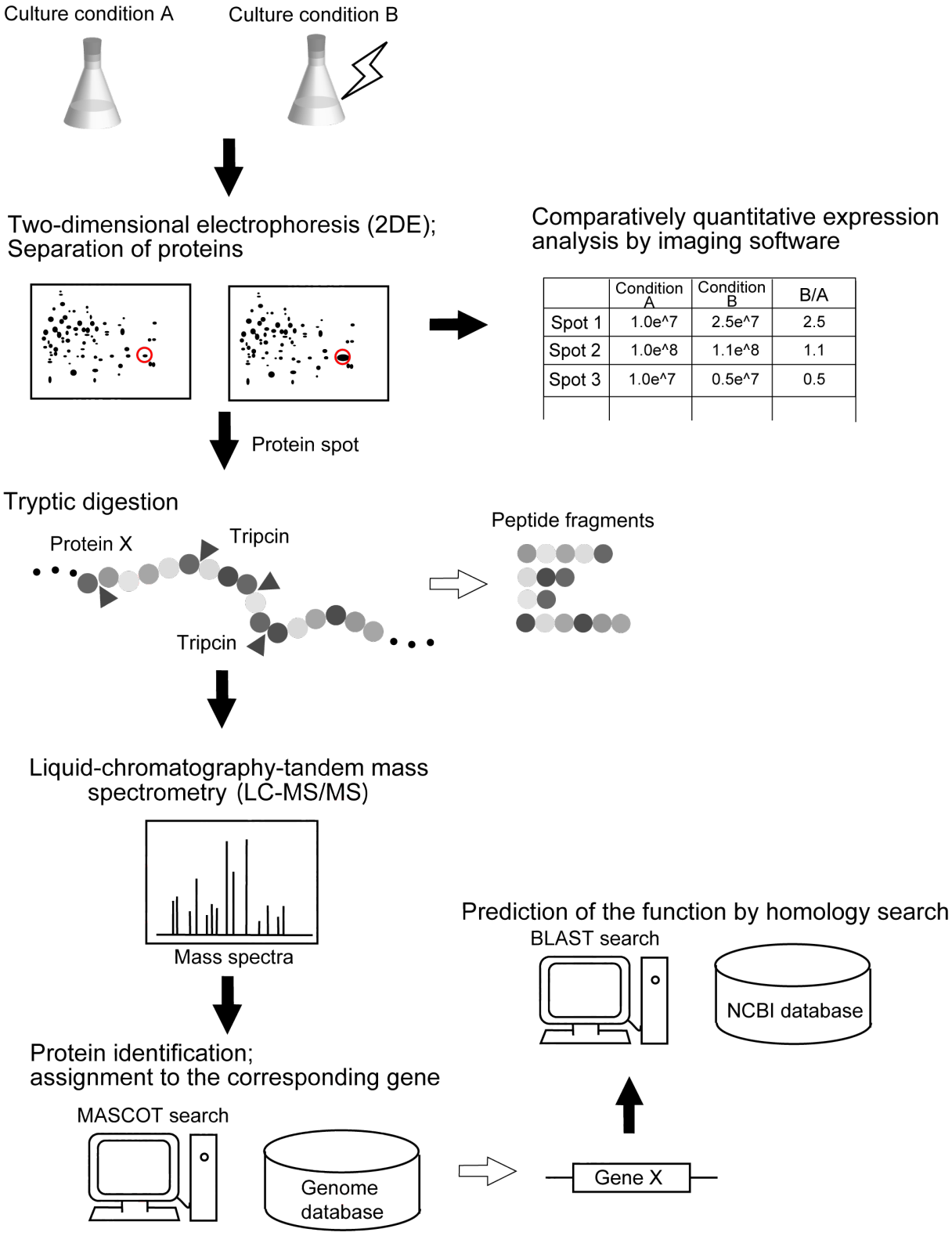
Effects of xylan on profile of extracellular enzymes produced from *P. chrysosporium* in cellulose-degrading culture

3.1 Introduction

P. chrysosporium is the first filamentous fungus for disclosing total genome sequences and the genome information makes proteomic analysis, including secretomic analysis, of this fungus available. In addition to the genome information, several techniques such as preparation of protein sample, separation and detection methodology of component proteins and mass-spectrometry for assigning component proteins to the genes are required for identifying comprehensive protein expression pattern and we need to choose suitable methods. Generally preparation of protein sample in filamentous fungi is hard step because electrophoresis was disturbed by the extracts from both fungi and lignocellulose when lignocellulose is used as a substrate (Fragner*, et al.*, 2009). In our case, extract disturbance is not tough so much since pure substrate is used and thus we chose simple treatment with ultrafiltration since general treatment such as acid precipitation occurs simultaneously with lacking some amount of proteins. Secondly, separation of component proteins is generally achieved by electrophoresis such as one-dimentional electrophoresis (1DE) or two-dimensional electrophoresis (2DE). In the case of 1DE, protein components are determined with lacking identification of the small amount protein while detectable spots on 2DE can be identified. In addition to separation method, kinds of staining methods such as Coomassie Brilliant Blue stain, silver stain and fluorescent stain determined the quality of proteomic analysis. Among them, fluorescent stain is most sensitive and accurate detection of proteins with extremely high cost. In regard to identification of protein using mass-spectrometry (MS), recently liquid chromatography-tandem mass spectrometry

(LC-MS/MS) with high accuracy and sensitivity is mainly used since it can determine de-novo sequence of protein, which is directly used as a query for genome information, called peptide-sequence tag method. Meanwhile, MS only allows peptide-fingerprinting method, which gained MS-spectra from fragmental peptides of one protein compare theoretical molecular weight derived from each gene of the genome information. After assigning a protein to a gene, function of the gene is computationally analyzed. We chose the process of proteomic analysis as shown in scheme 3-1

In this chapter, therefore, to elucidate the effects of xylan on cellulolytic enzymes produced from *P. chrysosporium* grown in cellulose-degrading culture (in chapter 2) at protein component level, secretomic analysis of *P. chrysosporium* using 2DE with fluorescent stain and LC-MS/MS was conducted to clarify the profile of the proteins up-regulated or down-regulated in the presence of xylan.



	Condition A	Condition B	B/A
Spot 1	1.0e^7	2.5e^7	2.5
Spot 2	1.0e^8	1.1e^8	1.1
Spot 3	1.0e^7	0.5e^7	0.5

Scheme 3-1. Process of secretomic analysis in this research

3.2 Materials and Methods

Fungal strain and cultivation conditions

Phanerochaete chrysosporium strain K-3 (Johnsrud & Eriksson, 1985) was cultivated in Kremer and Wood medium (Kremer & Wood, 1992) containing 2.0 % (w/v) cellulose (CF11; Whatman, Fairfield, NJ) and 2.0 % (w/v) cellulose + 0.2 % (w/v) xylan from oat-spelt (Nakarai Chemicals Ltd., Kyoto, Japan) as carbon sources. The culture medium (400 mL) was inoculated with 10^9 spores $liter^{-1}$ in 1 L Erlenmeyer flasks, incubated at 37 ˚C and shaken at 150 rpm for 2 days. After cultivation, culture filtrates were separated from mycelia and insoluble substrate using a glass filter membrane (ADVANTEC® GA-100; Tokyo Roshi Kaisya, Tokyo, Japan). Protein concentration of the culture filtrate was determined by means of the Bradford assay (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions.

Separation of proteins by two-dimensional electrophoresis

Culture filtrates from medium containing cellulose (C) and cellulose+xylan (CX) were centrifuged at 15,000 *g* for 5 min at 4˚C to remove insoluble materials. The supernatants were then concentrated using a 10 kDa Ultrafree®-0.5 Centrifugal Filter Device (Millipore, Billerica, MA) and washed with Milli-Q water three times. Samples were examined on a Multiphor system (GE Healthcare UK Ltd., Buckinghamshire, UK). Proteins (25 μg) were mixed with a rehydration buffer containing 7.5 M urea, 2 M thiourea, 4% CHAPS, 2% DTT, 0.5% IPG buffer (GE Healthcare), and a trace amount of bromophenol blue to a final volume of 330 μl and then loaded onto Immobiline Drystrips (18 cm, pH 3-10, non linear; **GE** Healthcare). After rehydration for 12 hour, proteins were isoelectrically focused under the following conditions: 500 V (gradient over 1 min); 3500 V (gradient over 90 min); 3500 V

(fixed for 6 h). These strips were equilibrated with buffer I (50 mM Tris–HCl pH 6.8, 6 M urea, 2 % (w/v) SDS, 30 % (w/v) glycerol, 2 % (w/v) DTT) and then buffer II (50 mM Tris–HCl pH 6.8, 6 M urea, 2 % (w/v) SDS, 30 % (w/v) glycerol, 2.5 % (w/v) iodoacetamide). These strips were placed on SDS-polyacrylamide gels (ExcelGel ^{TM} SDS XL 12-14; GE Healthcare) and electrophoresis was conducted under the following conditions: 12 mA for 60 min, 40 mA for 5 min, and finally 50 mA for 160 min. The gels were fixed in 10 % (v/v) acetic acid and 40 % (v/v) EtOH and then stained with SYPRO Ruby (Bio-Rad) for 1 h. The staining solution was removed, and the gels were washed in 10% acetic acid and 10 % (v/v) MeOH solution for 30 min. The stained 2DE gels were scanned with excitation at 532 nm using a Typhoon image scanner (GE Healthcare) and individual protein spots on different gels were matched and quantified using Progenesis SameSpots ver 4.0 (Nonlinear Dynamics Limited, Durham, NC).

Protein identification by LC-MS/MS and sequence analysis

The protein spots were excised, washed in 200 µl acetonitrile and then dried under vacuum. The proteins in the gel were reduced with 100 mM dithiothreitol in 0.1 M ammonium bicarbonate at 56˚C for 30 min and alkylated with 100 mM iodoacetamide in 0.1 M ammonium bicarbonate at 37˚C for 30 min in the dark. The gels were washed with 0.1 M ammonium bicarbonate, then acetonitrile, and dried. These gels were re-swollen with 12.5 ng/µl recombinant trypsin (proteomics grade; Roche Diagnostics Corporation, Indianapolis, IN) in 10 mM Tris-HCl buffer (pH 8.8) and then incubated at 37˚C for 12 h. After peptide extraction with extraction buffer (70 % (v/v) acetonitrile and 5 % (v/v) formic acid), the extracted peptide mixture was dried in a SpeedVac and dissolved in 20 µl of 0.1% trifluoroacetic acid. Peptides were subjected to high-performance liquid chromatography (HPLC) separation on MAGIC 2002 (Michrom BioResources, Auburn, CA) with a

reversed-phase capillary HPLC column (C_{18}, 200 A, 0.2 × 50 mm; Michrom BioResources). As solvents, 2 % (v/v) acetonitrile in 0.1 % (v/v) formic acid (solvent A) and 90 % (v/v) acetonitrile in 0.1% (v/v) formic acid (solvent B) were used, with a linear gradient from 5 % to 65 % of solvent B over 50 min. The chromatography system was coupled via an HTS-PAL (CTC Analytics, Zwingen, Switzerland) to an LCQ DECA XP ion trap mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA). The MS/MS spectra were collected from 50 to 4500 m/z and merged into data files. In-house-licensed Mascot search engine (Matrix Science, London, UK) identified peptides using 10,048 annotated gene models from *P. chrysosporium* v2.0 genome database (http://genome.jgi-psf.org/Phchr1/Phchr1.home.html). The deduced amino acid sequences thus obtained were subjected to BlastP search against the NCBI nonredundant database with default settings to confirm gene functions. The theoretical Mw and pI values were calculated using the protein parameter function calculation function on the EXPASY server (http://au.expasy.org/tools/pi_tool.html).

3.3 Results

Extracellular proteins from *P. chrysosporium* cultivated in the synthetic media, C and CX were separated by 2DE as shown Fig. 3-1 and 47 spots on the gels were subjected to LC-MS/MS analysis. Among 47 spots, 41 spots and 47 spots were detected on the 2DE gel in C and CX culture, respectively, and Table 3-1 shows a summary of the results. These results revealed that most of total 47 identified proteins were classified into GHs (37 spots) and CEs (5 spots), but a cellobiose dehydrogenase (CDH), a putative glutaminase and three hypothetical proteins were also included. When functionally classified, most of them were several Cellobiohydrolases (CBHs) and endo-glucanases (EGs) involved in cellulose degradation, and several xylanases and other xylanolytic enzymes related to xylan degradation. Major spots showing fluorescence intensity over 5.0×10^7 in all cultures were CBHs (Cel7C, Cel7D and Cel6A: spots 5, 7 and 8, respectively), EG (Cel5B: spot 15) and endo-xylanase and laminarinase (Xyn11A and lam16A: spot 24). Those three groups accounted for 39% and 37% amounts of total extracellular proteins in the C and CX media, respectively.

To investigate the effects of xylan on the ratios of protein components, the fluorescence intensity of each protein spot identified in CX cultures was compared with that in cellulose (C) culture using Progenesis SameSpots software. As shown in Fig. 3-2, 12 protein spots with more than 2-fold increase of intensity compared with C culture were detected in CX culture. Among them, 5 spots (spots 23, 30, 31, 32 and 42) were putative GH family 10 endo-xylanases (Xyn10C) and one spot was a putative glucuronoyl esterase belonging to CE family 15 (spot 9). Moreover, the spot assigned to the redox enzyme CDH (spot 3) was also increased 2-fold by addition of xylan. In addition, many protein spots of GH family 61s were increased almost 2-fold, compared with C culture.

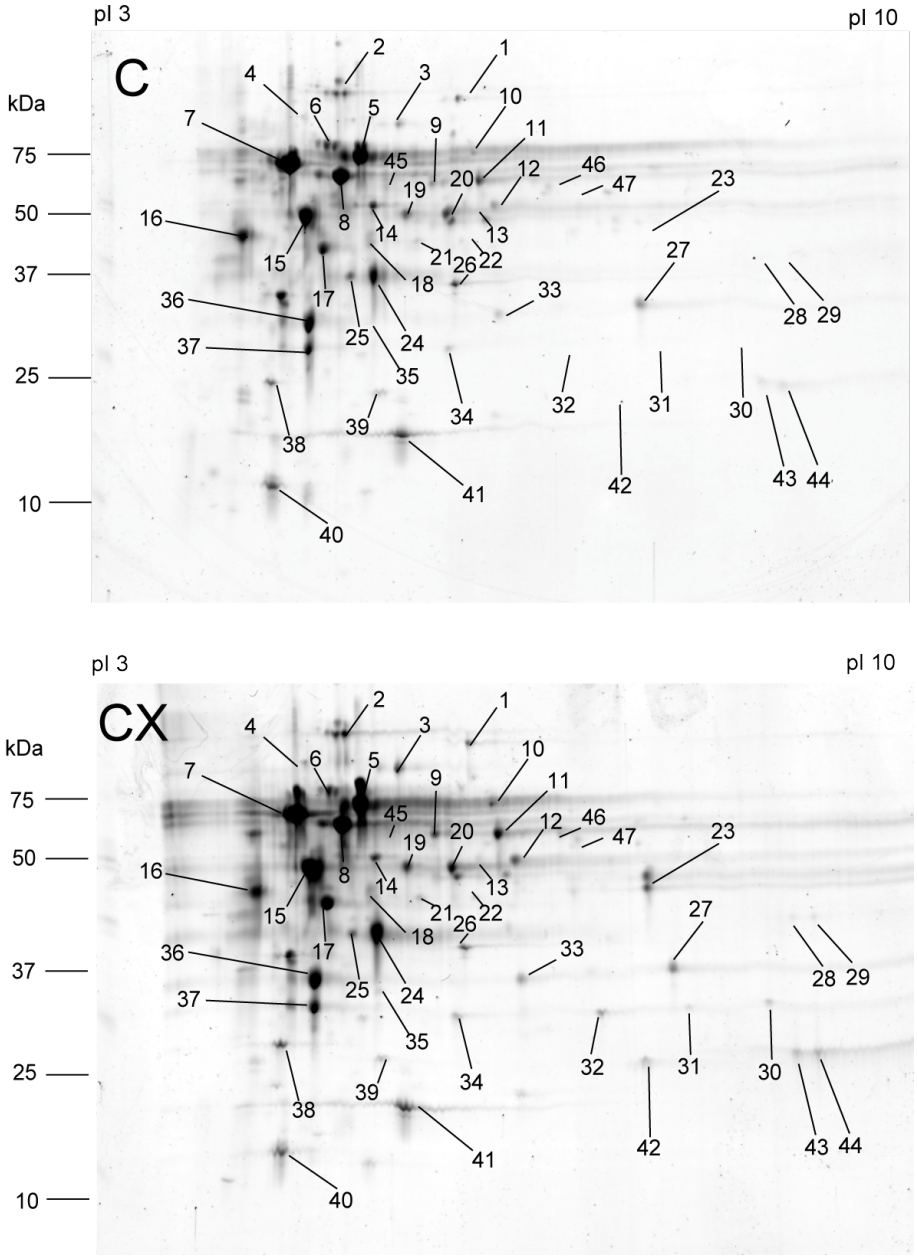


Fig. 3-1. Two-dimensional electrophoresis of extracellular proteins stained with fluorescent dye (SYPRO ruby). *P. chrysosporium* was cultivated in synthetic medium containing 2.0% cellulose (C) and 2.0% (w/v) cellulose + 0.2% (w/v) xylan (CX) for 2 days and an aliquot of extracellular proteins (25 μg) produced in each case was separated as described in Materials and Methods. The horizontal axis of the gel is from pH 3-10 and the vertical axis is from 250 to 5 kDa. Spot numbers correspond to the proteins in Table 3-1.

Table 3-1. List of identified proteins of *P. chrysosporium* in 2-day-incubated synthetic media containing 2.0% cellulose +0.2% xylan (CX). Spot number refers to figure 3-1.

Spot number	Score[a]	Protein ID[b]	Function (Gene or Domain)	Protein family	CBM[c]	Th Mw[d]	Th pI[d]	Sequence cover (%)	Reference(s)[e]	Protein expression[f]	
										C	CX
1	447	134658	Glucan β-1,3-glucosidase (bgl3)	GH3	N	85.053	5.43	16		+	+
4	164	129849	Putative β-glycosidase (gly3B)	GH3	-	87.136	4.72	8	1,2,4,5,6	+	+
15	172	4361	Endo-β-1,4-glucanase (cel5B)	GH5	N	49.721	6.21	11	2,6	+++	+++
17	257	6458	Endo-β-1,4-glucanase (cel5A)	GH5	N	39.851	5.04	32	2,6	++	++
22	137	135724	β-1,3-glucanase	GH5	-	46.864	5.55	9	6	+	+
47	117	5607	Putative β-glycosidase	GH5	-	49.195	5.4	5	2	+	+
8	484	133052	Cellobiohydrolase II (cel6A)	GH6	N	48.957	5.04	33	2,4,6	+++	+++
5	862	127029	Cellobiohydrolase 62 (cel7C)	GH7	C	56.107	5.03	48	2,4,5,6	+++	+++
7	780	137372	Cellobiohydrolase 58 (cel7D)	GH7	C	59.474	4.96	39	2,4,5,6	+++	+++
21	105	127029	Cellobiohydrolase 62 (cel7C) (Fragment)	GH7	C	56.107	5.03	4	2,4,5,6	+	+
14	110	138345	Endo-β-1,4-xylanase (xyn10A)	GH10	N	44.065	5.21	9	2,3,4,6	++	+
23	519	7045	Putative Endo-β-1,4-xylanase (xyn10C)	GH10	(N)*	31.21	6.89	59	2,3,6	+	+
30	360	7045	Putative Endo-β-1,4-xylanase (xyn10C)	GH10	(N)*	31.21	6.89	32	2,3,6	ND	+
31	260	7045	Putative Endo-β-1,4-xylanase (xyn10C)	GH10	(N)*	31.21	6.89	28	2,3,6	ND	+
32	410	7045	Putative Endo-β-1,4-xylanase (xyn10C)	GH10	(N)*	31.21	6.89	31	2,3,6	ND	+
42	224	7045	Putative Endo-β-1,4-xylanase (xyn10C)	GH10	(N)*	31.21	6.89	21	2,3,6	ND	+
18	155	133788	Endo-β-1,4-xylanase (xyn11A)	GH11	C	30.804	5.72	11	2,4,6	+	+
24	156	133788	Endo-β-1,4-xylanase (xyn11A)	GH11	C	30.804	5.72	11	2,4,6	+++	+++
36	87	8466	Endo-β-1,4-glucanase (cel12A)	GH12	-	26,459	4.79	8	2,6	++	++
37	93	8466	Endo-β-1,4-glucanase (cel12A)	GH12	-	26,459	4.79	8	2,6	++	++
38	92	7048	Putative endo-xyloglucanase (cel12B)	GH12	-	27.082	4.74	10	2,6	+	+
45	301	138813	Putative glucoamylase (gla15A)	GH15	C	60.991	5.24	22	2,6	+	+
24	144	10833	Endo-β-1,3-glucanase (lam16A)	GH16	-	33.715	4.94	12	6	+++	+++
25	187	10833	Endo-β-1,3-glucanase (lam16A)	GH16	-	33.715	4.94	16	6	+	+
39	90	136630	Putative lysozyme	GH25	-	24.571	5.63	15	6	+	+
35	272	3805	Putative endo-polygalacturonase (epg28A)	GH28	-	40.207	5.02	19	2,4,6	+	+
11	431	29397	Putative rhamnogalacturonase (rgh28C)	GH28	-	44.793	5.22	35	2,4,6	++	++
46	123	29397	Putative rhamnogalacturonase (rgh28C)	GH28	-	44.793	5.22	7	2,4,6	+	+
41			Endo-β-1,4-glucanase** (cel45)	GH45	-	18.169	5.04		2	++	++
26	76	138710	Putative arabinogalactan endo-β-1,4-galactanase	GH53	-	36.86	5.59	11		+	+
34	52	138710	Putative arabinogalactan endo-β-1,4-galactanase (fragment)	GH53	-	36.86	5.59	5		+	+
13	122	41563	Putative GH family 61 protein	GH61	C	33.195	5.39	16	2,6	+	+
28	160	41123	Putative GH family 61 protein (cel61C)	GH61	-	23.761	6.63	16	3,6	ND	+
29	212	41123	Putative GH family 61 protein (cel61C)	GH61	-	23.761	6.63	16	3,6	ND	+
43	238	41123	Putative GH family 61 protein (cel61C)	GH61	-	23.761	6.63	31	3,6	+	+

44	278	41123	Putative GH family 61 protein (cel61C)	GH61	-	23.761	6.63	29	3,6	+	+
6	480	138266	Putative xyloglucanase (gly74A)	GH74	-	77.79	4.72	18	2,6	++	++
2	455	134556	Xyloglucanase(xgh74B)	GH74	C	89.855	5.05	18	2,4,5,6	++	++
12	123	126075	Putative acetyl xylan esterase (axe1)	CE1	-	35.963	5.91	7	2,4,6	+	+
19	111	126075	Putative acetyl xylan esterase (axe1)	CE1	-	35.963	5.91	7	2,4,6	++	++
20	223	126075	Putative acetyl xylan esterase (axe1)	CE1	-	35.963	5.91	29	2,4,6	++	++
33	189	126075	Putative acetyl xylan esterase (axe1)	CE1	-	35.963	5.91	29	2,4,6	+	+
9	385	130517	Putative Glucuronoyl esterase	CE15	N	49.731	5.55	18	6	+	+
3	526	11098	Cellobiose dehydrogenase (cdh1)	LO3	-	82.185	5.19	16	2,6	+	+
10	448	140079	Putative glutaminase (gta1)		-	75.388	5.54	18	2,3	+	+
16	129	3097	Hypothetical protein (IPR001087 Lipolytic enzyme, G-D-S-L)		N	43.209	5.08	9	6	++	++
27	199	135606	Hypothetical protein		-	27.395	6.04	27	6	+	+
40	62	8221	Hypothetical protein (IPR010829 Cerato-platanin)		-	14.966	4.69	10	6	++	+

a) MASCOT score ($p>0.05$).
b) Protein model number ver. 2.1 (from *P.chrysosporium* genome database).
c) Deduced amino acid sequence contains CBM (carbohydrate binding module) or not. N; N-terminal, C; C-terminal.
d) Theoretical molecular weight (kDa) and pI.
e) Previous identification of protein in secretome analyses of *P.chrysosporium*. 1; (Abbas*, et al.*, 2005), 2; (Vanden Wymelenberg*, et al.*, 2005), 3; (Vanden Wymelenberg*, et al.*, 2006), 4; (Sato*, et al.*, 2007), 5; (Ravalason*, et al.*, 2008), 6; (Vanden Wymelenberg*, et al.*, 2009).
f) Spots showing fluorescence intensity over 5.0×10^7 (+++), under 5.0×10^7 and over 1.0×10^7 (++), under 1.0×10^7 (+) or not detected (ND)
*Manual examination revealed conserved CBM sequence at the N-terminus of this gene model.
**MS/MS dataset of spot 61 search on genome dataset ver. 2.0 in scaffold 6: 1798366-1797555 (Igarashi*, et al.*, 2008).

Spot Number	Protein family	Function (gene)	C	CX	Relative degree
23	GH10	Putative Endo-beta-1,4-xylanase (xyn10C)			5.6
30	GH10	Putative Endo-beta-1,4-xylanase (xyn10C)			unique
31	GH10	Putative Endo-beta-1,4-xylanase (xyn10C)			unique
32	GH10	Putative Endo-beta-1,4-xylanase (xyn10C)			unique
42	GH10	Putative Endo-beta-1,4-xylanase (xyn10C)			unique
35	GH28	Putative endo-polygalacturonase (epg28A)			2.6
28,29	GH61	Putative GH family 61 protein (GH61C)			unique
43,44	GH61	Putative GH family 61 protein (GH61C)			2.1
9	CE15	Putative glucuronoyl esterase			2.1
3	LO3	Cellobiose dehydrogenase			2.1

Fig. 3-2. List of the protein spots showing increase in intensity of more than 2-fold on the gel of CX culture comparing to C culture. Spot numbers refer to Fig. 3-1 and Table 3-1, and the intensity was analyzed by Progenesis SameSpots ver. 4.0.

3.4 Discussion

Among 47 spots analyzed, 2 spots are identified as two novel enzymes, while 45 spots are assigned to the protein components previously reported. (Abbas, *et al.*, 2005, Wymelenberg, *et al.*, 2005, Vanden Wymelenberg, *et al.*, 2006, Sato, *et al.*, 2007, Ravalason, *et al.*, 2008, Wymelenberg, *et al.*, 2009). Among 47 spots, 12 protein spots with more than 2-fold increase of intensity compared with C culture were detected in CX culture. Among them, 5 spots were Xyn10C, which may contribute to the significant increase of xylanase activity in CX medium in Chapter 2. Those spots assigned to the same gene, xyn10C, showed different pI and Mw values, possibly because of posttranslational modification and/or fragmentation, as described in a previous report (Dobozi, *et al.*, 1992). According to the total genomic sequence of *P. chrysosporium*, this fungus has six genes possibly coding GH family 10 proteins (Xyn10A-F), showing a maximum 92% identity of amino acid sequence. Although production of Xyn10A was not affected by the addition of xylan in the present study, production of Xyn10C was apparently increased by xylan, suggesting that this fungus produced xylanase isozymes differentially in response to different carbon sources. This fungus is known to have multiple genes coding GH family 7 cellulases, and they are secreted differentially in media containing different carbon sources (Wymelenberg, *et al.*, 2009). Transcriptional analysis has also revealed that they are expressed differentially at the transcript level in response to various carbon sources (Broda, *et al.*, 1995, Vallim, *et al.*, 1998, Suzuki, *et al.*, 2010). Similar expression studies should be performed for GH family 10 genes in order to clarify the role of each protein in the xylan-degrading system of this fungus.

Also, a putative glucuronoyl esterase belonging to CE family 15 was enhanced by addition of xylan. This protein has been postulated to hydrolyze ester linkages between the 4-*O*-methyl-D-glucuronic acid residue in xylan and the phenylpropane residue in lignin (Duranová, *et al.*, 2009). Enhancement of CE family 15 protein by xylan is consistent with

this postulation.

In addition of xylanolytic enzymes, CDH was also enhanced by xylan. CDH oxidizes cellobiose and cello-oligosaccharides to corresponding δ-lactones. Although many researchers have proposed various physiological functions for CDH (Henriksson, *et al.*, 2000), the precise role of this enzyme in degradation of plant cell wall remains to be established. Several recent transcriptional analyses have indicated that CDH is involved in cellulose metabolism (Yoshida, *et al.*, 2004). CDH may play a role in enhancing cellulase activity for cellulose degradation by relieving product inhibition (Igarashi, *et al.*, 2003). Dumonveaux reported that a CDH-deficient mutant of the wood-rotting basidiomycete *Trametes versicolor* grow poorly, not only on crystalline cellulose, but also on wood, implying that CDH may have role in invasion of the plant cell wall (Dumonceaux, *et al.*, 2001). Further transcriptional analysis of CDH under xylanolytic conditions will be necessary for a better understanding of its physiological function.

Moreover, many protein spots of GH family 61s were enhanced by addition of xylan, as shown in Fig. 3-2. Recently, Harris and co-workers have reported that the protein belonging to GH family 61 enhances the activity of cellulose hydrolysis in lignocellulose, but not in pure cellulose (Harris, *et al.*, 2010). Quite recently, moreover, Vaaje-Kolstad and co-workers demonstrated that Chitin-Binding Protein 21, which is structurally similar to GH family 61 protein with a conserved metal ion(s) in the protein (Karkehabadi, *et al.*, 2008), is an oxidative enzyme accelerating chitinase activity toward crystalline chitin (Vaaje-Kolstad, *et al.*, 2010). The present finding that CDH and GH family 61 proteins are upregulated by xylan suggests that the oxidative reaction is a critical step not only for the degradation of cellulose as proposed by Eriksson and co-workers in 1970's (Eriksson, *et al.*, 1974), but also for the degradation of other polysaccharides, and GH family 61 proteins may participate in the oxidation for degradation of plant polysaccharides. Although the biochemical function of

GH family 61 proteins is still unclear, enhancement of production of GH family 61 proteins by xylan is consistent with the recent evidences and provides a useful clue.

In conclusion, in cellulolytic culture of the basidiomycete *Phanerochaete chrysosporium*, the addition of xylan increases production of Xyn10C and a putative glucuronoyl esterase belonging to CE family 15, which may act in degradation of the main chain and side chain of xylan, respectively. Moreover, production of CDH and GH family 61 proteins, the potential oxidative enzymes accelerating enzymatic conversion of polysaccharides, is also increased in the presence of xylan. These results indicate that xylan is not simply an inducer of xylanolytic enzymes, but may promote the production of a variety of biomass-degrading enzymes by *P. chrysosporium*.

References

Abbas A, Koc H, Liu F & Tien M (2005) Fungal degradation of wood: initial proteomic analysis of extracellular proteins of *Phanerochaete chrysosporium* grown on oak substrate. *Curr Genet* **47**: 49-56.

Broda P, Birch P, Brooks P & Sims P (1995) PCR-mediated analysis of lignocellulolytic gene transcription by *Phanerochaete chrysosporium*: substrate-dependent differential expression within gene families. *Appl Environ Microbiol* **61**: 2358.

Dobozi MS, Szakács G & Bruschi CV (1992) Xylanase activity of *Phanerochaete chrysosporium*. *Appl Environ Microbiol* **58**: 3466-3471.

Dumonceaux T, Bartholomew K, Valeanu L, Charles T & Archibald F (2001) Cellobiose dehydrogenase is essential for wood invasion and nonessential for kraft pulp delignification by *Trametes versicolor*. *Enzyme Microb Technol* **29**: 478-489.

Duranová M, Spániková S, Wösten HAB, Biely P & de Vries RP (2009) Two glucuronoyl esterases of *Phanerochaete chrysosporium*. *Arch Microbiol* **191**: 133-140.

Eriksson KE, Pettersson B & Westermark U (1974) Oxidation: an important enzyme reaction in fungal degradation of cellulose. *FEBS Lett* **49**: 282-285.

Fragner D, Zomorrodi M, Kües U & Majcherczyk A (2009) Optimized protocol for the 2‐DE of extracellular proteins from higher basidiomycetes inhabiting lignocellulose. *Electrophoresis* **30**: 2431-2441.

Harris PV, Welner D, Mcfarland KC*, et al.* (2010) Stimulation of lignocellulosic biomass hydrolysis by proteins of glycoside hydrolase family 61: structure and function of a large, enigmatic family. *Biochem* **49**: 3305–3316.

Henriksson G, Johansson G & Pettersson G (2000) A critical review of cellobiose dehydrogenases. *J Biotechnol* **78**: 93-113.

Igarashi K, Tani T, Kawai R & Samejima M (2003) Family 3 beta-glucosidase from cellulose-degrading culture of the white-rot fungus *Phanerochaete chrysosporium* is a glucan 1,3-beta-glucosidase. *J Biosci Bioeng* **95**: 572-576.

Karkehabadi S, Hansson H, Kim S, Piens K, Mitchinson C & Sandgren M (2008) The first structure of a glycoside hydrolase family 61 member, Cel61B from *Hypocrea jecorina*, at 1.6 A resolution. *J Mol Biol* **383**: 144-154.

Ravalason H, Jan G, Mollé D*, et al.* (2008) Secretome analysis of *Phanerochaete chrysosporium* strain CIRM-BRFM41 grown on softwood. *Appl Microbiol Biot* **80**: 719-733.

Sato S, Liu F, Koc H & Tien M (2007) Expression analysis of extracellular proteins from *Phanerochaete chrysosporium* grown on different liquid and solid substrates. *Microbiol (Reading, Engl)* **153**: 3023-3033.

Suzuki H, Igarashi K & Samejima M (2010) Cellotriose and cellotetraose as inducers of the genes encoding cellobiohydrolases in the basidiomycete *Phanerochaete chrysosporium*. *Appl Environ Microbiol* **76**: 6164.

Vaaje-Kolstad G, Westereng B, Horn SJ, Liu Z, Zhai H, Sørlie M & Eijsink VGH (2010) An oxidative enzyme boosting the enzymatic conversion of recalcitrant polysaccharides. *Science* **330**: 219-222.

Vallim M, Janse B, Gaskell J, Pizzirani-Kleiner A & Cullen D (1998) *Phanerochaete chrysosporium* cellobiohydrolase and cellobiose dehydrogenase transcripts in wood. *Appl Environ Microbiol* **64**: 1924.

Vanden Wymelenberg A, Minges P, Sabat G*, et al.* (2006) Computational analysis of the *Phanerochaete chrysosporium* v2.0 genome database and mass spectrometry identification of peptides in ligninolytic cultures reveal complex mixtures of secreted

proteins. *Fungal Genet Biol* **43**: 343-356.
Wymelenberg AV, Gaskell J, Mozuch M, Kersten P, Sabat G, Martinez D & Cullen D (2009) Transcriptome and secretome analyses of *Phanerochaete chrysosporium* reveal complex patterns of gene expression. *Appl Environ Microbiol* **75**: 4058-4068.
Wymelenberg AV, Sabat G, Martinez D*, et al.* (2005) The *Phanerochaete chrysosporium* secretome: database predictions and initial mass spectrometry peptide identifications in cellulose-grown medium. *J Biotechnol* **118**: 17-34.
Yoshida M, Igarashi K, Kawai R, Aida K & Samejima M (2004) Differential transcription of beta-glucosidase and cellobiose dehydrogenase genes in cellulose degradation by the basidiomycete *Phanerochaete chrysosporium*. *FEMS Microbiol Lett* **235**: 177-182.

Chapter 4

Quantitave transcriptional analysis of the genes encoding cellulolytic enzymes of *P. chrysosporium* in the culture supplemented with xylooligosaccharides.

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Quantitave transcriptional analysis of the genes encoding cellulolytic enzymes of *P. chrysosporium* in the culture supplemented with xylooligosaccharides.

4.1 Introduction

In filamentous fungi, induction of polysaccharide-degrading enzymes with polymer is explained that soluble compounds transformed from polymer are true signal molecule as inducer. Moreover, it is known that extracellular enzymes to degrade polysaccharides are regulated at transcription level. Consequently, studies on gene expression of polysaccharide-degrading enzymes during growth on various soluble carbon sources related to polymer structure have been extensively investigated. In cellulose degradation of *P. chrysosporium*, it have been reported that cellotriose and cellotetraose induce transcripts of dominant cellulases, (Cel6A, Cel7C and Cel7D; the corresponding gene *cel6A, cel7C* and *cel7D*).

In chapter 2, it provided a hint that the effect of xylan on cellulolytic enzymes production could be caused by main chain but not side chains, suggesting that xylose or beta-1,4 xylosidic oligomer could directly affect production of cellulolytic enzymes at transcript level. In chapter 3, secretomic analysis elucidated that CDH were especially enhanced by the existence of xylan although CDH doesn't utilize xylose as a substrate. GH61 protein was also enhanced by xylan and it was reported to degrade cellulose chains with oxidative reaction. In this section, therefore, to elucidate the mechanism of these effects, we used reverse-transcription polymerase chain reaction (RT-PCR) to quantify transcript level of the gene encoding CDH and four GH61 isozymes (*cdh* and *gh61A-D*) as well as dominant

cellulases (*cel7C, cel7D* and *cel6A*). in cultures supplemented with xylose or xylooligosaccharides as well as glucose or cellooligosaccharides.

4.2 Materials and Methods

Preparation of xylooligosaccharides using gel-permeation chromatography.

10% (w/v) solution of unsubstituted xylooligosaccharides (Oji paper Co. Ltd., Tokyo, Japan) was filtered with syringe filter (MILLEX®-GV; Millipore, Billerica, MA) and then 10 mL sugar solution applied to the column system with two XK50 columns (GE Healthcare UK Ltd., Buckinghamshire, UK) in line. The first column is Toyopearl HW-50S (50 mm i. d., 1000 mm; Tosoh, Tokyo, Japan) and the second column is Toyopearl HW-40S (50 mm i. d., 800 mm; Tosoh). The xylooligosaccharides were eluted with milliQ water at a flow rate of 1.5 mL/min and fractionated into 9 mL. Total sugar amount of each fraction gained was roughly estimated by the orcinol-HCl method as previously described (Sun*, et al.*, 2002). The fractions containing sugar were applied to precoated silica gel 60 TLC plates (Merck, Darmstadt, Germany) and developed with EtOAc/CH_3COOH/water (3:2:1, by volume). Sugars were detected with orcinol reagent (1 % orcinol in 10 % H_2SO_4 dissolved in ethanol). These xylooligosaccharides separated into different DPs were collected respectively and concentrated using evaporator (TOKYO RIKAKIKAI Co. Ltd., Tokyo, Japan). The amount of xylooligosaccharides in the fluid concentrate was quantified by D-Xylose Assay Kit (Megazyme, Co. Wicklow, Ireland) after overnight incubation with commercial enzyme cocktail (Accellerase®DUET; Genencor, Jamsankoski, Finland).

Culture condition of the xylo/cello-oligosaccharides media for analysis of transcripts.

For gene transcript analysis under the cultivation with purified xylooligosaccharides, the spores of *P. chrysosporium* were inoculated in 80 ml of the medium containing 100 mM glucose (Wako Pure Chemical Industries Ltd., Osaka, Japan) as a carbon source under the same condition as described above. After 3-days precultivation, mycelium was collected,

washed with the medium containing no carbon source and transferred into the medium containing 20 mM glycerol (Wako) as a neutral carbon source. After 6-hours resting cultivation, 100 μM xylose (Wako), xylobiose, xylotriose or xylotetraose gained were added to the medium and the cultivation was continued. 5 ml aliquots were collected every hour and mycelia collected from the culture were immediately frozen in liquid nitrogen and sored at -80˚C to extract RNA. For gene transcript analysis under the cultivation with cellooligosaccharides, the resting-cultivated mycelia were also incubated in the medium supplemented 100 μM glucose (Wako), cellobiose, cellotriose, cellotetraose or cellopentaose (Seikagaku corporation, Tokyo, Japan) as described previously (Suzuki*, et al.*, 2010).

Primer design for specific amplification gh61 genes.

Among fifteen genes possibly encoding GH family 61 proteins in the genome, all four genes having the gene name of *gh61A, B, C* and *D* were selected as target genes. mRNA sequences of *gh61A* and *gh61D* (accession no. AY094489 (Wymelenberg*, et al.*, 2002) and AB670125 (Westereng, *et al*., 2011) respectively) and total genomic sequences of *gh61B and gh61C* (protein ID 121193 and 41123 respectively) were gotten from website (http://genome.jgi-psf.org/), showing 59-63 % identity each other. To confirm the sequences of *P. chrysosporium* strain K3, fragmental DNA was amplified using the primer sets of *gh61A, B and C* genes for RT-PCR as listed in Table 4-1. Those primers were designed in each region, which has no difference between the sequence gained from *P. chrysosporium* strain RP-78 and the transcriptome sequence of *P. chrysosporium* strain K3 in our laboratory. PCR was performed using KOD ver. 2.0 (Toyobo, Osaka, Japan) and the cDNA prepared from 2-day-old cellulose-grown mycelia as a template. The PCR reaction was as follows; 98˚C for 2 min and for 25 cycles, except 35 cycles for *gh61A,* of 98 ˚C for 10 s and 68˚C for 20 s. All PCR products were cloned in pCR4Blunt-TOPO using a Zero Blunt TOPO PCR cloning kit

(Invitrogen, Life Technology Japan Ltd., Tokyo, Japan) and *Escherichia coli* JM109 (Takara Bio Inc., Shiga, Japan), and sequenced with DNA sequencer CEQ 8800 according to manufacturer's instructions (Beckman Coulter, Inc., Brea, CA). And then the primer sets of *gh61A, B and C* for realtime RT-PCR, as listed in Table 4-1, were designed in the region of the obtained fragmental DNA sequence. For a primer set of *gh61D* for realtime RT-PCR, although it is not necessary to confirm the sequence because of this mRNA sequence from strain K3, pairwise sequence alignment with another gh61 gene (protein ID 4691) was submitted since this gene has partially high identities of *gh61D* and then in the low similar region, the primer set was designed as listed in Table 4-1. PCR was carried out using these primer sets under the same PCR condition for 25 cycles, except 35 cycles for *gh61A*. To verify the specificity, we checked no additionally amplified band with agarose gel electrophoresis and then the PCR products were cloned and sequenced, as described above.

Real-time RT-PCR analysis.

Frozen samples were broken into a fine powder using a Multi-Beads Shocker (Yasui Kikai, Osaka, Japan) and then total RNA were isolated from this sample using the RNAeasy Plant Mini Kit (Qiagen, Valencia, CA) according to manufacturer's protocol for plant cells and filamentous fungi with DNase digestion. Reverse transcription was performed using ReverTra Ace (Toyobo) and resulting first-strand cDNA were mixed with Brilliant Π Fast SYBR green QPCR master mix (Stratagene, La Jolla, CA) as described previously (Suzuki*, et al.*, 2010). Real-time PCRs were conducted by Mx3000P real-time QPCR system under the following condition; 95˚C for 2 min and for 40 cycles of 95˚C for 5 s and 60 ˚C for 20 s. After thermal cycling, PCR products were subjected to dissociation curve analysis to verify the specificity. All fluorescence data were analyzed using MxPro version 4.0 software (Stratagene). To quantify transcripts of cellulases and CDH (*cel7C, cel7D, cel6A* and *cdh*), we

used the primer sets as listed in the previous paper (Suzuki*, et al.*, 2008, Suzuki*, et al.*, 2009). For *gh61A-D*, the primer sets showing Table 4-1 were used. The transcript number of actin gene was used as an internal standard. The downstream and upstream primers for *actin* were 5'-GCATGTGCAAGGCTGGCTTTG-3' and 5'-AGGGCGACCAACGATGGATG-3'.

Table 4-1. Primer sets of the genes encoding GH61 isozymes

For RT-PCR			
	gh61A	F	CGCGTCGCTTGTGTTGCTC
		R	GCCTTGGCCATAGGTGTCAC
	gh61B	F	GATCGCCCTGCACTCCATG
		R	GGCCAGGGATGACATACGTC
	gh61C	F	TGACGGACGTGACGAGCAAG
		R	TTCGGGAGGGTGACGCTATG
For real-time RT-PCR			
	gh61A	F	CGTACCAGGCCGTCGATCC
		R	AGGTGTGTTTCCGCCAGGC
	gh61B	F	AGTGCGCGCAGATCCAGATC
		R	CGATGTTCAGGCCAGGGTC
	gh61C	F	ACGGGCGAGGACGCGATCT
		R	GTCGAACGACGATGCATCCG
	gh61D	F	CAGACCGCTACGGTTTCCG
		R	GGAGGCCGGGGACATCATGA

4.3 Results

Preparation of xylooligosaccharides.

The commercial mixture of unsubstituted xylooligosaccharides was fractionated by the tandem gel-permeation chromatography system. Chromatogram of total sugar concentration estimated by the orcinol-HCl method was shown in Fig. 4-1A. Degree of polymerization (DP) in each fraction exhibiting the peaks was determined by TLC analysis as shown in Fig. 4-1B. This result suggested that we could purify xylooligosaccharides up to DP=6 successfully. Among them, xylooligosaccharides with DP=2-4 were selected for further experiments since the number of single fraction was enough to yield.

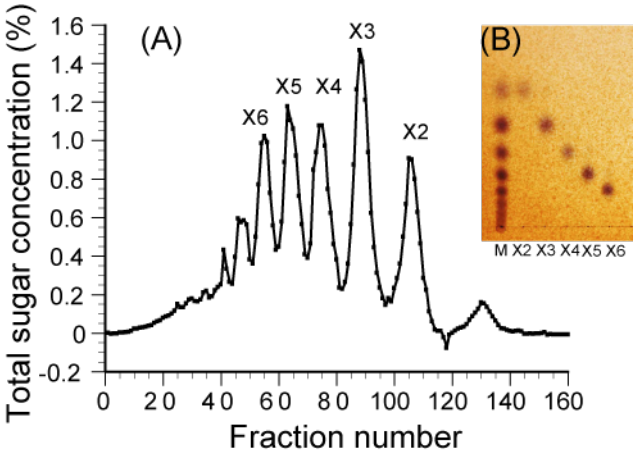

Fig. 4-1. Fractionation of xylooligosaccharides using gel-permeation chromatography. (A) Total sugar concentration of each fraction measured by the orcinol-HCl method as described in Materials and Methods. (B) Thin-layer chromatography of collected fractions. X2,xylobiose; X3, xylotriose; X4, xylotetraose; X5, xylopentaose; X6, xylohexaose; M, mixture of unsubstituted xylooligosaccharides used as a marker.

Transcript analysis of *cdh* gene.

P. chrysosporium was cultivated for 5 hours in the media containing xylose or xylooligosaccharides with DP=2-4 (X1, X2, X3 or X4) and transcript level of the genes encoding CDH was quantified by real-time RT-PCR. As showing in Fig. 4-2, transcript of *cdh,* increased in the existence of xylooligosaccharides (X2, X3 and X4) while transcript level was

not changed in the culture supplemented with xylose compared to control culture during cultivation. Transcripts of *cdh* reacted at almost the same value (7.0×10^2, 7.4×10^2 and 7.4×10^2) against X2 , X3 and X4 respectively. The transcripts showed the increase in 1 hour and after that apparent increases of the transcripts were hardly observed. On the other hand, *P. chrysosporium* was cultivated for 6 hours in the media containing glucose or cellooligosaccharides with DP=2-5 (C2, C3, C4 and C5) and transcript level of *cdh* was also quantified by real-time RT-PCR. As showing Fig. 4-3, when adding C3 and C4 to culture, the transcripts increased clearly at maximum level (1.0×10^4 and 1.6×10^4 respectively) in 2 hour and then decreased gradually. In the C2 culture, transcript level was raised slightly in 2 hour. Also C5 raised slightly transcript level but this reaction was more slowly than other substrates, suggesting that hydrolysis products might affect. The maximum transcript levels in cellooligosaccharides cultures showed 50-fold larger amount than that in xylooligosaccharides cultures.

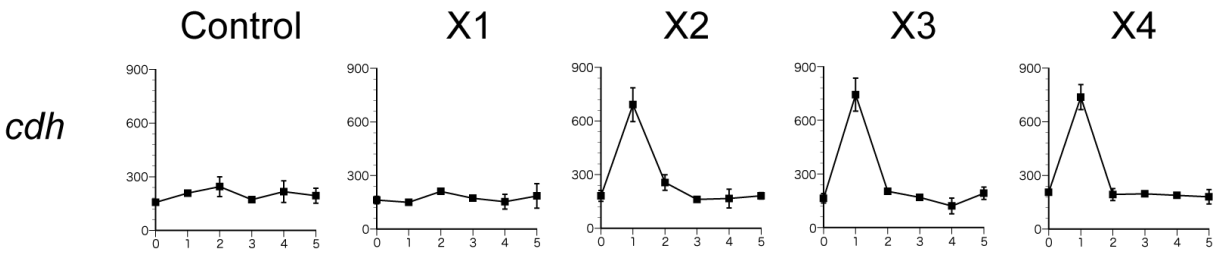

Fig. 4-2. Time course of *cdh* transcripts of *P. chrysosporium* grown in the culture supplemented with xylose (X1), xylobiose (X2), xylotriose (X3), xylotetraose (X4) and no addition (Control) during five hours. Error bars shows standard deviations from three tests.

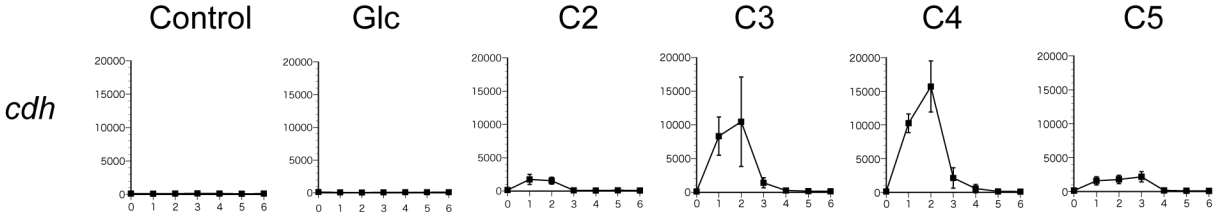

Fig. 4-3. Time course of *cdh* transcripts of *P. chrysosporium* grown in the culture supplemented with glucose (Glc), cellobiose (C2), cellotriose (C3), cellotetraose (C4), cellopentaose (C5) and no addition (Control) during six hours. Error bars shows standard deviation from three tests. All data is referred to the previous report (2011 Suzuki et al)

Transcript analysis of the genes encoding GH61 isozymes (*gh61A, gh61B, gh61C* and *gh61D*)

Transcripts of the genes encoding four GH61 isozymes (GH61A, B, C and D) were also investigated under the same condition as xylose or xylooligosaccharides (Fig. 4-4). All the transcript levels were also not changed in the culture supplemented with xylose although each gene reacted to xylooligosaccharides in quite different manner. Transcripts of *gh61C* increased in the existence of xylooligosaccharides while xylooligosaccharides slightly affected *gh61B* and didn't affect transcripts of *gh61A* and *gh61D* during cultivation. Transcripts of and *gh61B* increased in response to X2, X3 or X4 while *gh61C* respond to only X4, suggesting that the expression pattern of *gh61B* is similar to that of *cdh*. Transcripts of *gh61B* reached at maximum values in X2, X3 or X4 culture (1.8×10^2, 2.0×10^2 or 1.4×10^2 respectively) while *gh61C* reached at miximum value (2.4×10^3) in X4 culture. All the transcripts also exhibited the apparent responses in 1 hour. In the case of glucose or cellooligosaccharides (Fig. 4-5), all the transcripts strongly increased in C3 and C4 cultures in 2 hour and then decreased gradually in the same way as other cellulolytic enzymes. In the case of *gh61B*, transcript level raised slightly against C2 and C5 as well as *cdh*. Among the genes, maximum transcript level of *gh61C* was the highest (8.4×10^4), and subsequently that of *gh61B* followed (2.6×10^4), whereas in case of *gh61A* and *gh61D* maximum transcript levels were quite low (3.2×10^3 and 1.8×10^3 respectively). All the maximum transcript amounts of those genes during cultivation were remarkably larger than those in xylooligosaccharides.

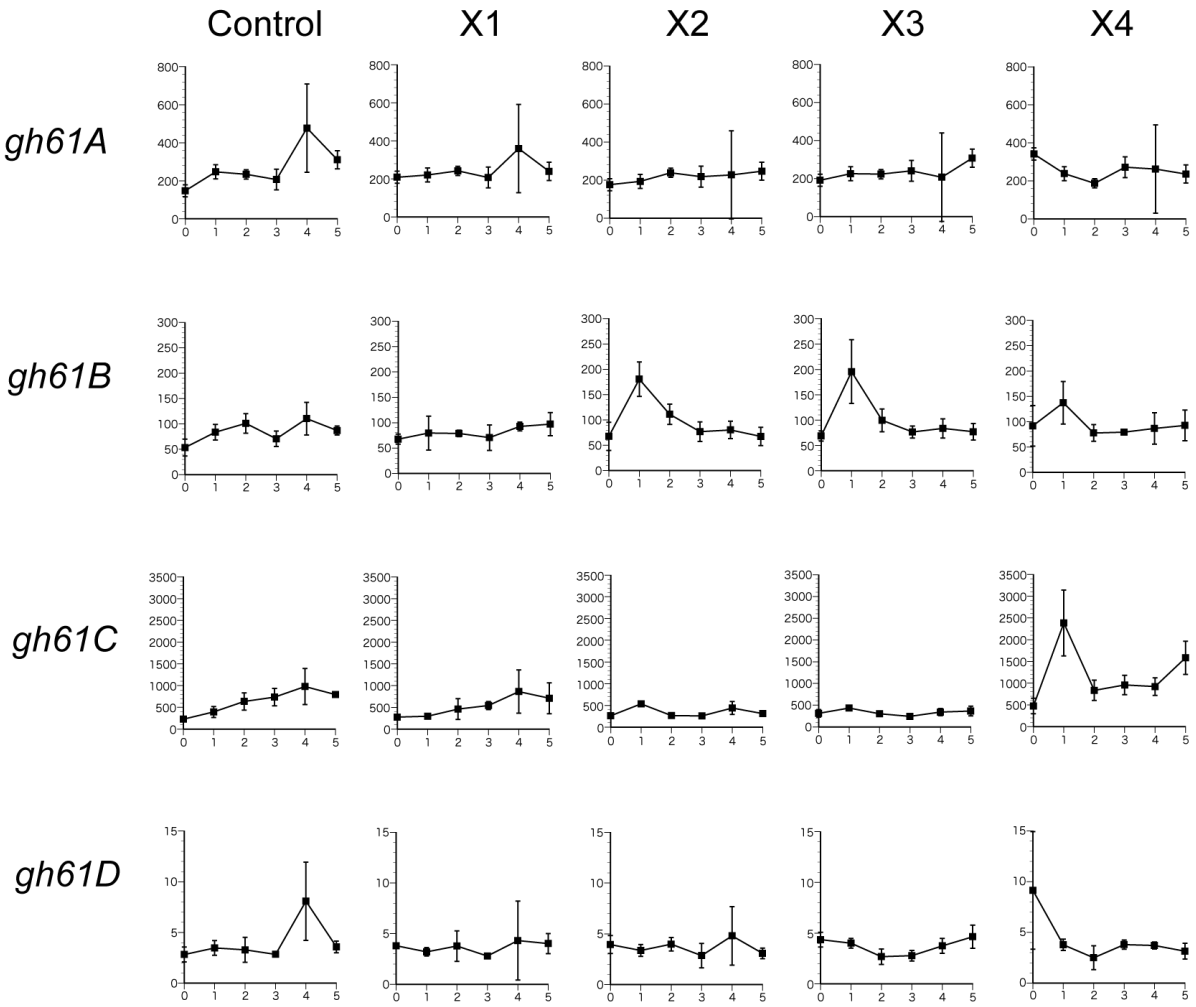

Fig. 4-4. Time course of *gh61A*, *gh61B*, *gh61C* and *gh61D* transcripts of *P. chrysosporium* grown in the culture supplemented with xylose (X1), xylobiose (X2), xylotriose (X3), xylotetraose (X4) and no addition (Control) during five hours. Error bars shows standard deviations from three tests.

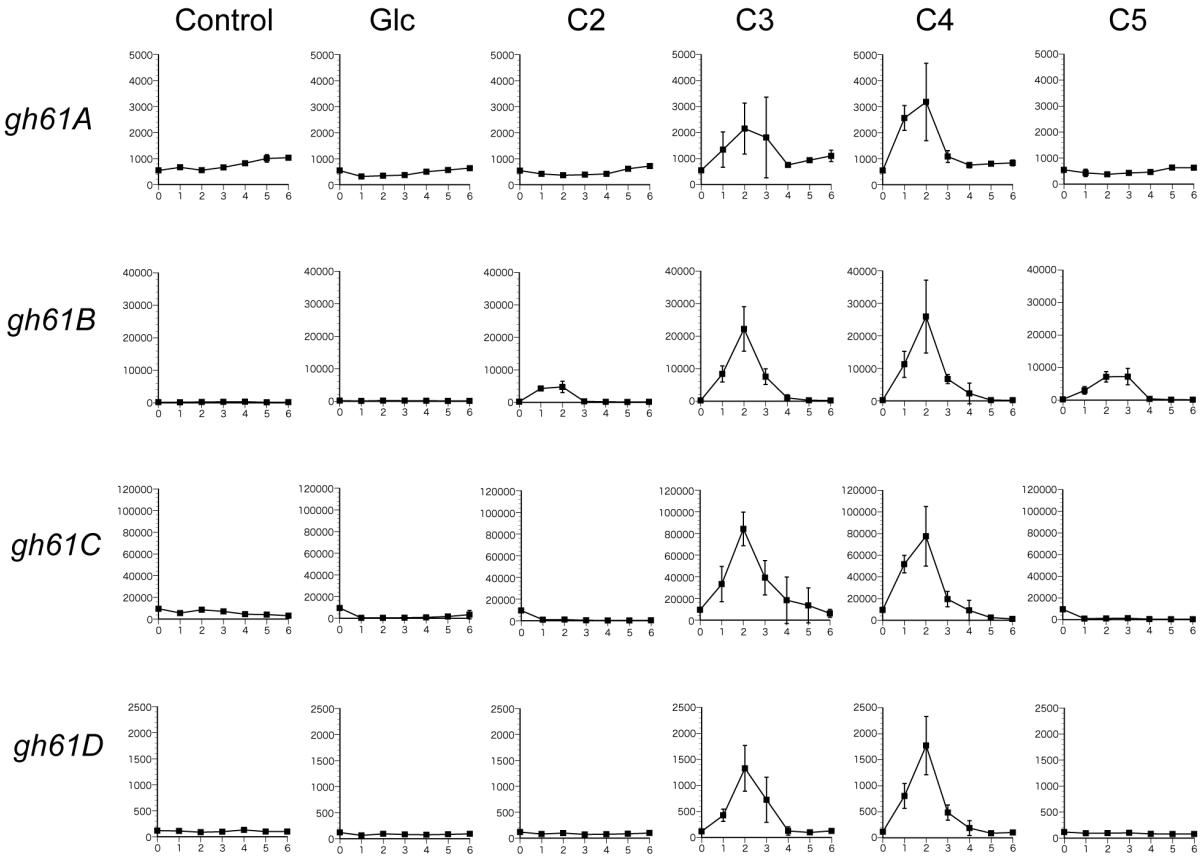

Fig. 4-5. Time course of *gh61A*, *gh61B*, *gh61C* and *gh61D* transcripts of *P. chrysosporium* grown in the culture supplemented with glucose (Glc), cellobiose (C2), cellotriose (C3), cellotetraose (C4), cellopentaose (C5) and no addition (Control) during six hours. Error bars shows standard deviation from three tests.

Transcript analysis of the genes encoding main cellulases (*cel6A, cel7C* and *cel7D*).

Transcript of the genes encoding some cellulases secreted mainly in the cellulose-degrading culture (*cel6A, cel7C* and *cel7D*) were also quantified by real-time RT-PCR in the cultures containing xylose or xylooligosaccharides. As showing in Fig. 4-6, each expression pattern is quite different among those genes. Although transcript of *cel6A* was not affected so much during cultivation, that of *cel7C* and *cel7D* increased in the existence of xylooligosaccharides and among them *cel7C* showed the best up-regulation. Transcripts of *cel7D* reacted at almost the same value (1.2×10^4, $1.2 \times 10^4$and 1.5×10^4) against X2 , X3 and X4 respectively, while *cel7C* reacted at relative high value (3.3×10^4 and 3.7×10^4) against X2 and X3 respectively and at relative low value (8.4×10^3) against X4. All the transcript levels didn't also changed in the presence of xylose, compared to control culture as the case of *cdh*. In addition, all the transcripts showed the apparent increase in 1 hour as well as *cdh*. On the other hand, when transcripts of *cel6A, cel7C* and *cel7D* were measured in the media supplemented with glucose and cellooligosaccharides with DP=2-5 (C2, C3, C4 and C5) in our previous report (Suzuki*, et al.*, 2010), all the transcripts were significantly up-regulated by C3 and C4, as showing in Fig. 4-7. Among them, only *cel7D* responded to C3 the best, whereas *cel6A* and *cel7C* responded to C4 the best as well as *cdh*. And only *cel7C* slightly responded to C2 and C5 in the same manner as *cdh*. The maximum transcript levels of *cel6A, cel7C* and *cel7D* in cellooligosaccharides cultures showed remarkably large amount compared to that in xylooligosaccharides cultures, too.

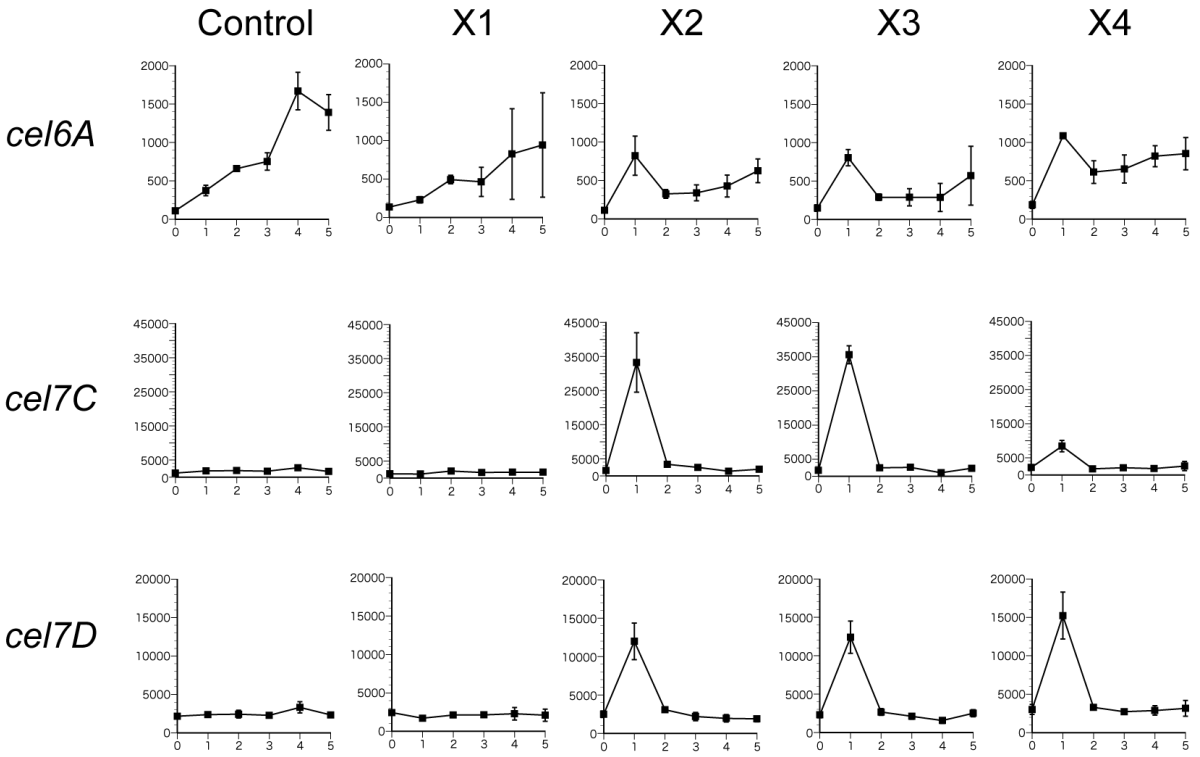

Fig. 4-6. Time course of *cel6A*, *cel7C* and *cel7D* transcripts of *P. chrysosporium* grown in the culture supplemented with xylose (X1), xylobiose (X2), xylotriose (X3), xylotetraose (X4) and no addition (Control) during five hours. Error bars shows standard deviations from three tests.

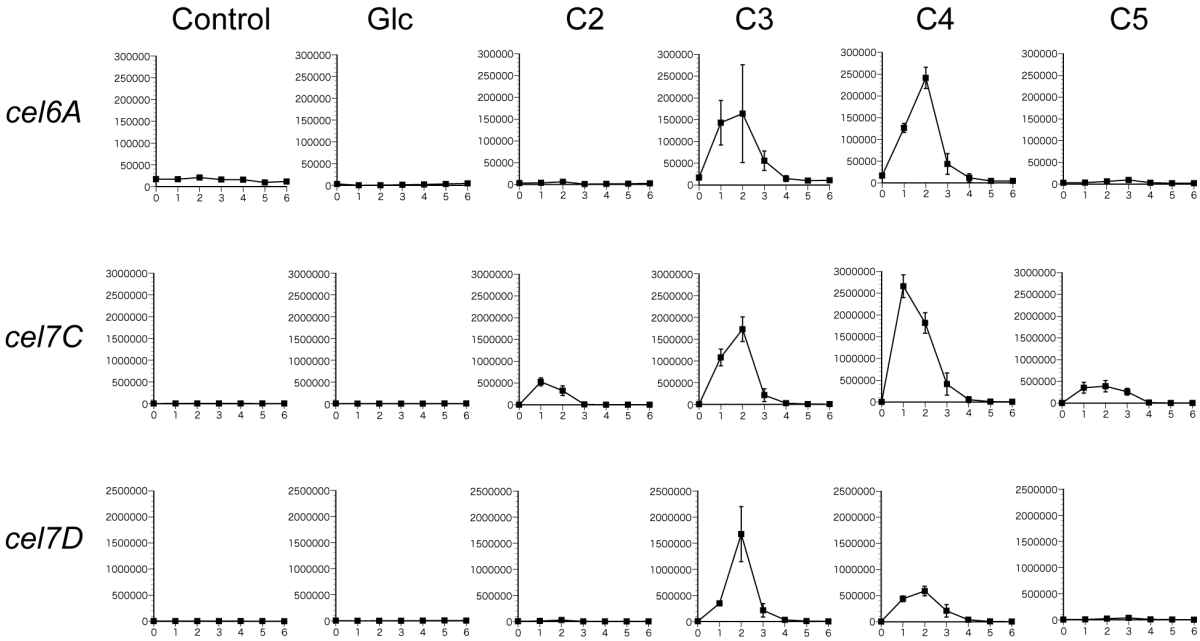

Fig. 4-7. Time course of *cel6A*, *cel7C* and *cel7D* transcripts of *P. chrysosporium* grown in the culture supplemented with glucose (Glc), cellobiose (C2), cellotriose (C3), cellotetraose (C4), cellopentaose (C5) and no addition (Control) during six hours. Error bars shows standard deviation from three tests. All data is referred to the previous report (2011 Suzuki et al)

4.4 Discussion

Our secretome analysis provided a clue about xylan to be associated with cellulolytic enzymes production, especially CDH, in Chapter 3. In addition, in Chapter 2, xylans with different side-chains similarly enhanced initial fungal growth, extracellular protein production and CDH activities, suggesting that those effects could be derived from main chain consisting of beta-1,4 xyloside residue rather than side chain. Accordingly, I purified unsubstituted xylooligosaccharides (DP=2-4) using the tandem gel-permeation chromatography system (Fig. 4-1), and then quantified and compared transcript pattern of the genes encoding CDH in the media supplemented with xylose or gained xylooligosaccharides as well as glucose and cellooligosaccharides (Fig. 4-2 and 4-3). As a result, *cdh* gene expression is induced more strongly by cellooligosaccharides (cellotriose and cellotetraose) than by xylooligosaccharides (xylobiose, xylotriose and xylotetraose). I previously found that production of glycoside hydrolase (GH) family 10 xylanase was enhanced in xylan-containing culture in Chapter 3, so I speculated that xylooligosaccharides produced by the xylanase promote CDH production in the culture. However, the results of the present study indicate that up-regulation of *cdh* transcription by short and unsubstituted xylooligosaccharides is not sufficient to account directly for the enhanced production of secreted protein. To reveal the target affected by xylooligosaccharides, I next focused on transcripts of the genes encoding GH family 61 proteins, which were also promoted by the existence of xylan. Among four GH61 isozymes, *gh61B*, *gh61C* were increased by xylooligosaccharides although all the transcriptional levels in xylooligosacchrides were much smaller than that in cellooligosaccharides as well as *cdh*. Therefore, I next examined the effect of xylooligosaccharides on the expression of cellobiohydrolase (*cel*) genes, *cel6A*, *cel7C* and *cel7D,* since the corresponding proteins are the dominant cellulases in medium containing cellulose. *cel6A*, *cel7C* and *cel7D* transcripts were all increased by xylooligosaccharides; in particular, *cel7C* transcripts were increased up

to 20-fold by xylobiose and xylotriose. Maximum transcription of *cel7C* was up-regulated by xylooligosaccharides at sufficient level, compared to those under repression and derepression condition in the previous reports (2008 Suzuki, 2009 Suzuki). These results indicated the induction of cellulolytic enzymes by xylan in cellulose-degrading culture is not simply due to xylooligosaccharides, but is more complex, involving induction by cellotriose and cellotetraose. Moreover, it is presumed that xylooligosaccharides enhance transcription of some cellulase gene (*cel7C*) and in turn its enzymatic products, cellooligosaccharides, induce cellulolytic genes predominantly. Indeed, in other fungi, the induction cascade is proposed that after xylose induction of some cellulases, the generating products such as cellobiose specifically induced cellulolytic genes (Hrmova*, et al.*, 1989, Royer & Nakas, 1990, de Vries & Visser, 2001).

Quite recently, GH61 protein is reported to act as a possible electron donor of CDH and those oxidative enzymes reacted synergistically for not crystalline region but amorphous region (Langston*, et al.*, 2011), which located in the cellulose surface adhesively connecting with xylan in nature. This finding is consistent with the enhancement of CDH and GH61 protein production in the existence of xylan in Chapter 2. In the present study, *gh61C* responded clearly to xylooligosaccharides although the expression pattern of *gh61B* is quite similar to *cdh* in both cellooligosaccharides and xylooligosaccharides cultures at same level. This various responses to different substrates in GH61 family are consistent with the previous reports (Wymelenberg*, et al.*, 2005, Vanden Wymelenberg*, et al.*, 2006, Wymelenberg*, et al.*, 2009). These results suggested that GH61 isozymes might have different functions, especially GH61B is one candidate for electron donor of CDH. The enzymatic associations of those GH61 proteins with CDH are of great concern. On the other hand, expression pattern of *cel7C* is similar to *cdh* in both cellooligosaccharides and xylooligosaccharides cultures, indicating that Cel7C plays as the producer of celloologosaccharide, which act as electron acceptors of

CDH.

Although there is no information about regulatory machinery in *P. chrysosporium*, some transcriptional factors were reported in some ascomycetes (Aro*, et al.*, 2005). In *Aspergillus spp*., the most famous transcriptional activator XlnR regulates some cellulolytic genes, but not all, with some xylanolytic genes via the inducer xylose (Gielkens*, et al.*, 1999, Hasper*, et al.*, 2000). In the present study, xylose didn't completely induced cellulolytic genes in this fungus, suggesting that this phenomenon was conducted under a control system unlike XlnR-mediated induction. Moreover, some reports indicated that not only xylose but also xylobiose and/or xylotriose are inducing compound independent on xylose for the plant polysaccharides-degrading enzymes in several filamentous fungi (Royer & Nakas, 1990, Zeilinger*, et al.*, 1996, Van Peij*, et al.*, 1997, Würleitner*, et al.*, 2003), suggesting that the case of *P. chrysosporium* may occur commonly in filamentous fungi.

In conclusion, this research revealed that transcription of some cellulase genes might be enhanced in the existence of xylooligosaccharides under a control system, which is unlike XlnR-mediated induction but possibly common in filamentous fungi. Moreover, cellooligosaccharides strongly induced the genes encoding oxidative enzymes, CDH and GH61 proteins, as well as cellulase whereas xylooligosaccharides only induced *cel7C* at sufficient level. These results suggested that xylooligosaccharides might enhance transcription of some cellulase gene (*cel7C*) and in turn its enzymatic products, cellooligosaccharides, induce cellulolytic genes predominantly. In addition, expression pattern of *cdh* was similar to *cel7C* and *gh61B* in both cellooligosaccharides and xylooligosaccharides culture, indicating that Cel7C and GH61B cooperate with CDH in degradation of plant cell wall. In this cooperation, Cel7C might act as the producer of cellooligosacchrides, which are an electron acceptor of CDH, and GH61B is one candidate for electron donor of CDH.

References

Aro N, Pakula T & Penttila M (2005) Transcriptional regulation of plant cell wall degradation by filamentous fungi. *FEMS Microbiol Rev* **29**: 719-739.

Carle-Urioste JC, Escobar-Vera J, El-Gogary S*, et al.* (1997) Cellulase induction in *Trichoderma reesei* by cellulose requires its own basal expression. *J Biol Chem* **272**: 10169-10174.

de Vries RP & Visser J (2001) *Aspergillus* enzymes involved in degradation of plant cell wall polysaccharides. *Microbiol Mol Biol Rev* **65**: 497-522, table of contents.

El-Gogary S, Leite A, Crivellaro O, Eveleigh DE & el-Dorry H (1989) Mechanism by which cellulose triggers cellobiohydrolase I gene expression in *Trichoderma reesei*. *P Natl Acad Sci Usa* **86**: 6138-6141.

Gielkens MM, Dekkers E, Visser J & de Graaff LH (1999) Two cellobiohydrolase-encoding genes from *Aspergillus niger* require D-xylose and the xylanolytic transcriptional activator XlnR for their expression. *Appl Environ Microbiol* **65**: 4340-4345.

Hasper AA, Visser J & de Graaff LH (2000) The Aspergillus niger transcriptional activator XlnR, which is involved in the degradation of the polysaccharides xylan and cellulose, also regulates D-xylose reductase gene expression. *Mol Microbiol* **36**: 193-200.

Hrmova M, Biely P & Vranskd M (1989) Cellulose- and xylan-degrading enzymes of *Aspergillus terreus* and *Aspergillus niger*. *Enzyme Microb. Technol.* **11**.

Langston J, Shaghasi T, Abbate E, Xu F, Vlasenko E & Sweeney M (2011) Oxidoreductive cellulose depolymerization by the enzymes cellobiose dehydrogenase and glycoside hydrolase 61. *Appl Environ Microbiol* **77**: 7007.

Royer JC & Nakas J (1990) Interrelationship of xylanase induction and cellulase induction of *Trichoderma longibrachiatum*. *Appl Environ Microbiol* **56**: 2535.

Seiboth B, Messner R, Gruber F & Kubicek CP (1992) Disruption of the *Trichoderma reesei* cbh2 gene coding for cellobiohydrolase II leads to a delay in the triggering of cellulase formation by cellulose. *Microbiol* **138**: 1259-1264.

Seiboth B, Hakola S, Mach RL, Suominen PL & Kubicek CP (1997) Role of four major cellulases in triggering of cellulase gene expression by cellulose in *Trichoderma reesei. J Bacteriol* **179**: 5318-5320.

Sun HJ, Yoshida S, Park NH & Kusakabe I (2002) Preparation of 1,4-beta-D-xylooligosaccharides from an acid hydrolysate of cotton-seed xylan: suitability of cotton-seed xylan as a starting material for the preparation of 1,4-beta-D-xylooligosaccharides. *Carbohydr Res* **337**: 657-661.

Suzuki H, Igarashi K & Samejima M (2008) Real-time quantitative analysis of carbon catabolite derepression of cellulolytic genes expressed in the basidiomycete *Phanerochaete chrysosporium*. *Appl Microbiol Biot* **80**: 99-106.

Suzuki H, Igarashi K & Samejima M (2009) Quantitative transcriptional analysis of the genes encoding glycoside hydrolase family 7 cellulase isozymes in the basidiomycete *Phanerochaete chrysosporium*. *FEMS Microbiol Lett* **299**: 159-165.

Suzuki H, Igarashi K & Samejima M (2010) Cellotriose and cellotetraose as inducers of the genes encoding cellobiohydrolases in the basidiomycete *Phanerochaete chrysosporium*. *Appl Environ Microbiol* **76**: 6164.

Tempelaars CA, Birch PR, Sims PF & Broda P (1994) Isolation, characterization, and analysis of the expression of the cbhII gene of *Phanerochaete chrysosporium*. *Appl Environ Microbiol* **60**: 4387-4393.

Vallim MA, Janse BJ, Gaskell J, Pizzirani-Kleiner AA & Cullen D (1998) *Phanerochaete chrysosporium* cellobiohydrolase and cellobiose dehydrogenase transcripts in wood. *Appl*

Environ Microbiol **64**: 1924-1928.

Van Peij NN, Brinkmann J, Vrsanská M, Visser J & de Graaff LH (1997) beta-Xylosidase activity, encoded by xlnD, is essential for complete hydrolysis of xylan by *Aspergillus niger* but not for induction of the xylanolytic enzyme spectrum. *Eur J Biochem* **245**: 164-173.

Vanden Wymelenberg A, Minges P, Sabat G*, et al.* (2006) Computational analysis of the *Phanerochaete chrysosporium* v2.0 genome database and mass spectrometry identification of peptides in ligninolytic cultures reveal complex mixtures of secreted proteins. *Fungal Genet Biol* **43**: 343-356.

Westereng, B., T. Ishida, G. Vaaje-Kolstad, M. Wu, V. G. H. Eijsink, K. Igarashi, M. Samejima, J. Ståhlberg, S. J. Horn, and M. Sandgren. (2011) The putative endoglucanase PcGH61D from *Phanerochaete chrysosporium* is a metal-dependent oxidative enzyme that cleaves cellulose. PLoS ONE **6** e27807.

Würleitner E, Pera L, Wacenovsky C, Cziferszky A, Zeilinger S, Kubicek CP & Mach RL (2003) Transcriptional regulation of xyn2 in *Hypocrea jecorina*. *Eukaryotic Cell* **2**: 150-158.

Wymelenberg AV, Gaskell J, Mozuch M, Kersten P, Sabat G, Martinez D & Cullen D (2009) Transcriptome and secretome analyses of *Phanerochaete chrysosporium* reveal complex patterns of gene expression. *Appl Environ Microbiol* **75**: 4058-4068.

Wymelenberg AV, Sabat G, Martinez D*, et al.* (2005) The *Phanerochaete chrysosporium* secretome: database predictions and initial mass spectrometry peptide identifications in cellulose-grown medium. *J Biotechnol* **118**: 17-34.

Wymelenberg AV, Denman S, Dietrich D*, et al.* (2002) Transcript analysis of genes encoding a family 61 endoglucanase and a putative membrane-anchored family 9 glycosyl hydrolase from *Phanerochaete chrysosporium*. *Appl Environ Microbiol* **68**: 5765-5768.

Zeilinger S, Mach RL, Schindler M, Herzog P & Kubicek CP (1996) Different inducibility of expression of the two xylanase genes xyn1 and xyn2 in *Trichoderma reesei*. *J Biol Chem* **271**: 25624-25629.

Chapter 5

Conclusion

Chapter 5

Conclusion

In chapter 2, to get an initial clue, we examined the effects of xylan on fungal growth and extracellular proteins production of *P. chrysosporium.* In addition, enzymatic activities of culture filtrates were measured. We presented the evidence that addition of xylan promoted initial fungal growth and extracellular protein production of *P. chrysosporium* in the cellulose-degrading culture. Also, xylan enhanced cellulase activities including cellobiose dehydrogenase (CDH) activities as well as xylanase activities. Moreover, xylans with different side-chains similarly enhanced initial fungal growth, extracellular protein production and production of CDH. These results suggested that those effects could be caused by main chain not side chain.

In chapter 3, to reveal the effects of xylan on cellulolytic enzymes production by *P. chrysosporium* at protein component level, we conducted secretomic analysis of *P. chrysosporium* grown in cellulose-degrading culture against the addition of xylan in chapter 2 using its genome information, two-dimensional electrophoresis and liquid chromatography-tandem mass spectrometry. As a result, in cellulolytic culture of the basidiomycete *Phanerochaete chrysosporium*, the addition of xylan increases production of Xyn10C and a putative glucuronoyl esterase belonging to CE family 15, which may act in degradation of the main chain and side chain of xylan, respectively. Moreover, production of CDH and GH family 61 proteins, the potential oxidative enzymes accelerating enzymatic conversion of cellulose, is also increased in the presence of xylan. CDH oxidize cellooligosaccharides, which produced by cellulases, but natural proton donor of CDH has remained uncertain. Recently, oxidative reaction involved in CDH and GH61 protein is

demonstrated to be important in cellulose degradation and in the participation, GH61 protein might be a proton donor of CDH.

In chapter 4, to reveal the effect of xylooligosaccharides from main chain on expression patterns of cellulolytic gene, especially encoding CDH and the related enzymes, *P. chrysosporium* was cultivated for 6 hours in the media containing xylose or gained xylooligosaccharides with DP=2-4 (X1, X2, X3 or X4) and transcript levels of the gene encoding CDH, GH61 isozymes and dominant cellulses (Cel6A, Cel7C and Cel7D; the corresponding gene *cel6A, cel7C* and *cel7D*) were quantified by real-time RT-PCR. In addition, under glucose and cellooligosaccharides-supplemented condition the same analysis was also investigated. As a result, transcription of some cellulase gene (*cel7C*) might be enhanced selectively in the existence of xylooligosaccharides under the control system unlike the famous XlnR-mediated induction in filamentous fungi. Meanwhile, cellooligosaccharides strongly induced all the cellulolytic genes including CDH and GH61 proteins rather than xylooligosaccharides.

These results suggested that xylan is degraded into xylooligosaccharides firstly (Fig. 5-1A) and they enhance transcription of some cellulase gene weekly (Fig. 5-1B) and in turn its enzymatic products (Fig. 5-1C), cellooligosaccharides, induce cellulolytic genes predominantly (Fig. 5-1D). In addition, expression pattern of *cdh* was similar to *cel7C* and *gh61B* in both cellooligosaccharides and xylooligosaccharides culture, suggesting that their corresponding proteins, Cel7C and GH61B, cooperate with CDH in degradation of the polysaccharides in plant cell wall, as shown in Fig. 5-2. In this cooperation, Cel7C might act as the producer of cellooligosacchrides, which are an electron acceptor of CDH, and GH61B is one candidate for electron donor of CDH. In addition, multiplicity of GH61 proteins raised the possibility of further function attacking on various polysaccharides in plant cell wall and

in that degradation of plant polysaccharides CDH might play a crucial role to supply electron to GH61 proteins.

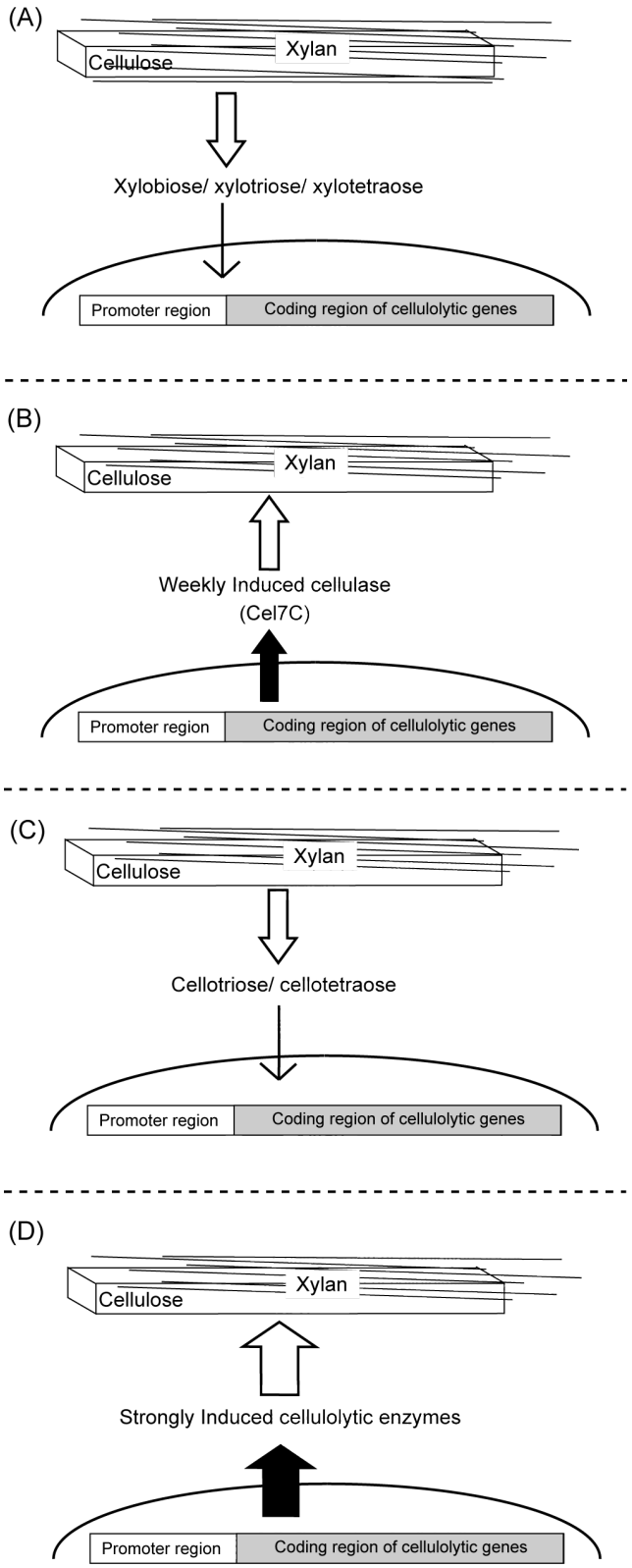

Fig. 5-1. Predicted induction cascades of cellulolytic enzymes production in *P. chrysosporium*

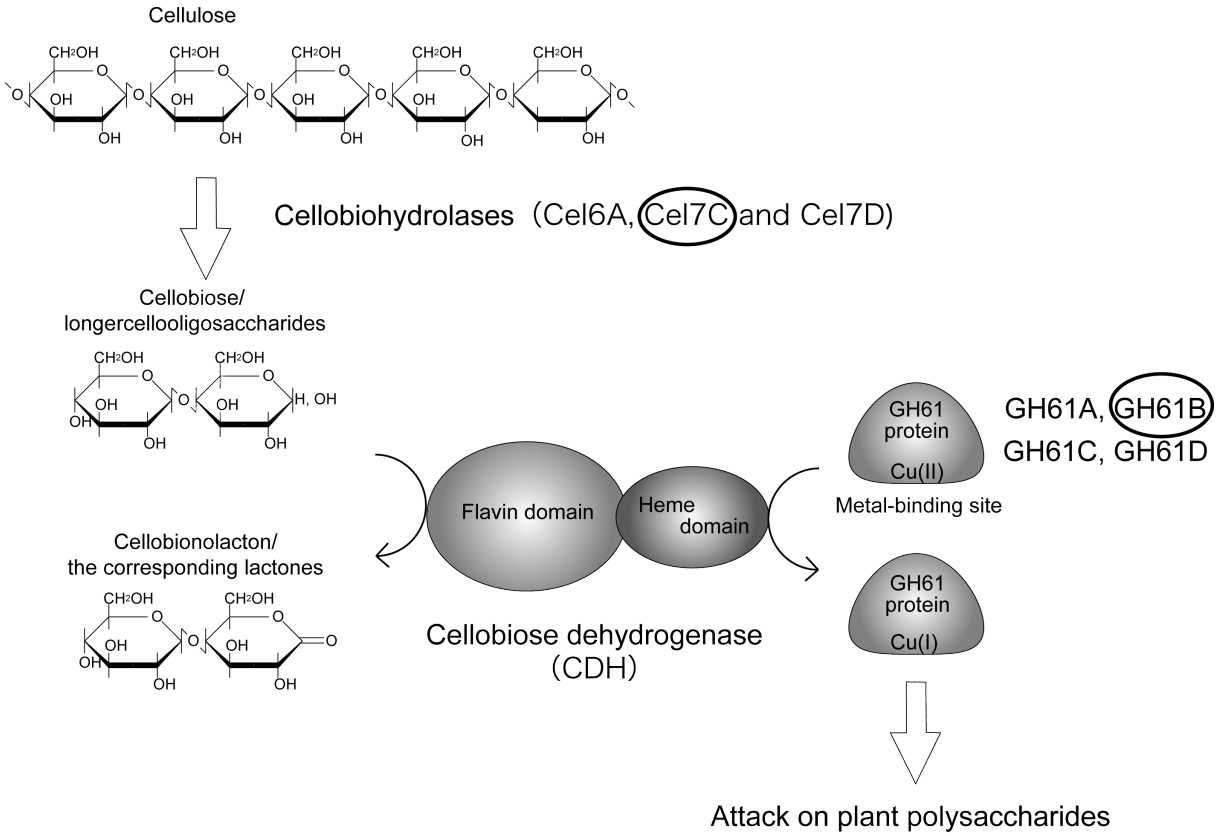

Fig. 5-2. Predicted extracellular redox system for degradation of plant polysaccharide in *P. chrysosporium*.

In conclusion, the induction of cellulolytic enzymes by xylan in cellulose-degrading culture is not simply due to xylooligosaccharides, but is more complex, involving induction by cellotriose and cellotetraose. One possible interpretation is that xylooligosaccharides promote the production of cellobiohydrolases, which produce cellooligosaccharides that in turn enhance the expression of other cellulolytic genes, including *cdh* and *gh61s* as shown in Fig. 5-1. As a result, degradation of cellulose was enhanced and thus the fungus grows faster in the existence of xylan. These results might be reflected by the circumstance that cellulose is adhesively surrounded with xylan in nature and xylan might be a signal to exhibit the presence of cellulose as preferable nutrient for this fungus.

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List of publications

1. Hori, C., Igarashi, K., Katayama, A., Samejima, M. Effects of xylan and starch on secretome of the basidiomycete *Phanerochaete chrysosporium* grown on cellulose.
   FEMS Microbiology Letters **321**(1)14-23 (2011)

2. Hori, C., Suzuki, H., Igarashi, K., Samejima, M. Transcriptional response of cellobiose dehydrogenase gene to cello- and xylooligosaccharides in the basidiomycete *Phanerochaete chrysosporium*.
   Applied and Environmental Microbiology [in press]

3. Igarashi, K., Ishida, T., Hori, C., Samejima, M. Characterization of endoglucanase belonging to new subfamily of glycoside hydrolase family 45 from the basidiomycete *Phanerochaete chrysosporium.*
   Applied and Environmental Microbiology **74** 5628-5634 (2008)

4. Ishiguro, M., Hori, C., Katayama, A., Igarashi, K., Takabatake, K., Kaneko, S., Samejima, M. Secretome analysis using transcriptomic sequence database of *Flammulina velutipes.*
   Mokuzaigakkaishi **56** 388-396 (2010)

5. Higashijima, K., Hori, C., Igarashi, K., Enomae, T., Isogai, A. First-aid for flood-damaged paper using saltwater -saltwater effect on inhibiting mold growth.
   Studies in conservation [in press]