Trichoderma reesei AS AN EXPRESSION SYSTEM FOR HOMOLOGOUS PRODUCTION OF INDIVIDUAL CELLULASES

by

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Trichoderma reesei AS AN EXPRESSION SYSTEM FOR HOMOLOGOUS PRODUCTION OF INDIVIDUAL CELLULASES

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ABSTRACT

Cellulases are a group of enzymes that can synergistically catalyze hydrolysis of cellulose into glucose, which is an essential process for conversion of huge amounts of dormant cellulosic biomass into fermentable sugar, one of the most potent alternative energy sources of the new world. Since purification is difficult and time-consuming, production of cellulases individually is more favorable for these applications that may require specific combination of different enzyme components.

In order to evaluate the filamentous fungus Trichoderma reesei as an expression system for production of individual cellulases, Endoglucanase I (EG1/Cel7B), Endoglucanase III (EG3/Cel12A) and Cellobiohydrolase I (CBH1/Cel7A) were

homologously expressed in the cellulase-negative mutant strain delta-xyr1 using two alternative promoters (tef1 and cdna1) on glucose medium. In this thesis we show that individual cellulase components (EG1, EG3 and CBH1) could be successfully overexpressed in active form in a cellulase negative T.reesei background under non-inducing conditions for the first time in the literature. We also show that cdna1 promoter resulted in higher expression levels of EG1 and EG3. Additionally, T.reesei was established and partially optimized as an expression system which can be employed for future applications.

SELÜLAZLARIN HOMOLOG ÜRETİMİ İÇİN BİR EKSPRESYON SİSTEMİ OLARAK Trichoderma reesei

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ÖZET

Selülazlar atıl durumdaki çok büyük miktarlardaki biyokütlenin geleceğin en etkili alternatif enerji kaynağı adayı olan mayalanabilir şekere dönüştürülmesi için elzem bir işlem olan selülozun glukoza hidrolize edilmesini sinerjik olarak katalize edebilen bir grup enzimdir. Enzimlerin saflaştırılması zor ve zaman isteyen bir işlem olduğu için, farklı bileşenlerin belirli oranlarda karışımını gerektirebilecek bu uygulamalar için enzimlerin tek tek üretilmesi daha tercih edilirdir.

Bir ipliksi mantar türü olan Trichoderma reesei'nin selülazların tek tek üretimi için bir ekspresyon sistemi olarak değerlendirilmesi amacıyla Endoglukanaz I (EG1/Cel7B), Endoglukanaz III (EG3/Cel7A) ve Sellobiyohidrolaz I (CBH1/Cel7A) enzimleri selülaz-negatif bir mutant olan delta-xyr1 soyunda, glukozlu ortamda yüksek

aktivite gösteren iki farklı promotor (tef1 ve cdna1) kullanılarak homolog olarak üretildi. Literatürde ilk defa bu tezde münferit selülaz bileşenleri (EG1, EG3 ve CBH1) selülaz-negatif bir T.reesei soyunda, indükleyici olmayan koşullarda, aktif halde yüksek miktarda başarılı bir şekilde üretildi. cbh1 promotoruyla EG1 ve EG3 enzimlerinin daha yüksek miktarda üretilebildiği gözlendi. Yanı sıra, T.reesei bir ekspresyon sistemi olarak tesis edildi ve ilerideki uygulamalar için kullanılabilecek şekilde kısmi olarak optimize edildi.

To my family with all my heart...

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ABBREVIATIONS

AmpR	Ampicillin Resistance Gene
bps	base pairs
ddH_2O	Double-distilled water or double deionized water
dNTP	Deoxynucleoside triphosphate
EtOH	Ethanol
g / μg	gram, microgram
h	hour / hours
hph	Hygromycin Phosphotransferase
IU	International Unit
kb	kilobases
LB / LB-Amp	Lysogeny Broth / Lysogeny Broth with Ampicillin
mL / μL	Milliliter / Microliter
mM / nM	Millimolar / Nanomolar
MetOH	Methanol
min	minute / minutes
MUC	4-Methylumbelliferyl-β-D-Cellobioside
MULAC	4-Methylumbelliferyl-β-D-Lactopyranoside
NaOAc	Sodium Acetate
NaOH	Sodium Hydroxide
Pcdna1	Promoter of cdna1 gene in *T.reesei*
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
Ptef1	Promoter of Translation Elongation Factor 1 alpha
rpm	Revolutions per minute
RT	room temperature

SDS Gel	Sodium Dodecyl Sulfate Polyacrylamide Gel
sec	second
spp	species (species pluralis)
T.reesei	*Trichoderma reesei*
TAE	Tris-Acetate-EDTA
TUWien	Vienna University of Technology, Austria
U	Unit
V	Volt
v/v	Volume / Volume ratio
w/v	Weight / Volume ratio
w/o	without

1. INTRODUCTION

Cellulose is a highly crystalline and unbranched polymer which is the most abundant biomass component on earth produced continuously by plants and trees (Kumar, 2008). In addition to these natural sources, people are involved in management of cellulosic biomass to a considerable extent by means such as agriculture and forestry (Demain, 2005). After harvesting and processing of crops, most of these cellulosic remnants are decomposed by microorganisms (Fang, 2010) which can produce cellulases that catalyze the hydrolysis of cellulosic material into their monomers (Sandgren, 2005). Some species, especially bacteria and fungi can secrete partial or complete set of cellulases and utilize lignocelluloses by degrading them into soluble sugar (Bisaria, 1981).

Increasing environmental concerns and exhausting non-renewable energy sources prompts people to search for alternative cheaper and environment friendly energy reservoirs, such as sugar. Feedstock of biofuels are naturally produced in extreme amounts yearly, only strategies are required to be able to utilize it. All of the cellulase components should be produced inexpensively in large amounts for conversion of cellulose to fermentable sugar and then to bioethanol, the energy supply of the future.

Complete hydrolysis of cellulosic material into glucose units requires synergistic action of three types of cellulases endo/exo-glucanases and β-glucosidases. These enzymes have different catalytic domains specialized for different regions of cellulose; endoglucanases can cut the cellulose fiber randomly while exoglucanases cut chain ends. β-glucosidases convert shorter cellooligosaccharides into glucose monomers (Kumar, 2008). Cellulases are already used in several industrial fields such as detergent, textile, pulp and paper industries for de-inking and refining (Kirk, 2002). The enzymes are used either as a mixture or individually depending on the aim (Becker, 2001). Biomass is utilized for production of bioethanol, sugars and other value added products

with established protocols employing cellulases (Fang, 2010) but the process is still expensive due to high prices of enzymes and lack of optimized bioreactors.

Hydrolysis rate of cellulose can be affected by internal properties of cellulases, such as adsorption capacity, as well as external factors such as cellulose crystallinity (Arantes, 2010). Rate of bioconversion of cellulose can be altered by pretreatments which usually are applied to increase accessible substrate area, physically or chemically (Cohen, 2005). Molecular biological techniques are employed to increase stability and activity of enzymes by random or site directed mutagenesis (Hong, 2007). Intervention with the secretory machinery of cellulolytic organisms can contribute to the yield as well (Archer & Pebedry, 1997).

Trichoderma reesei is a saprophytic filamentous fungus that can naturally produce a complete set of cellulases; endoglucanases, exoglucanases and β-glucosidase. It lives in several types of soils, utilizing plant and wood residues (Kubicek, 2003). There had been attempts to produce *T.reesei* proteins in other organisms and to produce heterologous proteins in *T.reesei*, since the presence of a strong secretory machinery makes it an attractive organism for overexpression of homologous or heterologous proteins.

2. OVERVIEW

2.1. Cellulose

Increasing demands for more energy and environmental awareness in the contemporary world have been prompting people to search for sustainable alternatives to non-renewable energy sources such as fossil fuels that will be exhausted soon. Cellulose is one of the most prominent candidates for alternative energy reservoirs of the future, being the major component of total biomass on earth (Kirk, 2002). $1.3x10^{10}$ tons (dry weight) of wood is produced by plants annually, corresponding to two-thirds of the energy need of the world, $1.8x10^{8}$ of which is available through agriculture and other sources (Demain, 2005). Availability of dormant lignocellulosic biomass produced in huge amounts by agriculture and forestry; and as a part of municipal solid waste makes it an attractive renewable target for production of bioethanol, sugars and other value-added substances such as organic chemicals, vanillin (Walton, 2003), xylitol (Rahman, 2007), furfural (Montane, 2002) and so on (Fang, 2010). Even though the production of bioethanol from sugar or starch is still much easier and cheaper than that of biomass, the process is still costly that biofuel becomes more expensive than fossil fuels. Hence, it is essential to bring forth the technology to convert cellulosic biomass into fermentable sugar efficiently and inexpensively for effective utilization (Kumar, 2008)

Cellulose, hemicelluloses and lignin are the components of wood and other celluloses (Kumar, 2008). Cellulose is a highly crystalline and unbranched polysaccharide consisting of β-(1-4)-linked glucose units with a length of several hundred to ten thousands (Mélanie, 2010). Even water cannot diffuse into the ordered regions of cellulose sometimes, because of the compact packing of fibers (Arantes, 2010). Crystal structure is formed by joint effects of hydrogen bonds, hydrophobic interactions and van der Waals forces keeping the fibers together (Sandgren, 2005).

Cellulases are the enzymes that catalyze the hydrolysis of plant biomass together with hemicellulases (xylanases, mannoses, pectinases i.e.) into smaller pieces which are subsequently degraded by α/β-glucosidases into their monomers (Sandgren, 2005).

2.2. Cellulases

Cellulases are hydrolytic enzymes that can cut β-1,4-glycosidic bonds of cellulose (Sandgren, 2005). Endoglucanase cuts the cellulose fibers randomly, mostly in the amorphous and disordered regions creating reducing or non-reducing flanking ends. Cellobiohydrolases (exoglucanases) cuts the cellulose fibers in these ends progressively producing cellobiose or short cellooligosaccharides. β-glucosidase finally converts these to individual glucose units (Figure 1) (Kumar, 2008).

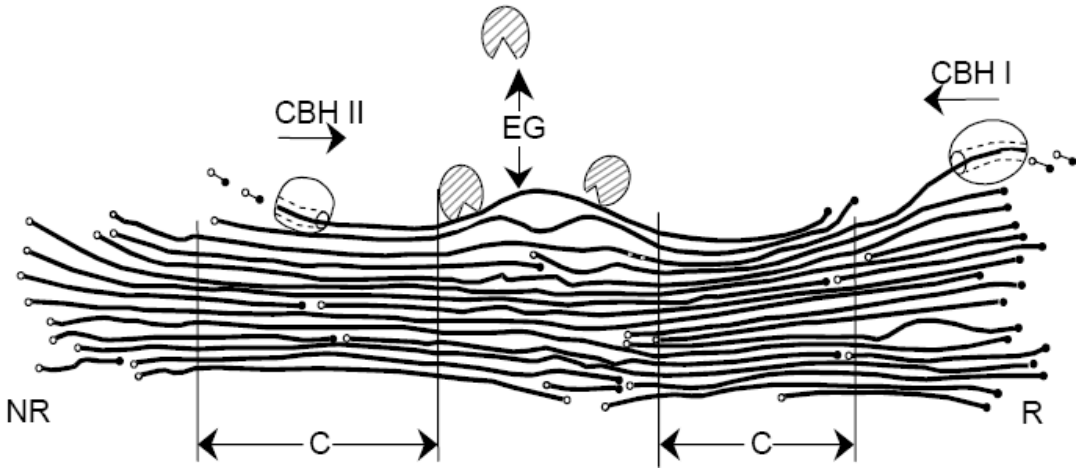


Figure 1: Cellulase activity on cellulose fibers. Endoglucanase hydrolyses amorphous regions, creating reducing and non-reducing ends which are targets of cellobiohydrolases that cut the chain ends producing cellobiose. C: crystalline region, R: reducing end, NR: non-reducing end, EG: endoglucanase, CBH: cellobiohydrolase.

Bacterial cellulosomes are multi-enzyme complexes with many (mostly different types of) subunits with diverse specificities, attached to a scaffold. They can contain about 50 proteins and have a weight of 2-6 Megadaltons (Sandgren, 2005). Cellulosomes are usually located close to bacteria in order to facilitate uptake of the

degradation products (Schwarz, 2001). Another system is secretion of individual cellulase components to the extracellular medium (Sandgren, 2005) as filamentous fungi. Filamentous fungi have a cellulolytic system consisting of endoglucanases, exoglucanases and β-glucosidases (Kumar, 2008).

Cellulose production ability of only a small percentage of organisms have been analyzed, some of which can produce only one or two types of cellulases, while the whole set secreted in adequate amounts is necessary for complete hydrolysis into glucose (Kumar, 2008). Relatively low quantity or lack of β-glucosidase in the enzyme complexes produced by *Trichoderma* spp. is a rate limiting step due to accumulation of cellobiose which in turn causes feedback inhibition of endo- and exo- glucanases (Bisaria, 1981). Some organisms such as *Pichia stipitis*, can produce Ethanol from lignocellulose (Jeffries, 2007).

Microorganisms require their optimum physical conditions such as pH and temperature, as well as chemical factors such as carbon, nitrogen, phosphorus sources in favorable amounts for maximum production of cellulases. There are also thermophilic fungi and anaerobic bacteria species that are capable of cellulase production, such as *Sporotrichum thermophile* and *Saccharophagus degradans* (Kaur, 2004; Taylor, 2006).

2.2.1. Mode of Action of Cellulases

Hydrolysis of cellulose into glucose monomers requires the synergistic action of three types of cellulases; endoglucanase creates flanking ends that are the substrates of cellobiohydrolase; and these two enzymes together produce cellobiose or cellooligosaccharides which are hydrolyzed by β-glucosidase (Kumar, 2008).

Several physical and chemical factors may affect the hydrolysis rate of lignocellulose by cellulases, such as pH, temperature, nitrogen, phenolic compounds (Kumar, 2008). Moreover, degree of crystallinity and accessibility of the cellulose fibers are significant parameters (Arantes, 2010).

Cellulases can be classified according to their catalytic mechanisms; that is, if configuration of anomeric carbon is retained after cleavage of the substrate, the mechanism is called retaining; while, invertion of configuration from α to β or vice versa will cause designation of the mechanism as inverting (Davies, 1995).

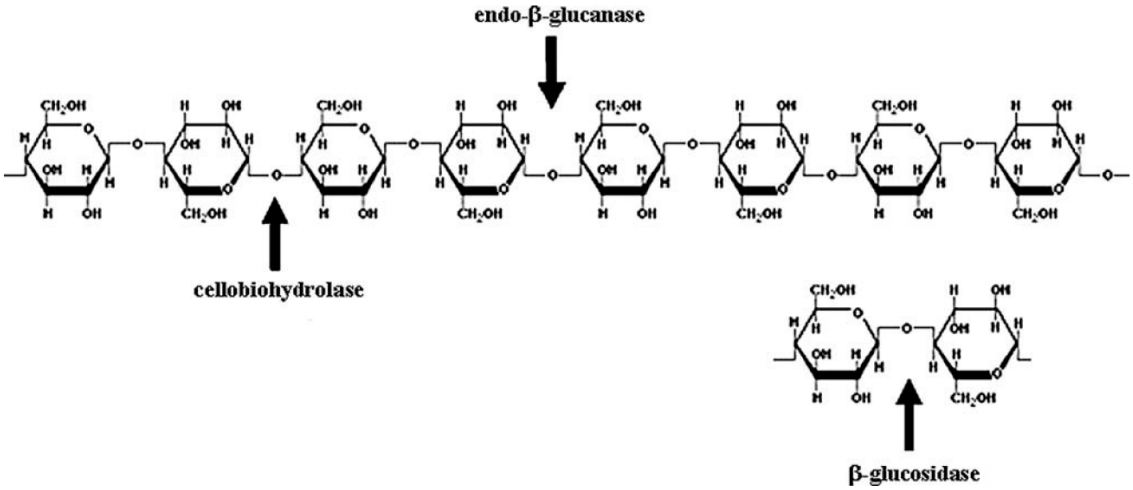


Figure 2: Hydrolysis of a cellulose fiber by cellulases. Endoglucanase randomly hydrolyzes amorphous regions. Cellobiohydrolase cuts chain ends producing cellobioses that is then digested to glucose monomers by β-glucosidase (Kumar, 2008)

2.2.2. Limitations and Solutions for Hydrolysis of Cellulose

2.2.2.1. Physical and Chemical Strategies

Structure of cellulose and applied pre-treatments can affect hydrolysis degree and rate of bioconversion of cellulose (Kumar, 2008). Crystallinity an important parameter of cellulose hydrolysis due to the fact that while amorphous regions of cellulose is accessible by hydrolytic enzymes and prone to degradation, crystalline parts could not be accessed easily, thus remain non-hydrolyzed (Cohen, 2005). Pretreatments with chemicals, such as sodium hydroxide, various acids and organic solvents, might be utilized to alleviate the inaccessibility of crystalline cellulose; yet, these procedures add to the cost of production (Martinez, 2005). Smaller particle sizes and larger accessible area can be derived by physical methods to overcome crystallinity such as milling and steam treatment (Smith, 1991; Weil, 1994). Steam explosion is a preferred method since it is 70% cheaper than other mechanical methods (Fang, 2010).

Adsorption capacity of enzymes to cellulose is yet another factor affecting hydrolysis rate of polymers; surface area and concentration of cellulose as well as pH

and temperature affect adsorption, thus bioconversion rate (Juhasz, 2004; Lambert, 2003). Optimum values for adsorption can be investigated for specific applications.

Systems can be designed that will recover the used enzymes from the environment/reactor (Bansal, 2009). Overall, three steps are essential for inexpensive and efficient conversion of biomass into fermentable sugar; size reduction, pre-treatment and hydrolysis (Zhang, 2006).

2.2.2.2. Molecular Strategies

Since cellulase production is regulated by several genetic and chemical factors, such as end product inhibition and induction; various strategies including strain improvement by mutagenesis or physical and chemical techniques are employed to improve the enzyme yield (Kumar, 2008).

Co-cultivation of microorganisms complementing each other's cellulase expression profiles has proven to be effective with some strains on various cellulosic substrates because each strain is having a rate limiting component when cultured alone (Kumar, 2008; Klyosov, 1986). When *T.reesei* and *A.niger* was cultivated together (after adjusting their delay time and ratios), cellulase production was improved (Fang, 2010) probably due to the complementary cellulolytic systems of two strains. That is, *T.reesei* is a good producer of endo- and exo- glucanases but poor in β-glucosidase production while *A.niger* is just the opposite, which allowed optimum utilization of the carbon source by compensating each other's deficiency. Bioreactors may be built that are optimized for one or a few organisms (Kumar, 2008). Finding different cellulases from new organisms by cloning and sequencing (Kumar, 2008) may facilitate finding new enzymes that are suitable for a particular demand.

Metabolic engineering strategies and mutagenesis techniques to produce strains which are unresponsive to end-product inhibition have been evaluated (Kumar, 2008). Alternative to traditional random mutagenesis and selection techniques, taking control over the cellulase inducing or repressing pathways would be more efficient (Kubicek, 2009). One another strategy is to increase gene dosage for enhanced gene expression which was proven to be effective in *A.niger*, yet, up to 20 copies (Archer, 1997). Expressing the gene at an active locus in the genome can also increase the yield.

Increased activity and stability in addition to efficient production of enzymes are the main goals to decrease the cost of production of enzymes (Kirk, 2002). Genetic manipulation of enzymes to change their pH-temperature optimum, stability, activity and substrate specificity is being implemented to design enzymes for targeted applications (Katahira, 2006; Hong, 2007). Fusion of target protein to 3' end of a homologous protein or a part of it might be effective for some heterologous expressions (Archer & Peberdy, 1997); the signal sequence can be cleaved by proteases later depending on the application.

Fungal proteins that are translated directly into the endoplasmic reticulum (ER) lumen are then translocated with vesicles either to other intracellular targets or to the cell membrane for secretion. Protein modifications such as cleavage of signal sequence, folding, disulfide bond formation and glycosylation take place during this process. Glycosylation of proteins are thought to contribute to thermal stability and thought to have a role in proper folding of proteins (Archer & Pebedry, 1997). Intervention with one or more of these steps can contribute to enzyme investigation further.

2.2.3. Use of Cellulases

Utilization of enzymes for production of foods such as cheese, wine, vinegar; and goods such as linen and leather has an ancient history. Although it was difficult to recover pure enzymes from the mixtures secreted by microorganisms or extracted from fruits and animal secretions in old times, strain improvement and large-scale fermentations facilitated obtaining purer and well-defined enzyme preparations nowadays, especially introduction of recombinant gene technology and protein engineering strategies allowed production of targeted enzymes (Kirk, 2002).

Cellulases and amylases constitute the second widespread group of enzymes used for industrial applications such as detergent, textile, pulp and paper industries for de-inking and refining (Kirk, 2002). The enzymes are used either as a mixture or individually depending on the aim (Becker, 2001). Pectinases and cellulases are used for softening and clarification of drinks. During animal feed production, cellulases together with other glycoside hydrolases are used to improve digestibility. In textile and detergent industries, cellulases are used for different applications such as creating stonewashed effect on jeans or depilling of textile surfaces (Sandgren, 2005).

2.2.4. Structural Features of Cellulases

Like most of the carbohydrate degrading enzymes, cellulases usually possess separately folded carbohydrate-binding modules (CBM) and catalytic domains (CD) (Arantes, 2010). CBMs have several functions that include increasing enzyme concentration on the surface, contributing to specificity and interruption of crystalline structure of fibers (Arantes, 2010). CBMs usually facilitate binding of enzymes to surface of crystalline cellulose, yet they do not have much effect on soluble substrates (Sandgren, 2005).

Although different cellulases might consist of completely diverse folds, they have common properties such as a substrate binding groove that can bind the sugar chain minimum 2 glucose units before and after the catalytic site (Sandgren, 2005). Binding clefts of endoglucanases usually are open, while cellobiohydrolases have tunnel-like clefts formed by loops that individual cellulose fibers can pass through (Figure 4) (Sandgren, 2005).

Inverting cellulases have two carboxylates acting as acid and base; while retaining enzymes again have two carboxylates acting as nucleophile and acid/base at their catalytic sites (Okada, 2000).

2.3. *Trichoderma reesei*

Trichoderma is a filamentous genus of fungi, belonging to *Ascomycota*, that can grow on various types of soils in all latitudes (agricultural, forest, desert) utilizing wood and plant material (Kubicek, 2003). In general, *Trichoderma* spp. grow well at 25-30 °C, but will not grow above 35 °C. Conidia (also called as conidiospores) appear after about one week on rich media and can be green, yellow or white. Some strains of *Trichoderma* are used as biocontrol agents to fight against unwanted species via competition (Harman, 2006).

Trichoderma reesei is an anamorph (asexually reproducing clonal line) derived from *Hypocrea jecorina* (Schmoll, 2009), with a genome size of 33 Megabases organized in seven chromosomes (Samuels, 2010). When its morphology is examined, phialides (small branches of mycelia), conidiophores (branched structures carrying conidia) and conidia (spores for reproduction) can be observed (Figure 3 and Figure 15-F, Figure 16). Color of the PDA medium will turn to yellow after being occupied by mycelia, usually in 3-4 days (See Figure 15-A, B, C).

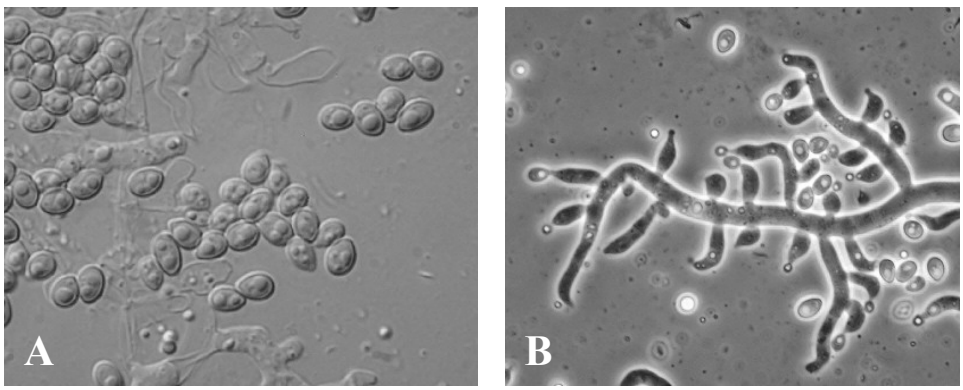


Figure 3: Conidia (A) and phialides (B) of *T.reesei* (Samuels, 2010).

First identified *T.reesei* strain was QM6a, from which several mutant strains were derived (Kubicek, 2009). It is an aerobic, filamentous, saprophytic fungus which is capable of secreting a complete set of cellulases in large amounts to degrade crystalline cellulose (Sandgren, 2005). It has an optimized mechanism to equilibrate the energy consumed according to the amount of accessible energy as substrates, which is entailed

upon its natural habitat comprising cellulose and hemicellulose. Interestingly, *T.reesei* genome has relatively low number of cellulose genes compared to close species, yet these genes are sometimes located close to the second metabolite managing genes, giving it an advantage to regulate expression effectively (Schmoll, 2008).

T.reesei have been used in several industrial fields mentioned before for a long time owing to its ability to secrete enzymes in large amounts that can hydrolyze plant polymers; which is why this species is called as "industrial workhorse" (Kubicek, 2009). Some industrial strains of *T.reesei* (CL847 i.e.) can secrete up to 40 g/L total protein (Verbeke, 2009).

2.3.1. *T.reesei* Cellulases

T.reesei can secrete a complete set of cellulases. Three enzymes used in this study are given in Table 1.

Table 1
T.reesei cellulases expressed in this study

Enzyme	Gene	Protein	EC Number	UniProt ID
Endoglucanase I	*egl1* / *cel7b*	EG1 / Cel7B	EC 3.2.1.4	P07981
Endoglucanase III	*egl3* / *cel12a*	EG3 / Cel12A	EC 3.2.1.4	O00095
Cellobiohydrolase I	*cbh1* / *cel7a*	CBH1 / Cel7A	EC 3.2.1.91	P62694

2.3.1.1. Cellobiohydrolases

T.reesei has two Cellobiohydrolase genes (*cel7a* and *cel6a*) encoding CBH1 and CBH2, belonging to glycoside hydrolase (GH) families 7 and 6 respectively (Kubicek, 2009). CBH1 constitutes 40-50 percent of total secreted protein by *T.reesei* (Sandgren, 2005). Both proteins have a carbohydrate-binding module (CBM) (CBH1, at carboxy-terminus; CBH2 at amino-terminus (Sandgren, 2005)), a catalytic domain (CD) and a heavily O-glycosylated linker peptide connecting these two domains. Cellulose chain is

hydrolyzed in its reducing end by CBH1 and in non-reducing end by CBH2 (Yui, 2010).

The procedure is as follows; adsorption of the enzyme to the substrate surface is followed by separation of a single cellulose chain end from the crystal structure and threading into the catalytic domain; after hydrolysis by catalytic residues cellobiose is extruded and the enzyme continues to move throughout the chain (Gregg, 2010; Mélanie, 2010). Crystal structure of Cel7A shows presence of minimum seven substrate-binding and two product-binding sites (Gruno, 2003). Deletion of CBM experiments revealed that it is needed for binding to and effective hydrolysis of crystalline cellulose (Gregg, 2010). Tunnel-like shape of active site is probably responsible for the higher stability and progressive movement of CBH1 on celloluse when compared to its homologue EG1 (Figure 4).

Calculated mass of CBH1 is 54,073 kDa (UniProt, 2010); yet, when glycosylated it is above 70 kDa. CBH1 has 4 potential glycosylation sites and 12 disulfide bonds; and is fully glycosylated in minimal media with low pH. Deglycosylation is observed in other conditions due to the presence of mannosidases and glucosidases. The linker peptide is O-glycosylated while N-glycosylation is seen in the core domain (Stals, 2004).

2.3.1.2. Endoglucanases

Eight endo-β-1,4-glucanases (EG) of *T.reesei* are identified up to now; Cel5A, Cel5B, Cel7B, Cel12A, Cel45A, Cel61A and Cel61B, Cel74A.

2.3.1.2.1. Endoglucanase I (Cel7B)

EG1, which belongs to family 7 glycoside hydrolases, is encoded by the gene *cel7b* (Kubicek, 2009) and is the major endoglucanase of *T.reesei* making up 5-10 percent of total secreted proteins (Sandgren, 2005). Calculated mass of EG1 is 48,208 kDa; it has 5 potential glycosylation sites and 8 disulfide bonds (Uniprot, 2010; PDBSum, 2009). EG1 has a CBM at the carboxy-terminus and has four sugar binding sites in the catalytic region. It has maximum activity at 50 °C (Becker, 2001) at pH 4-5;

and has a pI of 4.5. Glu197 is the nucleophile responsible for its catalytic activity and Glu202 is the acidic/basic residue (Kleywegt, 1997).

EG1 has an open active site in contrary to the tunnel-like shape of the homologous exoglucanase CBH1 (Figure 4); which makes it an endoglucanase that is able to cut mid-chains. The two proteins have a high sequence identity (45%) and they belong to the same family (Penttilä, 1986). EG1 is very active on soluble celluloses yet, it is slow on crystalline substrates (Henrissat & Bairoch, 1993).

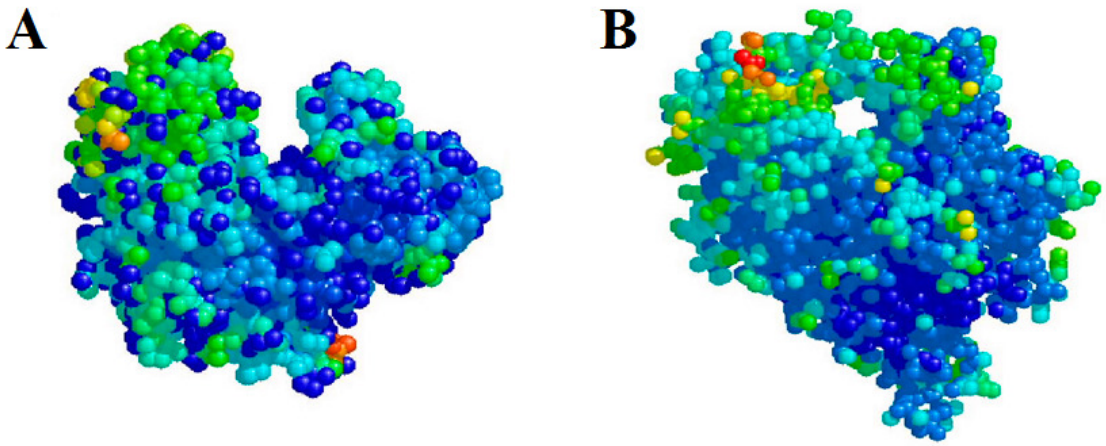


Figure 4: General structures of endoglucanases (A) and exoglucanases (B) (Bayer, 2010). Note the open active site of endoglucanase and tunnel-like active site of exoglucanase.

2.3.1.2.2. Endoglucanase III (Cel12A)

T.reesei EG3 (Cel12A) is the first identified member of family 12 glycoside hydrolases (Kubicek, 2009). It is usually not glycosylated and accounts for less than 1 percent of the total proteins secreted from *T.reesei* (Sandgren, 2005). EG3 protein has a weight of 25 kDa, pI of 7.5 and maximum activity at pH 5 at 50 °C (Karlsson, 2002). EG3 has lower affinity to cellulose substrates than other cellulases probably due to lack of a CBM (like Cel5B and Cel61B).

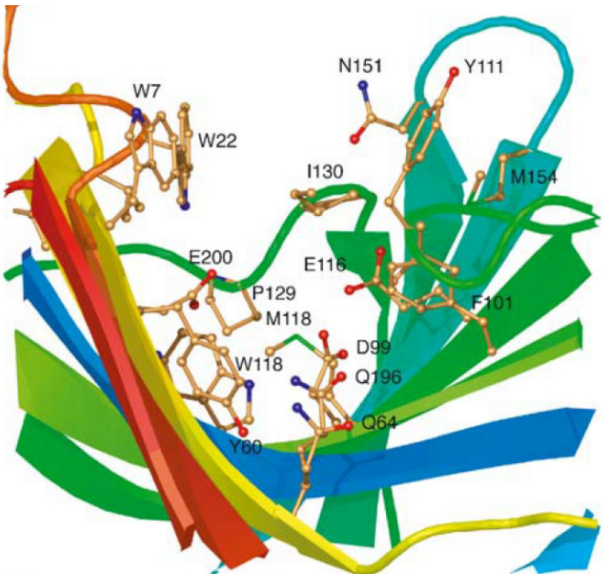


Figure 5: Active site and some important residues of T.reesei Cel12A. Catalytic residues E116 and E200 can be seen (Sandgren, 2005).

Cel12A is composed of 15 β-strands which fold into two anti-parallel β-sheets, twisting on top of each other. A single helix is present in the enzyme and only one disulfide bond is formed; between Cys 4 and Cys 32. N-terminus of the enzyme is cyclized to increase its resistance to proteolytic degradation and Asparagine 164 residue is glycosylated (Bower, 1998). As a retaining enzyme, two glutamic acid residues are necessary for its catalytic action; E116 as nucleophile and E200 as acid/base (See Figure 5) (Okada, 2000).

2.3.1.3. β-glucosidase

It is found out that *T.reesei* expresses intracellular (Saloheimo, 2002), extracellular (Fowler, 1992), membrane-bound (Umile, 1986) and cell wall-bound (Messner, 1990) β-glucosidases (Kubicek, 2009). As mentioned before, they catalyze the hydrolysis of cellobiose or cellooligosaccharides to glucose.

β-glucosidase usually acts as a rate limiter of cellulose hydrolysis due to lower production amount compared to other cellulases, although seven β-glucosidases are present in *T.reesei* genome (Kubicek, 2009). This causes accumulation of cellobiose

which in turn inhibits expression of enzymes (endo- and exo-glucanases) that produce cellobiose (end product inhibition).

2.3.2. Regulation of *T.reesei* Cellulases Expression

Cellulases are not expressed constitutively but their expression is regulated exquisitely, only some carbon sources, such as cellulose, sophorose and lactose can induce expression of cellulases; and monosaccharides inhibit cellulase expression, like glucose (Sandgren, 2005).

How cellulose can induce expression is an important point of interest since cellulose is an insoluble molecule and cannot diffuse into cells. There are a few theories on this, one of which is secretion of minute amount of enzymes, such as Cel5B, constitutively and upon encountering a substrate, release of oligosaccharides induce further expression (Kubicek, 2009) Basal expression of EG1 and CBH1 in uninduced cells was also shown (Carlos, 1997). Another suggestion and experimental fact is presence of anchored enzymes on conidial surface, such as Cel6A; since growth of conidia are halted after removal of enzyme activity on the surface by non-ionic detergents, yet growth was not affected negatively when respective deletion strains were grown on lactose. In both theories, basal cellulase activity produces small molecules that in turn induce expression of more enzymes (Kubicek, 2009).

It is also noteworthy that expression of most of the cellulases is proportional to each other (Ilman, 1997) except some hypercellulolytic mutants (Foreman, 2003), which supports co-regulation of them (Sandgren, 2005).

Cellulase expression in *T.reesei* is regulated at the transcriptional level. Depending on the carbon source that the fungi grow on, different inducers such as XYR1 and ACE2 that can bind to the same motif, and HAP2/3/5 complex that binds to CCAAT motif in the cellulase promoters affect cellulase expression positively. On the other hand, ACE1 and CRE1 are cellulase repressors. Carbon catabolite repression by glucose is known to depend on the Cys2His2 transcription factor CRE1 (Kubicek, 2009).

Light is another factor that regulates expression of some cellulases. For instance, *cel7a* gene has a higher transcription rate under constant light when compared to constant darkness. ENVOY and GNA1/3 proteins are thought to be involved in light perception (Kubicek, 2009).

2.3.3. Expression of T.reesei Cellulases in Other Systems

For the last 20 years *T.reesei* cellulases have been expressed in different species some of which are not naturally cellulase producers, such as *E.coli*, *S.cerevisiae*, *S.pombe*, *Aspergillus* spp. (Nakazawa, 2008**).** In several cases, yields were low due to inclusion body formation, proteolytic degradation and hyperglycosylation. Expression in *Aspergilli* was relatively advantageous since their transcriptional and translational mechanisms are comparable to *T.reesei* (Rose, 2002; Takashima, 1998).

Although CBH1 was detected by western blotting when expressed in *Ashbya gossypii* under *S.cerevisiae* PGK1 promoter, it had no activity towards MULAC. EG1 production with the same promoter resulted in higher amount of enzyme and specific activity. Maximum MULAC activity of EG1 was 400 μmol/min/L (1.3 nmol/min/μg secreted protein) and specific activity was 200-400 μmol/min/g dry weight, while cells were growing exponentially. 1000 μmol/min/L (2.2 nmol/min/μg secreted protein) was detected with *S.cerevisiae*. Overglycosylation compared to native *T.reesei* expression was observed when EG1 and CBH1 are expressed in *Ashbya gossypii* **(**Ribeiro, 2010).

Expression of EG1 in *Aspergillus oryzae* resulted in 59.8 U/mg and EG3 in 30.7 U/mg CMC activity. CBH1 activity was not detected (Takashima, 1998).

CBH1 was expressed in *Pichia pastoris*, with similar k_m and k_{cat} values to native CBH1, but with decreased hydrolysis rate of crystalline cellulose (70-80% of native). Produced enzyme had native-like thermostability and pH optimum. Hyperglycosylation of potential N-glycosylation sites were observed in *P.pastoris* expression, but lower than that of *S.cerevisiae* (Boer, 2000).

Full-length CBH1 could not be expressed in *E.coli* but only catalytic core domain expression could give a minute activity. Specific activities of EG1 core domain and EGIII towards CMC are stated to be estimated as 65 and 15 U/mL respectively for the *E.coli* expression (Nakazawa, 2008).

EG3 is expressed in *E.coli* using pAG9-3 vector. Although the proteins were aggregated as inclusion bodies in the cytoplasm they are later solubilized with urea and purified by chromatography. Maximum CMCase activity was measured as 58 mU/mL, at pH 5.5 with *E.coli* JM109 cells (Okada, 2000). EG3 is expressed by *Aspergillus niger* in hyperglycosylated form (Berka & Barnett, 1989).

Many other bacterial and fungal cellulases have been cloned to *E.coli* recently, in addition to successful expression of a number of cellulases in different bacteria and fungi such as *P.fluorescens*, *P.crysogenum* and yeast (Hong, 2007; Hou, 2007; Li, 2006).

2.3.4. *Trichoderma reesei* as an Expression System

Heterologous proteins were expressed in *T.reesei* previously; a few samples are given below:

- Bovine chymosin cDNA was expressed in T.reesei, between *cbh1* promoter and terminator, up to 40 mg/L. Chymosin was active and had same size with the native enzyme (Harkki, 1989).
- cDNA of Glucoseamylase P enzyme from *Hormoconis resinae* (fungus) was expressed in *T.reesei* under *cbh1* promoter. Although different sizes of enzyme were observed due to glycosylation, up to 700 mg/L active enzyme could be produced; that is 20 times higher than the *H.resinae* (Joutsjoki, 1993).
- When chromosomal gene and cDNA of Ligninolytic laccase enzyme of *Phlebia radiate* (fungus) was expressed under *cbh1* promoter, 20 mg/L active enzyme was obtained with similar weight (Saloheimo, 1991).

In addition to successful expression of several heterologous proteins in *T.reesei*, there are other advantages as well making this fungus an attractive host:

- It can be cultured in fermenters of sizes up to 230 m^3 using cheap carbon sources such as plant waste (Penttilä, 1998); that is an indication of its compatibility with fermentation conditions and resistance to contamination.
- Secretory machinery of *T.reesei* is very close to typical eukaryotic ones (Kruszewska, 1998), which brings it to a superior position than some other microorganisms like bacteria.
- It is non-pathogenic to healthy people under enzyme production conditions and does not produce antibiotics or toxins (Nevalainen, 1994).
- *Trichoderma* reesei is a natural hyperproducer strain (can secrete up to 40 g/L protein).
- Some industrial strains were already developed for improved production and lower protease activity (Mäntylä, 1998).

2.4. Methodological Background

2.4.1. Methods to Measure Cellulase Activities *in vitro*

There are several techniques to measure cellulase activities *in vitro*; total cellulase assays such as Filter Paper Activity (FPA) assay; as well as assays for individual cellulases are present, such as Cellobiose Assay (β-glucosidase), Carboxymethyl cellulose Assay (endoglucanase) and Avicel Assay (cellobiohydrolase). FPA Assay measures release of a certain amount of glucose from a certain amount of filter paper strip in defined conditions; while availability and susceptibility of substrate to hydrolysis makes this assay attractive, its non-linearity and susceptibility to operator errors are disadvantages (Zhang, 2009). Fluorescent substrates are also used that are more sensitive to cellulase activities, such as 4-methylumbelliferones.

2.4.1.1. 4-Methlumbelliferone Substrates

Among different activity assays, 4-Methylumbelliferyl-β-D glycosides offer a sensitive means to determine cellulase activities linearly. 4-Methlumbelliferly-β-D-cellobioside/-lactoside (abbreviated later as MUC and MULAC, respectively) release the fluorescent component methylumbelliferone when hydrolysed (Bailey & Tähtiharju, 2003). Their formula is $C_{22}H_{28}O_{13}$ with a molecular weight of 500.45 (Sigma-Aldrich). CBH1 shows activity on MULAC, yielding only lactose and phenol as products (Tilbeurgh, 1982). EG1 can as well liberate phenol from MUC (Claeyssens, 1992). Molecular structures of MUC and MULAC can be seen in Figure 6.

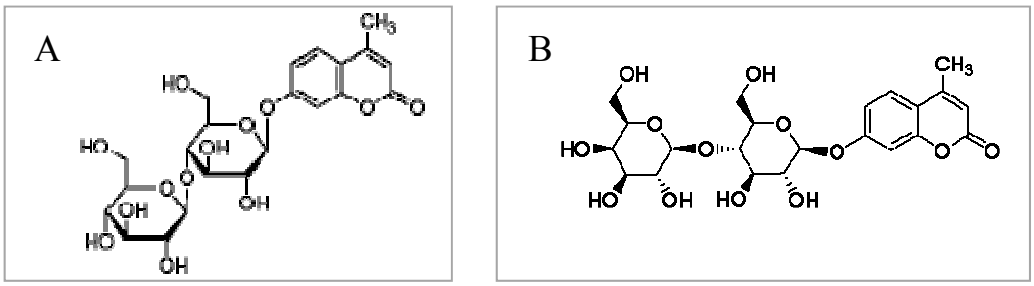


Figure 6: 4-Methlumbelliferly-β-D -cellobioside (A) and -lactopyranoside (B) (Sigma-Aldrich)

2.4.1.2. CMCase Assay

Carboxymethyl cellulose is a water-soluble viscous cellulose derivative. As an anionic substance, properties of CMC can change depending on the pH (Zhang, 2009). Since endoglucanases show higher activity towards water-soluble CMC, it is used to assay their activities; however, CMCase activity is non-linear. Since activity towards neither MUC nor MULAC was detectable with the conditions used for EG1 and CBH1, CMC assay was preferred for EG3 (Ghose, 1987).

CMCase activity is calculated by determining the enzyme amount needed to release a constant amount of glucose. 1 IU CMC is defined as 1 μmol min^{-1} reducing sugar liberation (Ghose, 1987). Three different dilutions of the enzyme is done to be able to determine the enzyme amount necessary to release 0.5 mg glucose in the reaction conditions (detailed description is present in methods). EDR is the dilution rate of the enzyme releasing 0.5 mg glucose. 0.185/EDR value gives the CMCase activity in IU/mL units.

2.4.2. *T.reesei* Strains and Phenotypes

QM9414: This strain is obtained by a two-step mutational procedure from QM6a (first isolated *T.reesei* strain) and can produce up to 4 times more cellulase than QM6a (Montenecourt, 1977). It is often called as wild-type strain.

Δxyr1: XYR1 (xylanase regulator 1) is a transcriptional regulator of *xyn1*, *xyn2* (xylanases), *cbh1*, *cbh2* and *egl1* genes regardless of inducer molecules. XYR1 is a zinc binuclear cluster protein that binds to GGCTAA motif in the *xyn1* promoter (Stricker, 2006). All inducible *T.reesei* cellulase promoters were found to contain consensus sequences for XYR1 binding. The deletion strain Δxyr1 is unable to induce cellulase production and grow on cellulose or sophorose (Kubicek, 2009). Δxyr1 strain grows and sporulates same as its parental strain QM9414 on low molecular weight carbon sources except D-xylose (Stricker, 2006).

Rut-C30: This hypercelluloytic strain can escape from carbon catabolite repression caused by glucose. It is a mutant of QM6a and has a truncated *cre1* gene

(Ilmén, 1996). However, the strain needs an inducer for overproduction of cellulases (Kubicek, 2009).

2.4.3. Expression Vectors and Promoters

pPtef1: This plasmid is modified from pUC19; it has a Hygromycin B Phosphotransferase gene (hph) and promoter of Translation Elongation Factor 1-α inserted in between XhoI and ClaI sites that is followed by a multiple cloning site (APPENDIX E). After transformation, the vector can integrate into several locations of the the genome as multiple copies (Joutzjoki, 1993). Hygromycin B is an antibiotic that kills bacteria, fungi and eukaryotic cells by inhibiting protein synthesis (Pittenger, 1953). The cells that possess hph enzyme (also called as Hygromycin B kinase) are resistant to Hygromycin B since they can convert it to 7"-O-phosphohygromycin (Zalacain, 1987). ***tef1*** **Promoter:** Translation Elongation Factor 1-α helps entry of the aminoacyl tRNA into a free site of the ribosome during translation and its promoter (called Ptef1 throughout this thesis) is known to be derepressed on glucose medium (Nakari, 1993)

pPcdna1: This plasmid was obtained by replacing the *tef1* promoter in pPtef1 with cDNA1 promoter via the XhoI-ClaI sites. ***cdna1*** **promoter:** *cdna1* is an unknown gene but *cdna1* promoter was previously found to be highly active on glucose-containing media (Nakari, 1993; Nakari-Setälä, 1995) (up to 50-fold of Ptef1). *cdna1* promoter is found in scaffold 23:43726-44652 of *T.reesei* genome (Dubchak, 2006) preceding a high number of Expressed Sequence Tags.

3. PURPOSE OF THE STUDY

Cellulases have been of important industrial importance due to their ability to catalyze hydrolysis of lignocellulosic materials into their monomers. *Trichoderma reesei* is considered as the workhorse of such applications owing to its ability to secrete a complete set of cellulases in large quantities naturally on a variety of lignocellulosic substrates, which is advantageous for large scale production facilities. Although various physical, chemical and molecular techniques have been applied to increase cellulase production of *T.reesei* further; inexpensive hyperproduction of proteins might not be sufficient since diverse applications may require single or specific combinations of enzymes rather than a complete set; which necessitates purification of them -a costly process-. Combination of enzymes after separate production is much economical than purification from a mixture, especially for large scale utilization.

In this study, we evaluate the potential of *Trichoderma reesei* as an expression system for production of individual cellulases. Homologous expression of the enzymes EG1, EG3 and CBH1 under two different strong promoters in glucose, Ptef1 and Pcdna1, in cellulase-deficient *T.reesei* strain Δxyr1 was studied. For this purpose, transformation vectors were constructed with either promoter followed by a cellulase gene that was amplified from genomic DNA of wild type strain QM9414. After transformation and selection of antibiotic-resistant strains, protein expression is done in minimal medium using glucose as the carbon source, to confirm production of cellulases. Supernatants from expression cultures were analyzed with SDS-PAGE for enzyme presence. Activities of supernatants towards fluorogenic substrates or carboxymethyl cellulose were assayed, and strains were compared according to their expression efficiencies.

4. MATERIALS AND METHODS

4.1. Materials

4.1.1. Chemicals

4.1.1.1. General Chemicals

All the chemicals used are listed in Appedix K.

4.1.1.2. Enzymes

All the enzymes used are listed in Appedix J.

4.1.1.3. Buffers and Solutions

2% CMC in 50 mM NaOAc Buffer: 2% CMC (w/v) in 50 mM NaOAc Buffer; dissolved by stirring and heating to 50-60 °C.

3M NaOAc Buffer (pH 5.2): 24.6 g NaOAc is dissolved in 50 mL PCR water, pH is adjusted to 5.2 with Acetic Acid and the volume is completed to 100 mL.

6X Laemmli Buffer: 6X Laemmli Buffer was prepared according to the protocol described in *Molecular Cloning: A Laboratory Manual, Sambrook et.al, 2001.*

50 mM NaOAc Buffer (pH 4.8): 50 mM NaOAc in ddH_2O; pH is adjusted to 4.8 with Acetic Acid.

Coomassie Staining Solution: 8 g Ammonium Sulfate is dissolved in 80 mL 2% Phosphoric Acid Solution, stirring. 1.6 mL 5% Coomassie G-250 Solution (w/v, in ddH_2O) is added and mixed well. This solution can be kept at RT. 20 mL MetOH is added just before use, and the solution is then kept at +4 °C. Final concentrations: 0.08% Coomassie G-250, 1.6% Phosphoric Acid, 8% Ammonium Sulfate, 20% MetOH.

DNS Reagent: 0.53 g 3,5-Dinitrosalicylic acid and 0.99 g NaOH are dissolved in ddH_2O. 18 g Rochelle Salts, 0.38 mL Phenol, 0.415 g Sodium metabisulfite are added and dissolved stirring. The reagent is kept at +4 °C.

MiniPrep Buffers

Buffer 1: 50mM Tris (pH 8.0), 10 mM EDTA, 100 µg/mL RNase A; stored at 2-8 °C after RNase addition. **Buffer 2:** 200 mM NaOH, 1% SDS; stored at RT. **Buffer 3:** 3 M Potassium Acetate (pH 5.5); stored at 2-8 °C or RT.

Physiological Salt Solution: 0.8 % (w/v) Sodium Chloride and 0.05 % (w/v) Tween 80 in ddH_2O; autoclaved before using.

Solutions for T.reesei transformation

- **Tris-HCl (1M, pH 7.5):** 1M Tris base in ddH_2O; pH is adjusted to 7.5 with Hydrochloric acid.
- **Solution A:** 1.2 M Sorbitol and 0.1 M Potassium dihydrogen Phosphate in ddH_2O; pH is adjusted to 5.6 with Potassium Hydroxide; autoclaved.
- **Solution B:** 1 M Sorbitol, 50 mM Calcium Chloride dihydrate and 10 mM Tris-HCl (1M, pH 7.5) in ddH_2O; pH is adjusted to 7.5 with Hydrochloric acid; autoclaved.
- **PEG Solution:** 25% PEG 6000 (w/v), 50 mM Calcium Chloride dehydrate, 10 mM Tris-HCl (from 1M Tris, pH 7.5 solution); pH is adjusted to 7.5 with Hydrochloric acid; autoclaved.

4.1.2. Molecular Biology Kits

Molecular biology kits are listed in Appendix M.

4.1.3. Growth Media

Bottom Medium: 30 g/L Malt Extract, 15 g/L Agar Agar, 182.17 g/L (1M) Sorbitol in ddH_2O. After autoclaving and cooling down to 50 °C, Hygromycin B is added to final concentration of 50 μg/mL and poured into plates, slightly thinner than usual agar plates.

Lysogeny Broth (LB) Medium / LB Agar Medium: LB Medium: 10 g/L Pepton, 5 g/L Yeast Extract, 10 g/L NaCl in ddH_2O. 15 g/L Agar Agar is included to obtain LB Agar medium. Ampicillin in 50% EtOH is added to autoclaved media after cooling down to 50-60 °C to a final concentration of 100 μg/mL to obtain LB-Amp medium and LB-Amp agar plates.

MEX medium: MEX medium: 30g/L Malt Extract in ddH_2O. MEX Plates: 30g/L Malt Extract and 20 g/L Agar Agar in ddH_2O.

MEX-Cellophane Plates: 8-10 cellophane discs with size of petri dishes are cut and placed on MEX plates using two sterile forceps. With the help of a Drigalski spatula, cellophanes are smoothened preventing air bubbles.

Overlay Medium: Same as bottom medium, except Agar Agar is replaced by 15 g/L Agar Noble. After addition of Hygromycin B, 4-5 mL aliquots in culture tubes are kept in 48-50 °C water bath.

PDA Plates: 39 g/L PDA in ddH_2O. **PDA-TritonX plates**: After autoclaving PDA medium, Hygromycin B and TritonX-100 are added to final concentrations of 50 μg/mL and 0.1% (v/v) respectively.

4.1.4. Strains

JM109 competent bacteria and *T.reesei* strains QM9414, Δxyr1 and Rut-C30 were kindly provided by Molecular Biotechnology Group, Technical University of Wien, Austria.

Transformants obtained were named as "Promoter – Gene Name – Strain Number" and promoter name is also abbreviated occasionally. Hence, strain names such as Ptef1-egl1-1, tef1-egl1-1, t-egl1-1 specify the same transformant. Δxyr1 strain is sometimes written as Δxyr.

4.1.5. Vectors and Genomic DNA

pPtef1 vector and QM9414 genomic DNA were kindly provided by Molecular Biotechnology Group, Technical University of Wien, Austria. (See Appendices E and G for sequence and map of the vector).

4.1.6. Primers

Primers were purchased from Sigma-Aldrich and described in 4.2.2.1.1 in detail.

4.1.7. Equipment

Laboratory equipments used are listed in Appendix N.

4.1.8. Software

Quantity One Basic (v 4.6.9) is used to take gel photos and calculate DNA concentrations.

GeneRunner (v 3.05) is used to design and analyze primers and vectors.

BVTech Plasmid (v 4.1) is used to draw vectors.

SoftMax Pro 4.3 is used to run and analyze the fluorogenic assays.

4.1.9. Unlisted Materials

Glass Wool Eppendorfs and Glass Wool Funnels

Eppendorfs: Bottoms of the eppendorf is pierced and a piece of rolled up glass wool is inserted inside. Eppendorfs are sterilized by autoclaving.

Funnels: Larger amount of glass wool is rolled up and placed inside the glass/plastic funnel close to the stem. Funnel is covered completely with aluminum foil and autoclaved.

4.2. Methods

4.2.1. General Methods

Agarose Gel

Agarose Gels were prepared with a w/v ratio of 8%, unless stated otherwise; using 1X TAE as the solvent; and contains Ethidium Bromide for visualization. TAE was prepared according to the protocol described in *Molecular Cloning: A Laboratory Manual, Sambrook et.al, 2001*.

EtOH Precipitation of DNA

1 volume of DNA was mixed with 2.5 volumes of 96% EtOH and 0.1 volume of 3M NaOAc (pH5.2), and incubated at -20 °C for at least 1 hour or at -80 for 30 min. The mixture was then centrifuged at 13200 rpm for 20 min at +4 °C. Supernatant was poured off, 800 μL 70% EtOH was added and tube was centrifuged for at least 2 minutes. After pouring off the supernatant and evaporating remaining EtOH, pellet was resuspended with PCR water.

EtOH Precipitation of Proteins

500 μL culture supernatant (w/o cells) and 1 mL 96% EtOH were mixed in a 1.5 mL eppendorf, and kept at -20 °C at least one day. Samples were pelleted at 13200 rpm, for 20 min, at 4-10 °C. Supernatant was poured off and remaining EtOH was removed with pipette. Proteins were resuspended with ddH_2O.

Ligation

Vectors and inserts were digested and purified with QIAQuick Gel Extraction Kit. Fragments were run on Agarose gel and concentrations were determined with Quantity One. Unless stated otherwise, Vector 1:3 Insert molar ratio was used for ligation with an equal volume of TaKaRa Ligation Solution I. The reaction was incubated at 16 °C for at least 1 hour or overnight.

MiniPrep

Approximately 2 mL LB-Amp was added to sterile glass/plastic culture tubes and bacterial colony picked from the plate with sterile toothpick/plastic micropipette tip was dropped into the medium. Tubes were incubated at 37 °C, shaking at 250 rpm, overnight. Next day, 1.5 mL culture was transferred into 1.5 mL eppendorfs and centrifuged at 12000 rpm for 1 min. After pouring off the supernatant, 100 µL Buffer 1 was added and tube was vortexed. 200 µL Buffer 2 was added, tube was inverted 4-6 times to mix and transferred on ice. 150 µL Buffer 3 was added, tube was inverted 4-6 times to mix and incubated on ice for 5-10 min. Tubes were centrifuged at 13000-15000 rpm, at +4 °C, 10 min. Supernatant was transferred to 1.5 mL eppendorfs containing 900 µL 96% EtOH, and inverted 2-3 times. Tubes were centrifuged at 13000-15000 rpm, at +4 °C, 10 min. After pouring off the supernatant, 800 µL 70% EtOH was added on pellet, centrifuged a few minutes at RT. After supernatant was poured off and remaining EtOH was taken out with pipette or evaporated; pellet was resuspended with 50 µL PCR water.

Restriction Enzyme Digestion

Reaction mixtures were prepared in 1.5 mL tubes with following final concentrations unless stated otherwise; 1X Restriction Buffer, 0.25 U/µL of each enzyme, DNA; and PCR water up to final volume. Fast enzymes of Fermentas were preferred for cloning purposes whereas conventional enzymes were used for restriction analyses. All the reactions were incubated at 37 °C.

SDS-PAGE

Gels and samples were prepared and run according to the protocol described in *Molecular Cloning: A Laboratory Manual, Sambrook et.al, 2001*. 12% resolving gel and 5% stacking gel was used for all SDS-PAGEs. Gels were run with constant current (Gel number x 15mA) using Mini-PROTEAN® Tetra Cell. Gels were stained with Coomassie Staining Solution overnight and destained with ddH_2O.

Spore Collection

PDA plate was inoculated with relevant strain placing either a few microliters of spore solution or 0.5cm x 0.5 cm agar piece cut from the stock plate containing spores to the middle of the plate. Plates were incubated at 28 °C up to two weeks for production of sufficient number of spores. Depending on spore concentration, 5-15 mL of Physiological Salt Solution was poured on the plate and spores were dislocated with a Drigalski spatula. The solution was then passed through a glass wool eppendorfs to a sterile falcon and kept at +4 °C. Spore solution can be used for transformation maximum 2 weeks and for culture inoculation up to 4-5 months after collection.

Sterilization

All liquid and agar containing media were autoclaved at 121 °C for 15 minutes after preparation, unless stated otherwise. Solid materials were either autoclaved at 121 °C for 20 minutes or sterilized in 121 °C oven for at least one day.

In all experiments including bacteria and fungi either on the bench or in the laminar flow, work was done near flame to maintain sterility.

Transformation of Competent Bacteria

50 µL or 100 µL competent bacteria were mixed with intact plasmid or ligation product. The mixture was incubated on ice for 30 min. Heat-shock was applied at 42 °C for 2 minutes and cells were incubated on ice for 2 min. Adding 400 µL LB, cells were recovered at 37 °C for 30 min. The mixture was either spread to LB-Amp-Agar Plates and incubated in 37 °C incubator overnight, or used to inoculate 50 mL LB-Amp liquid culture and incubated shaking at 250 rpm at 37 °C.

4.2.2. Transformation of Trichoderma reesei

4.2.2.1. Construction of Transformation Vectors

4.2.2.1.1. Amplification of Cellulase Genes and *cdna1* Promoter

Primers

Primers were designed to start approximately 10-20 bases before start codon and extend 400-600 bases after the start codon to include each gene's own terminator. Restriction sites indicated in Table 2 were added with each primer to the beginning and end of PCR products in order to be able to insert them into expression vectors. Egl3 reverse primer already had an internal HindIII site. *cel7b* (*egl1*) was amplified between 25th base before start codon and 560th base after stop codon; *cel12A* (*egl3*) was amplified between 10th base before start codon and 293rd base after stop codon; *cel7a* (*cbh1*) was amplified between 13th base before start codon and 474th base after stop codon.

Table 2
8 Primers designed for amplification of cellulase genes and *cdna1* promoter

Target	Target Length (bps)	Direction	Restriction site added	Primer
egl1	2110	Forward	ClaI	5' - GTTATCGATTCTTAGTCCTTCTTGTTGTCCC - 3'
		Reverse	HindIII	5' - GATAAGCTTGGTGGGAGAAGACTTTGGAC - 3'
egl3	1138	Forward	ClaI	5' - GTTATCGATTAGCGTCGCAATGAAGTTCC - 3'
		Reverse	(HindIII)	5' - GGAAAGCTTGCCGTGAGAATTGTAC - 3'
cbh1	2183	Forward	ClaI	5' - GTTATCGATTCCGGACTGCGCATCATGTATC - 3'
		Reverse	SalI	5' - CATGTCGACTTGTCTCCCTATGGGTCATTAC - 3'
Pcdna1	943	Forward	XhoI	5' - TGACTCGAGCAGACAATGATGGTAGCAGC - 3'
		Reverse	ClaI	5' - AGTATCGATGAGAGAAGTTGTTGGATTGATC - 3'

* Highlighted regions show extra bases added and underlined bases are restriction sites *

Polymerase Chain Reaction

PCR was performed using genomic DNA of *T.reesei* strain QM9414 as the template. Polymerase Chain Reactions were prepared with the following ingredients and final concentrations; 1X Green GoTaq Flexi Buffer, 2.5 mM $MgCl_2$, 0.4 mM dNTPs, 400 nM forward primer, 400 nM reverse primer, DNA, 0.025U/μL GoTaq DNA Polymerase in a total volume of 50 μL. Reactions are performed in duplicates. PCR cycles are described in Table 3. 3 μL of each PCR product was run on 8% Agarose gel (Figure 8).

Table 3

PCR cycles used for amplification of genes and Pcdna1

	egl1* / *cbh1		***egl3***		**Pcdna1**		
	Temp.	Time	Temp.	Time	Temp.	Time	Repeat number
Initialization	95 °C	90 sec	95 °C	90 sec	95 °C	90 sec	1
Denaturation	95 °C	60 sec	95 °C	60 sec	95 °C	60 sec	
Annealing	56.5 °C	60 sec	58.5 °C	60 sec	54.5 °C	60 sec	30
Elongation	72 °C	180 sec	72 °C	120 sec	72 °C	120 sec	
Final Extension	72 °C	600 sec	72 °C	600 sec	72 °C	600 sec	1

4.2.2.1.2. Construction of pPtef1- Vectors

4.2.2.1.2.1. Preparation of pPtef1 Vector

Restriction sites that are not present inside the genes were selected for insertion into the pPtef1 vector; ClaI-HindIII pair was used for *egl1* and *egl3*, ClaI-SalI pair was used for *cbh1*. XhoI-ClaI pair was used for replacement of *tef1* promoter in the gene inserted vectors with *cdna1* promoter (See Appendix G for vector maps).

Transformation of JM109 Cells with pPtef1

Transformation was done as described in 4.2.1.

MIDI-Prep of pPtef1

MIDI-Prep DNAs were obtained with PureYield™ Plasmid Midiprep System protocol. EtOH precipitation of DNAs was done after the procedure.

Digestion of pPtef1 with restriction enzymes and Purification

Two separate digestions of pPtef1 were done overnight; HindIII-ClaI digestion using Buffer Tango and ClaI-SalI digestion using Buffer Fast. Digested vectors were run on Agarose gel and purified using QIAQuick Gel Extraction Kit.

4.2.2.1.2.2. Preparation of Genes and *cdna1* Promoter

Purification of PCR Products

PCR products were cleaned-up using QIAquick PCR Purification Kit.

Amplification of PCR Products with pGEMT-Easy Vector

In order to amplify the genes and to be able to digest them properly, genes were cloned into pGEMT-Easy Vector and amplified. Digestion of MiniPrep DNAs was done to confirm the presence of insert.

Ligation: 6 μL purified PCR product and 1 μL pGEM-T Easy Vector were ligated as described before.

Transformation: JM109 cells were transformed with ligation mixture which then were spread to (IPTG and X-Gal containing) LB-Amp plates for blue-white screening.

MiniPrep: Overnight grown bacteria were held at +4 °C for 1 hour in case some colonies would turn to blue; then, 5 white colonies for each transformation (egl1, egl3, cbh1, cdna1) were selected and MiniPrep protocol was applied.

Control digestion: HindIII-ClaI digestion of pGEMT-egl1/egl3 vectors were done with Buffer Tango; SalI-ClaI digestion of pGEMT-cbh1 and XhoI-ClaI digestion of

pGEMT-cdna1 were done with Buffer Fast as described in 4.2.1, with 2 μL MiniPrep DNA in a final volume of 20 μL for 2.5 hours. All of the digestion products were run on Agarose gel (Figure 9). Two positive colonies of each vector were chosen for the next step.

Digestion of PCR products and Purification

In order to obtain sticky-ended fragments, 5 μL of each of the pGEM-T Easy vectors containing PCR fragment was digested as described in 4.2.1, in a final volume of 40 μL, overnight. All of the digestion products were run on Agarose gel which were then cut out and purified using QIAQuick Gel Extraction Kit.

4.2.2.1.2.3. Cloning of genes into pPtef1 Vector

ClaI-HindIII digested *egl1*/*egl3* genes are ligated to pPtef1 vector having same sticky ends. ClaI-SalI pair is used instead for *cbh1*. Vector-gene ligation products are used for transformation of JM109 cells and 10 colonies were selected randomly for MiniPrep. Presence of the genes in MiniPrep DNAs was checked with relevant enzyme pair (Figure 10, Figure 11). Among the positive ones, colonies 6 and 10 for egl1, 7 and 10 for egl3, 6 and 7 for cbh1 are selected for transformation of *T.reesei*.

MIDI-Prep of these 6 vectors was done which were then used for transformation of *T.reesei*.

Restriction Analyses of Ptef1- Vectors

After selection of vectors and obtaining their MIDI-Prep DNAs, restriction analyses of pPtef1- vectors were done before transformation to make sure that correct genes are in correct orientation (Figure 12). Total length of each vector is written in parentheses (bps), enzymes used for analyses and expected fragment lengths (bps) are shown after enzyme names below:

pPtef1-egl1 (7849)	XhoI: 6402, 1447	XhoI-HindIII: 5009, 1447, 1393
pPtef1-egl3 (6877)	BamHI: 3995, 2882	XhoI-HindIII: 5009, 1868
pPtef1-cbh1 (7940)	HindIII: 6667, 1269	XhoI-SalI: 5027, 2913

4.2.2.1.3. Construction of pPcdna1- Vectors

4.2.2.1.3.1. Construction of pPcdna1-egl3 and pPcdna1-cbh1

tef1 promoter of previously constructed pPtef1-egl3/cbh1 vectors were replaced with *cdna1* promoter to construct pPcdna1-egl3/cbh1 vectors. Initially, Ptef1 was extracted from vectors via XhoI-ClaI sites; remanining vector was purified by gel extraction and ligated with purified Pcdna1 prepared in 4.2.2.1.2.2. Three positive colonies of pPcdna1-egl3 were obtained; yet, pPcdna1-cbh1 vector could not be constructed due to the problems with ligation.

4.2.2.1.3.2. Construction of pPcdna1-egl1

Since *egl1* gene contains an XhoI site, pPcdna1-egl1 vector could not be constructed by replacing Ptef1 with Pcdna1 in pPtef1-egl1 vector. Instead, pPtef1 was digested with XhoI-HindIII to extract Ptef1, remaining linear vector was ligated with Pcdna1 and *egl1* simultaneously. Out of ten colonies obtained after a few trials, four were chosen and tested for accuracy of ligation.

Restriction Analyses of Pcdna1- Vectors

Following the construction of pPcdna1-egl1/egl3 vectors, XhoI-HindIII digestion of egl3 vectors; and HindIII and XhoI-HindIII digestion of egl1 vectors were done to check their accuracy (Figure 13). *cdna1* promoter has an internal HindIII restriction site and *egl1* has an XhoI (See Appendix G for plasmid maps). Total length of each vector is written in parentheses (bps), enzymes used for analyses and expected fragment lengths (bps) are shown after enzyme names below:

pPcdna1-egl1 (8038) HindIII: 5622, 2416 XhoI-HindIII: 5009, 1393, 1023, 613

pPcdna1-egl3 (7066) XhoI-HindIII: 5008, 1445, 613

4.2.2.2. Transformation of *T.reesei*

Δxyr1 was used as parental strain for all transformations. Vectors used for transformation were; pPtef-egl1, pPtef-egl3, pPtef-cbh1, pPcdna1-egl1 and pPcdna1-egl3.

Transformation has two main steps; protoplasting, which is degradation of cell walls and obtaining competent cells (protoplasts), and transformation, in which several incubations of protoplasts mixed with DNA are done.

4.2.2.2.1. Protoplasting

10-100 μL spores, depending on spore concentration, were spread to each of 8-10 MEX-cellophane plates with Drigalski spatula. Next day, after mycelia have reached to adequate density, cellophane layers were collected with forceps to an empty petri dish, adding 1.5-2 mL Protoplasting Solution between layers. Discs were incubated at 28 °C for 90 minutes. Mycelia were collected into the petri plate by removing cellophane discs one by one. Mycelia were filtered through glass wool funnel into the Corex tube or 50 mL falcon using cut tips. Cells were centrifuged in swing-out rotor at 2000 rpm, for 10 min, at +4 °C. Supernatant was poured off; cells were resuspended gently with 4 mL Solution B (+4 °C) and centrifuged at 2000 rpm, for 10 min, at +4 °C. After supernatant was poured off, cells were resuspended with 0.5-2 mL Sulution B (+4

°C) depending on protoplast amount. Protoplasts should be kept on ice and used immediately.

Protoplasting Solution: 0.075 g Lysing Enzymes from *Trichoderma harzianum* was dissolved gently in 15 mL Solution A. Sterilized with 45 µm filter.

4.2.2.2.2. Transformation Procedure

200 µL protoplast suspension, 10 µL purified DNA, 50 µL PEG Solution (+4 °C) were mixed gently and incubated on ice for 20 minutes. 2 mL PEG solution (RT) was added to this mixture and incubated at room temperature for 5 min. 4 mL Solution B (RT) was added and mixed gently. 0.2-1 mL of this solution was added to 4-5 mL aliquoted overlay medium, mixed shortly and poured on bottom medium immediately.

4.2.2.2.3. Selection and Purification of Positive Transformants

One week after transformation, colonies started to grow on MEX-Sorbitol plates. ❶ Selected colonies were passed to small (35mm) PDA plates with Hygromycin B. ❷ After they sporulate, a few microliter of spores were transferred to PDA-TritonX plates and spread for single spore selection. ❸ A single spore was taken to a small PDA plate again, to confirm stabilty of resistance. ❹ The transformant was transferred to PDA plate without antibiotic to allow growth and sporulation.

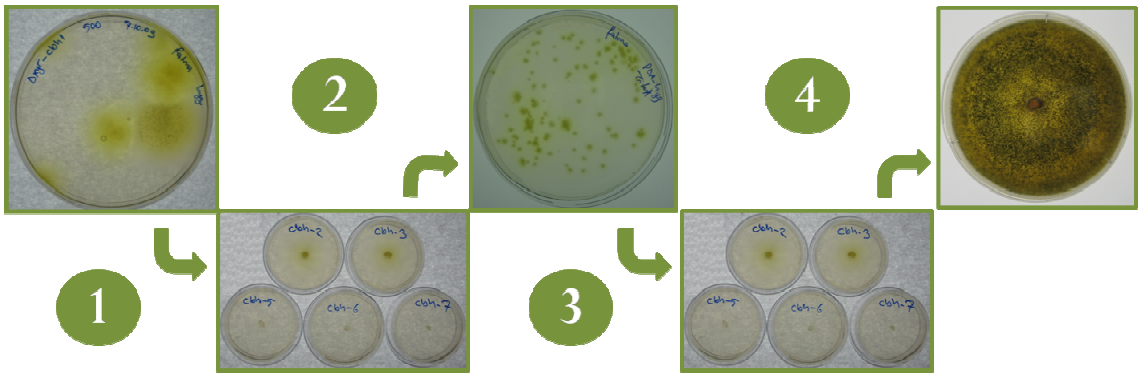


Figure 7: Purification of Hygromycin B resistant transformants.

4.2.3. Expression of Cellulases

After stabilization of Hygromycin-resistant transformants, they were expressed in MA Medium (See Appendix H). All the expression cultures are started with 2 x 10^6 spores/mL and glucose is used as the sugar source.

QM9414 expression on cellulose: QM9414 (2 x 10^6 spores/mL) was pregrown on MA Medium with Glycerol as the sugar source for 18 hours. Mycelia were pelleted at 2000 g, at +4 °C, 10 min and washed once with ddH_2O. MA Medium with cellulose as the carbon source was inoculated with these mycelia.

4.2.3.1. Protein Expression in T.reesei

Strains were grown on PDA plates and spores were collected after 10-14 days. Spore count was determined either with Turbidity Meter or Hemocytometer. All cultures were started with a total medium volume of 250 mL and with a concentration of 2 x 10^6 spores/mL. Glucose and spores were added to the medium simultaneously just before starting cultures. Flasks were incubated shaking with 250 rpm, at 28 °C. Samples were taken at 0^{th}, 20^{th}, 28^{th}, 36^{th}, 44^{th}, 52^{nd} and 72^{nd} hours after inoculation and placed on ice immediately to prevent proteolytic degradation.

Exactly 20 mL sample was taken for each strain at the specified time-point, in order to be able to determine dry weight accurately. Samples were centrifuged at 5000 g for 10-20 min, at +4 °C. Supernatants were aliquoted to 2 mL eppendorfs and kept at -20 °C. Pellet was washed to get rid of salts by resuspending with 40 mL ddH_2O and centrifuged at 5000 g for 10-20 min, at +4 °C. Pellet was dried in 70 °C oven for 1 week or more -until complete evaporation of water- and weighed with precision balance.

4.2.3.2. Coffee Filters as Shake Flask Closures

Coffee filters were assessed in terms of oxygen permeability and sterility. Shake-flask cultures were done with stitched cotton pad-type closures, coffee filters and coffee-filter closed empty medium (not inoculated with spores) for 52 hours. Dry

weights of cultured strains were determined (Figure 17). Sterility of empty medium was checked with 8-hour intervals under microscope.

4.2.3.3. Comparison of Smooth and Baffled Flasks

Since baffled flask is known to provide more oxygen to the culture, smooth and baffled flasks were compared for *T.reesei* shake-flask cultures by growing same strain in both types of flasks simultaneously (Figure 18).

4.2.3.4. Extra Sugar Addition

Based on the fact that glucose is used as both sugar source and expression inducer; in order to see whether exhaustion of sugar is a limiting factor for growth rate and protein expression, extra 10 g/L glucose is added to the expression medium of the best producer strain, c-egl1-6, at 20^{th} hour. Smooth and baffled flask comparison together with sugar addition was done (Figure 22, Figure 26).

4.2.4. Analysis of Expression

Almost all of the expressions were done in duplicates at different times, but only single copies of data is shown in this thesis as a representative of other data, which was pretty similar.

4.2.4.1. Growth Rates

Growth rates were calculated and compared by measuring dry weights as described in (Figure 20).

4.2.4.2. SDS Gel Analysis

Initially, supernatants were precipitated with EtOH and concentrated 10-fold to check the presence of cellulases on the gel (data not shown). After detection of producing strains, supernatants of these were run with respect to time to see the course of protein expression and to compare strains (Figure 21, Figure 23, Figure 24). Different amount of supernatants were used for each strain to prevent over/under-loading of wells depending on the protein amount.

4.2.4.3. Activity Assays

4.2.4.3.1. Fluorogenic Substrates

4-Methylumbelliferyl-β-D-Cellobioside/Lactoside were dissolved in DMSO. Activity assays were done with 250 μM MUC/MULAC (50 nmol/well), 50 mM NaOAc buffer (pH 5.0) and 50 μL supernatant in a reaction volume of 200 μL/well. Results were measured with Gemini XS Spectrofluorometer with 1 minute intervals for 1 hour at 40 °C, with the following parameters; excitation: 330 nm, emission: 456 nm, cutoff: 455 nm. ddH_2O was used as blank and RFU/min values were calculated from the initial linear release rate of fluorescence.

4.2.4.3.1.1. 4-Methylumbelliferyl-β-D-Cellobioside

4-Methylumbelliferyl-β-D-Cellobioside (MUC) was used to determine the activity of EG1 producing strains (Figure 26)

4.2.4.3.1.2. 4-Methylumbelliferly-β-D-Lactoside

4-Methylumbelliferyl-β-D-Lactoside (MUC) was used to determine the activity of cbh1 producing strains (Figure 26).

4.2.4.3.2. Carboxymethyl Cellulose Assay

Since no degradation of fluorogenic substrates (MUC/MULAC) were detected with *egl3* culture supernatants, Carboxymethyl Cellulose **(**CMC) Assay was done to compare activities of EG3 producers. Supernatants were precipitated with EtOH and resuspended with ddH_2O prior to assay in order to prevent signals coming from glucose in the culture supernatant. All experiments were done in quadruplets, reagents were pre heated to 50 °C and 96-well plate was covered with plastic mat during incubations to prevent evaporation.

30 µL supernatants were added to 96-well PCR plates and heated to 50 °C. 30 µL 2% CMC in NaOAc Buffer (pH 4.8) was added to wells simultaneously, mixed quickly and incubated for 30 min at 50 °C. 60 µL DNS reagent was added to wells, mixed shortly and incubated at 95 °C for 5 min for color development. PCR plate was then placed on ice and absorbances of 100 µL of mixtures were read at 550 nm after transferring them to flat bottomed 96-well microplate.

CMCase activity: CMCase activity was calculated as described previously (Ghose, 1987). Activities were calculated assuming a linear relationship between enzyme concentration and final glucose concentration, since excess amount of cellulose was used in the assay. 1 IU/mL CMCase Activity corresponds to 1 µmol/min of liberated glucose (**Hata! Başvuru kaynağı bulunamadı.**).

CMCase Activity (IU/mL) = 0.185 / EDR

Enzyme Dilution Rate (EDR) = 0.5 / (mg/mL glucose liberated)

4.2.4.4. Determination of Total Protein Concentrations

Total protein concentrations in the culture supernatants were measured by Bradford Assay as described by Bradford (1976) (Table 4).

4.2.5. BLAST Analysis of cdna1 gene

BLAST search for nucleotide sequences of *cdna1* promoter and gene were done in nucleotide collection (nr/nt) with megablast or discontiguous megablast. Nucleotide seauence of the predicted gene was translated into protein sequence using GeneRunner and BLAST search for protein was done among non-redundant protein sequences with blastp algorithm.

5. RESULTS

5.1. Construction of Transformation Vectors

5.1.1. Amplification of Cellulase Genes and *cdna1* Promoter

3 μL of each PCR product is seen in Figure 8. The genes were found to be in expected sizes (*egl1*: 2110, *egl3*: 1138, *cbh1*: 2183, Pcdna1: 943) and no extra bands were present in the gel.

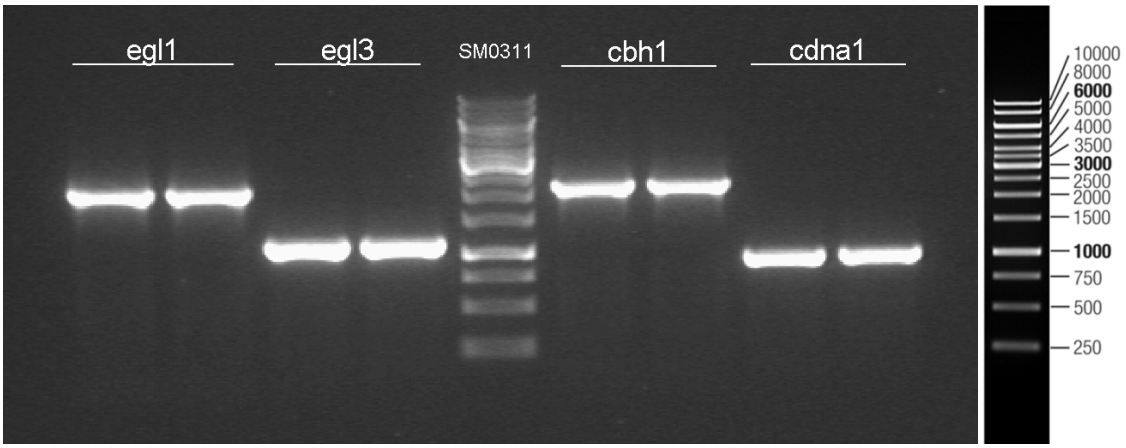


Figure 8: Agarose gel electrophoresis of fragments obtained by PCR. 3 μL fragment/well and 5 μL Ladder are run on 8% Agarose gel. **Right**: SM0311.

5.1.2. Three Cellulase Genes and Pcdna1 in pGEM-T Vector

In order to check the presence of cloned fragments in pGEMT vectors constructed, they were digested; HindIII-ClaI digestion of pGEMT-egl1/egl3; SalI-ClaI digestion of pGEMT-cbh1 and XhoI-ClaI digestion of pGEMT-cdna1. All of the clones seem to have correct fragment (Figure 9).

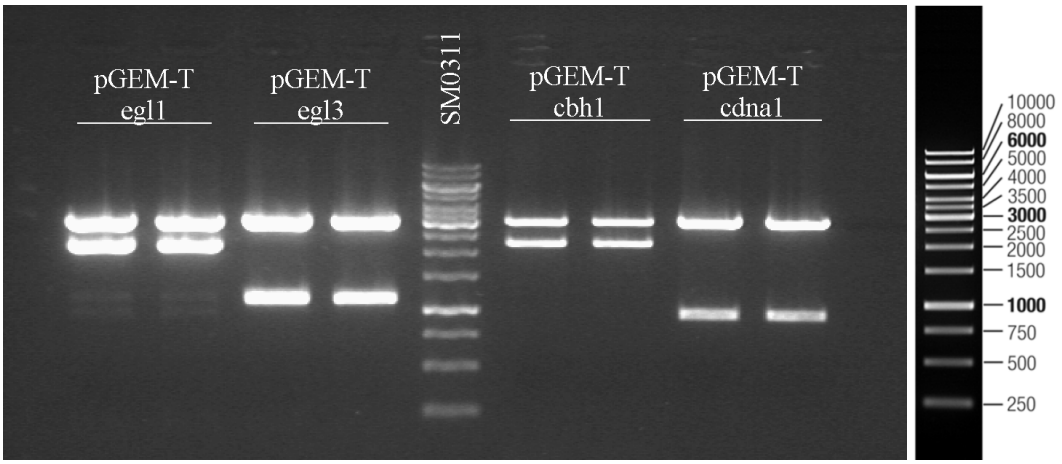


Figure 9: Control digestion of pGEM-T clones. *egl1/egl3*: ClaI-HindIII, *cbh1*: ClaI-SalI, Pcdna1: XhoI-ClaI. 2 µL DNA/well and 5 µL Ladder were run on 8% Agarose gel.

5.1.3. pPtef1- Vectors

After MiniPrep DNAs of selected pPtef1- colonies were obtained, control digestion was done to check the presence of inserts. When pPtef1-egl1/egl3 DNAs were digested with ClaI-HindIII and pPtef1-cbh1 with ClaI-SalI, the results in Figure 10 and Figure 11 were seen, respectively. All of the egl1vectors, eight egl3 vectors and seven cbh1 vectors seem to be positive. Colonies 6 and 10 of egl1, colonies 7 and 10 of egl3, colonies 6 and 7 of cbh1 were selected to obtain MIDI-Prep DNA for *T.reesei* transformation.

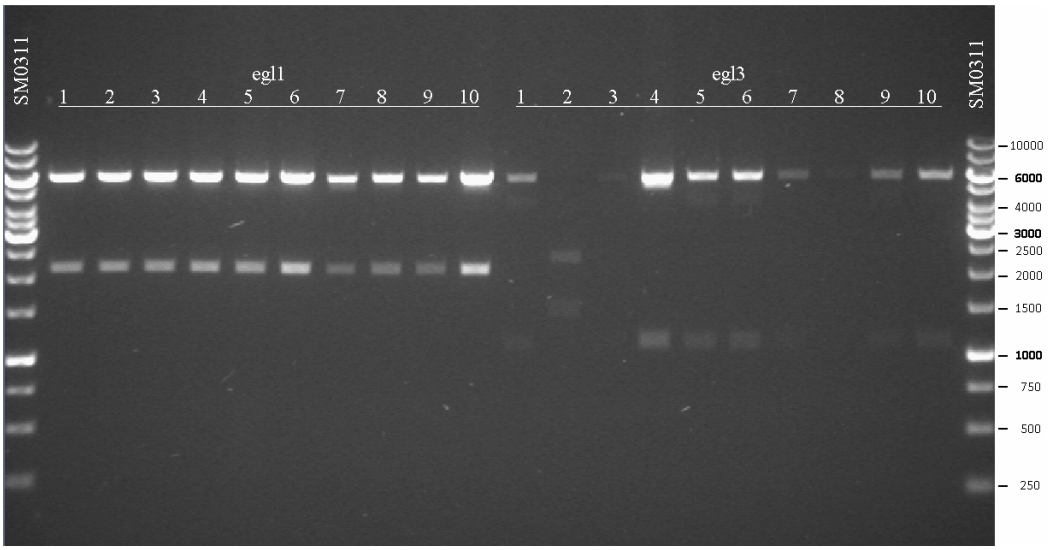


Figure 10: Control digestion of pPtef1-egl1 and pPtef1-egl3 MiniPrep DNAs. 2 μL DNA/well and 5 μL Ladder were run on 8% Agarose gel.

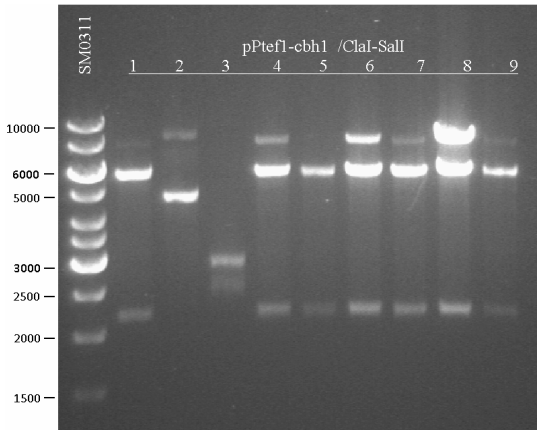


Figure 11: Control digestion of pPtef1-cbh1 MiniPrep DNAs. 2 μL DNA/well and 5 μL Ladder were run on 8% Agarose gel.

5.1.4. Restriction Analyses of pPtef1- Vectors

In Figure 12, Agarose gel electrophoresis of digested pPtef1- vectors is seen. Expected fragment sizes were as follows; pPtef1-egl1/XhoI: 6402, 1447; /XhoI-HindIII: 5009, 1447, 1393; pPtef1-egl3/BamHI: 3995, 2882; /XhoI-HindIII: 5009, 1868; pPtef1-cbh1/HindIII: 6667, 1269; /XhoI-SalI: 5027, 2913. All of the fragments seem to be in correct sizes, except, cbh1 vector has an extra band approximately 8 kb which might be

incompletely digested linear vector, therefore these vectors will be used for transformation of *T.reesei.*

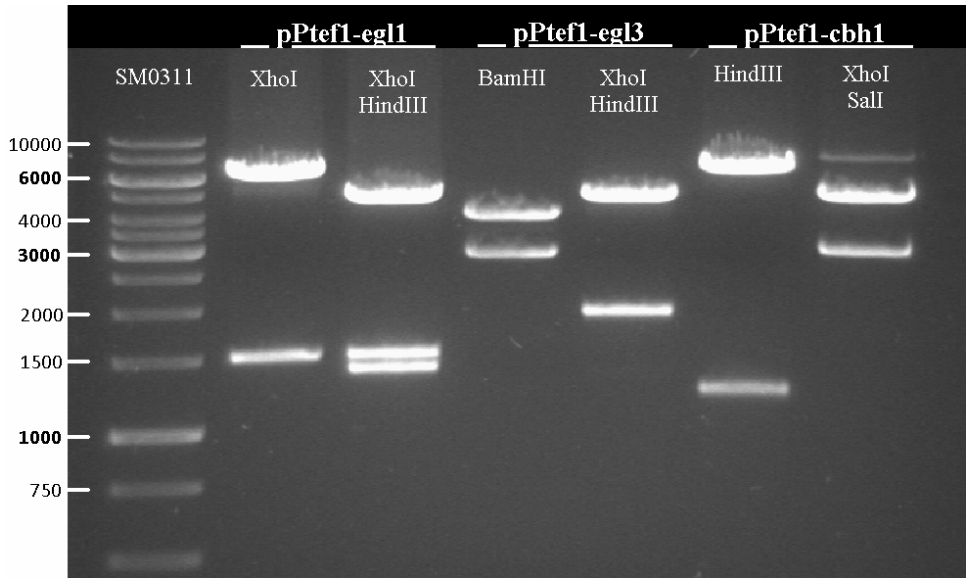


Figure 12: Restriction analyses of pPtef1- MIDI-Prep DNAs. 0.5 µL DNA/well and 5 µL Ladder were run on 8% Agarose gel.

5.1.5. Restriction Analyses of pPcdna1- vectors

Two fragments with lengths of approximately 2416 and 5622 bps; and four fragments with sizes of 613, 1023, 1393 and 5009 were expected from HindIII and XhoI-HindIII digestion of pPcdna1-egl1 respectively. These fragments were obtained when four vectors are digested with mentioned enzymes (Figure 13-A).

Three fragments with lengths of 613, 1445 and 5008 bps were expected from digestion of pPcdna1-egl3 vectors with XhoI-HindIII, since Pcdna1 has an internal HindIII site (see Appendix G for plasmid maps), which were detected in Figure 13-B.

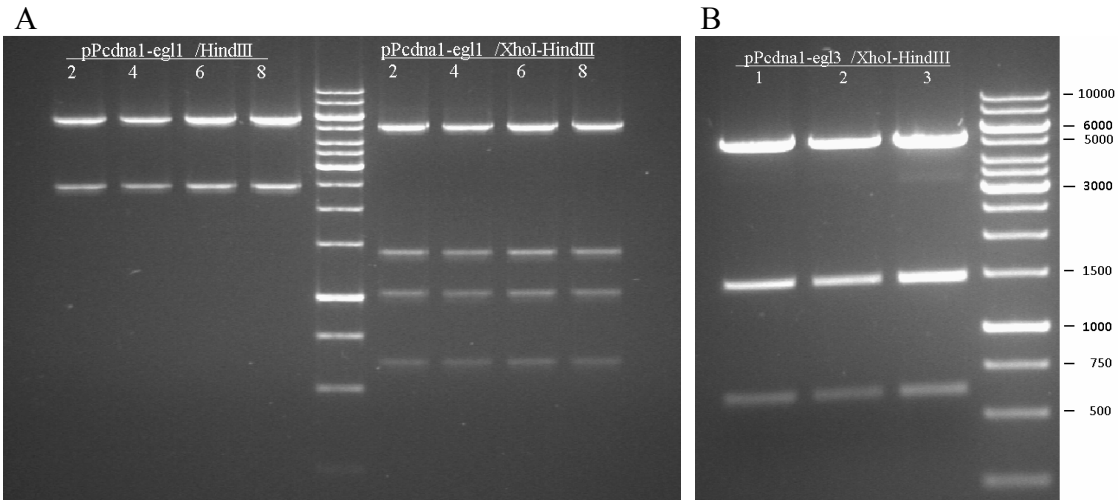


Figure 13: Restriction analyses of pPcdna1-egl1 (A) and pPcdna1-egl3 (B) vectors.

5.2. Transformation of *T.reesei* and Purification of Strains

Total 23 transformants were found to be stable and Hygromycin-B resistant after selection and purification steps following transformation of *T.reesei*. Initially, colonies on transformation plates were selected semi-randomly; positive clones were growing quite fast and became whitish (Figure 14). When they were transferred to PDA-Hygromycin plates, some of them were still resistant (Figure 14-B). Since TritonX restricts colony growth, it helped selection of colonies coming from a single spore. Since not all of the Hygromycin-resistant transformants were producers, expression on MA Medium was done to see their expression profiles.

Growth of all strains and transformants (except Rut-C30) on PDA plate were the same; in Figure 15-(A-C) an example is seen showing growth of QM9414 strain from 1st day until sporulation.

Spore plates and spores are exemplified in Figure 15-(D-F); a fully sporulated plate (A) and its detail (B). After collection, spores on hemocytometer can be seen in (C); note the oval-like shapes and greenish colors of spores.

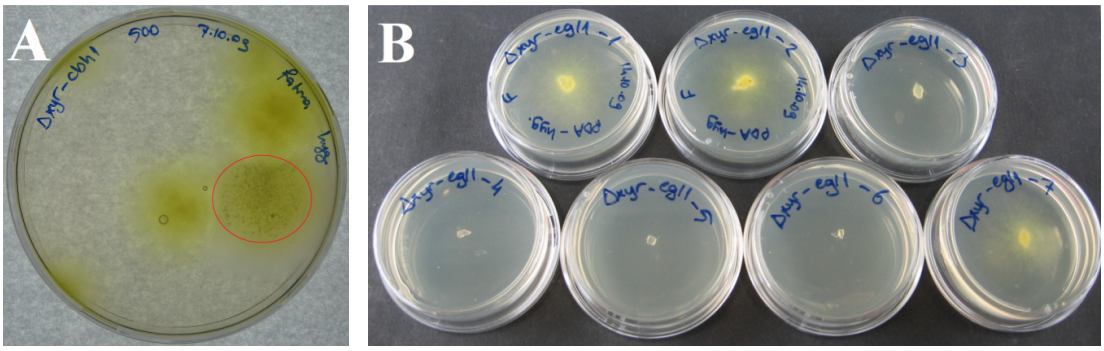


Figure 14: Sample plates for transformation. (A) A transformation plate 5 days after transformation; a probably positive colony is encircled, (B) small plates for PDA-Hygromycin B selection 1 day after selected colonies transferred; upper left two and lower rightmost colonies seems to be resistant.

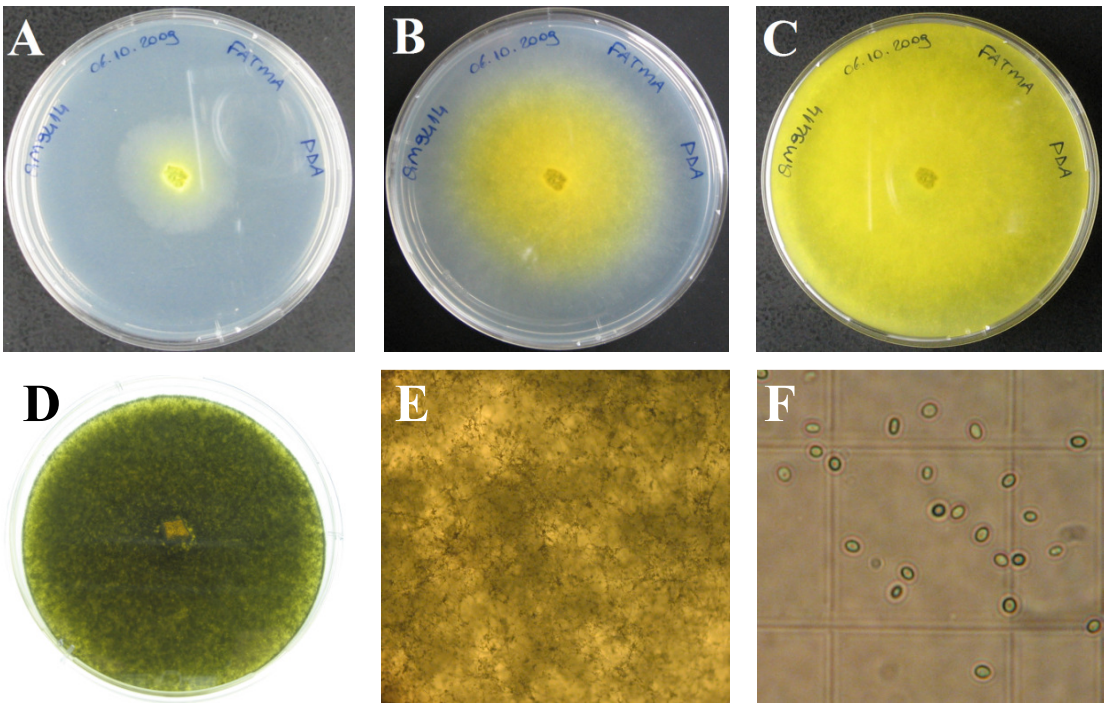


Figure 15: Growth of *T.reesei* on PDA (A, B, C, D) and *T.reesei* spores (D, E, F). (A) 1 day, (B) 2 days, (C) 3 days after transfer to plate; (D) fully sporulated plate after 10 days, (E) a closer look to the sporulated plate, (F) spores on hemocytometer, one side of square in F is 50 μm.

5.3. Expression of Cellulases

Given that Δxyr1 is a cellulase-negative mutant, it cannot grow on cellulose; in addition, since the promoters used are highly active on glucose, glucose was used as the carbon source for protein expression.

In Figure 16, mycelia samples are seen. A is taken at 32nd hour of culture (100X), B is 32nd hour (400X) magnification and C is 48th hour (100X). Magnification values might be slightly different due to different zoom values of the camera.

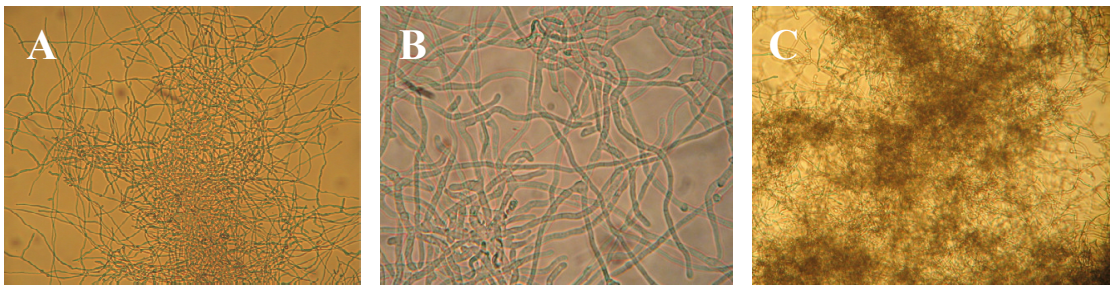


Figure 16: Mycelia in expression culture. (A) 32 hours after inoculation (100X), (B) 32 hours after inoculation (400X), (C) 48 hours after inoculation (100X)

5.3.1. Coffee Filters as Shake Flask Closures

Coffee filter was proven to be effective for maintenance of culture sterility and oxygen permeability (Hartman, 1987). Samples were taken from empty culture just like others at the specified time-points and no contamination was observed till the end of 52 hours. When growth patterns of cultured strains are compared (Figure 17), only a shift of maximum weight from 28th hour to 36th hour is seen (which might as well be due to experimental variations), yet maximum and mean growth rates are quite close.

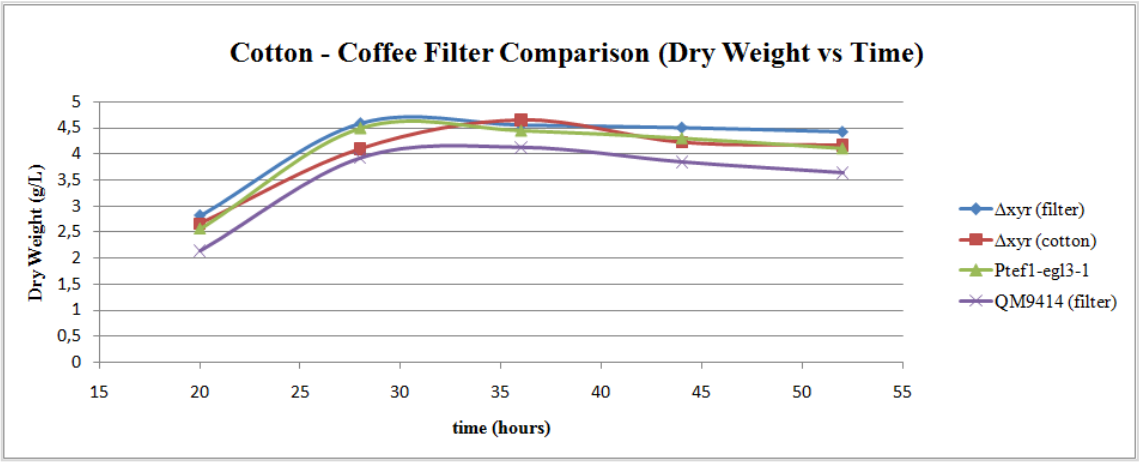


Figure 17: Effect of using coffee filter or cotton as shake-flask closure to culture growth. Strains are grown on MA Medium with glucose.

5.3.2. Comparison of Smooth and Baffled Flasks

Ptef1-cbh1-1 strain was grown in both smooth and baffled flasks. In Figure 18 the strain seems to outgrow other strains (about 1.5-fold) at 28th hour, but then it decreases to the same level with others probably due to exhaustion of sugar. In Figure 19, when protein expression profiles in smooth and baffled flasks are compared, they show similar patterns to strain growth; such that, in baffled flask protein amount reached maximum at 28th hour and then decreased. In addition, expression of other proteins seems to have increased as well.

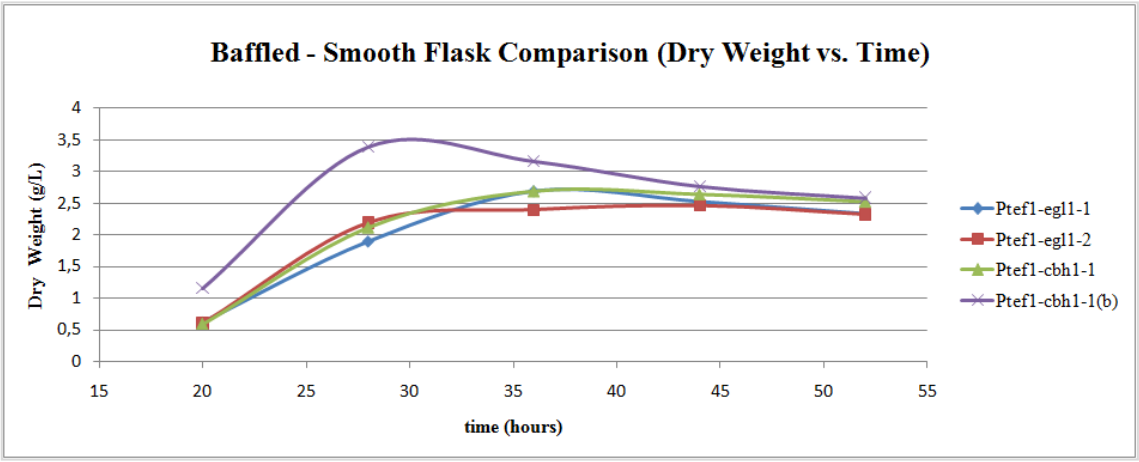


Figure 18: Growth of *T.reesei* in baffled and smooth flasks. Ptef1-cbh1-1(b): baffled, other strains: smooth flask. Strains are grown on MA Medium with glucose.

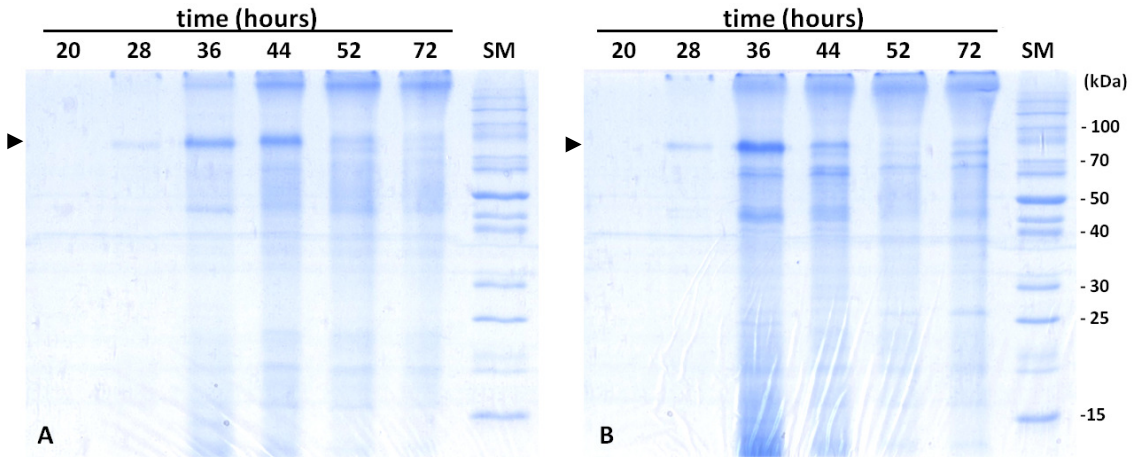


Figure 19: Comparison of protein expression in smooth (A) and baffled (B) flasks. Wells contain EtOH-precipitated supernatant corresponding to 250 µL. SM: 5µL SM0661

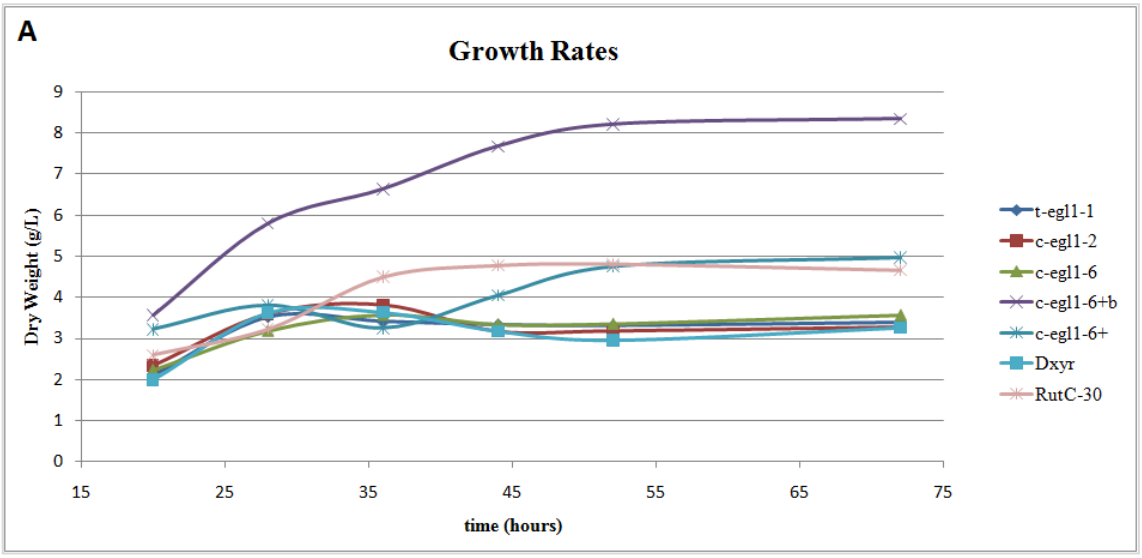
5.3.3. Growth Rates

Cell dry weights of cultures were calculated in order to be able to correlate protein expression to growth of strains. All of the strains but QM9414 (cel) were grown on MA Medium with glucose as carbon source (see 0). Groups that were cultured simultaneously are written in the same parentheses: (t-egl1-1, c-egl1-2, c-egl1-6, Δxyr, t-egl3-1, c-egl3-5) - (c-egl1-6+b, RutC-30, QM9414 (cel), t-cbh1-1, t-cbh1-4) - (QM9414 (glu)) - (c-egl1-6+) - (c-egl3-2).

In Figure 20-A, growth rates of egl1 producers, parental strain Δxyr and hypercellulolytic mutant RutC-30 are seen. Growth patterns of simultaneously grown strains are exactly the same, which are also same with parental strain and QM9414 (glu) (B). When c-egl1-6 strain in baffled flask was supplemented with additional sugar (c-egl1-6+b), its growth increased almost 2-fold; yet, this is not the case with smooth flask (c-egl1-6+). RutC-30 grows better than other strains, except sugar-added ones.

In Figure 20-B, growth rates of EG1 and CBH1 producers, Δxyr and wild-type QM9414 (grown on glucose or cellulose) strains are seen. Dry weights of simultaneously grown strains are close again, together with the parental strain and QM9414. Since c-egl3-2 was cultured another time, its growth curve is different (other strains of that expression had exactly the same growth curves, data not shown).

Although QM9414 is similar to transformants when glucose is the carbon source, growth on cellulose is quite unusual that it seems to decrease.



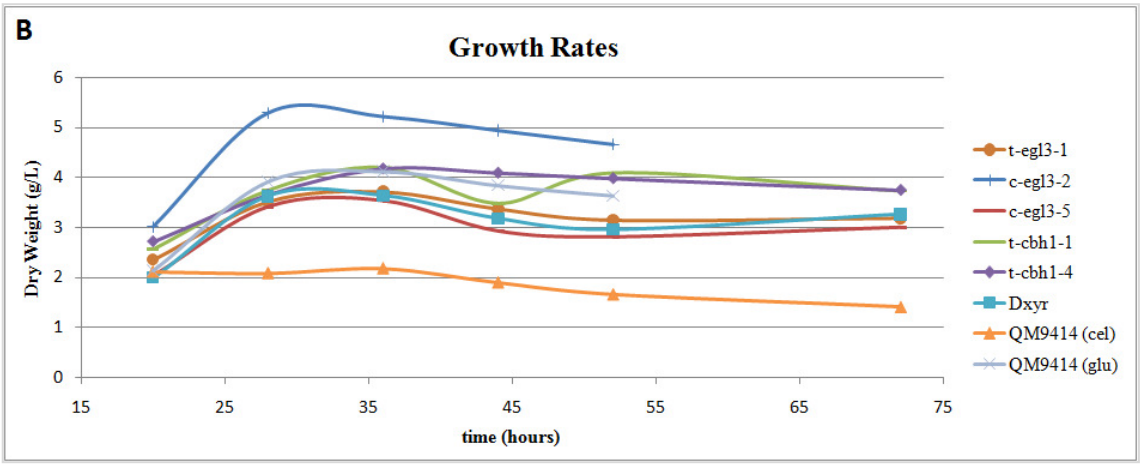


Figure 20: Growth rates of transformants, and Δxyr, QM9414 and RutC-30. c-egl1-6+: glucose was added at 20th hour, c-egl1-6+b: glucose is added at 20th hour and grown in baffled flask, QM9414 (glu): grown on glucose, QM9414 (cel): grown on cellulose.

5.3.4. SDS Gel Analysis

In Figure 21, SDS-PAGE results of three transformants t-egl1-1 (A), c-egl1-2 (B), c-egl1-6 (C), which were found out to be EG1 producers, and Δxyr strain (D) as a negative control are shown. Total protein concentrations of the supernatants can be

checked from Table R-1-A. EG1 is detected in the middle of 60 and 70 kDa (about 65 kDa).

A neat gel image of the samples taken from QM9414 when grown on cellulose could not be obtained since residual cellulose disrupted running of the gel causing distortion of bands; even if supernatants were EtOH precipitated, cellulose precipitated, too. RutC-30 was concentrated as well, yet no considerable bands were present, therefore its SDS gel is not included.

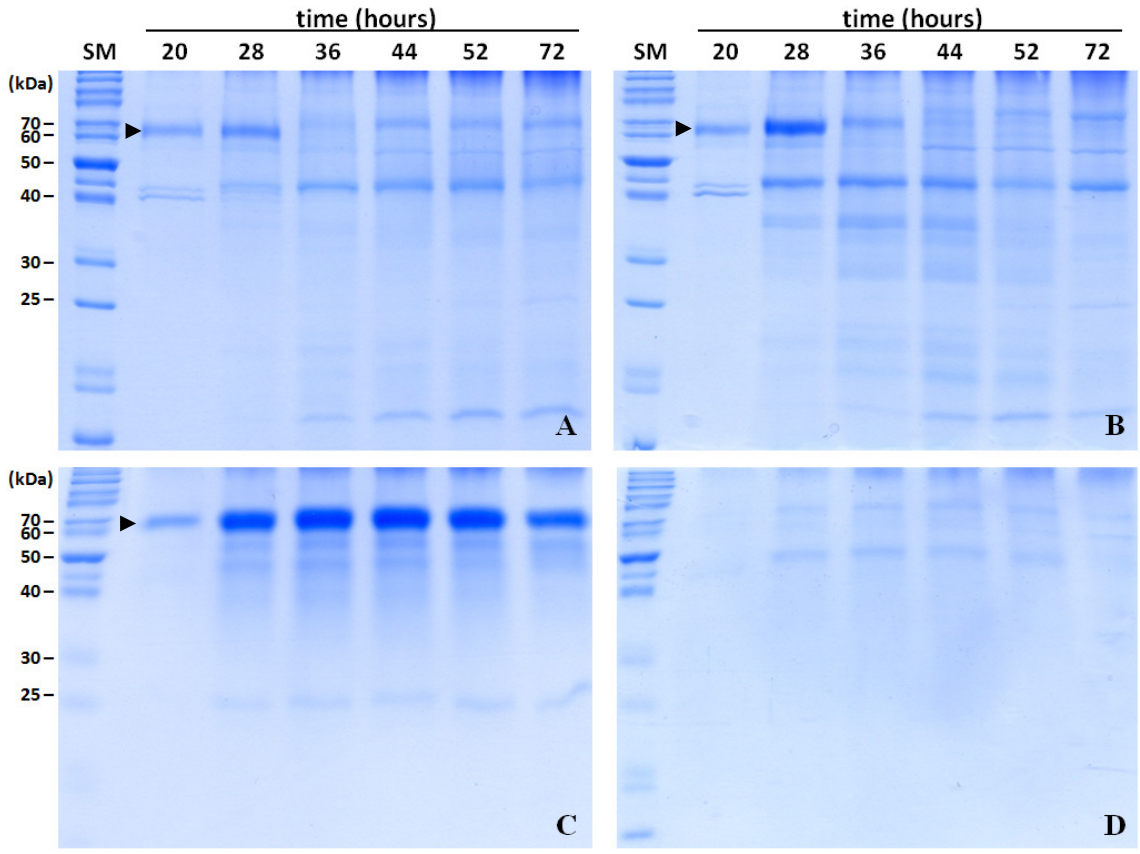


Figure 21: Endoglucanase I was expressed under both promoters. (A) Ptef1-egl1-1 (100 µL), **(B) Pcdna1-egl1-2** (125 µL), **(C) Pcdna1-egl1-6** (125 µL), **(D) Δxyr1** (125 µL). Inside the parentheses amounts of supernatants loaded to the gel are indicated. Time: hours after culture inoculation. SM: SM0661 (5 µL)

In order to see whether exhaustion of sugar is a limiting component for protein expression, extra 10 g/L glucose was added to c-egl1-6 strain (c-egl1-6+) at 20th hour. In Figure 22, supernatants corresponding to 250 µL are run on SDS gel with maximum amount at 36th hour.

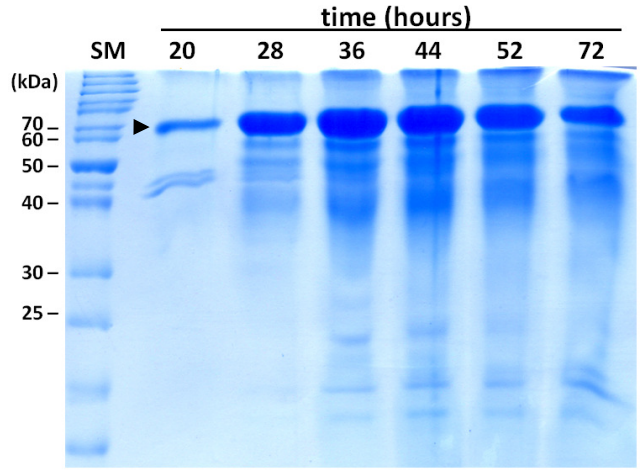


Figure 22: Endoglucanase I expression by c-egl1-6 strain has increased upon addition of extra glucose (c-egl1-6+). 250 μL supernatant/well. SM: SM0661 (5 μL)

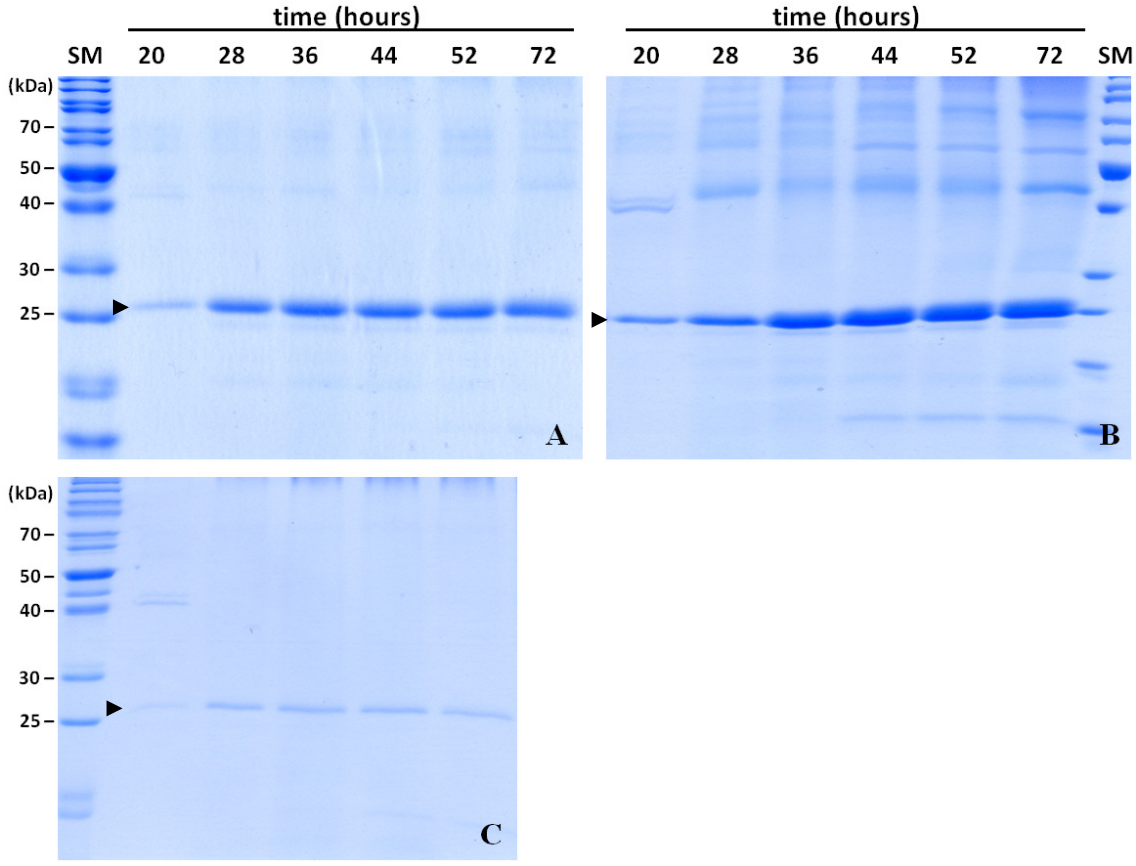


Figure 23: Endoglucanase III was expressed under both promoters. (A) Pcdna1-egl3-5 (15 μL), **(B) Ptef1-egl3-1** (75 μL), **(C) Pcdna1-egl3-2** (25 μL). Inside the parentheses amounts of supernatants loaded to the gel are indicated. Time: hours passed after culture inoculation. SM: SM0661 (5 μL)

In Figure 23, SDS-PAGE results of transformants c-egl3-5 (A), t-egl3-1 (B) and c-egl3-2 (C), which were found to express EG3 (note the arrowheads), are shown. Total protein concentrations of the supernatants can be checked from Table 4. All of the strains show increasing amount of enzymes until 36th hour which then persists till 72nd hour.

SDS-PAGE results of transformants t-cbh1-1 (A) and t-cbh1-4 (B) are shown in Figure 24. Previous findings (Hall, 2010) reporting CBH1 to be around 67 kDa supported our data with bands slightly above 70 kDa. Both strains show stable CBH1 expression until 72nd hour with very similar expression profiles. In Figure 25, CBH1 produced by QM9414 when grown on glucose at 36th hour is indicated. Although, being an end product, glucose inhibits cellulase synthesis, residual CBH1 expression can be detected in the gel. It is obvious that this band and the CBH1 bands in Figure 24 are of the same size.

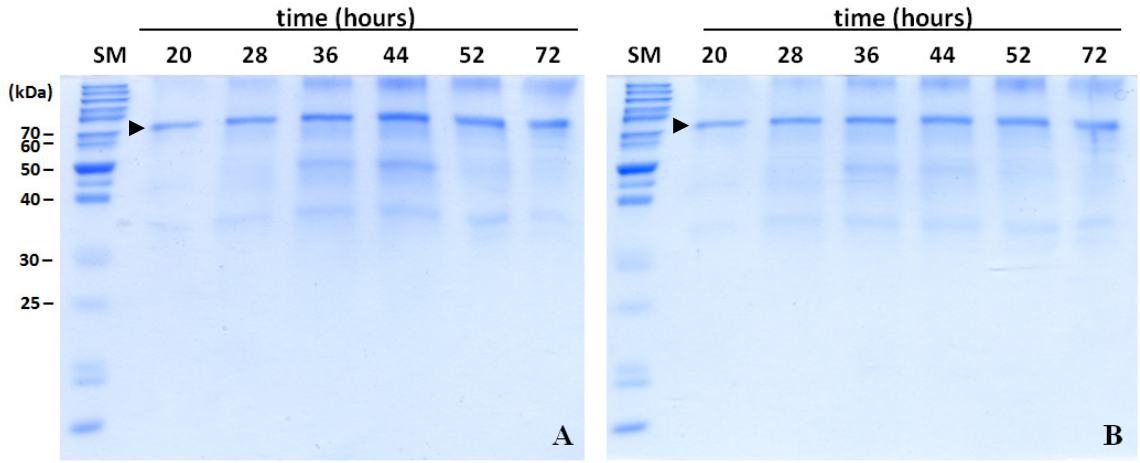


Figure 24: Cellobiohydrolase I was produced under *tef1* promoter (slightly above 70 kDa). (A) Ptef1-cbh1-1 (125 µL), **(B) Ptef1-cbh1-4** (125 µL). Inside the parentheses amounts of supernatants loaded to the gel are indicated. Time: hours passed after culture inoculation. SM: SM0661 (5 µL)

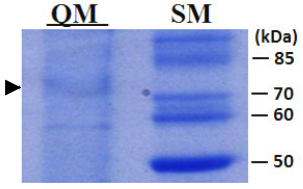


Figure 25: CBH1 produced by QM9414 at 36th hour when grown on glucose. Contrast was increased to intensify the band. QM: QM9414 supernatant (250 µL). SM: SM0661 (5 µL)

5.3.5. Activity Assays

5.3.5.1. Activity of Endoglucanase I towards MUC

Activities of EG1 producing strains, Δxyr, QM9414 and RutC-30 towards fluorogenic substrate 4-Methylumbelliferyl-β-D-Cellobioside (MUC) were measured and compared.

In Figure 26-A, activities are drawn with respect to sampling time. Δxyr, QM9414 and RutC-30 strains have activities close to zero. t-egl1-1 and c-egl1-2 have very similar activities, while latter is slightly higher. c-egl1-6 supernatant shows increasing activity till 36th hour and when extra glucose was added 20th hour (c-egl1-6+), activity has increased more than 2-fold.

Addition of glucose did not have a striking effect on expression in baffled flask (c-egl1-6+b), in fact it decreased the activity compared to smooth flask.

5.3.5.2. Activity of Cellobiohydrolase I towards MULAC

Activities of cbh1 producing strains, Δxyr, QM9414 and RutC-30 towards fluorogenic substrate 4-Methylumbelliferyl-β-D-Lactoside (MULAC) were measured and compared.

In Hata! Başvuru kaynağı bulunamadı.-B, activities are drawn with respect to sampling time. Again Δxyr has shown no activity; and neither QM9414 nor RutC-30. In fact their activities are not absolute zero, but they are negligible when compared to cbh1s'. t-cbh1-1 and t-cbh1-4 has very similar activities.

5.3.5.3. Activity of Endoglucanase III towards CMC

Activities of egl3 producing strains towards CMC were measured and compared after EtOH precipitation. Protein concentrations before and after EtOH precipitation is shown in Table 4. CMCase activities with respect to sampling times are drawn in Hata! Başvuru kaynağı bulunamadı.-C. Supernatants of all of the strains have CMCase activity with varying degrees. Maximum activity is displayed by c-egl3-5 at 36th hour, 0.55 IU/mL

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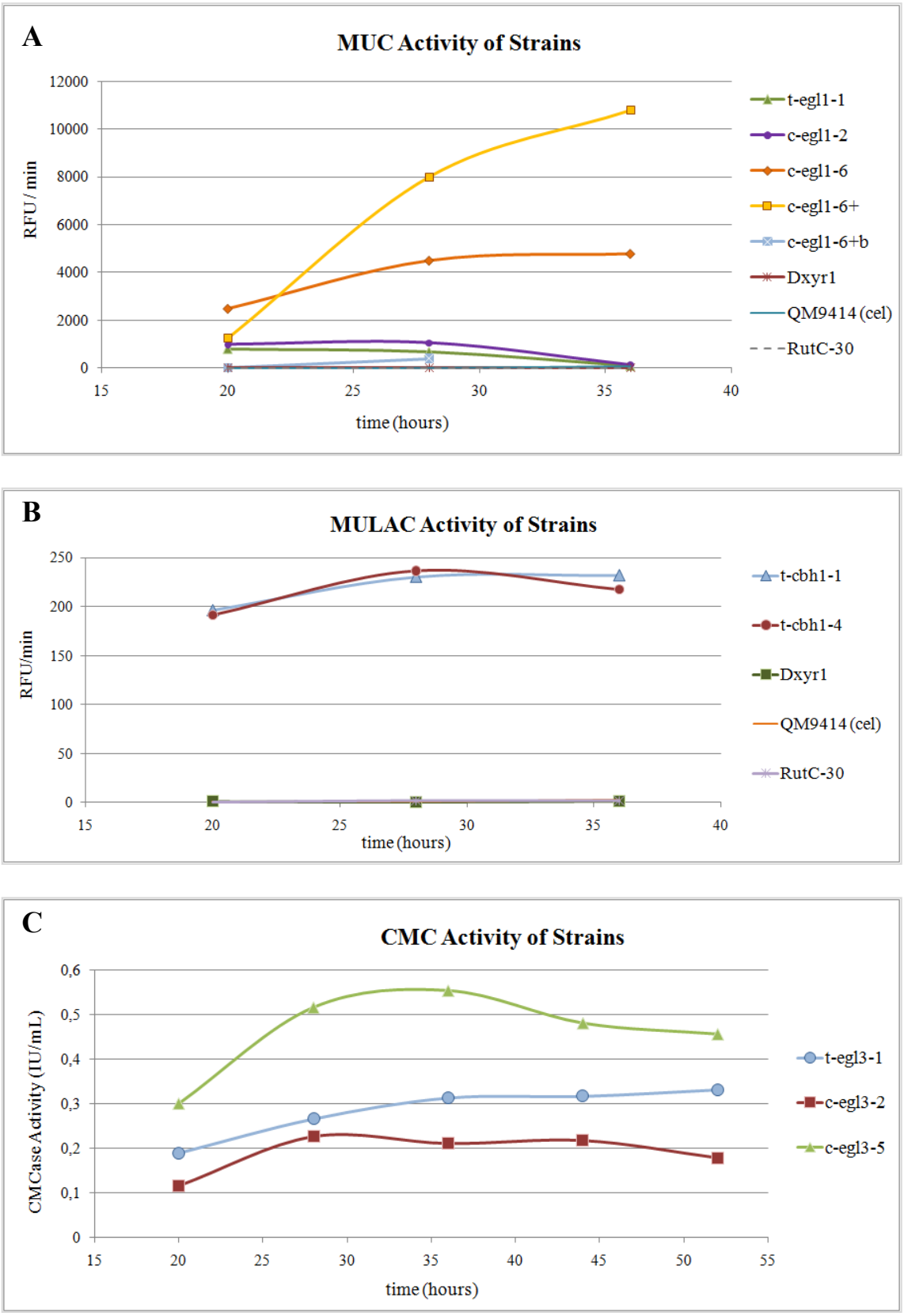

Figure 26: MUC activity of EG1 producers (A), MULAC activity of CBH1 producers (B) and CMC Activity of EG3 producers. RFU/min values for MUC and MULAC; IU/mL values for CMCase activities were shown.

5.3.6. Analyses of Protein Concentrations and Activities

Total protein concentrations in the culture supernatants were measured for each strain and sampling time, shown in Table 4. Specific Activity (Activity/Total Protein) is determined for each assay to gain insight in purity of the enzymes in the culture supernatants (Figure 27); for this, activities were assumed to directly correlate with enzyme amount. Highest activity time of each strain is indicated by dark background and highest specific activity values are written in bold.

In Figure 27-A, specific activities of EG1 producers towards MUC, in Figure 27-B specific activities of CBH1 producers towards MULAC and in Figure 27-C specific activities of EG3 producers towards CMC (right part) are compared to each other.

Table 4: Total protein concentrations (mg/L) of culture supernatants. E: EtOH precipitated samples.

	Total Protein Concentration (mg/L)					
time (h)	**20**	**28**	**36**	**44**	**52**	**72**
t-egl1-1	5,1	13,0	22,4	15,4	9,8	4,8
c-egl1-2	4,5	23,4	25,3	21,7	13,9	7,1
c-egl1-6	6,3	31,5	45,9	41,7	36,5	30,9
c-egl1-6+	7,4	22,2	49,3	56,3	44,5	35,8
c-egl1-6+b	2,1	45,9	23,1	16,8	13,6	9,9
t-egl3-1	4,8	17,3	20,5	16,1	10,5	8,3
t-egl3-1 E	2,6	11,8	15,8	11,5	10,4	7,8
c-egl3-2	13,3	32,1	43,7	27,9	17,2	-
c-egl3-2 E	2,5	11,7	20,7	10,3	4,9	-
c-egl3-5	4,2	9,7	21,8	19,5	15,0	9,3
c-egl3-5 E	1,4	3,9	14,2	12,4	9,9	6,8
Δxyr1	3,4	13,2	18,2	10,2	8,6	4,4
QM9414 (cel)	10,8	26,5	38,2	45,6	22,8	22,1
RutC-30	3,9	77,9	64,7	42,4	25,3	12,9

Specific activities were calculated as follows: Each well of microplate contained 50 nmol MUC and upon complete hydrolysis, 93000 RFU is reached; meaning that 1 RFU = 5.376×10^{-13} mol hydrolyzed MUC. Therefore,

a RFU min^{-1} μg^{-1} = a x [5.376×10^{-4}] µmol min^{-1} mg^{-1} (MUC)

can be used for specific activity conversion into IU units.

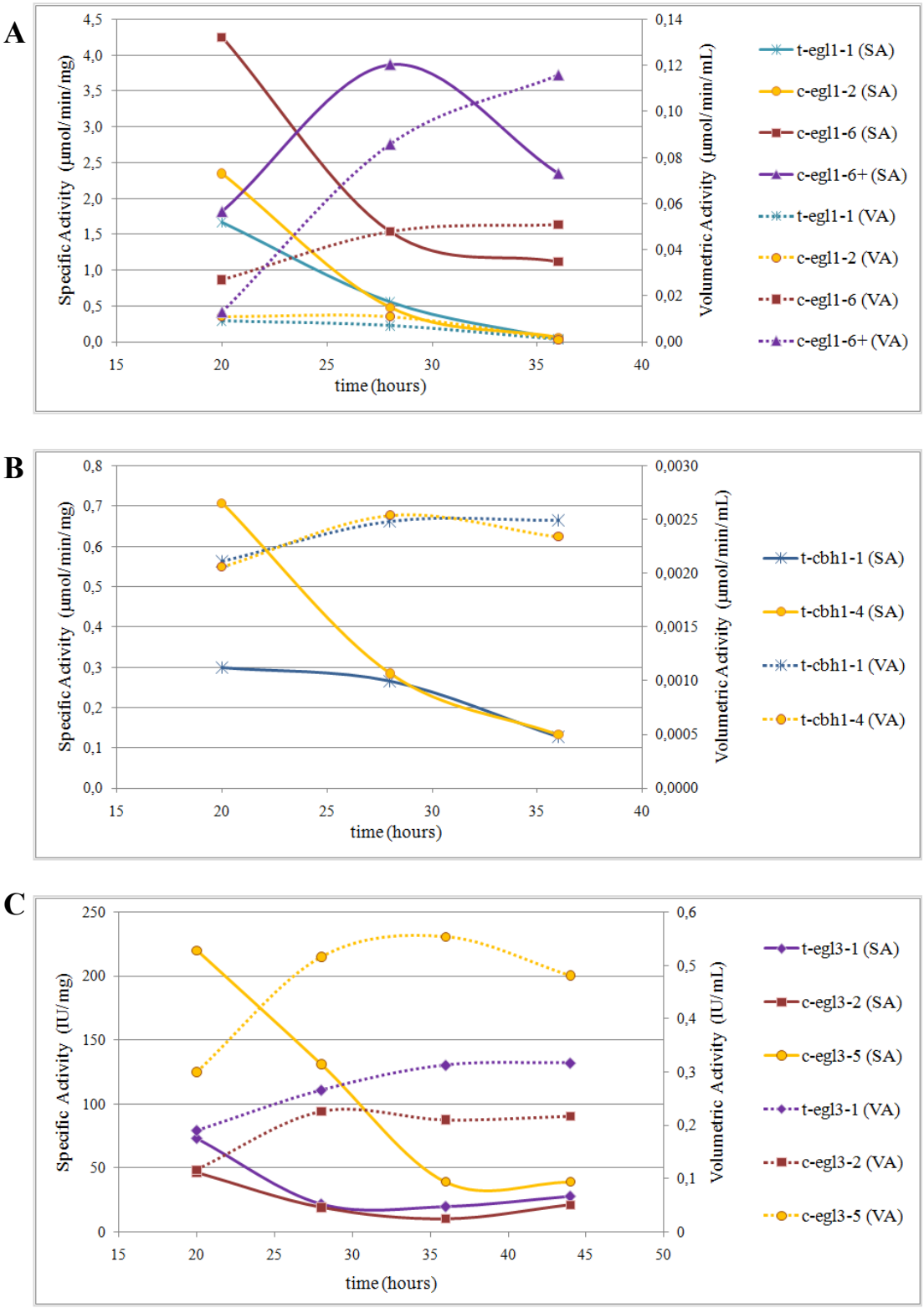

Figure 27: Volumetric and Specific MUC (A), MULAC (B) and CMC (C) Activities of transformants. Volumetric activities (VA) are µmol/min/mL for fluorogenic substrates and IU/mL for CMC. Specific activities (SA) are µmol/min/mg for fluorogenic substrates and IU/mg for CMC.

As a summary, maximum activities and specificities observed for each enzyme among all transformants were given in Table 5 and Table 6.

Table 5
Maximum volumetric activity observed for each enzyme

Enzyme	timepoint	Maximum Activity
c-egl1-6+	36	0.116 μmol min^{-1} mL^{-1}
c-egl3-5 (E)	36	0.554 IU/mL
t-cbh1-4	28	0.00254 μmol min^{-1} mL^{-1}

Table 6
Maximum specific activity observed for each enzyme

Enzyme	timepoint	Maximum Specificity
c-egl1-6	20	4.25 μmol min^{-1} mg^{-1}
c-egl3-5 (E)	20	220 IU/mg
t-cbh1-4	20	0.707 μmol min^{-1} mg^{-1}

5.3.7. BLAST Analysis of *cdna1* gene

BLAST search of nucleotide sequences of *cdna1* promoter and gene did not give any results, even if they were trimmed and search was re-done. In the jgi-*T.reesei* website, there are two hypothetical proteins in this region with IDs 110879 and 123515. We have translated the nucleotide sequence of the gene (scaffold 23:44654-44994) into protein sequence *in silico* resulting in a 80 amino acid sequence given in Appendix F. BLAST search of this polypeptide sequence among non-redundant protein sequences with blastp algorithm gave several results, five of which with highest E-values had 39-46% identities and 53-63% positives. All of these five results were hypothetical proteins from species; *Haematonectria haematococca*, *Gibberella zeae*, *Sclerotinia sclerotiorum*, *Botryotinia fuckeliana* and *Verticillium alboatrum* which are all plant pathogenic fungi (Kirk, 2001).

6. DISCUSSION

6.1. Construction of Transformation Vectors

Three cellulase genes encoding Endoglucanase I (*egl1*), Endoglucanase III (*egl3*) and Cellobiohydrolase I (*cbh1*) were amplified from genomic DNA of wild type strain QM9414 with PCR reaction which was successful in the first trial with correct sizes of fragments and no unspecific bands or smear in the agarose gel. Since restriction of PCR products would result in the fragments of almost same sizes with unrestricted ones, it was not a good idea to cut PCR products directly. They were cloned into the pGEM-T vector instead, in order to be able to keep them for a long time and to make sure that when we cut the fragments from pGEM-T vector, all would have sticky ends. pPtef1 vector was prepared by restriction digestion with the enzymes that flanked each gene's PCR product. Digested PCR products and pPtef1 vector were purified by gel extraction in order to get rid of unwanted materials that would interfere with ligation procedure and to have more concentrated DNA. Ligation of fragments to pPtef1 vector was straightforward and at least 7 positive colonies for each plasmid (pPtef1-egl1, pPtef1-egl3 and pPtef1-cbh1) were obtained, two of which were selected for MIDI-Prep amplification in order to have higher concentration and purity for *T.reesei* transformation.

Construction of pPcdna1- plasmids were planned to be done by extracting *tef1* promoter from pPtef1- vectors and ligating *cdna1* promoter instead, via Xho-ClaI sites (Appendix G). This was achieved for pPtef1-egl3 to pPcdna1-egl3 conversion. However, presence of XhoI site inside *egl1* gene eliminated this replacement possibility for the pPtef1-egl1 vector. When presence of a HindIII site in the *cdna1* promoter was considered, initial replacement of Ptef1 in pPtef1 with Pcdna1 was not a reasonable choice either (see Appendix G). In order to construct pPcdna1-egl1 vector; triple ligation of Ptef1 extracted pPtef1, *egl1* gene and Pcdna1 was done. 10 positive colonies

were obtained at 4th trial. Although the replacement strategy was expected to work with pPtef1-cbh1 – pPcdna1-cbh1 conversion, no colonies were obtained after several trials; which might be due to loss of activity of the ligation solution used for cloning.

After obtaining MIDI-Prep DNAs of these vectors, restriction analyses of constructed vectors were done using either internal or flanking restriction sites, all of which gave positive results (Figure 12 and Figure 13) showing that the promoters and the genes were there with correct orientations. These vectors were used then for transformation of *T.reesei*.

6.2. Transformation of *T.reesei* and Purification of Strains

Protoplasting is the process of degrading cell walls of mycelia, which is a required step prior to transformation of *T.reesei* since cell walls block DNA entry (Ping, 2005). Degradation was done by using Lysing Enzymes from *Trichoderma harzianum* that hydrolyzes poly(1-3)-glucose in the cell wall glucan (Sigma-Aldrich). DNA is then introduced to the cells in the presence of Polyethylene Glycol (Mach, 1998). PEG is necessary for anchoring of vector DNA to cell membranes of protoplasts and it is also thought to assist intake of DNA by the cells (Ping, 2005). Sorbitol is included in bottom medium that the protoplasts are spread on and overlay medium that covers on top of them after transformation to provide osmotic support since the protoplasts are very fragile not possessing a cell wall. Hygromycin B was used in transformation and purification plates for selection of positive transformants since it can kill eukaryotic cells, so that untransformed cells were eliminated. After complete purification and glycerol stock preparation, strains were maintained on media without any antibiotics in order to allow their growth and sporulation. All the transformants were observed to grow with almost the same rates and patterns on agar media, together with the parental strain Δxyr1 and QM9414 (Figure 15-ABC); yet Rut-C30 has a lower growth rate and limited sporulation. This might be due to maintenance of the stock agar pieces at +4 °C for a long time before re-culturing them.

Approximately 2-3 days after transformation, although tiny, mycelia started to be seen on plates and it took about 1 week for growth of colonies. Positive transformants usually could be identified on transformation plates, they grew very fast, acquired a

whitish color and started to produce white spores (Figure 14-A). Some other colonies grew fast but had a patchy appearance, and were usually proven to be negative later. Nevertheless, very small pieces of several colonies were passed to individual small PDA-Hygromycin B plates to confirm their resistance; yet, not all of them were resistant usually (Figure 14-B). Colonies on these small plates were allowed to grow until sporulation and a few spores were collected with a few drops of Physiological Salt Solution which then are spread to PDA-TritonX-Hygromycin B plates for single spore selection. Since Triton X is a surfactant, it restricted colony growth so that single colonies could be isolated.

6.3. Expression of Cellulases

Expression cultures were started with equal number of spores (2 x 10^6 spores/mL) in order to be able to standardize cell numbers and total dry weights to some extent. Spores were counted in at least four big squares on each side of hemocytometer, usually after 20-fold dilution, to obtain a statistically correct value.

Minimal medium described by Mandels & Andreotti (1978) with glucose as the carbon source was used in all standard expressions, except one expression of QM9414 with cellulose as carbon source. Using glucose as the carbon source had two advantages, first of which is due to choice of promoters that are highly active on glucose medium. Secondly, even if residual expression of cellulases in Δxyr1 background somehow arises, glucose will act as the warrantor of cellulase-negative background.

6.3.1. Optimization of Culture Conditions

Coffee filters are evaluated as flask closures; as they are disposable, sterility is maintained better than washed and re-used cotton closures in addition to being more available and cheaper. Since it was previously tried and has proven to be successful for cultivation of bacteria (Hartman, 1987), we tested them for growth of fungi. In Figure 17, when growth rate of Δxyr1 strain in flasks either closed with stitched cotton and 2-

fold coffee filter are compared, no difference in maximum dry weight and trends was visible, except a shift of maximum from 28th hour to 36th hour. Other strains in the figure were grown with coffee filters, too. Eventually, coffee filters were used as flask closures in all of the subsequent expressions.

Baffled flasks were compared with smooth flasks for growing *E.coli* and found to be effective in oxygen absorption rates (McDaniel, 1965). Thus, we compared baffled and smooth flasks for growth of *T.reesei* strains. Effect of growing Ptef1-cbh1-1 strain in baffled or smooth flasks is seen in Figure 18; maximum dry weight was attained at 28th hour which is about 40 % higher relative to maxima of strains growing in smooth flasks. Protein expression profiles of these two cultures were also examined on SDS gel (Figure 19). Although maximum CBH1 amount seems to be obtained at 36th hour in baffled flask, other proteins and more smear are present in the lane, which will decrease purity of the enzyme in the supernatant. It seems that cells grow faster in baffled flask, protein is expressed shortly and glucose is exhausted soon, which stops growth and protein expression. Glucose addition to baffled flasks at 20th hour is tested later to confirm this. Baffled flasks can be used for other applications where enzyme amount is more important than its purity. We decided to do subsequent expressions in smooth flasks for the sake of long-term purer expression.

In order to check whether sugar exhaustion is a limiting factor for growth and protein expression; extra glucose was added to c-egl1-6 cultures in smooth or baffled flasks; since it is already a hyper-producer, we wondered if this can increase expression further (Figure 20-A). As estimated, sugar addition increases growth further in both types of flasks. Their growth continued till 52nd hour and in baffled flask dry weight has doubled others, probably owing to presence of enough oxygen supported by additional glucose, which was not the case with baffled flasks without extra glucose (Figure 18). Although expression in smooth flask has increased until 36th hour (Figure 22), to our surprise, no protein production in baffled flask was observed upon addition of glucose, even though the expression was repeated (data not shown). We claim that this is due to excessive growth of strains since the translation machinery was occupied by expression of proteins necessary for cell growth and division; or *cdna1* promoter might not be functional in very high growth rates. Additionally, if SDS gels are compared (Figure 21-C and Figure 22) more that 2-fold change can be deduced for c-egl1-6+ relative to c-egl1-6 although supernatant amount was two-fold, indicative of expression increase upon glucose addition.

6.3.2. Growth Rates

Strains growing simultaneously usually had the same growth pattern, but this usually changes from expression to expression due to limited control of ambient temperature. As can be followed from dry weight – time graphs, strains that were cultured simultaneously show almost same pattern (Figure 17, Figure 18, Figure 20) except a few outliers; which was not the case when different sets of cultures were compared. This is probably because of our limited control over ambient conditions; internal temperature of the shaker had been shifted 2-3 °C above or below 28 °C occasionally for instance. Since light has an impact on growth and cellulase expression of *T.reesei* (Schmoll, 2009) inability to keep the cultures under same level of light might have contributed to set to set variations. As the shaker used for expression was not optimized for *T.reesei*, oxygen circulation could be variable and oxygen access of each culture might have been inconsistent.

Similar growth patterns were observed for all strains in general; maximum dry weight is gained between 28th and 36th hours, which is approximately 4 g/L and then it decreases. Maximum values differed probably due to the reasons mentioned above. There were four exceptions to this trend and one of them was when QM9414 grown on cellulose (Figure 20-B) dry weight seems to have decreased as time passed. The data can be biased due to precipitation of cellulose in the medium together with the cells, reflecting incorrect initial weights. Alternatively and more probably; taken into account that the MA-glucose pregrown mycelia were transferred to MA-cellulose medium at 20th hour, cells might not have adapted easily to the new environment to induce cellulases and utilize the cellulose in the medium, because at 20th hour it has same dry weight with MA-glucose grown strains. Second exception was Rut-C30 that its dry weight continues to increase until 44th hour, probably since it is a mutant obtained for the sake of hypersecretion, thus it grows better as well. Other two exceptions were seen when extra glucose was added to expression culture of c-egl1-6 strains (either in smooth or baffled flasks: c-egl1-6+ and c-egl1-6+b) at 20th hour (Figure 20-A).

6.3.3. SDS Gel Analysis

For initial screening of the supernatants with SDS-PAGE, they were concentrated 10 times in order to magnify the band intensity and see even minute amounts of expressed proteins, if present. After detecting the enzymes, original supernatants were concentrated this time according to the protein amount they contain in order to prevent over/under-loading of the wells. Presence of too much protein interfered with electrophoresis by changing local current and causing smile effect, or some of the proteins were unable to enter the gel; and presence of less concentrated proteins were undetectable.

EG1 was produced by three strains; approximately 65 kDa for all strains (Figure 21, arrowheads) rather than being close to 61 kDa stated previously (Collen, 2001). This might be due to full/hyper-glycosylation of the enzyme which has 5 glycosylation sites; it is noteworthy that proteins are at the same level for all three strains. While EG1 production makes a peak at 28th hour in t-egl1-1 (Figure 21, A) and c-egl1-2 (B), it increases and persists till the end of culture for the c-egl1-6 (C) which is a superior producer. Δxyr strain is included as a negative control (D), in which no band is perceptible at the same level.

EG3 was expressed by three strains (Figure 23**)** with an approximate size of 25 kDa in accordance with previous findings (Karlsson, 2002) which is probably due to presence of only one glycosylation site that does not affect the molecular weight much. All of the strains show increasing amount of enzymes until 36th hour which then are stable until 72nd hour. Note that although 15 μL supernatant is loaded, c-egl3-5 has very dense bands. Although c-egl3-2 was also a strong producer, due to mixing up of strains, supernatants from previous expression were used that is probably why its bands are so faint.

CBH1 was expressed under *tef1* promoter by two strains. Since pPcdna1-cbh1 vector could not be constructed due to the problems mentioned before, CBH1 was not expressed under *cdna1* promoter. Although CBH1 was reported to be around 67 kDa (Hall, 2010), proteins were detected slightly above 70 kDa in Figure 24, probably again due to the difference in glycosylation degree of protein. Both strains show stable CBH1 expression until 72nd hour and their expression profiles are very similar. QM9414, when grown on MA-glucose, had residual CBH1 expression (Figure 25) which is detected at

the level corresponding to same level with Ptef1-cbh1 strains; showing that our proteins are processed native-like. In addition, Rut-C30 had a band at the same level mentioned before, although due to smearing of the wells it was not so clear (data not shown).

Rut-C30 was known as a hypercellulolytic strain but no remarkable expression was observed in our experiments; probably since glucose is not an inducer and RutC-30 needs inducing conditions for hyperproduction (Kubicek, 2009). Additionally, decreasing enzyme amounts in SDS gels in general might be due to precipitation of enzymes together with mycelia during sampling.

6.4. Activity Assays

Activity assays were done using MUC and MULAC for EG1 and CBH1 supernatants, respectively. CMCase Assay was preferred for EG3 since it showed no activity towards aforementioned fluorogenic substrates. Activities of protein concentrations were considered proportional to amount of enzyme in the supernatant, assuming they are completely active.

While temperature optima of EG1 and CBH1 are around 50 °C, fluorogenic assays were performed at 40 °C due to the capacity of spectrofluorometer; therefore, measured RFU/min values might not be indicative of precise activities.

MUC activities of strains were measured (Figure 26-A) and they were consistent with the protein amounts detected on SDS gels. t-egl1-1 and c-egl1-2 have very similar activities; c-egl1-2 was slightly higher in concordance with its SDS gel data (Figure 21). c-egl1-6 strain was already a good producer and this was reflected in the activity curve. Remarkably, when extra glucose was added to the culture at 20^{th} hour (c-egl1-6+), activity has increased more than 2-fold, which is an indication of an increase in egl1-6 expression; which was previously observed in the SDS gels (Figure 21-C and Figure 22). As discussed above, this was not the case with glucose addition to baffled flask (c-egl1-6+b); which was also supported with activity data here. Since glucose is not an inducer, Rut-C30 supernatant showed very low activity as expected. Interestingly, QM9414 had, too, very low activity although it was grown on cellulose - inducer of cellulases- maybe because it takes a long time to induce cellulases, which is supported by the growth data that cell dry weight has decreased till 72^{nd} hour.

MULAC is used to compare activities of CBH1 producing cells (Figure 26-B); both of which were quite similar, consistent with their SDS gel data (Figure 24). QM9414 had negligible activities compared to CBH1s; possibly due to same reasons stated for MUC. It is also notable that MULAC activities are quite lower than MUC activities, which can be explained with lower activities of CBH1 enzymes; because in the raw data, fluorescent emittance was linear throughout the assay with a constant slope. One contributor of this can as well be the use of 455 nm as emittance value, while 445 nm was the true parameter for MULAC. Since the aim is comparison of strains with each other, this did not pose a problem.

CMCase activities of EG3 supernatants (Figure 26-C) were, although variable, comparable and consistent with the SDS gel data (Figure 23). EtOH precipitation of samples was done in order to get rid of background signal coming from glucose in the expression medium since glucose liberation is measured with CMCase Assay. It seems that protein concentration decreases with EtOH precipitation up to 5-fold (Table 4).

CMCase activity of EG3 in *E.coli* expressions were reported as 0.058 IU/mL (Okada, 2000) and 15 IU/mL (Nakazawa, 2008) while our maximum in *T.reesei* is 0.55 IU/mL. *Aspergillus oryzae* could express 30.7 IU/mg *T.reesei* EG3 (Takashima, 1998) and we could express 220 IU/mg by c-egl3-5. It should be noted that our values are of culture supernatants, not that of concentrated or purified proteins and much higher than *A.oryzae* expression.

6.5. Analyses of Protein Concentrations and Activities

Specific activity is an indication of enzyme purity in the solution; however, as division cancels out total protein amount and activity; specific and volumetric activities were drawn together and concentrations were also shown in Table 4. Total protein concentrations paralleled the cellulase expression in transformants and they made a peak at 36^{th} hour.

Specific activities were defined as μmol min^{-1} mg^{-1}; since MULAC had never reached to complete hydrolysis of product, its maximum was approximated by that of MUC.

Cellulase amount in the supernatant could have been deduced by subtracting concentrations of Δxyr1 from that of transformants; however, since the values are not standardized and might change from expression to expression, specific activities were preferred. This is confirmed when concentration values of Δxyr1 are subtracted from transformants; activity and net protein concentration was not directly correlated (data not shown), supporting our claim.

Protein concentrations and specific activities of EG1 producers towards MUC are compared to each other to identify the best conditions (strain, timepoint) and to obtain general information about activity trends. Although RutC-30 and QM9414 had quite high concentration of total proteins, they showed almost no specific activity towards MUC and MULAC. SDS gel photo of Rut-C30 was not included due to smearing and unclear bands; however, its total protein concentration seems to be quite high (Table 4). c-egl1-6 is better than t-egl1-1 and c-egl1-2 in terms of total protein concentration and specific activity, consistent with previous data. It is remarkable that c-egl1-6+ has almost reached the same protein levels of Rut-C30 and exceeded QM9414. The instant peak of c-egl1-6+b at 28th hour and dry weight data are quite meaningful when combined; as the strain grows faster, protein secretion decreases, probably due to occupation of translation machinery with transcription/translation of cell cycle related proteins. Increasing activity~decreasing purity problem is overcame to some extent by addition of glucose at 20th hour (c-egl1-6+) that the enzyme is still purer and has relatively high activity at 28th hour.

Protein concentrations and purities of CBH1 producers towards MULAC are compared (Table 4, B). Δxyr1, QM9414 and RutC-30 strains have no activity, once more. Although protein amounts of CBH1 producers were quite similar on SDS gel (Figure 24) t-cbh1-4 seems to produce purer enzyme at the beginning but then they are equalized.

CMCase activities and enzyme purities of EG3 producers are compared. Once again, purity is higher at early hours (20th for all), although the enzyme amounts increase later. It should be noted that EtOH precipitation caused lose of a significant percentage of proteins. c-egl3-5 produces purer enzyme in higher amounts.

In general, it can be concluded that the supernatants of strains tend to contain purer enzymes at the beginning of cultures but as more proteins are secreted as time passes, purity of the enzyme in the supernatant decreases even if secreted enzyme

amount increases as well. One can take advantage of this observation depending on the ultimate aim; “pure” enzyme or “less pure but more” enzyme. Additionally, as proven with c-egl1-6+ trial, changing some factors might help superposing maximum purity and maximum activity in some timepoint.

6.6. BLAST Analysis of *cdna1* gene

cdna1 promoter precedes a high number of Expressed Sequence Tags in the *T.reesei* genome. Calculated protein was found to be contained in these ESTs, with same size as the predicted protein by the jgi website. Hypothetically translated protein of *cdna1* is 80 aa long. Top scoring BLAST results are from five different fungi species, three of which belong to the same family with *T.reesei* (*Hypocreaceae*) and other two belong to *Sclerotiniaceae* family that converges with *T.reesei* in the phylum *Ascomycota* (Dubchak, 2006). It is noteworthy that all of these species are plant pathogenic fungi; which might be an indication of importance of cdna1 protein in growth, which is also supported by expression of proteins in the growing phase in general; however, it should be further investigated why cdna1 is expressed highly in glucose medium.

6.7. General Comments

Compared to other pre-established expression systems, like bacteria or yeast, *T.reesei* is a complex organism that can be affected by environmental factors, oxygen supply even light. Therefore, a highly optimized system should be available to work with *T.reesei.* This is further supported by experiments done in this thesis; different set of expression cultures can show dissimilar growth patterns and expression profiles. Some additional work should be done for complete optimization of the expression system.

Initially it might seem paradoxical that we are using glucose as the inducer and sugar source of fungi to produce enzymes in order to convert cellulosic material into

glucose; however thermostability and long-life of enzymes allows us to use them several times, thus it is quite advantageous to invest some glucose to gain much more of it later. Nevertheless, expression of these three homologous cellulases opened a window for utilization of T.reesei as an expression system for production of other proteins as well.

7. CONCLUSION

Our aim was to overexpress individual cellulases homologously in a cellulase-negative background under highly active promoters. We have obtained eight producer strains that express the desired enzymes with different amounts and purities. The enzymes have proven to be active towards either fluorogenic substrates or carboxymethyl cellulose.

Due to the difficult nature of chemical strategy, high throughput transformation could not be achieved; yet, hyperproducer strains could be obtained among such a small number of transformants.

To our knowledge, this is the first time that active individual cellulases in a cellulose negative background under non-inducing conditions could be overexpressed.

Finally, *Trichoderma reesei* was established as an expression system in our laboratory. Most of the basic protocols are optimized (transformation, expression etc.) to fit the laboratory facilities here and they are sometimes modified and standardized to some extent.

8. FUTURE PROJECTIONS

Although *T.reesei* could successfully be transformed and expressed individual cellulases, alternative high throughput transformation strategies can be employed (such as electroporation) to have higher number of transformants that will possibly contain much stronger producers.

Addition of sugar source and use of baffled flask experiments indicates that fine-tuning of conditions can allow present or future strains to produce higher amount of enzymes. For instance fed-batch strategy can increase production by elongating growth phase and increasing maximum growth amount since the enzymes produced in general during the growth phase. In addition to sugar, other limiting factors of growth could be investigated.

cdna1 gene is still uncharacterized, although its promoter was shown to be quite active on glucose compared to *tef1* promoter. The hypothetical protein can be expressed and characterized. Homology modeling of the protein is another strategy to find structural similarity to any other proteins and deduce its function in fungi.

Other promoter-mutant-carbon source combinations can be tried to obtain new expression systems. Another strong promoter that is active on an abundant carbon source could be tried. After optimization of this system, overexpression and purification of these enzymes will lead to the determination of best combinations necessary for specific applications. In addition, this strategy can be used to study effect of modifications or mutations in cellulase structure and function, since expression in large amounts is achieved. This strategy can be adapted to express heterologous enzymes or to overexpress and characterize mutant *T.reesei* cellulases, which is much more advantageous over using a heterologous system.

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10. APPENDICES

APPENDIX A

DNA sequence of *Cel7b* (Endoglucanase I)

>egl1_scaffold_10|665650|667800 (2151 bp)

TCTTAGTCCTTCTTGTTGTCCCAAAATGGCGCCCTCAGTTACACTGCCGTTGACCACGGCCATCCTGGC
CATTGCCCGGCTCGTCGCCGCCCAGCAACCGGGTACCAGCACCCCCGAGGTCCATCCCAAGTTGACAAC
CTACAAGTGTACAAAGTCCGGGGGGGTGCGTGGCCCAGGACACCTCGGTGGTCCTTGACTGGAACTACCG
CTGGATGCACGACGCAAACTACAACTCGTGCACCGTCAACGGCGGCGTCAACACCACGCTCTGCCCTGA
CGAGGCGACCTGTGGCAAGAACTGCTTCATCGAGGGCGTCGACTACGCCGCCTCGGGCGTCACGACCTC
GGGCAGCAGCCTCACCATGAACCAGTACATGCCCAGCAGCTCTGGCGGCTACAGCAGCGTCTCTCCTCG
GCTGTATCTCCTGGACTCTGACGGTGAGTACGTGATGCTGAAGCTCAACGGCCAGGAGCTGAGCTTCGA
CGTCGACCTCTCTGCTCTGCCGTGTGGAGAGAACGGCTCGCTCTACCTGTCTCAGATGGACGAGAACGG
GGGCGCCAACCAGTATAACACGGCCGGTGCCAACTACGGGAGCGGCTACTGCGATGCTCAGTGCCCCGT
CCAGACATGGAGGAACGGCACCCTCAACACTAGCCACCAGGGCTTCTGCTGCAACGAGATGGATATCCT
GGAGGGCAACTCGAGGGCGAATGCCTTGACCCCTCACTCTTGCACGGCCACGGCCTGCGACTCTGCCGG
TTGCGGCTTCAACCCCTATGGCAGCGGCTACAAAAGGTGAGCCTGATGCCACTACTACCCCTTTCCTGG
CGCTCTCGCGGTTTTCCATGCTGACATGGTTTTCCAGCTACTACGGCCCCGGAGATACCGTTGACACCT
CCAAGACCTTCACCATCATCACCCAGTTCAACACGGACAACGGCTCGCCCTCGGGCAACCTTGTGAGCA
TCACCCGCAAGTACCAGCAAAACGGCGTCGACATCCCCAGCGCCCAGCCCGGCGGCGACACCATCTCGT
CCTGCCCGTCCGCCTCAGCCTACGGCGGCCTCGCCACCATGGGCAAGGCCCTGAGCAGCGGCATGGTGC
TCGTGTTCAGCATTTGGAACGACAACAGCCAGTACATGAACTGGCTCGACAGCGGCAACGCCGGCCCCT
GCAGCAGCACCGAGGGCAACCCATCCAACATCCTGGCCAACAACCCCAACACGCACGTCGTCTTCTCCA
ACATCCGCTGGGGAGACATTGGGTCTACTACGAACTCGACTGCGCCCCCGCCCCCGCCTGCGTCCAGCA
CGACGTTTTCGACTACACGGAGGAGCTCGACGACTTCGAGCAGCCCGAGCTGCACGCAGACTCACTGGG
GGCAGTGCGGTGGCATTGGGTACAGCGGGTGCAAGACGTGCACGTCGGGCACTACGTGCCAGTATAGCA
ACGACTGTTCGTATCCCCATGCCTGACGGGAGTGATTTTGAGATGCTAACCGCTAAAATACAGACTACT
CGCAATGCCTTTAGAGCGTTGACTTGCCTCTGGTCTGTCCAGACGGGGGCACGATAGAATGCGGGCACG
CAGGGAGCTCGTAGACATTGGGCTTAATATATAAGACATGCTATGTTGTATCTACATTAGCAAATGACA
AACAAATGAAAAAGAACTTATCAAGCACTGTACCAAGGAAGCTCATTATGCGTCTGGCGGGTTCAAATG
ATCCGTGATAGGTTATGCCAGCTGATTGTTTGCCCGCAGGGTTGACACCACCAGGGGATAATGGCCACT
TTCATCTGAATCAAGGACAGGAGCCGTTGATGATTTCCAGATATCCTATCTTCATCGCTAGTACTAATA
ACAAGTAAGCAAACAGCCAGCTACACTCGTACACACCGCTCATGAAAACATAAGACAAAGCTCAAGCCA
TGTCAAGCACCACCCCATCGTACACGTACATCTTCTACCAATCTGTCCAGATGCCGGCTACGTCAGCCT
CCAACCCATTGCGATACAATGACGGATGTCGTATAGACCATGGCAACGGCTCCCACCACCATTGTCACT
GCGTCCAAAGTCTTCTCCCACCTGCTCTCAGCAACGCCCTTGAAATGCAGGAATGCCGGATACATGTAG
ACCAATGGAATG

Underlined regions: primer binding sites

Dark background: START and STOP codons

Retrieved from: http://genome.jgi-psf.org/cgi-bin/getScaffold?db=Trire2&scaffold=scaffold_10&start=665650&end=667800

APPENDIX B

DNA sequence of *Cel12a* (Endoglucanase III)

>egl3_scaffold_19|258400|260012 (1613 bp) – reverse complement

TGGCCAAATCGTGATCGATTGATACTCGCATCTATAAGATGGCACAGATCGACTCTTGATTCAC
AGACATCCGTCAGCCCTCAAGCCGTTTGCAAGTCCACAAACACAAGCACAAGCATAGCGTCGCA
ATGAAGTTCCTTCAAGTCCTCCCTGCCCTCATACCGGCCGCCCTGGCCCAAACCAGCTGTGACC
AGTGGGCAACCTTCACTGGCAACGGCTACACAGTCAGCAACAACCTTTGGGGAGCATCAGCCGG
CTCTGGATTTGGCTGCGTGACGGCGGTATCGCTCAGCGGCGGGGCCTCCTGGCACGCAGACTGG
CAGTGGTCCGGCGGCCAGAACAACGTCAAGTCGTACCAGAACTCTCAGATTGCCATTCCCCAGA
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CATCCGCGCTAATGTTGCGTATGACTTGTTCACCGCAGCCAACCCGAATCATGTCACGTACTCG
GGAGACTACGAACTCATGATCTGGTAAGCCATAAGAAGTGACCCTCCTTGATAGTTTCGACTAA
CAACATGTCTTGAGGCTTGGCAAATACGGCGATATTGGGCCGATTGGGTCCTCACAGGGAACAG
TCAACGTCGGTGGCCAGAGCTGGACGCTCTACTATGGCTACAACGGAGCCATGCAAGTCTATTC
CTTTGTGGCCCAGACCAACACTACCAACTACAGCGGAGATGTCAAGAACTTCTTCAATTATCTC
CGAGACAATAAAGGATACAACGCTGCAGGCCAATATGTTCTTAGTAAGTCACCCTCACTGTGAC
TGGGCTGAGTTTGTTGCAACGTTTGCTAACAAAACCTTCGTATAGGCTACCAATTTGGTACCGA
GCCCTTCACGGGCAGTGGAACTCTGAACGTCGCATCCTGGACCGCATCTATCAACTAAAACCTG
GAAACGTGAGATGTGGTGGGCATACGTTATTGAGCGAGGGAAAAAAAGCATTGGATCCATTGAA
GATGTTAGTCATATACAGACTTAGAAGATTTACATGAATGTCAAACGATGAGCGACTGTGGAAC
GTTATGAATAATAGACTGGAACCGGGCCCTTTGATTGACGACTCCATATTTTGTAGATGTAGCA
ACTCGGCAAGAGCATTATGTGCAATACATTTGTTACCATACAAAGGCAGCTGCCAGACGACTTG
TATTGCGTACAATTCTCACGGCAAGCTTTCCAGGTGTTATGCATTATGCGCAAATGCTTGATGC
TTACCGCAGGATTAATCTCGGAAGAAGCGCTGCAAGCTATATGGGTGTAGTAGATATGTAGATG
TACCAACCAATGAAGAACATTTATGGTCTAGAACGTAGTGATGAAGGTTTTGAGTAATTTGTAT
CAAGTAAGACGATATTATTGATATAATACCAAGCATATATTCATGATAAATTACTTGGAACCAC
CCTTGCGTCCGGCCTCACGAGCCTTCTCACTGCCGGGCTCGAAGGAGCCACTGGAGGCCTGTCC
ACCCTTGGATGCGATTTCCTGCACCTTTTCCTTGGGCCTGCACGTCGATTAGACATGATTCAAA
TCGAGATCTTGGA

Retrieved from: http://genome.jgi-psf.org/cgi-bin/getScaffold?db=Trire2&scaffold=scaffold_19&start=258400&end=260012

APPENDIX C

DNA sequence of *Cel7a* (Cellobiohydrolase I)

>scaffold_29|332250|334750 (2501 bp)

TTAGCCAAGAACAATAGCCGATAAAGATAGCCTCATTAAACGGAATGAGCTAGTAGGCAAAGTC
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CAACTCAGATCCTCCAGGAGACTTGTACACCATCTTTTGAGGCACAGAAACCCAATAGTCAACC
GCGGACTGCGCATCATGTATCGGAAGTTGGCCGTCATCTCGGCCTTCTTGGCCACAGCTCGTGC
TCAGTCGGCCTGCACTCTCCAATCGGAGACTCACCCGCCTCTGACATGGCAGAAATGCTCGTCT
GGTGGCACGTGCACTCAACAGACAGGCTCCGTGGTCATCGACGCCAACTGGCGCTGGACTCACG
CTACGAACAGCAGCACGAACTGCTACGATGGCAACACTTGGAGCTCGACCCTATGTCCTGACAA
CGAGACCTGCGCGAAGAACTGCTGTCTGGACGGTGCCGCCTACGCGTCCACGTACGGAGTTACC
ACGAGCGGTAACAGCCTCTCCATTGGCTTTGTCACCCAGTCTGCGCAGAAGAACGTTGGCGCTC
GCCTTTACCTTATGGCGAGCGACACGACCTACCAGGAATTCACCCTGCTTGGCAACGAGTTCTC
TTTCGATGTTGATGTTTCGCAGCTGCCGTAAGTGACTTACCATGAACCCCTGACGCTATCTTCT
TGTTGGCTCCCAGCTGACTGGCCAATTCAAGGTGCGGCTTGAACGGAGCTCTCTACTTCGTGTC
CATGGACGCGGATGGTGGCGTGAGCAAGTATCCCACCAACACCGCTGGCGCCAAGTACGGCACG
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GATGGATATCTGGGAGGCCAACTCCATCTCCGAGGCTCTTACCCCCCACCCTTGCACGACTGTC
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TGGCTCAAGCTTTACCCTCGATACCACCAAGAAATTGACCGTTGTCACCCAGTTCGAGACGTCG
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GCTCAGGTCGAATCTCAGTCTCCCAACGCCAAGGTCACCTTCTCCAACATCAAGTTCGGACCCA
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CATGCATTTTAAGATAACGGAATAGAAGAAAGAGGAAATTAAAAAAAAAAAAAAAAACAAACATC
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GATCGTTCAATGTTGATATTGTTCCCGCCAGTATGGCTCCACCCCCATCTCCGCGAATCTCCTC
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CAACAGTGGAAATTAGTGGCGCAATAATTGAGAACACAGTGAGACCATAGCTGGCGGCCTGGAA
AGCACTGTTGGAGACCAACTTGTCCGTTGCGAGGCCAACTTGCATTGCTGTCAAGACGATGACA
ACGTA

Retrieved from: http://genome.jgi-psf.org/cgi-bin/getScaffold?db=Trire2&scaffold=scaffold_29&start=332250&end=334750

APPENDIX D

DNA Sequences of Promoters: *tef1* and *cdna1*

tef1 Promoter

>scaffold_6|766900|767850 (951 bp) - reverse complement

GATATGACGGCCACCAATCGGAAGGGTGTCGTATCTCACATCACCAGCGTCAGGACACGCTTCACAAAATTCCC
ACAAGTTCCAGTTGAAATGACCCGTAGGAGAGCGCCTCGAGGGACAGAATGTACAGTACTATACTACCTACCTT
AGATGCCATCACCCCTTCGCAGGCCATGGAATTTCTGGCAAGTCGCACCCTTCAGCCATGCCTGTCCGCCATCA
TCATACCAGATCGTGTCGTGGCGGGCACTTGGGAAGGACGAGCTCCAACCGCCGCCGCTGATTGGCCTGATCCG
CCACAGCCACTGGCGCTCGCGGCGAGGAAAAAAAAAAATCTTGCGGGTGTGCAGCAGCATTGAGTGGAAAGCGG
GAGCCGCTGCACGACGCTGATTCGTCCGGGCTGCCTCGTTAGGGCTAGACCAGCAGGCGGGCGCCGCTTATTTT
GCCCCTCCTCAGGCTTTTTTTCACAAGCCCCGTTTTGCTGCCCCGCGCGTGGCAGAAAAAAAAAATACAGCACG
AGCCACTGGCACTTGCACCCCTCCACCCCTCCGCCGACCACCTTTTAAATCTCCTCCTCCCACCATCTGCTTTC
GAAATTTTCTCTCTTCTGCTCTTCGTCTCGCATACCCGGTTCAAGCATCCGATCTGCGAATTTGTATGTGCTGC
CTCTCCCTCTGACCTTCTGGTCTGGTGATACCATCCTCCCTCAGTTTGGATCATCGCCTTATTCTTCTTCCCTC
TTCTGCATCTGCTTCCTGCTCGTTTGAGGAACATCGCCAGCTGACTCTGCTTGCCTCGCAGCGATCTAGTCAAG
AACAACACAGCTCTCACGCTACATCACACAAACCGTCAAAATGGGTAAGGAGGACAAGACTCACATCAACGTGG
TCGTCATCGTACGTATTTTCCGATCCCTCATCGGCGTCATCTGCCAGTCTGATTCCAAGAATC

Retrieved from: http://genome.jgi-psf.org/cgi-bin/getScaffold?db=Trire2&scaffold=scaffold_6&start=766900&end=767850

cdna1 Promoter

>scaffold_23|43454|44994 (1541 bp)

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GACAAGAGTTGCCTGGCTATTGAGCTCTGGTACATGGATCTCGAACTGAGAGCGTACAAGTTACATGTAGTAAA
TCTAGTAGATCTCGCTGAAAGCCCTCTTTCCCGGTAGAAACACCACCAGCGTCCCGTAGGACAAGATCCTGTCG
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GGAAGAACCCGGTTGTTCGGAATGTCCTTGTGCTAACAGTGGCATGATTTTACGTTGCGGCTCATCTCGCCTTG
GCACCGGACCTCAGCAAATCTTGTCACAACAGCAATCTCAAACAGCCTCATGGTTCCCAGATTCCCTGATTCAG
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GGAAGCGATTCCAGGTCGTCTTTTTTTGGTTCATCATCACAGCATCAACAAGCAACGATACAAGCAATCCACTG
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GATTTTTCCCAAGCCGGCTTCAAAACACACAGATAAACCACCAACTCTACAACCAAAGACTTTTTGATCAATCC
AACAACTTCTCTCAACATGTCTGCTGCAACCGTCACCCGCACTGCAACCGCCGCTGTTCGCAGACCCGGCTTCT
TCATGCAAGTCCGACGGATGGGACGCTCATTCGAGCACCAGCCCTTTGAGCGACTCTCCGCCACCATGAAGCCT
GCACGACCCGACTATGCTAAGCAAGTCGTCTGGACGGCTGGCAAGTTTGTCACGTACGTACAACCACAGGACTT
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Retrieved from: http://genome.jgi-psf.org/cgi-bin/getScaffold?db=Trire2&scaffold=scaffold_23&start=43454&end=44994

APPENDIX E

Partial DNA Sequence of pPtef1 Vector

>Ptef1 (5775 bp)

1200- TCTCGTTTCTTCCTAACAAACAACCACCACCAAAATCTCTTTGGAAGCTCACGACTCACGCAAG

CTCAATTCGCAGATACAAATCTAGAATGAAAAAGCCTGAACTCACCGCGACGTCTGTCGAGAAGTTTCTGATCG
AAAAGTTCGACAGCGTCTCCGACCTGATGCAGCTCTCGGAGGGCGAAGAATCTCGTGCTTTCAGCTTCGATGTA
GGAGGGCGTGGATATGTCCTGCGGGTAAATAGCTGCGCCGATGGTTTCTACAAAGATCGTTATGTTTATCGGCA
CTTTGCATCGGCCGCGCTCCCGATTCCGGAAGTGCTTGACATTGGGGAATTCAGCGAGAGCCTGACCTATTGCA
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GCGGAGGCCATGGATGCGATCGCTGCGGCCGATCTTAGCCAGACGAGCGGGTTCGGCCCATTCGGACCGCAAGG
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CAGGCTTTTTTTCACAAGCCCCGTTTTGCTGCCCCGCGCGTGGCAGAAAAAAAAAATACAGCACGAGCCACTGG
CACTTGCACCCCTCCACCCCTCCGCCGACCACCTTTTAAATCTCCTCCTCCCACCATCTGCTTTCGAAATTTTC
TCTCTTCTGCTCTTCGTCTCGCATACCCGGTTCAAGCATCCGATCTGCGAATTTGTATGTGCTGCCTCTCCCTC
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AGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAA
ACCTGTCG ... AmpR ...

Name	Sequence	Location	Description
hph		1289-2313	Hygromycin Phosphotransferase Gene
XhoI	CTCGAG	2772	Restriction Site
pTef1		2769-3512	Translation Elongation Factor 1 alpha Promoter
ClaI (Bsu15I)	ATCGAT	3514	Restriction Site
SalI	GTCGAC	3520	Restriction Site
HindIII	AAGCTT	3538	Restriction Site
AmpR		4715-5575	Ampicillin Resistance Gene

APPENDIX F

Hypothetical cdna1 protein and BLAST Results

> Cdna1 protein (translated)

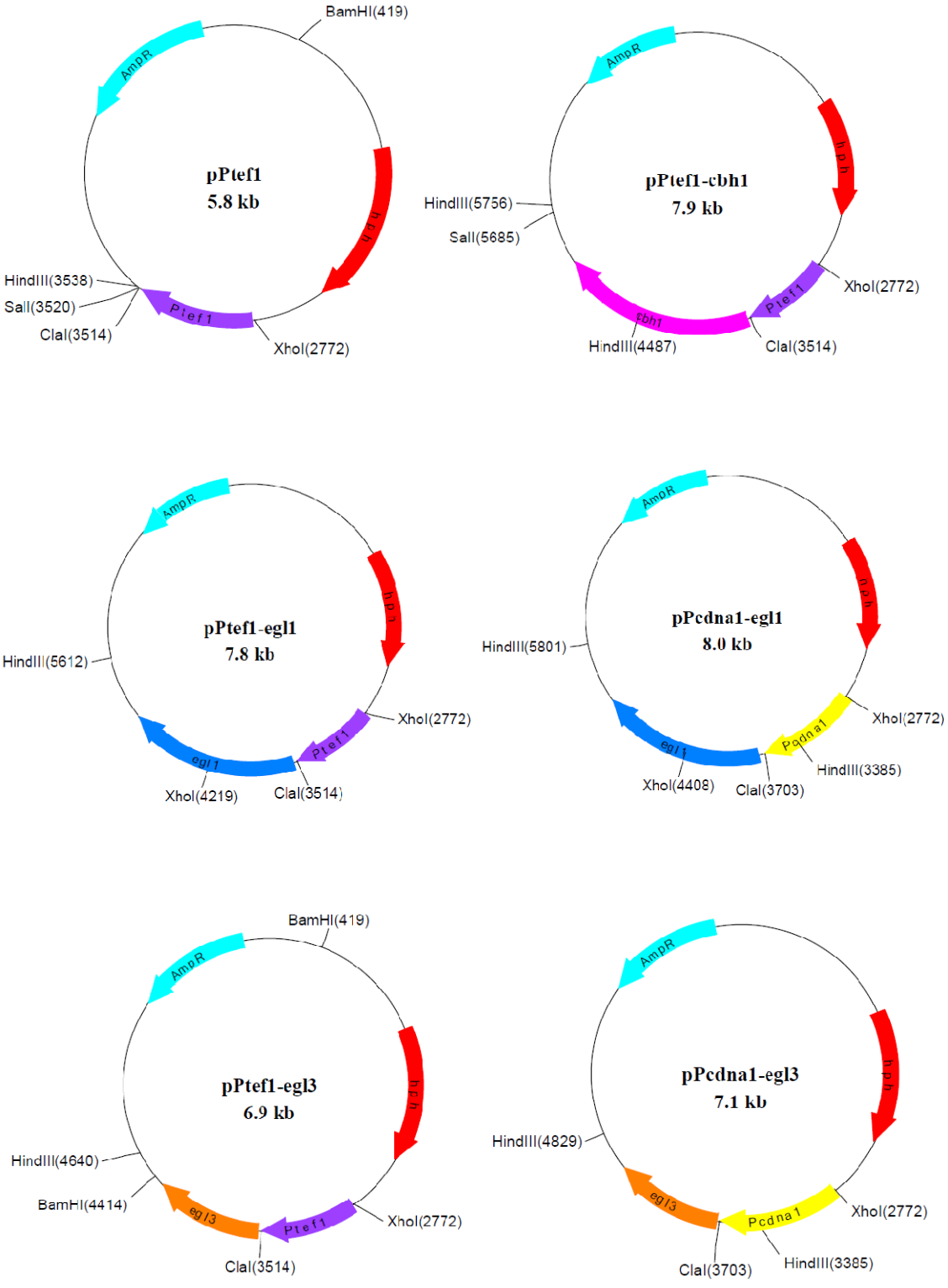
MSAATVTRTATAAVRRPGFFMQVRRMGRSFEHQPFERLSATMKPARPDYAKQVVWTAGK
FVTYVQPQDLMHPYIQYDLVD

BLAST Results of cdna1 Protein

```
>gb|EEU45561.1|  predicted protein [Nectria haematococca mpVI 77-13-4]
Length=86

 Score = 43.5 bits (101),  Expect = 0.007, Method: Compositional matrix adjust.
 Identities = 23/49 (46%), Positives = 31/49 (63%), Gaps = 2/49 (4%)

Query  5   TVTRTATAAVRRPGFFM--QVRRMGRSFEHQPFERTSATMKPARPDYAK  51
           V R+A  AV+R  F +   +R +GRS E  PFER   + +PA+PDYAK
Sbjct  4   IVVRSAARAVQRRQFSLLSSMRTVGRSMESHPFERLPISQQPAKPDYAK  52


>ref|XP 390400.1|  hypothetical protein FG10224.1 [Gibberella zeae PH-1]
Length=86

 GENE ID: 2792053 FG10224.1 | hypothetical protein [Gibberella zeae PH-1]

 Score = 39.3 bits (90),  Expect = 0.15, Method: Compositional matrix adjust.
 Identities = 22/49 (44%), Positives = 26/49 (53%), Gaps = 2/49 (4%)

Query  5   TVTRTATAAV--RRPGFFMQVRRMGRSFEHQPFERTSATMKPARPDYAK  51
           V R+A  A   R+      +R  GR+ E  PFER   T KPA PDYAK
Sbjct  4   IVARSAARAFQTRQFSLLTAMRNAGRAMESHPFERLPITQKPASPDYAK  52


>ref|XP 001598779.1|  predicted protein [Sclerotinia sclerotiorum 1980]
 gb|EDN91465.1|  predicted protein [Sclerotinia sclerotiorum 1980]
Length=82

 GENE ID: 5495022 SS1G 00868 | hypothetical protein
[Sclerotinia sclerotiorum 1980 UF-70]

 Score = 38.9 bits (89),  Expect = 0.20, Method: Compositional matrix adjust.
 Identities = 21/53 (39%), Positives = 30/53 (56%), Gaps = 0/53 (0%)

Query  11  TAAVRRPGFFMQVRRMGRSFEHQPFERTSATMKPARPDYAKQVVWTAGKFVTY  63
           A+ R     F QVR++ R FE  PFER   + + A+ D++K V  TAG  V +
Sbjct  8   VASRRTFSIFTQVRQLARGFEPHPFERYPISQQAAKADWSKLVKRTAGNAVFF  60


>ref|XP 001552840.1|  predicted protein [Botryotinia fuckeliana B05.10]
 gb|EDN29076.1|  predicted protein [Botryotinia fuckeliana B05.10]
Length=81

 GENE ID: 5433362 BC1G 09022 | hypothetical protein
[Botryotinia fuckeliana B05.10]

 Score = 38.5 bits (88),  Expect = 0.25, Method: Compositional matrix adjust.
 Identities = 19/42 (45%), Positives = 25/42 (59%), Gaps = 0/42 (0%)

Query  22  QVRRMGRSFEHQPFERTSATMKPARPDYAKQVVWTAGKFVTY  63
           Q R++ R FE   PFER   + + A+ D+AK V  TAG  V Y
Sbjct  19  QARQLARGFEPHPFERYPISQQAAKSDWAKLVKRTAGNAVLY  60


>ref|XP 003009002.1|  hypothetical protein VDBG 00684 [Verticillium albo-atrum VaMs.102]
 gb|EEY14576.1|  hypothetical protein VDBG 00684 [Verticillium albo-atrum VaMs.102]
Length=85

 Score = 36.6 bits (83),  Expect = 0.94, Method: Compositional matrix adjust.
 Identities = 22/51 (43%), Positives = 32/51 (62%), Gaps = 3/51 (5%)

Query  3   AATVTRTATAAVRRPGFFM--QVRRMGRSFEHQPFERTSATMKPARPDYAK  51
           +A V R+A  A +R  F +   +R M R+FE  PF+R + T + A+PDYAK
Sbjct  2   SAFVARSALRATQRRQFSLLTNMRNMARTFEPHPFQRMT-TAQAAKPDYAK  51
```

APPENDIX G

Vector Maps



APPENDIX H

Mandels-Andreotti (MA) Medium

	1 L	250 mL	FINAL
$(NH_4)_2.SO_4$	1,4 g/L	0,35 g	
KH_2PO_4	2,0 g/L	0,50 g	
$MgSO_4.7H_2O$	0,3 g/L	0,075 g	
$CaCl_2.2H_2O$	0,4 g/L	0,10 g	
Trace elements (50X)	20 mL	5 mL	1X
Pepton[1]	1 g/L	0,25 g	0.1% (w/v)
Urea (5 mM)	0,3 g/L	0,075 g	5 mM
10% Tween 80	2 mL	0,5 mL	0,2 g/L
Sub-TOTAL	**900 mL**	**225 mL**	
After preparing this mixture, pH is adjusted to 5.0 with citric acid. 225 mL medium is added to each 1L flask. Flasks are closed with cotton cloth or coffee filters and bound with rubber rings. Top of flasks are covered with aluminum foil and flasks are autoclaved. If necessary, they can be kept at +4°C until use.			
Sugar	10 g/L	2,6 g	1 % (w/v)
Sub-TOTAL	**100 mL**	**26 mL**	
Sugar solutions are prepared separately in 1/10 volume of final mixture (+1 mL is done usually to compensate the solution adhering the walls) in 100 mL flasks. Autoclaved and can be kept at +4°C. Sugar solution is added to the medium just before starting the expression together with spores.			
TOTAL	**1000 mL**	**250 mL**	

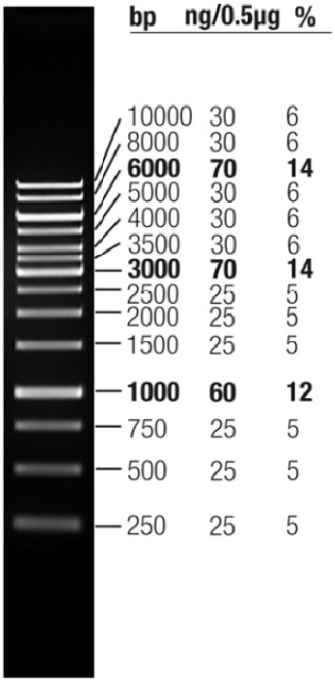
For the buffered solutions; $Na_2HPO_4.2H_2O$ (50mM final) is used (adjust pH with citric acid)

TRACE ELEMENTS (50X)			
	1 L	**500 mL**	**100 mL**
$FeSO_4.7H_2O$	250 mg	125 mg	25 mg
$MnSO_4.H_2O$	80 mg	40 mg	8 mg
$ZnSO_4.7H_2O$	70 mg	35 mg	7 mg
$CoCl_2.2H_2O$	100 mg	50 mg	10 mg

[1] Pepton from casein pancreatically digested (Merck – 1.02239.0500)

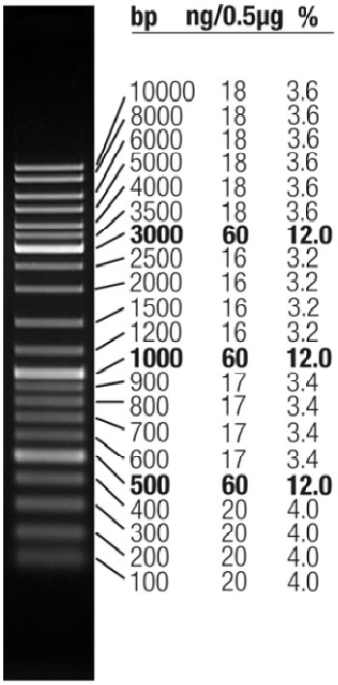
APPENDIX I

Size Markers



0.5 µg/lane

Figure 28: SM0311 GeneRuler 1kb DNA Ladder



0.5 µg/lane

Figure 29: SM0331 GeneRuler DNA Ladder Mix

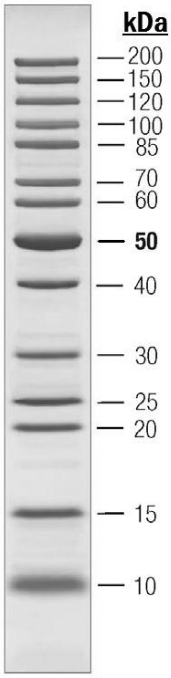


Figure 30: SM0661 PageRuler™ Unstained Protein Ladder

APPENDIX J

Buffers, Loading Dyes, Size Markers, Antibiotics, Enzymes

GoTaq DNA Polymerase	Promega	M830A (M3171)
5X Green GoTaq Flexi Buffer	Promega	M891A (M8291)
RNase A	QIAGen	19101
HindIII	Fermentas	
ClaI (Bsu15I)	Fermentas	
SalI	Fermentas	
XhoI	Fermentas	
Lysing Enzymes from Trichoderma Harzianum	Sigma	L1412-5G
10X Buffer Tango	Fermentas	BY5
10X Fast Digest Buffer	Fermentas	

Unstained Protein Ladder	Fermentas	SM0661
GeneRuler 1kb DNA Ladder	Fermentas	SM0311
6X DNA Loading Dye	Fermentas	R0611
GeneRuler DNA Ladder Mix	Fermentas	SM0331

Ampicillin	Roth	K029.2
Hygromycin B	Calbiochem	400051
Hygromycin B	Roth	CP13.3

APPENDIX K

General Chemicals

3,5-Dinitrosalicylic acid	Fluka	42260
4-Methylumbelliferyl-β-D-Cellobioside	Sigma	M6018
4-Methylumbelliferyl-β-D-Lactoside	Sigma	M2405
Acetic Acid, Glacial	Roth	3738-1
Acrylamide-Bisacrylamide	RPI	A11410-500.0
Agar Agar	Merck	1.01614.1000
Agar Noble	Difco	214230
Agarose Low EEO Standard	Star Lab	N3101-0500
Agarose Low EEO Standard	Applichem	A2114,0500
Ammonium Persulfate	Sigma	A-6761
Ammonium Sulphate ($(NH_4)_2SO_4$)	Roth	3746.1
Ammonium Sulphate ($(NH_4)_2SO_4$)	Merck	1.01217.1000
Calcium Chloride dihydrate ($CaCl_2.2H_2O$)	Sigma	C-2536
Cellulose	Acıselsan	SY-1000
Coomassie Brilliant Blue G-250	Merck	1.15444.0025
EDTA	Sigma	E5134-500G
Ethanol (96%)	Merck	1.00971.2500
Ethidium Bromide	Merck	OCO28942
Glucose	Sigma	G-7021
Glycerol	Roth	3783.1
Glycerol	Riedel-de-Haen	15523
Isopropanol	Applichem	A3928
Magnesium Chloride Solution (25 mM)	Promega	A351B (M8291)
Magnesium Sulphate Heptahydrate ($MgSO_4.7H_2O$)	Roth	T888.2
Magnesium Sulphate Heptahydrate	Fluka	63142
Malt Extract	Merck	1.05391.0500

Methanol (for analysis)	Merck	1.06009.2500
Rochelle Salts (Potassium sodium tartrate)	Fluka	60412
Phenol	Applichem	A0889
Sodium Chloride (NaCl)	Roth	3957-2
Sodium Chloride (NaCl)	Merck	1.06400.5000
Sodium Hydroxide	Merck	1.06462.1000
Sodium Dodecyl Sulphate	Sigma	L-4390
D-Sorbitol (97%)	Acros	132730025
D-Sorbitol (97%)	Sigma	A2222,5000
PEG 6000	Merck	8.07491.1000
Pepton from Casein Pancreatically Digested	Merck	1.02239.0500
Phosphoric Acid 85%	Merck	1.00563.1000
Potassium dihydrogen Phosphate (KH_2PO_4)	Merck	1.04873.1000
Potassium dihydrogen Phosphate (KH_2PO_4)	Riedel-de-Haen	4243
Potassium Hydroxide (KOH)	Riedel-de Haen	06009
Potato Dextrose Agar	Difco	213400
Potato Dextrose Agar	Merck	1.10130.0500
Sodium Acetate	Merck	1.06264.0500
TEMED	Roth	2367.1
Tris	Fluka	93349
Tris	Amresco	0826
TritonX-100	JTBaker	2840
TritonX-100	Applichem	A1388
Tween80 for synthesis	Merck	8.22187.1000
Tween80 for synthesis	RPI	P20390-0.5
Water GR for Analysis (PCR Water)	Merck	1167545000
Yeast Extract	Roth	2363-2
Yeast Extract	RPI	Y20025-1000.0

APPENDIX L

Instruments and Consumables

1.5 mL PP Micro tubes	Sarstedt	72.690.001
0,2 mL PCR Tubes	Star Lab	B1402-5500
5mL (Makro) tips	Roth	
Aluminum Foil	Burpak	
Filter	Sartorius	
Cellophane		
Chromatography Paper 3MM Chr 100 sheets	Whatman	3030917
Chromatography Paper 3MM Chr 100 sheets	Schleicher-Schuell	
Corex tube (50 mL)	Corex	
Eppendorfs	TreffLab	
Falcon	TPP	
Filter: Asahi Techno Glass CO, 25mm 0.45 micron	Asahi Techno	2053-0.25
Flat-bottomed 96-well microplate	Globe Scientific	LP120038
Flat-bottomed 96-well microplate	Costar	3915
Glass Funnel	Schott-Mainz	
Glass Wool		
Glass Microfibre Filters, GF/C 47mmØ Circles	Whatman	1822047
Hemocytometer	Thoma	HB6
Micropipette tips	Axygen	
Needles (100 (0,90x40mm))	Sterican	
Needles (100 (0,55x25mm))	Sterican	
Petri Dishes (100mm)	Grenier Bio-one	
Petri Dishes (35 mm))	Grenier Bio-one	627160
Syringe (10ml - 12mL)	Norm-Ject	4100.000V0
Syringe	SetMedikal	

APPENDIX M

Molecular Biology Kits

BioRad SDS Kit	BioRad	
QIAQuick PCR Purification Kit (250)	QIAGen	28106
QIAQuick Gel Extraction Kit	QIAGen	28706
pGEM®-T Easy Vector System	Promega	A1360
PureYield™ Plasmid Midiprep System	Promega	A2492
TaKaRa Ligation Kit, Version 2.1	TaKaRa	6022

APPENDIX N

Equipment

70 °C incubator	Memmert	Model: 600 D06062
37 °C incubator	Memmert	Model: 300 D06059
28 °C incubator	Nüve	EN120
120 °C incubator	Binder	
80 °C incubator	Binder	
37 °C incubator	Heraeus	
28 °C incubator	Heraeus	
50 °C water bath	Fischer Scientific	Isotemp
37 °C water bath	GFL	1083
37 °C water bath	Memmert	
Autoclave	Priorclave	
Autoclave: Hiclave	Hirayama	HV110
Autoclave	Nüve	OT032
Balance	Denver Instrument	TP303
Balance	Sartorius	BP211D
Balance	Sartorius	BP221S
Balance	Kern EMB	
Calibrated Densitometer (Scanner)	BioRad	GS800
Cooling Centrifuge	Eppendorf	5415 R
Centrifuge: Multifuge	Heraeus	3L
Refrigerated Microfuge	Sigma	1-15K
Cooling Centrifuge: Allegra	Beckman Coulter	X-15R
TableTop Centrifuge	Fischer Scientific	3722L
TableTop Centrifuge: Minispin Plus	Eppendorf	

Centrifuge: Bench Top	Sigma	3K-30
Centrifuge Rotor: 19776	Sigma	
Fume Hood	Trox Technik	
Micropipette (1000 μL, 200 μL, 20 μL)	VWR	
Micropipette (1000 μL, 200 μL, 20 μL)	Eppendorf	
Microwave	Samsung	M1712N
Microwave	Bosch	
Spectrometer	Schimadzu	UV-1208
Laminar Flow: Thermo Scientific KS9 1/PE AC	Thermo Fisher	
Laminar Flow: HeraSafe	Heraeus	HS12
Laminar Flow	Nüve	MN120
Mini-PROTEAN® Tetra Cell	BioRad	165-8001
Millipore	MilliQ	QGARD00R1
Shaker: Unitron A6	Infors	Unitron 108527
Ice machine	Scotsman	AF20
Magnetic Stirrer	Velp Scientifica	
Orbital Shaker	Forma Scientific	4520
Precise Balance	Acculab	
Power Supply: PowerPac Basic	BioRad	164-5050
Power Supply	Wealtech	Elite 300
Power Supply	Pharmacia Biotech	EPS300
Refrigerator (-80 °C)	Thermo-Forma	
Refrigerator (-20 °C)	Bosch	
Spectrofluorometer	SpectraMax	GeminiXS
Speed Vacuum	Savant	Sc100A
Swingout Rotor: 11390/13150	Sigma	3K30
Thermocycler	Biometra	T3000

Thermocycler	Biometra	T3
Thermocycler	MD Research	PTC-100
Thermocycler	PE Applied Biosystems	9700
Thermomixer Compact	Eppendorf	T1442
Turbidity Meter	BioLog	ID: 200-7849 Model: 21907
UV-Transilluminator	BioRad	GelDoc 2000
Vacuum Pump	Imvac	
Vortex	Velp Scientifica	

Publications in preparation arising from this thesis:

"Production of individual cellulase components by *Trichoderma reesei* in a cellulase-negative background"

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