Chapter 4
The Saccharification Step: *Trichoderma Reesei* Cellulase Hyper Producer Strains

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Abstract One of the major applications of cellulases is to produce fermentable sugars from lignocellulosic biomass for biofuels production. The filamentous fungus *Trichoderma reesei* is known to be hyper producer of cellulases and hemicellulases and it is widely used for commercial scale production of these enzymes using novel fermentation techniques. Some of the *T. reesei* industrial strains produce over 100 g/l of cellulases. However, there are still technical and economic constraints to the development of cheap commercial cellulase production process. Here, we bring together and discuss the results on *T. reesei* as cellulase producer, the different kinds of enzymes it expresses, recent genomic, genetic, and metabolic engineering approaches that have helped to improve the biomass degrading enzyme mixture and the strategies adopted to reduce the cost of enzymes during fermentation process. Current efforts and some future perspectives for reducing the cost of enzymes by using cheaper substrates, recycling enzyme during the hydrolysis and fermentation process, and on-site enzyme production in the biorefinery facility are also discussed.

Contents

4.1 Introduction 66
4.2 Cellulase and Hemicellulase Genes in *Trichoderma reesei* 68
4.2.1 *T. reesei* Genome: General Characteristics 68
4.2.2 *T. reesei* Genome Encoded Cellulases and Hemicellulases 71
4.3 Random Mutagenesis of *T. reesei*—Cellulase Hyper-Producer Strains 73
4.4 Metabolic Engineering and Protein Engineering 74
4.4.1 Transcriptional Regulators 74
4.4.2 Rational Design and Direct Evolution of Individual Cellulase/Hemicellulase 76

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4.5 Fermentation Technologies for Enzyme Production 77
4.5.1 Nutrients for *T. reesei* Growth and Enzyme Production 77
4.5.2 Inducers for Cellulase and Hemicellulase Production 78
4.5.3 Fermentation Conditions 79
4.5.4 Morphology 79
4.6 Current Efforts and Future Perspective of Reducing the Cost of Enzymes 80
4.6.1 Enzyme Production Using Lignocellulosic Biomass 80
4.6.2 Improvement of the Fermentation Process 82
4.6.3 Fast Downstream Processing for Maximum Product Recovery 83
4.6.4 Recycling Enzymes During Bioconversion 83
4.6.5 Other Approaches to Reduce the Cost of Enzyme Production 83
References 84

4.1 Introduction

Import of crude oil in United States (US) and European Union (EU) alone constituted 60 and 80 %, respectively, in 2009. Other developing countries like India and China are completely dependent on foreign oil to meet their liquid transportation fuel demand. In recent years, an increase in the cost of crude oil has initiated extensive research and development to produce large-scale alternate liquid transportation fuels from renewable resources. In the US, the Department of Energy (DOE) has set a goal of producing 60 billion gallons of renewable fuels per year by 2030. In the EU there is a mandate to produce 25 % of its transportation fuel using renewable resources by 2030 (Himmel et al. 2007). Production of ethanol from sugarcane juice (Brazil) and corn starch (US) has almost reached its full capacity and both methods are often criticized for using feedstock for food to make biofuels. The only sustainable alternative substrate for making ethanol is lignocellulosic biomass. The primary sources of lignocellulosic biomass include agricultural wastes (corn stover, sorghum, sugarcane bagasse, rice straw, wheat straw, empty fruit bunch from oil palm and date palm, Agave bagasse from tequila industry), Perennial grasses (switchgrass, miscanthus), woody biomass, and municipal solid waste (Gomez et al. 2008).

A biorefinery is a facility that produces fuels and chemicals from lignocellulosic biomass using a combination of process technologies (Fig. 4.1). The sugar polymers (cellulose and hemicellulose) present in biomass are depolymerized by efficiently degrading glycosidic bonds using microbial enzymes followed by microbial fermentation of sugars to fuels and chemicals (Menon and Rao 2012). This concept resembles a petroleum refinery, which produces different fuels and chemicals from crude oil. Compared to a petroleum refinery, producing fuels and chemicals in a biorefinery has several advantages: energy security, environmental benefits, and sustainability (Huber and Dale 2009; Fitzpatrick et al. 2010). Technologies for several different aspects of the biorefinery process are currently being developed. They include: (i) biomass production (breeding, cultivation, harvesting); (ii) transportation of biomass; (iii) biomass storage and preprocessing;

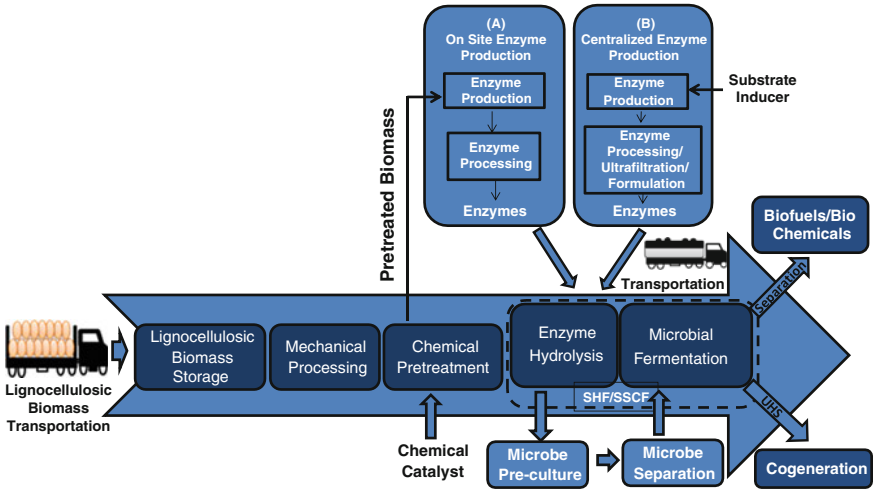

Fig. 4.1 Biorefinery process flow diagram for producing biofuels and biochemical. Here, two different scenarios of enzyme production are shown (**a**) On-site enzyme production and (**b**) Centralized enzyme production. SHF: separate hydrolysis and fermentation; SSCF: simultaneous saccharification and co-fermentation; UHS: unhydrolyzed solids

(iv) pretreatment; (v) enzyme hydrolysis; (vi) microbial fermentation, and (vii) product separation (Balan et al. 2012). Though ethanol is projected to be widely produced in the biorefinery, other fuels and chemicals are also pursued using biological processing route. Some of the chemicals produced using biological route could be transformed to higher value products using a hybrid combination of biochemical and catalytic routes (Ohara 2003). Depending on geographical location and availability of feedstock, the biorefinery can be operated as a large centralized facility or as smaller decentralized facility (Sanders et al. 2007; Lyko et al. 2009).

Several microbes could naturally grow on a wide variety of biomass substrates by efficiently degrading cellulose and hemicellulose and are a good source for new lignocellulosic biomass degrading enzymes. Enzymes that are needed to hydrolyze sugar polymers are cellulases and hemicellulases (Bouws et al. 2008; Kumar et al. 2008). Scientists have been looking for several hypercellulase/hemicellulase producing strains for quite some time. One of the best hyper producers of extracellular cellulolytic enzymes is the mesophilic, filamentous fungus, *Trichoderma reesei (Trichoderma viride)*, first recognized during World War II, when it destroyed cotton fabric US Army tents (Cherry and Fidantsef 2003a, b). The genome sequence of *T. reesei* QM6a was published in 2008 (Martinez et al. 2008a, b). For commercial production of enzymes, several *T. reesei* strains (Rut-30, RL-P37, and MCG-80) have been developed and are currently being used in the industry (Merino and Cherry 2007). Some of these industrial strains produce more than 100 g/l of cellulase/hemicellulase enzymes (Cherry and Fidantsef 2003a, b).

Typical enzyme concentrations used to hydrolyze cellulose are 15–30 filter paper units of cellulase per g of cellulose. On the other hand, for starch hydrolysis 0.2 k Novo unit of amylase per g and 3.26 amyloglucosidase units of glucoamylase per g are needed. This shows that enzyme concentration needed for cellulose degradation is 40-fold to 100-fold higher than that of starch hydrolysis. As a result, the cost of enzymes for cellulose degradation is much higher and it is considered as one of the key bottlenecks for producing fuels and chemicals from lignocellulosic biomass. Several efforts are underway to reduce the cost and maximize enzyme production yield (Carroll and Somerville 2009; Wilson 2009). Some of the strategies include (i) improving the performance of the enzymes by increasing the specific activity (through direct evolution and site directed mutagenesis) and thereby minimizing enzyme dosage (ii) reducing the cost of enzyme production improving cellulase titers during fermentation (through process engineering approaches using cheap substrates including biomass, producing enzymes near biorefinery or expressing enzymes in plants). An overview of *T. reesei* as a cellulase producer, different kinds of enzymes they express, recent genomic, genetic, and metabolic engineering approaches that have helped to improve the biomass degrading enzyme mixture, and strategies pursued to reduce the cost of enzymes are presented in this chapter.

4.2 Cellulase and Hemicellulase Genes in *Trichoderma reesei*

4.2.1 T. reesei Genome: General Characteristics

T. reesei has a genome with a sequence length of around 34 Mbp (Martinez et al. 2008a, b). Gene modeling using a combination of homology and ab initio method predicted 9,129 genes in the genome with an average gene length of 1,793 (bp), and 3.1 exons per gene. Among the 9,129 genes, only 200 genes encode glycoside hydrolases (GHs). The GH genes' number was surprisingly low considering the plant polysaccharide degradation efficiency of *T. reesei.* It was also indicated that the set of plant degrading enzymes of *T. reesei* is smaller than any other sequenced plant cell wall degrading fungus. *T. reesei* also has less carbohydrate-binding module (CBM)-containing proteins among the Sordariomycetes. Totally, ten cellulases are encoded in the *T. reesei* genome including two cellobiohydrolases (CBHI/CEL7A and CBHII/CEL6) and eight endoglucanases (EGII/CEL5A, EGVIII/CEL5B, EGI/CEL7B, CEL12A/CEL12A, EGV/CEL45A, EGIV/CEL61A, EGVII/CEL61B) (Table 4.1). The *T. reesei* genome also contains 16 hemicellulases and among the pectin degrading enzymes, only members of the GH28 family were found. Only seven cellulases (CBHI, CBHII, EGI, EGII, EGIII, EGIV, and EGV) and two β-glucosidases (BGLI and BGLII) have been characterized (Amore and Faraco 2012; Aro et al. 2005). Two major endo-β-1,4-xylanases XYNI and

Table 4.1 Cellulase and hemicellulase enzymes characterized from *Trichoderma reesei*

Enzyme	Former name	CAZY nomenclature	GH family	Amino acids	Mol Wt (kDa)	P.I	Structure (PDB ID)	Ref
β-glucosidase	BGLII	CEL1A	1	466	52.2	5.3	3AHY	Ouyang et al. (2006), Seiboth et al. (2011)
β-glucosidase	BGV	CEL1B	1	484	55.1	5.5	NA	Ouyang et al. (2006), Seiboth et al. (2011)
β-glucosidase	BGLI	CEL3A	3	744	78.4	6.4	NA	Ouyang et al. (2006), Seiboth et al. (2011)
β-glucosidase	CEL3B	CEL3B	3	874	93.9	5.7	NA	Ouyang et al. (2006), Seiboth et al. (2011)
β-glucosidase	BGLIV	CEL3C	3	834	91	5.5	NA	Ouyang et al. (2006), Seiboth et al. (2011)
β-glucosidase	CEL3D	CEL3D	3	700	77.1	6	NA	Ouyang et al. (2006), Seiboth et al. (2011)
β-glucosidase	CEL3E	CEL3E	3	765	83	6	NA	Ouyang et al. (2006), Seiboth et al. (2011)
Endoglucanase	EGII	CEL5A	5	418	44.15	5	3QR3	Ouyang et al. (2006), Seiboth et al. (2011)
Endoglucanase	EGVIII	CEL5B	5	438	46.9	4.3	NA	Ouyang et al. (2006), Seiboth et al. (2011)
Cellobiohydrolase	CBHI	CEL6A	6	471	49.7	5.1	NA	Ouyang et al. (2006), Seiboth et al. (2011)
Cellobiohydrolase	CBHII	CEL7A	7	514	54.1	4.6	2V3I, 1EGN	Ouyang et al. (2006), Seiboth et al. (2011)
Endoglucanase	EGI	CEL7B	7	459	48.2	4.7	1EG1	Ouyang et al. (2006), Seiboth et al. (2011)

(continued)

Table 4.1 (continued)

Enzyme	Former name	CAZY nomenclature	GH family	Amino acids	Mol Wt (kDa)	P.I	Structure (PDB ID)	Ref
Endoglucanase	CEL12A	CEL12A	12	234	25.16	6.9	1OLQ, 1H8V	Ouyang et al. (2006), Seiboth et al. (2011)
Endoglucanase	EGV	CEL45A	45	242	24.4	4.2	NA	Ouyang et al. (2006), Seiboth et al. (2011)
Endoglucanase	EGIV	CEL61A	61	344	35.5	5.3	NA	Ouyang et al. (2006), Seiboth et al. (2011)
Endoglucanase	EGVII	CEL61B	61	249	26.8	7.8	2VTC	Ouyang et al. (2006), Seiboth et al. (2011)
Xyloglucanase	EGVI	CEL74A	74	818			NA	Ouyang et al. (2006), Seiboth et al. (2011)
Xylanase	XYNI	XYN11A	11	223	24.1	8.1	NA	Ouyang et al. (2006), Seiboth et al. (2011)
Xylanase	XYNII	XYN11B	11	229	24.6	5	3LGR, 2DFB	Ouyang et al. (2006), Seiboth et al. (2011)
Xylanase	XYNIII	XYN10A	10	347	38.1	7.1	1FH7, 1CLX, 1XYZ…	Ouyang et al. (2006), Seiboth et al. (2011)
Acetyl Xylan Esterase	AXE1	AXE1	CE5	302	47	7	NA	Margolles-Clark et al. (1996a)
α-L-arabino-furanosidase	ABFI	ABFI	54	500	51	7.5	NA	Poutanen (1988)
α-Galactosidase	AGLI	AGLI	27	444	50	5.2	1SZN	Margolles-Clark et al. (1996b)

NA not available

XYNII (EC 3.2.1.8) (Törrönen et al. 1992; 1994); and one β-xylosidase, BXLI (EC 3.2.1.37) (Herrmann et al. 1997a, b) have been characterized. The secretory mechanism of extracellular enzymes is not yet fully understood, but an analysis of the membrane trafficking system suggests that *T. reesei* has a very diverse system (Martinez et al. 2008a, b).

4.2.2 *T. reesei* Genome Encoded Cellulases and Hemicellulases

Cellobiohydrolases (CBHs): CBHs comprise 80–85 % of the total secreted cellulase protein and the major cellobiohydrolase component, CBHI, accounts for 50–60 % (Gritzali and Brown Ross 1979). CBHI and CBHII have a molecular weight of 57 and 56 kDa, respectively, and act on cellulose chains from non-reducing and reducing ends (Sun et al. 2008a, b; Teeri 1997). They both have a cellulose-binding domain (CBD) and a catalytic domain (CD), which is also observed in many other cellulases. CBHII contains two essential Gly residues located at positions 212 and 217 which take part in the initial binding sites with predominantly hydrogen bonds (Divne et al. 1998). There are two steps for glycosidic hydrolysis by CBHI producing cellobiose as the main product. A nucleophilic substitution at the anomeric C1 atom is involved in both steps. In the first step, a negatively charged carboxyl group in Glu 217 acts as a nucleophile with general acid-catalytic assistance from the carboxyl residue forming a glycosyl-enzyme intermediate. In the second step, water attacks this intermediate with general base-catalytic assistance from the deprotonated carboxyl residue, displacing the nucleophile (Divne et al. 1998). In the tunneled active site of CBHI, there are many H-bonds and indole-glycosyl interactions create a large steric confinement which may be the main factor explaining why substituted celluloses, like carboxymethyl cellulose (CMC), are poor substrates for CBHI (Divne et al. 1998).

Endoglucanases (EGs): EGs comprise around 10–15 % of the total secreted protein by *T. reesei* (Gritzali and Brown Ross 1979). EGI has a sequence that is 45 % identical to CBHI and the respective proteins have similar folding pattern suggesting a common ancestor (Penttilä et al. 1986). The EGI protein contains 437 aminoacidic residues and has a molecular weight of around 46–58 kDa (Messner et al. 1988; Penttilä et al. 1986). The secretion of this protein is directed by a signal peptide of 22 aa which is cleaved in the mature protein at the N-terminal. It is heavily glycosylated near the C-terminal (Penttilä et al. 1986). EGIII is believed to have evolved by divergent evolution from a common ancestor from *Schizophyllum commune* (Saloheimo et al. 1988). It contains relatively long introns and encodes a protein (EG3) whose main product is cellodextrins with a degree of polymerization of 2–5 with a turnover rate of 10–200 per minute (Saloheimo et al. 1988). Like CBHI and EGI, EGIV contains a CBD near the C-terminal and has a MW of $\sim$56 kDa. Unlike most EGs, EGIV's main byproduct is cellobiose, but it was

deemed as an EG because of its ability to hydrolyze CMC, which cannot be hydrolyzed by CBHs (Karlsson et al. 2001; Saloheimo et al. 1997). EGIV has a molecular mass of 57 kDa and has an endoglucanase activity that is very small compared to EGI (Karlsson et al. 2001). EGV encodes a relatively small protein of 242 amino acids (aa) (MW of 22.8 kDa) whose catalytic core is also small compared to other cellulases (Saloheimo et al. 1994). Its shape is believed to be rod-like, which may enable the enzyme to penetrate the cellulose fibers better than other cellulases (Saloheimo et al. 1994).

β-glucosidases (BGLs): BGLs do not technically belong to the cellulases because they do not directly act on cellulose but are commonly included in the cellulase complex because of their synergetic effect with CBHs and EGs. The role of BGLs is hydrolysis of cellobiose and short cello-oligomers into glucose. It appears that *β*-glucosidase gene expression is regulated separately from CBHs and EGs (Jackson and Talburt, 1988). *T. reesei* produces intracellular (Inglin et al. 1980), extracellular (Chen et al. 1992) and mycelium associated *β*-glucosidase (Jackson and Talburt 1988). The *bgl1* gene encodes an extracellular *β*-glucosidase while the *bgl2* gene product is an intracellular *β*-glucosidase (Mach et al. 1995, 2006; Saloheimo et al. 2002). It has been shown that the *bgl1* gene product is required for rapid induction of the cellulase complex (Fowler and Brown 1992). When sophorose is used as an inducer, the *β*-glucosidase from the *bgl1* gene is secreted. However, when methyl- *β*-o-glucoside or gentiobiose is used as an inducer, *β*-glucosidase is typically mycelium-associated and the protein produced was not associated with *bgl1* gene (Mach et al. 1995). The levels of *β*-glucosidase are also correlated to fungal morphology. In vegetative hyphae there are very low levels of *β*-glucosidase while in conidiogenous cells or germinating conidia high levels of *β*-glucosidase are found. Based on this, it was postulated that conidia formation and germination may be coupled with *β*-glucosidase formation (Jackson and Talburt 1988).

Xylanases (XYNs): The two endo-*β*-1,4-xylanases, XYNI and XYNII, contribute around 90 % of the xylanase activity in the *T.reesei* enzyme complex (Rauscher et al. 2006). XYNI and XYNII have a MW of 19 and 21 kDa, and an optimum pH range of 2.5–4.0 and 4.5–5.5, respectively (Törrönen et al. 1992). The *xyn2* gene codes for a protein of 223 aa having two N-glycosylation sites and contains one intron of 108 nucleotides (Saarelainen et al. 1993). *Xyn1* and *xyn2* have very similar gene sequences except for the first 100 N-terminal aa's, and the secondary structure of both xylanases consists of primarily *β*-sheets (Törrönen et al. 1992). Throughout most of these types of xylanases, the aa's are typically conserved at the positions of the *β*-turns, suggesting a common ancestor (Törrönen et al. 1992). *β*-xylosidase production by *T. reesei* was also reported (Herrmann et al. 1997a, b).

4.3 Random Mutagenesis of *T. reesei*—Cellulase Hyper-Producer Strains

Random mutagenesis by treating a microorganism with mutagens (e.g. N-nitroguanidine or UV light) and then screening for the mutants with desired features is a widely used method and an efficient way to obtain a desired microbial strain. This method has been successfully applied to *T. reesei* strains development. Because of the worldwide interests in alternative fuels production from cellulosic biomass, cellulase production and *T. reesei* strain development was of great interest in 1970 s and 1980 s (Peterson and Nevalainen 2012). Random mutagenesis was widely used at that time for improving cellulase production/activity, reducing catabolite repression and alleviating end-product inhibition.

The success of random mutation highly depends on the screening/selection method used. Montenecourt and Eveleigh (1979) summarized the screening methodologies for cellulases production strains. Different substrates together with colony inhibitors oxgall and Phosfon D were used for screening the mutants with desired cellulase activities. For instance, to screen a mutant with enhanced production of all the cellulases, acid swollen cellulose was used as the substrate, while CMC was used as the substrate for selection of a mutant with high endoglucanase activities. Several efficient plate screening techniques were developed for selecting β-glucosidase producer mutants. For instance, one method was developed using esculin and ferric ammonium citrate in the agar medium. β-glucosidase splits esculin into glucose and esculetin which reacts with ferric ammonium citrate and forms a black precipitate. Another method applied cellobiose and 2-deoxyglucose. The mutants that could not produce β-glucosidase must use 2-deoxyglucose which is toxic and causes the death of the mutants, while the mutants producing β-glucosidase are able to utilize cellobiose. For screening a mutant with the catabolite de-repression feature, high concentrations of a catabolite repressor (e.g. glucose or glycerol) were applied in the selection medium. The use of 2-deoxyglucose is particularly useful because it can be used as a catabolite repressor as well as an antimetabolite (Montenecourt and Eveleigh 1979).

A successful case of random mutagenesis on *T. reesei* was conducted by Mandels et. al. (1971), who irradiated the conidia of QM6a with high energy electrons generated by a linear accelerator and then screened the mutants in a cellulose medium. A mutant strain QM9123 was isolated, which secretes twice as much cellulase as the wild type, QM6a (Mandels et al. 1971). Later, another mutant QM 9414 was obtained, which has an even higher capacity of cellulase production (Mandels 1975). Another random mutagenesis effort generated the renowned hypercellulolytic strain RUT-C30 (Peterson and Nevalainen 2012). The native strain QM6a was first treated by UV light and then a catabolite repression resistant mutant M7 was isolated. Further mutagenesis by N-nitroguanidine and screening on an acid swollen cellulose plate with oxgall, Phosfon D and 5 % glycerol led to the isolation of NG14, which produced around 20 times the filter paper activity compared to QM6a (Montenecourt and Eveleigh 1977, 1979;

Peterson and Nevalainen 2012). Subsequent mutagenesis on NG14 using UV light and selection on cellobiose and a 2-deoxyglucose medium obtained RUT-C30, which is a catabolite de-repression strain that produces 15–20 times higher cellulase activity than QM6a (Montenecourt and Eveleigh 1979).

4.4 Metabolic Engineering and Protein Engineering

Several transformation systems (e.g., agrobacterium-mediated transformation) have been developed for efficient manipulation of *T. reesei* (Guangtao et al. 2010; Steiger et al. 2011; Yao et al. 2007). Metabolic engineering of *T. reesei* for cellulase and hemicellulase production have been focusing on study and manipulation of transcriptional regulators (Kubicek et al. 2009). Protein engineering research for improving individual cellulase/hemicellulase includes rational design and direct evolution (Zhang et al. 2006; Wen et al. 2009). Several detailed reviews have been published in the past on protein engineering (Chandel et al. 2012; Peterson and Nevalainen 2012; Elkins et al. 2010). In some cases, point mutations were done to improve the thermo tolerance or specific activity of the enzymes. In many other instances, cellulose and hemicellulose degrading enzymes with multiple activities could be produced using gene-fusion techniques. Since cellulase comprise of catalytic, linker, and cellulose-binding domains, there can be n-number of possibilities of mixing and matching different domains to create novel enzymes with superior activities. Though *T. reesei* have 12 β-glucosidases in their genome, most of them were found to be intracellular and secreted enzyme had lesser cellobiase activities. Companies like Novozyme (http://www.novozymes.com) and Genencor International Inc. (http://www.genencor.com) have already developed a genetically modified *T. reesei* strain that shows higher β-glucosidase activity. Though several synergistically acting enzymes from other organisms are expressed in *T. reesei* for improving their enzyme activity, the details are yet to be revealed (Gusakov 2011).

4.4.1 Transcriptional Regulators

Production of cellulases and hemicellulases by *T.reesei* is tightly regulated by transcriptional regulators and requires an inducer (e.g., cellulose and lactose) for cellulase/hemicellulase gene expression to occur (Kubicek et al. 2009). Currently, three positive transcriptional activators (Xyr1, Ace2, and the Hap2/3/5 complex) and two negative regulators (Ace1and Cre1) have been identified. Xyr1 is a central regulatory protein which belongs to the class of zinc binuclear cluster proteins. All inducible cellulase promoters were found containing *consensus sequences* for Xyr1 (Kubicek et al. 2009). Xyr1 not only activates the most important hydrolase genes involved in the degradation of xylan and cellulose, including *cbh1, cbh2, egl1, bgl1, xyn1, xyn2* and *bxl1* (Mach-Aigner et al. 2008; Pucher et al. 2011), but

it is also involved in the regulation of xylose and lactose metabolism (Seiboth et al. 2007; Stricker et al. 2006). It was found by Mach-Aigner et al. (2008) that the *xyr1* gene was not induced by any cellulases inducers. However, Portnoy et al. (2011) indicated that it was induced by lactose and D-galactose. *Xyr1* transcription was repressed by Ace1 as well as by glucose through carbon catabolite repression mediated by Cre1 (Mach-Aigner et al. 2008). Deletion of the *xyr1* gene results in elimination of all cellulase and some hemicellulase production (Akel et al. 2009). Although it is well known that Xyr1 is the main regulator for cellulase and hemicellulase production, how to use this transcriptional regulator for producing cellulase and hemicellulase at a higher yield is still under investigation (Kubicek et al. 2009).

Cre1 has two zinc fingers of the C2H2 type involved in base recognition (Ilmen et al. 1996; Strauss et al. 1995) and it confers negative regulation of transcription. Unlike Xyr1, Cre1 only has direct control of some major hydrolases, most notably *cbh1* and *xyn1*, whereas other hydrolytic genes such as *cbh2, xyn2,* and *bgl1* are not Cre1 regulation dependent (Ilmen et al. 1996; Mach et al. 1995; Margolles-Clark et al. 1997). It has been illustrated that in the presence of glucose, Cre1 binds to specific sites in the *cbh1* promoter and hence it represses cellulase production (Ilmen et al. 1996). Deletion or modification of *cre1* is a way to resolve the catabolite repression issue (Nakari-Setala et al. 2009). The hypercellulolytic strain *T. reesei* Rut-C30 was found having a truncated *cre1* gene which is the reason for its catabolite derepression property (Ilmen et al. 1996; Nakari-Setala et al. 2009; Peterson and Nevalainen 2012). Zou et al. (2012) substituted the binding sites of Ace2 and Hap2/3/5 for the Cre1 binding sites within the *cbh1* promoter and thus improved the efficiency of the promoter.

Transcription factors Ace1, Ace2, and Hap2/3/5 complex are also involved in the regulation of cellulase formation in *T. reesei.* Ace1 has been described as a cellulase repressor (Aro et al. 2003) while Ace2 is described to promote cellulase production (Aro et al. 2001). Ace1, containing three Cys2His2 –type zinc fingers, is a repressor of cellulase and xylanase genes (Aro et al. 2003). It binds to eight sites in the *cbh1* promoter containing the core sequence 5'AGGCA (Saloheimo et al. 2000). An increase of all the main cellulase and xylanase expression was observed for the Δ*ace1* strain when cultured on sophorose and cellulose (Aro et al. 2003), which may be due to the fact that Ace1 also acts as a repressor of *xyr1*, as mentioned before (Mach-Aigner et al. 2008). Ace1 also competes with Xyr1for binding sites in the *xyn1* promoter and thus it represses the transcription of *xyn1* (Rauscher et al. 2006).

Ace2 is a zinc binuclear cluster protein like Xyr1. In contrast to Ace1, expression of Ace2 helps to increase cellulase expression (Aro et al. 2001). Deletion of *ace2* leads to lower transcript levels of major cellulases (CBHI, CBHII, EGI, and EGII) and xylanase (XYNII) when cellulose was used as an inducer, but it was unaffected when sophorose was used as an inducer (Aro et al. 2001). Ace2 binds to the strong *cbh* promoter at the 5'-GGCTAATAA site (Aro et al. 2001), and it has been suggested that phosphorylation and dimerization are needed for the binding of Ace2 to the target promoter (Stricker et al. 2008).

Hap2/3/5 complex binds to a CCAAT box of *cbh2*-activating element (CAE) in the *cbh2* promoter and acts as a transcriptional enhancer (Zeilinger et al. 2001).

4.4.2 Rational Design and Direct Evolution of Individual Cellulase/Hemicellulase

The idea of rational design is to modify the aminoacidic sequence of a protein in order to achieve dramatic impacts on the protein performance (Sheehan and Himmel 1999). Rational design for improving cellulases requires detailed information of the protein structure, protein structure/function relationships and how the protein interacts with the substrate to make the catalysis reaction to occur (Zhang et al. 2006). Site-directed mutagenesis, secondary structure element exchange, and whole domain exchange of fusion proteins are examples of how protein modifications can be achieved. The success of enzyme enhancement (e.g., increased activity) is usually limited to well-understood proteins, and is commonly applied to the amino acidic sites near the active site or the binding pocket in the 3-dimensional structure (Zhang et al. 2006). Based on the limited understanding of insoluble cellulose substrates, reaction complexity of the cellulase enzymes and the arsenal of enzymes needed to work in synergy to degrade lignocellulosic biomass, the cellulase complex needs much more investigation before effective methods can be developed. Cellulases working on the insoluble substrates have a complex mechanism to degrade the cellulose. For instance, six steps were proposed for CBHI of *T. reesei* to work on cellulose: binding to substrate via CBM (carbohydrate-binding module), recognizing a reducing end of a cellulose chain, threading the cellulose chain, forming a catalytically active complex, hydrolyzing the cellulose, and expulsing the product (Chundawat et al. 2011). Because of the complexity of their action, site-directed mutagenesis has met some difficulties toward improving the properties of the cellulases (Zhang et al. 2006). However, there are few successful cases concerning cellulases from fungi. For example, Wohlfahrt et al. improved the pH stability of the CBHII from *T. reesei* by mutagenesis of the non-active site residues (Wohlfahrt et al. 2003). Voutilainen et al. enhanced the thermo stability and activity of a CBHI from another fungus, *Talaromyces emersonii,* by introducing an additional disulfide bridge to the catalytic module (Voutilainen et al. 2010). Chen et al. increased the thermo stability of a xylanase from *Aspergillus niger* F19 by introducing five arginine substitutions and a disulfide bond to the enzyme (Chen et al. 2010).

Direct evolution mimics the natural random mutation and selection through recombinant DNA technology. It does not require the knowledge of enzyme structure and enzyme-substrate interactions, but relies on the screening method for evaluating the mutants (Zhang et al. 2006). CMC plus Congo red staining is a widely applied screening method for endoglucanase mutants (Lin et al. 2011). The Endoglucanase activity can be determined by the “halos” on the solid agar plates

in which higher hydrolysis correlates with the size of the halos. DNA techniques that have been applied for directed evolution include family shuffling, DNA shuffling, error-prone Polymerase Chain Reaction (PCR), and SCHEMA (Lin et al. 2011). Improved enzyme activity, thermostability, and pH adaptability are the desired characteristics (Goedegebuur et al. 2005; Han et al. 2009; Lin et al. 2011; Trivedi et al. 2011; Wang et al. 2005; Xia and Wang 2009). For instance, Nakazawa et al. (2009) carried out directed evolution of *T. reesei* EGIII using error-prone PCR and selected a mutant exhibiting broader pH stability, better thermo stability as well as higher activity when compared to the wild type EGIII. Moreover, Hokanson et al. (2011) enhanced the thermo stability of the GH11 xylanase II from *T. reesei* through a directed evolution method.

4.5 Fermentation Technologies for Enzyme Production

4.5.1 Nutrients for T. reesei Growth and Enzyme Production

T. reesei is a mesophilic fungus having the metabolic pathways to utilize all the lignocellulose carbohydrates (Amore and Faraco 2012) and little nutrient requirements for growth (Mandels and Weber 1969). Hence, it can grow fairly easily on most carbon sources. Its growth rate is rapid on glucose, fructose, and glycerol and relatively slow on cellulose and lactose (Messner and Kubicek 1991). Glucose was shown to give higher cell biomass yield when compared to lactose, arabinose, or their mixtures (Xiong et al. 2004a). Nevertheless, glucose is a repressor of cellulase production via the action of Cre1. However, cellulase expression was still seen in a glucose medium after glucose was depleted with no inducer present (Ilmen et al. 1997). Oligosaccharides released from the cell walls of the starving fungus or sophorose generated from the glucose by the action of β-glucosidase were possibly acting as inducers in this case (Ilmen et al. 1997). When grown on carbohydrates, the fungus produces acidic compounds and the growth continues until pH drops below 2.5. In contrast, when grown on peptone the pH increases and growth continues until the pH reaches 7.5 (Mandels and Weber 1969). *T. reesei* can produce cellulases in a defined medium with simple nutrient salts and cellulose, but the addition of peptone has been shown to improve protein production. In another study, peptone was shown to have little effect on improvement of cellulase activity but increased β-glucosidase activity (Esterbauer et al. 1991). Tween 80, a commonly used surfactant, has been shown to increase cellulase production. It was theorized that this increase is due to loosening of the *T. reesei* cell wall and thus facilitating the entrance and exit of compounds from the cell (Reese and Maguire 1969).

4.5.2 Inducers for Cellulase and Hemicellulase Production

Cellulose and xylan were thought to be the natural inducers for cellulase and hemicellulase production. When considering the insoluble nature of xylan and cellulose, which are unable to enter the fungal cell, it has been suggested that the natural inducer was low molecular weight hydrolysis products (such as oligosaccharides and their derivatives), which can penetrate the cell and affect the fungal metabolism (Haltrich et al. 1996). Commonly used inducers including sophorose, lactose, and sorbose are discussed here.

The disaccharide sophorose is a very powerful soluble inducer of *T. reesei* cellulases and it was suggested to be the natural inducer (Sternberg and Mandels 1979; Sternberg and Mandels 1982). It has been demonstrated that the formation of sophorose from cellobiose was mediated by the trans-glycosylation activity of β-glucosidase (Vaheri et al. 1979). In addition, when β-glucosidase was inhibited, a substantial decrease in the synthesis of EGs was observed with cello-oligodextrins but not with sophorose as inducers (Kubicek 1987). These results further indicate the importance of β-glucosidase and sophorose in the induction of hydrolytic enzymes.

Lactose (D-galactosyl- β-1,4-D-glucoside) is another widely used cellulase inducer. A clear advantage of lactose compared to cellulose as an inducer is its solubility, yet lactose consumption has been shown to be slower and cellulase yields were lower compared to cellulose (Warzywoda et al. 1983). Because natural lactose only occurs in the milk of mammals, it is unlikely that it is the natural inducer for cellulose production (Kubicek et al. 2009). Extracellular hydrolysis of lactose into D-galactose and D-glucose is the initial step of lactose catabolism in *T. reesei.* The extracellular hydrolysis of lactose raised the question of the potential of the monomers ability to induce cellulase expression (Kubicek et al. 2009). Seiboth et al. (2003) found that neither D-glucose nor D-galactose or any mixture of these two resulted in cellulase induction even when the carbon catabolite repressor Cre1 was absent. Based on this, it is believed that the stereospecificity of the D-galactopyranose which is released from the cleavage of lactose by β-galactosidase plays a key role in the induction of cellulase by lactose (Kubicek et al. 2009).

Monosaccharides generally inhibit cellulase expression through carbon catabolite repression via the action of Cre1 or through end-product feedback inhibition. The only monosaccharide found to have a cellulase-inducing effect is L-sorbose (Kawamori et al. 1986; Nogawa et al. 2001). Sorbose affects cellulase formation at a transcriptional level and has been proposed to inhibit β-1,3-glucan synthetase, which changes the composition of the fungus cell wall and reduces the degradation of inducers (Nogawa et al. 2001).

XYNI and XYNII are not co-regulated, but are both formed in the presence of xylan or xylobiose, but only one is formed in the presence of sophorose (Hrmová et al. 1986; Senior et al. 1989). Cellulose, sophorose, xylan, xylobiose, and L-arabitol can induce expression of most of the tested hemicellulase genes including

two β-xylanases, β-mannase, acetyl xylan esterase, β-xylosidase, and many others (Margolles-Clark et al. 1997). In the presence of glucose, most of the hemicellulase genes are repressed but de-repressed expression was observed once glucose was depleted (Margolles-Clark et al. 1997). *Xyn2* transcription occurs at a low basal level when the fungus is grown on glucose as the sole carbon source and is elevated in the presence of xylan, xylobiose, or sophorose (Zeilinger et al. 1996).

4.5.3 Fermentation Conditions

The culture medium pH is a critical factor affecting many aspects of *T. reesei* fermentation including germinating time, growth rate, morphology, and enzyme production. Germination time is the shortest in the pH range of 3–5 and elongated when the pH becomes too acidic or alkaline (Lejeune et al. 1995). Maximal growth rate is also in the pH range of 3–5 with decreased growth rate in more acidic or alkali medium (Brown and Zainudeen 1977; Lejeune et al. 1995). On cellulose and xylan-based media, it was observed that cellulase production is favored at a pH around 4.0 while xylanase production favored at a pH around 7.0 (Bailey et al. 1993). In another study on lactose medium, the highest xylanase activity was observed at pH 6.0 and optimum cellulase production at a pH range of 4.0–5.0 (Xiong et al. 2004b). Different xylanases also favor different pH. Xiong et al. (2004b) found that XYNI favors pH 4.0, XYNIII favors pH 6.0, and XYNII favors both. Relatively high production of β-glucosidase was also found at high pH values (Juhász et al. 2004).

Agitation rate also plays a crucial role in enzyme production. Enzyme production is an energy intense reaction and requires a lot of ATPs. High agitation rate typically results in high dissolved oxygen level and hence facilitates aspiration and ATP production. However, high agitation rate also causes high shear stress and affects hyphae growth (Ahamed and Vermette 2010). When a 2.6 L fermenter is used, the optimal agitation speed for cellulase production was determined to be 300 rpm (Mukataka et al. 1988). In contrast to the total cellulase activity, the optimal agitation speed for endoglucanase and β-glucosidase production was 200 and 400 rpm, respectively (Mukataka et al. 1988). Effect of culture media composition is also known to have significant impact on cellulase production by *T. reesei* (Ahamed and Vermette 2008, 2010).

4.5.4 Morphology

Filamentous fungi are able to develop three major morphologies during submerged fermentation: pellets, mycelial aggregates (clumps), and filamentous mycelia (Cox et al. 1998). In bioreactors, the filamentous or clump mycelia are undesirable because they increase the viscosity of the medium and also wrap around the

impellers. The pellet form is a desirable morphology, especially for industrial production, not only because of the reduced viscosity but also due to the improved culture rheology (enhanced mass and oxygen transfer) and the reduced energy cost for agitation and aeration (Suijdam et al. 1980). Numerous factors in the fermentation affect the fungus morphology, including agitation speed (see above), medium, pH, polymer additives, surface active agents, and inoculum size (Metz and Kossen 1977; Papagianni 2004; Ferreira Susana et al. 2009). It has been postulated that increasing the number of tips of hyphae increases protein production (Juge et al. 1998; Pluschkell et al. 1996) because it is believed that in filamentous fungus, protein secretion occurs at the tip of growing hyphae (Peberdy 1994; Punt et al. 1994). Since the tips are more porous, they allow proteins to exit more easily through the cell wall (Punt et al. 1994; Wosten et al. 1991). Correlation between fungal physiology and cellulase production is yet to be clearly understood. Several articles have shown that cellulase production is directly influenced by the fungal morphology during fungal fermentation (Grimm et al. 2005 and Ferreira Susana et al. 2009).

4.6 Current Efforts and Future Perspective of Reducing the Cost of Enzymes

Costs for producing enzymes used to hydrolyze pretreated biomass represent about one-third of the total hydrolysis processing cost (Walker and Wilson 1991; Lynd et al. 2005). It has been projected that the cost of enzymes is 2665 dollars per mega gram. Over the past 30 years, the cost of enzymes has been considerably decreased, but it is still considered to be high. About 10–20 Filter Paper Unit (FPU) of enzymes is required per gram of cellulose to achieve 90 % sugar conversion in 72 h (Chandel et al. 2012; Peterson and Nevalainen 2012). Though some of the reported *T. reesei* QM 6a strains produce an enzyme concentration of about 20 FPU ml^{-1} at a rate of 150 FPU $L^{-1}h^{-1}$, the cost of pure cellulose substrate used in the process is very high. Several efforts have been taken to lower the cost of enzymes as described below.

4.6.1 Enzyme Production Using Lignocellulosic Biomass

One of the main sources for the high cost of cellulase production is the substrate cost. Currently, pure cellulose power and inorganic salts are used as media with artificial inducers (Qu et al. 1991). Several artificial inducers (like lactose, cellobiose) are used in the industry to induce cellulase and hemicellulase production in *T. reesei* (Table 4.2). In order to reduce the cost of enzyme production, these artificial inducers could be replaced by natural inducers. The oligosaccharides

Table 4.2 Protein production using *T. Reesei* Rut-30 strain on different substrates

Substrate	Cellulase (FPU/ml)	Xylanase IU/ml	Reference
Cellulose+ yeast extract	5.02		Ahamed and Vermette 2008
Cellulose-Yeast nitrogen base-CMC	1.4		Ahamed and Vermette 2008
Plasma-assisted pretreated wheat straw-unwashed-sterilized by autoclave	0.1	84.0	Rodriguez-Gomez et al. 2012
Plasma-assisted pretreated wheat straw-washed-sterilized by autoclave	0.4	106.0	Rodriguez-Gomez et al. 2012
Wet oxidized wheat straw	<0.37		Thygesen et al. 2003
Rice straw	0.38		Colina et al. 2009
Sticks of rice straw	0.6		Sun et. al. 2008
Sticks of rice straw (alkaline pretreatment)	1.07		Sun et. al. 2008
Sticks of rice straw (acid pretreatment)	0.3		Sun et. al. 2008
Steam-pretreated spruce	0.45	13.2	Juhasz et al. 2005
Steam-pretreated spruce	0.8		Szengyel et al. 2000
Steam-pretreated willow	0.56	57.0	Juhasz et al. 2005
Steam-pretreated willow	1.6		Szengyel et al. 1997; Zacchi 1996
Steam-pretreated willow	0.64		Palmqvist 1997
Steam-pretreated willow + hydrolisate	0.6		Chahal 1982
Steam-pretreated corn stove	1.2	64.4	Juhasz et al. 2005
Steam-exploded poplar nonwashed	1		Szakacs and Tengerdy, 1997
Steam-exploded poplar washed	3.7		Szakacs and Tengerdy, 1997
Pretreated poplar wood	1.4		Shin et al. 2000
Steam-exploded wood	4.3		Xiong et al. 2005
Oat husk hydrolysate (acid)	0.5	276.0	Xiong et al. 2005
Spruce fiber hydrolysate (acid)		30.0	Xiong et al. 2005
Homogenized dairy manure (optimized)	1.72		Wen et al. 2005
Bagasse pretreated with hot water	0.6		Bigelow and Wyman 2002
Pulverized newspaper sludge	1.7		Shin et al. 2000
Old corrugated cardboard	2.27		Szijarto et al. 2004

produced during the hydrolysis of cellulose play important roles in the natural cellulase induction (Ladisch et al. 1981). Pure cellulosic materials (avicel, cotton, filter paper) have often been used both as substrates and as the source of inducers during fermentation processes for cellulase production (Lee and Fan 1983; Ahamed and Vermette 2008). Several limitations do exist while using solid substrates, such as an increase in viscosity and problematic agitation and reduced oxygen transfer efficiency of the bioreactors. To overcome these problems, lower solid concentrations are often used to get higher cellulase yields (Szengyel et al. 1997). If a cheap source of oligosaccharides is available, they could be directly used for

induction. In many cases both native and pretreated biomass are used for induction (Dashtban et al. 2011). For pretreated biomass, a longer lag phase was observed when compared to controls with media containing glucose (Lo et al. 2005; Juhasz et al. 2005; Lau et al. 2012). These lag phases were attributed to the presence of inhibitory products produced during pretreatment (Palmqvist et al. 1997; Chundawat et al. 2010). When Ammonia Fiber Expansion (AFEX^{TM}) pretreated wash stream was used as inducing medium, more hemicellulases were produced when compared to cellulases. Also, continuous culture produces more enzymes than batch culture, since oligosaccharides are known to be hydrolyzed before the cell concentration is high enough to produce a high concentration of cellulases (Lo et al. 2010).

Many researchers are looking for cheap sources of substrate like clarifier sludge and digester fine from paper mill, pretreated sugarcane bagasse, sorghum straw, corn stover (Yu and Koo 1999; Szakacs and Tengerdy 1997) for producing enzymes. In a larger lignocellulosic biorefinery, pretreated biomass could be used to produce enzymes that will substantially reduce the cellulase production costs.

4.6.2 Improvement of the Fermentation Process

4.6.2.1 Batch *Versus* Fed Batch Substrate Loading

Concentration of substrate and how the substrates are loaded during fermentation can influence the cellulase enzyme productivity. It has been reported that as the substrate concentration in the fermentation tank increases, the cellulase productivity raises: 2–3FPU/ml at 2 % substrate loading and 1–15 FPU/ml at 8 % substrate loadings (Esterbauer et al. 1991). Also, higher cellulase concentrations and volumetric rates were reported for fed batch fermentation when compared to batch fermentation (Hendy et al. 1984).

4.6.2.2 Solid State Fermentation (SSF) *Versus* Submerged Fermentation (SmF)

SSF process is done by growing the microbes on moist solid materials in the absence of free water. Here the substrate used in the process is used slowly and steadily. Although this method is best suited for fungus, the residence time for the process is too long and product separation is tedious. On the other hand, SmF process is done using free flowing liquid substrate or in substrate slurry. This is best studied for bacteria and the separation and purification of product is much easier (Bailey and Tähtiharju 2003; Subramaniyam and Vimala 2012). Most of the cellulase production in the industry is done using SmF. However, most of the aerobic microorganisms produce cellulases at high titers during SSF which is similar to natural environment. About 10-fold reduction in production cost has

been reported when cellulases were produced in SSF when compared to SmF (Singhania et al. 2010). This is due to several advantages SSF have which include high volumetric productivity, higher titer of enzymes, lesser waste generation, low catabolic repression. Given its advantages, SSF could be promising technology for the future.

4.6.3 Fast Downstream Processing for Maximum Product Recovery

Downstream processing of enzymes could account for 50 % of the total cost of enzyme production. Traditional technologies used in the industry are centrifugation (solid liquid separation), filtration, and ultrafiltration (to remove waste and concentrate the enzymes) (Beilen and Li 2002). Efficient enzyme bioseparation process will bring down the cost of enzyme production. Some of the advanced technologies include two-phase extraction, reverse micelle extraction, cloud-point extraction, and field-assisted (electric, magnetic, and acoustic) separation methods (Keller et al. 2001; Karumanchi et al. 2002).

4.6.4 Recycling Enzymes During Bioconversion

One of the strategies to reduce the cost of enzyme is recycling the enzyme during the enzyme hydrolysis step (Tu et al. 2007). The most economical way to recover the enzyme after hydrolysis is done by re-adsorption of free cellulases onto fresh lignocellulosic substrates. About 80–85 % of enzymes activities could be removed using this approach (Lee et al. 1995). In few cases, ultrafiltration methods were also used to retain almost all the enzyme which could be used for subsequent cycle of enzyme hydrolysis. In another study, about 30–50 % of enzymes have been demonstrated to be recycled after each cycle of hydrolysis process using fast Separate Hydrolysis and Fermentation (SHF) and Simultaneous Saccharification and Co-Fermentation (SSCF) process which takes just two days to complete both hydrolysis and fermentation process when compared to traditional process which takes close to 10 days (Galbe and Zacchi 1993; Lynd et al. 2005; Jin et al. 2012).

4.6.5 Other Approaches to Reduce the Cost of Enzyme Production

There are two models widely followed for supplying enzymes to the biorefinery (Fig. 4.1). The first includes production of enzymes in a centralized large-scale processing facility which concentrates and formulates the enzymes and ships them to the different biorefinery locations. There are several drawbacks to this approach.

Pure substrates, like cellulose and inducers, add additional costs to the process, ultrafiltration techniques used to concentrate the enzymes are energy intensive and shipping enzyme solution are very inefficient. The second approach includes production of enzymes at the site of the biorefinery. In this approach, there are several advantages, such as concentration of the enzymes is unnecessary, the pretreated substrate available in the biorefinery can be used as substrate for enzyme production and there are no shipping costs. Many enzyme companies are trying to adopt the second approach in order to reduce the cost of enzymes for making biofuels.

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