SOUTHERN ANALYSIS OF *TORULA THERMOPHILA* GENOME FOR THE MULTIPLICITY OF THE CELLUASE GENES

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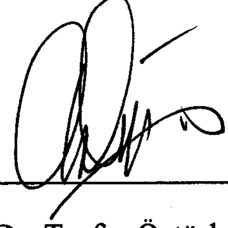

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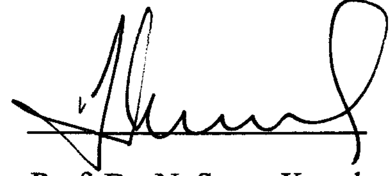

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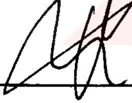

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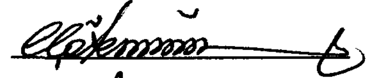
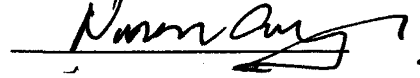
Prof. Dr. Cumhur Çökmüş 

Assoc. Prof. Dr. Nursen Çoruh 

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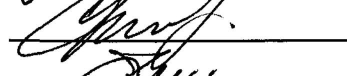
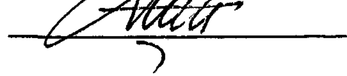
Assoc. Prof. Dr. Candan Gürakan 

Assoc. Prof. Dr. Zümrüt B. Ögel 

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ABSTRACT

SOUTHERN ANALYSIS OF *TORULA THERMOPHILA* GENOME FOR THE MULTIPLICITY OF CELLULASE GENES

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The objective of this study was analyzing of the *Torula thermophila* genome to determine the number of the cellulase genes with a cellulase specific probe. To achieve this the 100 bp PCR product of *T. thermophila* was firstly analyzed with Southern hybridisation of *Trichoderma reesei* with respect to its specificity. The similarity of hybridisation patterns of endoglucanase III gene of *T. reesei* and 100bp probe on *T. thermophila* indicates the specificity of the probe to cellulase genes. Multiplicity of cellulases was shown by the homologous genomic hybridisation *of T. thermophila* with 100bp cellulase specific probe. By performing RNA dot blot heterologous analysis with the 100bp DNA fragment of *T. thermophila*, specificity of

the probe was confirmed and it was shown that the probe is belong to the expressed region of cellulases.

Keywords: Cellulase, gene multiplicity, *T. thermophila, S. thermophilum*

ÖZ

TORULA THERMOPHILA GENOMUNUN SELÜLAZ GEN ÇOKLUĞUNUN TESPİTİNE YÖNELİK SOUTHERN İZLEŞTİRME ANALİZİ

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Bu çalışmada *Torula thermophila* selülaz genlerinin sayısının bu genlere özgün gen sondaları kullanılarak tespiti amaçlanmıştır. Buna yönelik olarak 100 baz çiftlik Polimeraz Zincir Tepkimesi ürünü *T. reesei* de Southern izleştirme yöntemiyle selülazlara özgünlüğü açısından analiz edilmiştir. Bu aşamada *T. reesei* endoglukanaz III geni ile homolog *T. thermophila* 100 baz çiftlik DNA fragmanı ile de heterolog izleştirme yapılmış ve bu DNA fragmanının *T. thermophila* selulaz genlerine özgünlügü doğrulanmıstır. Selulaz genlerinin çokluğu ise *T. thermophila* da 100bp selülaz spesifik gen sondası kullanılarak homolog Southern genom izleştirmesi ile gösterilmiştir. Bunlara ek olarak 100 baz çiftlik selülaz spesifik gen sondası *T. thermophila* toplam RNA sı ile de hetrolog izleştirmeye tabi tutulmustur.

Bu analizin sonucu gen sondasının selulaz genlerinin ifade edilen bölgesine ait olduğunu göstermiş ve selülazlara özgünlügünü doğrulamıştır.

Anahtar Sözcükler: Selülaz, gen çokluğu, *T. thermophila, S. thermophilum.*

To My Mother

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ABBREVIATIONS

A	Adenine
A	Alanine (in aminoacid structure)
Bp	Base pair
BSA	Bovine serum albumin
C	Cytosine
C	Cysteine (in aminoacid structure)
CBD	Cellulose binding domain
CTAB	Cethyltrimethylammoniumbromide
D	Aspartate
DEPC	Diethyl pyrocarbonate
DNTP	Deoxynucleotidetriphosphate
E	Glutamate
EDTA	Ethylenediaminetetraaceticacid
EG	Endoglucanase
EtBr	Ethidium Bromide
F	Phenylalanine
G	Guanine
G	Standard acceleration of gravity
G	Glycine (in aminoacid structure)
H	Histidine

H. insolens	*Humicola insolens*
I	Inosine
I	Isoleucine (in aminoacid structure)
K	Lycine
kb	Kilobase
L	Leucine
M	Methionine
N	Asparagine
P	Proline
PCR	Polimerase chain reaction
PD	Potao dextrose
PDA	Potato dextrose agar
Q	Glutamine
R	Arginine
rpm	Revolutions per minute
S	Serine
T	Threonine
T. reesei	*Trichoderma reesei*
T. thermophila	*Torula thermophila*
TE	Tris-EDTA buffer
V	Valine
W	Tryptophan
Y	Tyrosine

CHAPTER I

INTRODUCTION

1.1 Enzyme Technology:

Enzymes are valuable in manufacturing because of their rapid and efficient action at low concentrations under mild pH values and temperatures, their high degree of substrate specificity, their low toxicity, and ease of stopping their action by mild treatments.

A large number of enzymes are produced by microorganisms and among them filamentous fungi are the most promising group for industrial enzyme production.

1.1.1 Filamentous Fungi in Industry:

The secretion of enzymes by many species of filamentous fungi is an essential feature of their life style, whether it is to support saprophytic growth or pathogenicity (Archer and Wood. 1995). Due to the ability of producing a wide range of enzymes, filamentous fungi have been used for more than fifty years in industrial enzyme production. Most fungi secrete several enzymes, and hence the crude products that have been used for industrial applications contain multiple enzymes. The possibility

of cloning and expressing selected enzymes has facilitated a switch from the production of enzyme mixtures to recombinant single enzyme components (Dalboge and Heldt-Hansen, 1995).

1.1.2 Industrial Importance of Thermophilic Fungi:

Enzyme systems of thermophilic fungi are stable to higher temperatures, e.g. up to 70 °C (Ali *et al.*, 1993, Takashima *et al.*, 1996) and they have an optimum pH around neutrality (Takashima *et.al.*, 1996). They have also a lower risk of contamination by allowing higher fermentation temperatures (Rodriguez *et.al.*, 1991). Thus all these advantages make these enzymes preferable in industry.

1.2 Cellulases:

The term cellulase encompasses a variety of hydrolytic plus some oxidative and phospholytic enzymes that interactively promote the degradation of cellulose (Table 1.1) (Goyal *et.al.*,1991). Functionally complete cellulase enzyme systems can be produced by a large diversity of microorganisms, such as aerobic and anaerobic bacteria, white rot fungi, and anaerobic fungi (Beguin and Aubert, 1994, Kubicek *et.al.*,1993). Cellulose-degrading protozoan have also been identified in rumen (Beguin, 1990). The soft-rod fungi, *Trichoderma reesei* and *Trichoderma viride* are by far the most extensively studied ones. Among prokaryotes, the *Corynebacterium Cellulomonas fimi* and the anaerobe *Clostridium thermocellum* are the best known examples (Cullen and Kersten, 1992).

Table 1.1. Enzymes involved in cellulose degradation

EC number	Enzyme
1.1.5.1	Cellobiose dehydrogenase (quinone)
1.1.99.18	Cellobiose dehydrogenase (acceptor)
2.4.1.20	Cellobiose phosphorylase
2.4.1.49	Cellodextrin phosphorylase
3.1.1.17	Lactonase
3.2.1.4	*Endo*-glucanase
3.2.1.21	*β*-Glucosidase (Cellobiase)
3.2.1.74	*Exo*-glucohydrolase
3.2.1.91	*Exo*-cellobiohydrolase

1.2.1 Cellulose as Substrate:

Cellulose is by far the most abundant renewable carbohydrate source with an estimated synthesis rate of $4x10^7$ tons per year (Kraulis *et.al.*, 1989,Goyal et.al.,1991,Davies *et.al.*, 1993, Divne *et.al.*,1994). As such, it has attracted the interest of many microorganisms, which use it as carbon source, and, more recently, of biotechnologists, who wish to use it as a renewable source of fuels and chemicals.

1.2.2 Structure of cellulose:

Cellulose is a linear homopolymer made of glucose subunits linked by β-1,4 bonds (Figure 1.1) (Kubicek *et.al.*,1992, Linder and Teeri, 1997). Each glucose residue is rotated by 180° relative to its neighbors, so that the basic repeating unit is

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in fact cellobiose. Chain length varies between 100 and 14000 residues (Beguin and Aubert, 1993, Coughlan, 1990).

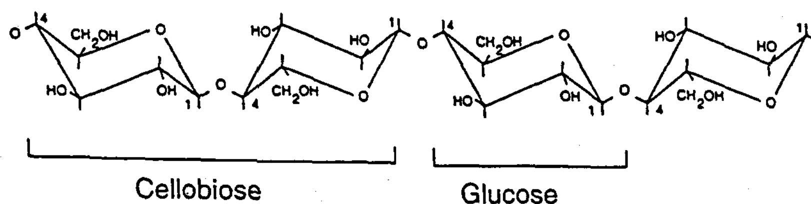

Figure 1.1. Structure of cellulose

Cellulose differs from other glucose polysaccharides by its insoluble, rigid structure leading to a natural resistance to biological degradation (Beguin and Aubert, 1993, Pentila *et.al.*, 1986). This is due to the propensity of cellulose chains to pack together, and to form long crystals stabilised by intramolecular forces (Linder and Teeri, 1997, Beguin and Aubert, 1993). Furthermore, the fibres are embedded in a matrix of hemicellulose and lignin, which further reduces their accessibility to cellulolytic enzymes (Beguin, 1990).

Since cellulose is a natural polymer, its crystalinity is rarely perfect and various imperfections and amorphous regions have been observed in different cellulose samples (Figure 1.2) (Linder and Teeri, 1997, Goyal *et.al.*, 1991). Therefore, different cellulosic materials exhibit very different properties depending on their source and method of extraction (Linder and Teeri, 1997).

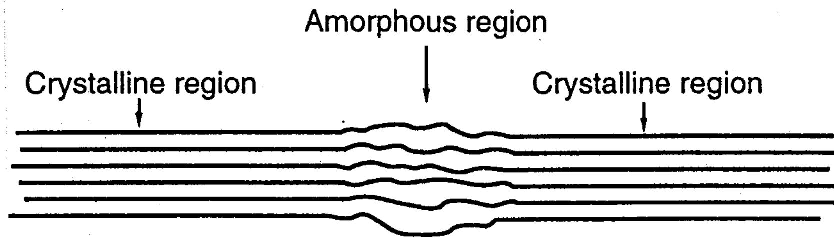

Figure 1.2. Schematic structure of cellulose fibrils.

Because of the rigid and variable nature of cellulose, cellulolytic microorganisms meet the challenge of their complex, insoluble substrate by generating a remarkable variety of enzymes acting in different ways on different parts of substrates.

1.2.3 Fungal Cellulases:

In submerged culture, most cellulolytic fungi secrete a complex array of degradative enzymes and the classical fungal system is based primarily on *Trichoderma* cellulases, which are recognized on the basis of substrate specificity (Cullen and Kersten, 1992, Goyal *et.al.*, 1991):

- Endo-glucanases (EG), (endo-1,4-β-D-glucan 4 glucanohydrolase, EC 3.2.1.4);
- Cellobiohydrolase (CBH), (Exo-1,4-β-D-glucan cellobiohydrolase, EC 3.2.1.91);

- Cellobiase (β-glucosidase), β-D-glucoside glucohydrolase, EC 3.2.1.21) (Beguin,1990, Goyal *et.al.*, 1991, Henrissat *et.al.*, 1989, Pentilla *et.al.*,1986, Takashima *et.al.*, 1996).

In the degradation of cellulose by *Trichoderma* species, at least two cellobiohydrolases (CBHI and CBHII), two ore more endoglucanases and one β-glucosidase operate synergistically (Kubicek, 1992, Cullen and Kersten, 1992, Jennings, 1995).

1.2.3.1 Endoglucanases:

Endoglucanases are extracellular enzymes possessing a more open active site cleft when compared to cellobiohydrolases (Azevedo *et.al.*, 1990, Teeri *et.al.*, 1994). They hydrolyse the disordered, amorphous regions of cellulose by randomly cutting at internal β-1,4-glycosidic linkages (Beguin, 1990, Divne *et.al.*, 1994, Goyal *et.al.*, 1991, Liner and Teeri, 1997, Pentilla *et.al.*, 1986).

Endoglucanases have been classified into two groups; one group having cellulose binding domain attached to the catalytic domain, and the other lacking a cellulose binding domain. This structural difference causes the difference in their activities towards different cellulosic substrates. Endoglucanases having a cellulose-binding domain bind and degrade crystalline cellulose, while endoglucanases lacking a cellulose binding domain cannot bind this substrate (Arifoglu, 1999). Endoglucanases have very little activity towards cellobiose hydrolysis, but hydrolyse

cellodextrins, phosphoric acid swollen cellulose and substituted cellulose such as carboxymethyl- and hydroxyethyl-cellulose (Biely *et.al.*, 1991, Kubicek *et.al.*, 1992)

1.2.3.2 Cellobiohydrolases:

Cellobiohydrolases (CBH), like endoglucanases, are also extracellular enzymes (Azevedo *et.al.*, 1990). They split off glucose and cellobiose from the non-reducing ends of cellulose chains, which enables the total degradation of crystalline cellulose into glucose by cellobiase activity (Beguin, 1990, Kubicek *et.al.*, 1997, Pentilla *et.al.*, 1986). That is, cellobiohydrolase activity has a central role in the degradation of crystalline cellulose. They have virtually no activity on carboxymethylcellulose (Schulein, 1997).

1.2.3.3 Cellobiase (β-Glucosidase):

Cellobiase is an intracellular enzyme, believed to be bound to the cell wall of cellulolytic organisms (Azevedo *et.al.*, 1990, Kubicek *et.al.*, 1992). It hydrolyses cellobiose and low molecular weight cellodextrins into glucose and cellobionic acid into glucose and glucanolactone (Beguin, 1990, Pentilla *et.al.*, 1986). It is believed that it may have a role in controlling the accumulation of cellulase inducers (Kubicek *et.al.*, 1992).

1.2.3.4 Synergistic Interaction Between Endo- and Exo-acting Enzymes:

Fungal cellulases have frequently been reported to act synergistically in the degradation of crystalline cellulose. (Caughlan, 1990, Cullen and Kersten, 1992, Kubicek *et.al.*, 1993, Nidetzky *et.al.*, 1994, Baker *et.al.*, 1994). On the basis of biochemical analysis of cellulase systems produced by *P. chrysosporium* and *Trichoderma konigii,* a model was proposed in the late seventies. Endoglucanases would first hydrolyse amorphous regions of the cellulose fibers. The non-reducing ends generated could then be attacked by CBHs, which would then proceed with the degradation of the crystalline regions. β-glucosidases would prevent the accumulation of cellobiose, which inhibits CBHs (Figure 1.3) (Beguin, 1990). Neither type of enzyme, acting alone, can affect extensive hydrolysis of crystalline cellulose. However, in appropriate mixtures of components, the endoglucanases affect random scission of glycosidic linkages and so provide chain ends from which the exoglucanases cleave cellobiose. It follows from this theory that chain ends are not available in quantity for cellobiohydrolase action in the absence of endoglucanases. Because of the high degree of crystalline cellulose, bonds cleaved by endoglucanases would reform spontaneously in the absence of cellobiohydrolases (Coughlan, 1990). This model is, however, most likely an over-simplification because, for example, it does not explain the synergism between CBHs or, conversely, the absence of synergism between some CBHs and some EGs. The interpretation of these observations is still debated (Beguin, 1990).

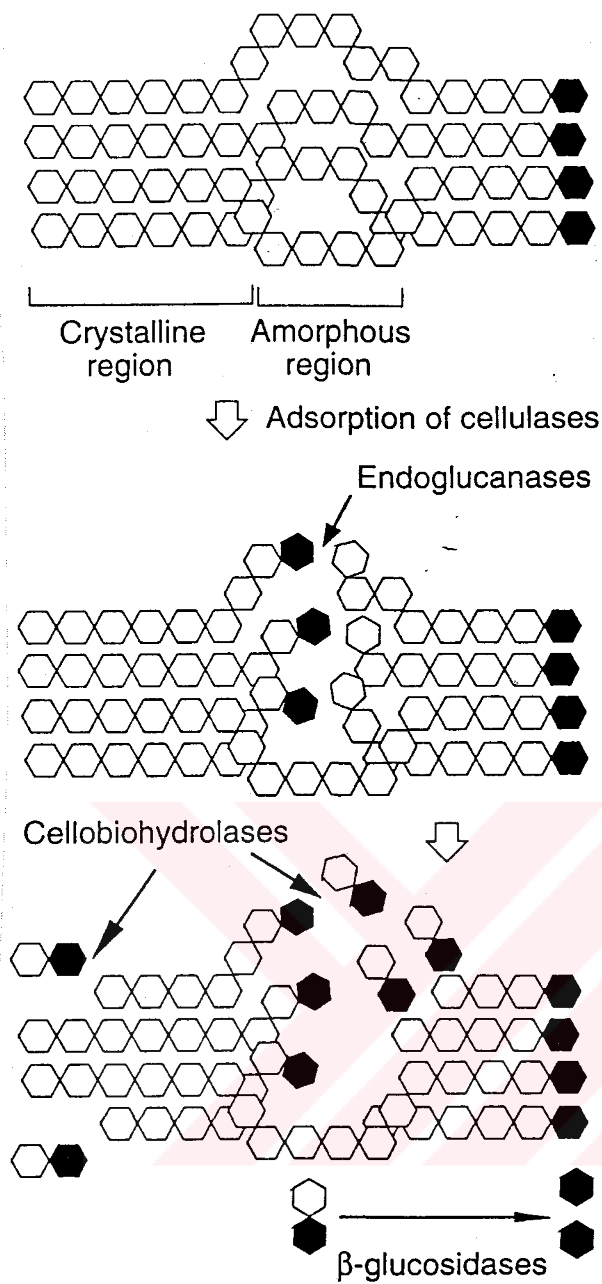

Figure 1.3. Synergistic interaction between endoglucanases, cellobiohydrolases and β-glucosidases in fungal cellulase systems.

1.2.4 Structural Elements of Fungal Cellulases:

Sequence analysis of cellulase genes and the biochemical characterisation of wild type and truncated enzymes have shown that many cellulolytic enzymes are multifunctional proteins composed of distinct domains which can be arranged in various combinations (Beguin and Aubert, 1994). The structure of cellobiohydrolases and endoglucanases of *T. reesei* is composed of three domains, namely catalytic domain, hinge and cellulose binding domain (Figure 1.4, 1.5) (Cullen and Kersten, 1992, Kubicek 1992). In literature, the cellulose-binding domain is abbreviated as A and the hinge region as B (Stahlberg *et.al,* 1988).

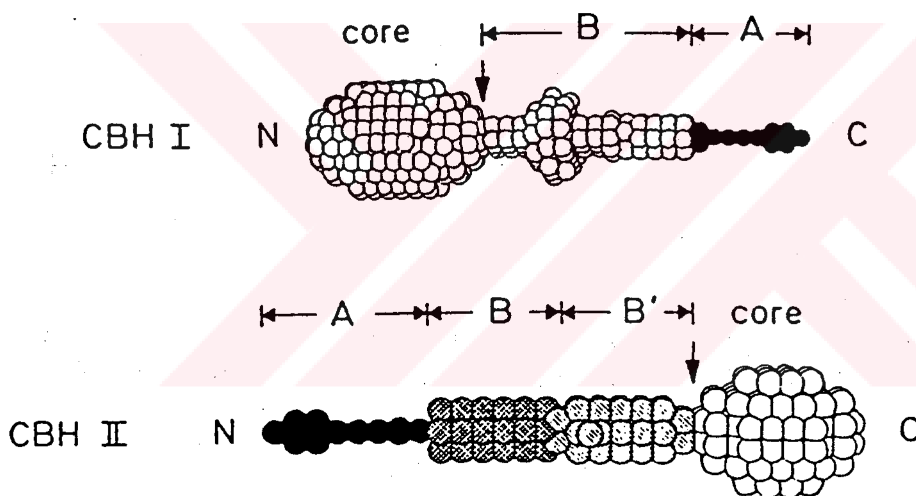

Figure1.4. Model structure of CBHI and CBHII from *T. reesei.*

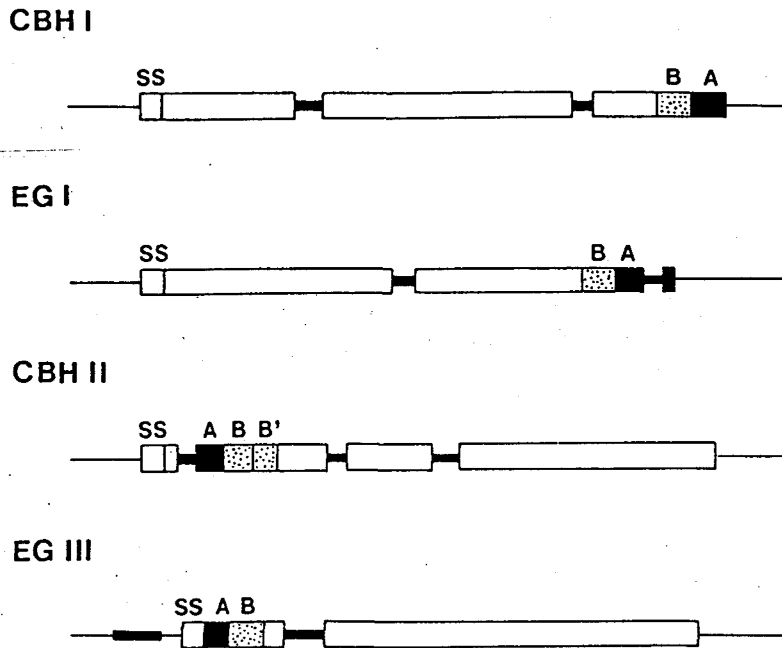

Figure 1.5. Domain structure of four major cellulase genes of *T. reesei.*

1.2.4.1 The Catalytic Domain:

The catalytic domain is the largest domain, which behaves as an independent entity (Beguin, 1990). It contains the active site, which carries out general acid catalysed hydrolysis of the β-1,4-glycosidic bonds in cellulose. The catalytic domain has a defined specificity towards small soluble oligosaccharides (Srisoduk *et.al.*, 1993). In *T. reesei* the catalytic domain is present either at N- or C-terminus of cellobiohydrolases and endoglucanases (Henrissat *et.al.*, 1989, Kraulis *et.al.*, 1989, Liner *et.al.*, 1996, Teeri *et.al.*, 1987).

1.2.4.2 The Cellulose Binding Domain:

The CBDs (the A region) of fungal cellulases are small peptides (Figure 1.6) folding into a rigid, disulfide-stabilized structure that has a distinct cellulose binding face required for the substrate recognition and formation of hydrogen bond (Linder *et.al.,* 1996).

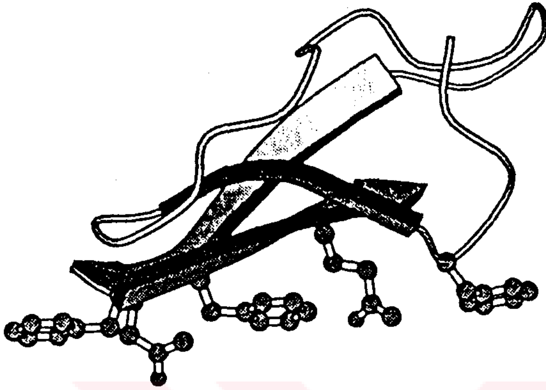

Figure 1.6. The backbone structures of the CBDs from the cellobihydrolase I from *T. reesei*.

CBDs are widespread in all types of cellulases and some hemicellulases (Linder and Teeri, 1997) and they appear to be highly conserved with respect to some amino acids (Table1.2) (Denman *et.al.,* 1996). For example the overall homology between CBDs of *Humicola grisea, Trichoderma reesei* and *Phanerochaete chrysosporium* is about 60%. Comparison of amino acid sequences of T. *reesei cbhl, cbh2, egl,* and *eg2* have shown that a high sequence conservation within block A (70% identical) (Aho *et.al.*, 1991, Coughlan, 1991, Salohiemo *et.al.*, 1988, Sthalberg *et.al.*, 1988, Teeri *et.al.*, 1987, Tomme *et.al.*, 1981). This region is rich in glycine and cysteine with the number and position of which are strictly conserved (Koch *et.al.*, 1993).

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Table 1.2. Conserved, noncatalytic domains of cellulases and xylanases.

Domain type	Function	Present in	Enzyme family	Position within the protein
		T. reesei CBHI	C	COOH end
		T. reesei CBHII	B	NH_2 end
Trichoderma reesei	Substrate binding	*T. reesei* EGI	C	COOH end
		T. reesei EGIII	A	NH_2 end
		P. chrysosporium CBHI	C	COOH end
		C. fimi EXO	F	COOH end
		C. fimi EGA	B	NH_2 end
Cellulomonas fimi	Substrate binding	*M. bispora* EGA	B	NH_2 end
		B. fibrisolvens EG	A	COOH end
		P. fluorescens EGA	E	COOH end
		P. fluorescens EGB	?	NH_2 end
		P. fluorescens XYNA	F	COOH end
		C. thermocellum EGA	D	COOH end
	Substrate binding?	*C. thermocellum* EGB	A	COOH end
		C. thermocellum EGD	E	COOH end
Clostridium thermocellum		*C. thermocellum* EGE	A	Middle of protein
	Binding to cellulosome?	*C. thermocellum* EGH	A	COOH end
		C. thermocellum XYNZ	F	Middle of protein
		C. cellulolyticum EGA	A	COOH end
		B. subtilis EG	A	COOH end
Bacillus subtilis	Secretion?	*B. circulans* EG1	?	COOH end
		C. saccharolyticum	A/F	Middle of protein

There is a strong correlation between the catalytic capacities of cellulolytic enzymes to degrade crystalline cellulose. In addition, all of the cellulases that are active against crystalline cellulose, and whose sequences have been determined, posses a CBD or are associated with a cellulose binding protein (Arifoglu, 1999, Beguin and Aubert, 1993, Gilkes *et.al.*, 1991, Black *et.al.*, 1995).

1.2.4.3 The Hinge:

The hinge is an other consecutive block in fungal cellulases. It is flexible, relatively long linker peptide of 6 to 59 amino acids (Davies *et.al.*, 1993, Srisoduk *et.al.*, 1993).

Deletion of the linker (the B region) does not affect the activity of the enzyme against soluble substrates, but dramatically reduces the rate of crystalline degradation even though the enzyme still binds to substrate (Black *et.al.*, 1995). It is concluded that sufficient spatial separation and the correct conformation of A and B blocks is required for proper function (Linder and Teeri, 1997).

1.3 Multiplicity and Gene Structure of Cellulases:

Cellulolytic microorganisms have long been known to secrete a variety of cellulolytic enzymes. However, to what extent such multiple cellulases arose from the expression of different genes or from post-translational modifications of the same gene product is not clear (Beguin, 1990, Jennings, 1995). Post translational modification, such as glycosylation or proteolysis occurring in aging cultures should not be neglected as a source of cellulase diversity (Beguin, 1990). The role of proteolysis has taken a enhanced significance with the recent demonstration of binding site deficient EGs and CBHs (Pocas-Fonseca *et.al.*, 1997, Stahlberg *et.al.*, 1988). This emphasised the concept of post-translational modification to yield novel enzymes with new specificities. Gene cloning studies have shown there to be relatively few fungal genes. Thus the multiple cellulase components arise primarily via artefactual or biosynthetic routes (Goyal *et.al.*, 1991).

In many cases, cloned cellulase genes are not clustered, and transcription studies have so far failed to detect polycistronic mRNAs (Goyal *et.al.*, 1991).

1.4 Regulation of Cellulases:

The cellulolytic system in fungi is adaptive. Synthesis of cellulases appears to be controlled by two basic regulation mechanisms. All known cellulase systems are repressed in the presence of low molecular weight carbon sources that are more easily metabolised than cellulose. In addition, in many systems, cellulase biosynthesis is induced in the presence of cellulose or its degradation products (Jennings, 1995, Beguin, 1993, Kubicek *et.al.*, 1993).

Addition of easily metabolised substrates blocks the synthesis of cellulase observed with cultures grown in the presence of cellulose alone (Beguin and Aubert, 1993). Addition of glucose, other mono- or disaccharides, or of compounds that are related to glucose metabolism, arrest cellulase formation. However derepression of cellulases is not observed under conditions of slow-glucose feeding or after glucose has been exhausted from the medium (Morawetz, *et.al,* 1991). The molecular mechanisms of "carbon source control" have been shown to act at a pretranslational level (Kubicek *et.al.*, 1993). Also a motive, homologous to the CRE A (carbon catabolite repressor protein) binding site has been found in the promoter region of cbh1, whose deletion results in expression CBH I on glucose (Carle- Urioste *et. al.*, 1997, Ilmen *et.al.*, 1996).

It is not clear an insoluble molecule such as cellulose can trigger induction of the requisite enzymes, however it has been proposed that the fungus synthesized a low level of an enzyme or enzymes that, on contact with cellulose, releases a soluble inducer of increased cellulolytic activity (Carle-Urioste *et.al.*, 1997, Kubicek *et.al*

1993, Beguin and Aubert, 1994, Coughlan, 1990, Jennings 1995). Induction of cellulases appears at the level of mRNA transcription. It is also reported that distinct mechanisms of induction and repression are operating in the cellulase expression (Ilmen *et.al.*, 1997).

1.5 Industrial Applications of Cellulases:

In food industry cellulase and hemicellulase preparations are used to clear fruit juices from remaining pulp particles. Cellulases can also help the extraction of juice and oil from fruit or seed. They are also preferred in brewing to hydrolyse β-1,3-β-1,4-glucan, which is present in high amounts in low grade barley and hampers the filtration of beer. Cellulases are also expected to have an impact on the processing of animal feed (Beguin and Aubert, 1994).

Textile processing is another area where cellulases have been successfully put in to use. Cotton fabrics are made softer and brighter by limited hydrolysis (Beguin and Aubert, 1994, Linder and Teeri, 1997). They are also added to laundry powder to help restore softness. Cellulases have been used to remove excess dye from the denim fabric in pre-faded blue jeans ('biostoning') (Beguin and Aubert, 1994).

Use of cellulosic wastes, which are produced in increasing amounts either as municipal solid waste or agricultural waste, is gaining increasing acceptance (Beguin and Aubert, 1994, Linder and Teeri, 1997).

The industrial feasibility of the enzymatic processing of cellulose does not simply depend on large-scale production of well-identified enzymes, but requires further basic knowledge to find out how existing cellulase systems work (Beguin 1990).

1.6 Genome Analysis By Southern Hybridisation Techniques:

Detection of specifically targeted nucleic acid sequences has evolved to be a highly versatile and useful method for different purposes in recent years. Techniques such as Southern and Northern hybridisation have become routine methods for analysis of DNA and RNA. Several other more sophisticated methods such as RFLP and gene sequencing rely on the same principles of detection (During, 1993). In molecular biological research as well as biomedical analysis these techniques have proven to be of great potential specificity and sensitivity.

Localisation of particular sequences within genomic DNA is usually accomplished by the transfer techniques described by Southern (1975). Genomic DNA is digested with one or more restriction enzymes and resulting fragments are separated according to the size by electrophoresis through an agarose gel. The DNA is then denatured *insitu* and transferred from the gel to a solid support (nitrocellulose or nylon membranes). The relative positions of the DNA are preserved during their transfer to the filter. The DNA attached to the filter is hybridised to labeled DNA or RNA, and a detection technique is used to locate the positions of bands complementary to the probe (Sambrook *et.al.*, 1993). Determination of target

sequence can be proceeded by radioactive probes or non-radioactive probes that is detected by enzymatic systems.

1.6.1 Non-Radioactive Labeling and Detection of Nucleic Acids:

For many years, radioactive labeling of nucleic acids has been the only applicable method. No other efficient label was known until the mid-1980s (During, 1993). The emergence of molecular biological techniques with interest for a broad spectrum of fields of research and analysis has been a reason to initiate a search for new labeling techniques avoiding radioactivity. In recent years a highly innovative development has taken place, beginning with the first non-radioactive labeling and detection system (based on the interaction of biotin with avidin) for Southern hybridizations and achieving a well elaborated set of methods offering a broad spectrum of applicability sensitivity equal to that of radioactive labeling. Different non-radioactive methods have been introduced since 1980s as possible alternatives to radioactive hybridisation assays, using probes labeled with fluorescent agents, enzymes, fluorescein, biotin or digoxigenin (DIG) (Holtke et.al., 1990).

1.6.2 Use of Non-Radioactively Labeled Probes:

In molecular biological research as well as biomedical analysis hybridisation techniques have proven to be of great potential, specificity and sensitivity. In genomic blots single –copy genes can be detected with only 0.5 to 5 µg human DNA depending on the type of probe and the length of the hybridizing region (Seibl *et.al.*,

1990). Detection of 30 fg homologous DNA in a dot blot is possible (Holtke, *et.al.*, 1992)

Other advantage of non-radioactive labeling of nucleic acids with biotin or digoxigenin is the possibility to store labeled probes for some years without loss of activity. This saves a lot of work in comparison with radioactive labeling, which requires probes to be prepared freshly and used within some days.

Besides their potentially greater sensitivity and stability, non-radioactive systems lack the health hazards, cost, and disposal problems of radioactive detection, implying that radiolabeled probes can be replaced by non-radiolabeled counterparts.

1.6.3 Principles of Nucleic Acid Labeling and Detection With DIG System:

The most frequently used non-radioactive label is digoxygenin (DIG). DIG is an artificial hapten that is not present in most relevant tissues and, therefore does not lead to unspecific signals which can occur with natural labels like biotin, especially in hybridization to un-purified DNA and *in situ* hybridizations (Holtke *et.al.*, 1992; Solanas and Escrich, 1997). DIG is bound *via* spacer arm to uridine nucleotides (DIG-11-dUTP) (Figure 1.6) and incorporated enzymatically at a defined density into nucleic acid probes by random primed DNA labeling, nick translation, PCR, *in vitro* RNA transcription, or 3'-end labelling/tailing (Holtke, *et.al.*, 1992).

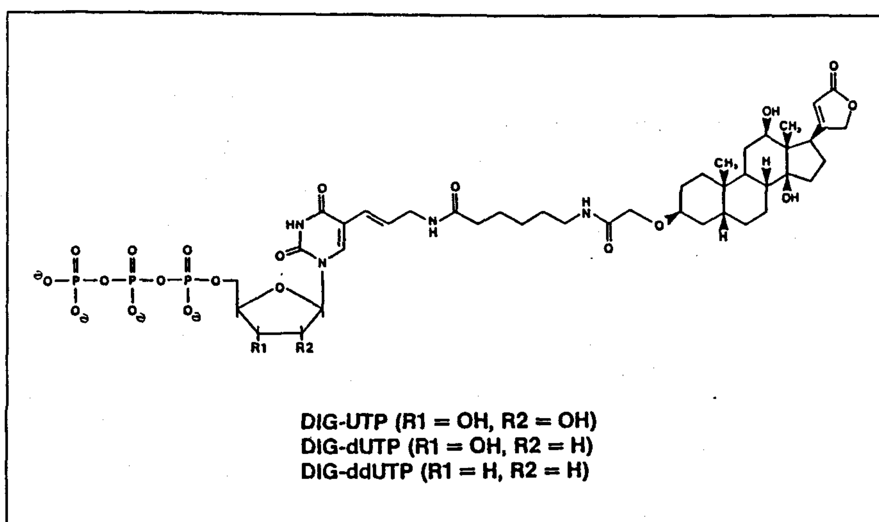

Figure 1.7. Structure of digoxygenin (DIG)-modified nucleotides

The DIG-labeled probes are hybridized to a membrane-bound nucleic acid on a Northern Blot, Southern Blot, Dot/Slot Blot or colony/plaque lift. The DIG label is detected by polyclonal anti-digoxigenin Fab fragments, which are conjugated to alkaline phospatase. Most enzyme-based DNA detection assays utilize alkaline phosphatase the preferred label, due to unusual resistance to environmental conditions and its thermal stability (Engler-Blum, *et.al.*, 1993). The enzymatic reaction can be visualized by either a color or chemiluminescent substrate.

1.7 The Aim of the Study:

In this study, it was aimed at analyzing the multiplicity cellulase genes of the thermophilic fungus *Torula thermophila* by using genomic restriction digests and Southern hybridization technique. To determine the number of cellulase genes and the cellulase specificity of 100bp probe previously obtained by PCR from Torula

thermophila (Öztürk, *1999)*, homologous and heterologous hybridization techniques were applied. Northern analysis with total RNA of *Torula thermophila obtained* from the cells grown on avicel (microcrystalline cellulose) was also performed to ensure specificity of the probe to the endoglucanase I gene of Trichoderma reesei was used. Experiments were performed in parallel with T. reesei genomic digests both as a control and for the sake of comparison.

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Fungal strains

Torula thermophila strain 3A was isolated in METU food engineering department from mushroom compost (Arifoğlu, 1999). *Trichoderma reesei* ATCC 66589 was kindly supplied by ORBA.

2.1.2 Chemicals and Enzymes

The list of chemicals and enzymes used and their suppliers are given in Appendix A.

2.1.3 Culture media, Buffers and Solutions

The preparation of the culture media, buffers and solutions used in experiments are given in Appendix B.

2.2 Methods

2.2.1 Maintenance of strains

Stock culture of Torula *thermophila* was grown on YpSs agar slants (Appendix B) at 40°C and maintained at room temperature, while that of *Trichoderma reesei* was prepared by growing cells on PDA (Appendix B) Stock cultures were subcultured every months.

2.2.2 Cultivation of strains for DNA and RNA isolation

For DNA isolation *Torula thermophila* was cultivated in two different media; YEPD broth (Appendix B) and YEPD with 0.5 % glycerol in 500ml erlenmayer flasks containing 200 ml medium at 45°C and 150 rpm for three days. Culture medium with glycerol was used to decrease pigmentation and enhance nucleic acid purification (Section 2.2.3).

Trichoderma reesei was cultivated in 200 ml PD broth (Appendix B) at 140 rpm for three days.

For RNA isolations *Torula thermophila* was cultivated in 2L erlenmeyer flasks containing 1L YpSs broth (Appendix B) with avicel as the carbon source instead of starch. After incubation at 45°C and 155 rpm for three days filtered mycelia, were used in RNA isolation by the method described in section 2.2.6.

2.2.3 Genomic DNA isolation

In genomic DNA isolation the method of Zang et.al. (1996), based on the use of phenol extraction with the CTAB was used with slight modifications.

Approximately 5g of mycelia was harvested by filtering through Whatman 3MM filter paper, rinsed with distilled sterile water and dried at 45°C only for 3-4 hours (over-dried mycelia suspends in the lysis buffer and decreases the extraction efficiency of DNA). Dried mycelia were ground into a powder using a mortar and pestle after liquid nitrogen treatment. Powdered mycelia were either used immediately or stored at –80°C until use.

Powdered mycelia were transferred in a 50 ml falcon tube. Then 15 ml lysis solution (Appendix B) was added and mixed by inversion. To this suspension, 3 ml of 5 M NaCl (Appendix B) and 2ml of 10% CTAB (Appendix B) were added. The resulting mixture was incubated at 65°C, for 45 min and 37°C for15 min by occasional inversion.

1:1 volume of phenol:chloroform:isoamylalcohol (25:24:1) (Appendix B, number 9) was added to the mixture, mixed by inversion and centrifuged at 2000g (5500 rpm) for 5 minutes. The upper phase was transferred carefully into a new tube. Phenol:chloroform extraction was repeated one more times until the interphase was sufficiently clear.

To the upper phase after the second phenol:chloroform extraction 100 µl 0.5 mg/ml DNase free RNase (Appendix B, number 10) was added and the mixture incubated at 37°C for 30 min.

Phenol:chlorophorm:isoamylalcohol extraction was repeated once and the upper phase was carefully transferred to a Sorwall tube.

1:1 volume of isopropanol was added slowly and than mixed well by inversion and incubated at –20°C at least for 1 h. After incubation, tubes were centrifuged at 4°C, and 5500 g (10000 rpm). The supernatant was discarded.

The pellet was washed with 10 ml 70% ice-cold ethanol, which was discarded after centrifugation at 2000 g (6000 rpm) for 3 minutes. Pellet was dried for 5 to 20 min at room temperature.

Approximately 250 µl TE buffer (Appendix B, number 11) was added to the pellet and, DNA was dissolved, at 4°C overnight.

DNA concentration and purity were determined either by spectrofotometric methods or by comparing the intensity of λ DNA/Hind*III* fragments of known concentration.

2.2.4 Genomic DNA restriction enzyme digestion of *T. thermophila* and *T. reesei.*

T. thermophila and *T. reesei* genomic DNA was digested with different restriction enzymes in suitable buffers and proper incubation conditions (Appendix C). Double digestions of *T. thermophila* genomic DNA were also performed.

For single digests, 5μg genomic DNA was digested with 5 units of restriction enzyme per μg DNA in a total volume of 20μl, containing 10X reaction buffer supplemented with BSA (Bovine Serum Albumin) to give a final concentration of 1X; injection grade water was used to make up the required volume.

Concentrations and the type of buffers for double digestion reactions were determined (Appendix D) precisely according to the manufacturers instructions to eliminate star activity.

2.2.5 The Polymerase Chain Reaction (PCR)

2.2.5.1 Primers used in PCR

To amplify *egl1* gene of *T. reesei* coding for the endoglucanase I enzyme (Saloheimo *et.al.*, 1988), primers EG-1A and EG-1B (Appendix E), corresponding to the start and end of *egl1* (Öztürk, 1999) were used. The degenerate primers TEG-deg1 and TEG-deg2 (Appendix E), were designed according to the conserved

regions in the cellulose binding domains of *Humicola insolens* and *Trichoderma reesei* endoglucanase genes and were used in the amplification of the 100bp cellulase gene probe from T. thermophila genomic DNA (Öztürk, 1999).

2.2.5.2 Amplification of *T. reesei egl3* gene

A 50 µl reaction mixture contained:

- injection grade water to give a final volume of 50µl
- 1X reaction buffer
- 1.5 mM $MgCl_2$
- 0.25 mM dNTP mix
- 2 unites of Taq DNA polymerase
- 100pmolesEG-1A, 100pmoles EG-1B
- 0.25µg *T. reesei* genomic DNA

10µl mineral oil was added to prevent evaporation. Amplifications proceeded with the following cycle; 95°C x 3min., 35 x {95°C x 1min., 55°C x 1 min., 72°C x 2 min.}, 55°C x 3 min.

2.2.5.3 Amplification with Degenerate Primers

A 50 µl reaction mixture contained:

- injection grade water to give a total volume of 50µl
- 1X reaction buffer

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- 1.5 mM $MgCl_2$
- 0.25 mM dNTP mix
- 2 units of Taq DNA polymerase
- 100 pmoles TEGdeg-1 and 100 pmoles TEGdeg-2
- 0.25µg T. thermophila genomic DNA.

10 µl mineral oil to prevent evaporation. Amplifications were proceeded with the following cycle; 95°C x 3 min., 35 x {95°C x 1 min., 50°C x 1 min., 72°C x 30 sec.}, 50°C x 3 min.

2.2.6 RNA Isolation

Total RNA from *T. thermophila* was isolated by the Promega RNAgents Total RNA isolation Kit according to the manufacturers instruction protocol supplied with the kit without any modification.

Homogenization of cells was performed by proper mixing with vortex for 4-5 min in eppendorf tube at 4°C.

Isolated RNA was stored at –80 in 95% ethanol. Before use RNA was precipitated at 1200 rpm and 4°C, washed with 75% ethanol, dried for 5 min at room temperature and dissolved in TE buffer (Appendix B). Concentration and purity of the RNA was determined by spectrophotometric analysis.

All plasticware, glassware and solutions used in RNA isolation were made RNase free by DEPC treatment.

2.2.7 Agarose Gel Electrophoresis

To analyze the restriction fragments of genomic DNA, 1% agarose gel (Appendix B) was used to achieve a good resolution. For PCR products 1.5% agarose gel (Appendix B) was used. For electrophoresis of genomic digests 60cm^2 mini-gel was used.

Agarose gel (Appendix B) was and cooled to 50-60°C before the addition of ethidium bromide and was poured into mould and allowed to solidify for 30 min. Agarose gel was placed into the electrophoresis tank filled with 1X TAE buffer (Appendix B). Electrophoresis was carried out at constant voltage; genomic restriction digests were run at 40 V, while PCR products were migrated at 60V. Gels were visualized on a UV transilluminator at 312 nm and photographed with an aperture of 8 and exposure time of 4 seconds.

2.2.8 Hybridization Analysis

2.2.8.1 Probe isolation

T. thermophila 100 bp DNA fragment and *T. reesei egl* gene were used in hybridisation analysis. Both types of probes were recovered from the agarose gel by using Wizard minicolumns (Promega) with a modified procedure;

The samples were run on agarose gels in TAE buffer (Appendix B) and were stained with ethidium bromide.

Fragment of interest were excised from the gel under UV light with a sharp razor blade and were washed by immersing in distilled water for 5 min to remove ethidium bromide and salts present in the gel.

Then the excised gel, containing the probe, was treated with liquid nitrogen and was put into a Wizard minicolumn, which had been placed on top of a 1.5 ml microcentrifuge tube.

Minicolumn was centrifuged at 13000 rpm for 10 min, and DNA in TAE buffer (Appendix B) was collected at the bottom of the tube

10% the volume of 4M sodium acetate (Appendix B, number 15) and 2-2,5X volume of 96% ice-cold ethanol were added into TAE buffer (Appendix B) containing the DNA fragment.

After incubation at –20°C for at least 1 hour, the sample was centrifuged at 12000 rpm for 10 min and washed with 75% ethanol. The pellet was then dried for 5 min at room temperature and dissolved in 10 μl TE (Appendix B).

2.2.8.2 Labelling of Probes

The probes were labeled by random priming with Boehringer Mannheim DIG DNA Labelling Kit according to the manufacturers instructions.

2.2.8.3 Southern Blotting

Genomic DNA from *T. thermophila* and *T. reesei* digested with different restriction enzymes were run on a 1% agarose gel (Appendix B), at 40v, for 3 h, and the resolved DNA fragments were transferred onto the positively charged Boehringer Manheim nylon membrane according to following procedure.

The gel was placed in to a tray containing 250mM HCl and held until the color of bromophenol blue turned into yellow by gentle agitation at room temperature.

After rinsing in distilled water the gel was placed into the denaturing solution (Appendix B) and was held for 30 min at room temperature.

The gel was rinsed with distilled water and placed into the neutralization solution (Appendix B) and was washed twice with the same solution by gentle agitation for 15 min at room temperature.

The capillary blot was set up (Figure 2. 1) onto a tray filled with 20X SSC (Appendix B) and transfer was allowed to proceed overnight. The membrane was baked at 100°C for 30 min to fix the DNA onto the membrane.

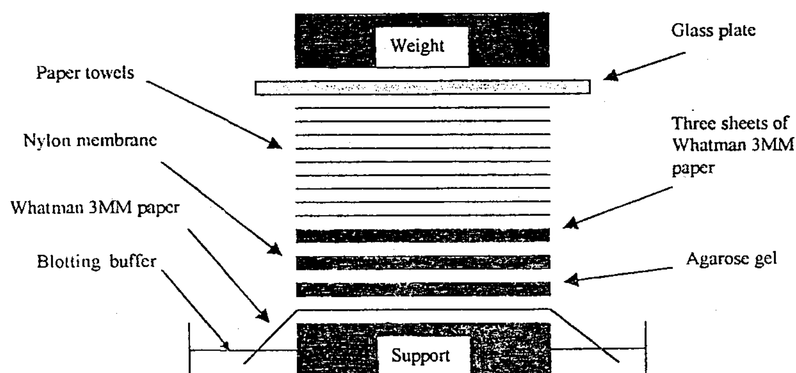

Figure 2.1. Set up for capillary transfer of DNA from agarose gel to nylon membrane.

Membrane was immediately used in hybridisation analysis.

2.2.8.4 Dot blot analysis

The RNA samples isolated from 3rd , 4th , 5th , 6th , 7th days of incubation on avicel as the carbon source (Section 2.2.6) were spotted onto the Boehringer Manheim N+ membrane, baked at 100°C for 10 min to fix RNA and were used in hybridization analysis.

2.2.8.5 Hybridization

Hybridizations were made according to the protocol supplied by manufacturers (Boehringer Manheim). The only modification was the use of 0.5X SSC instead of 0.1X in the stringency washing step. For homologous hybridisation 68°C hybridisation temperature was used, while 55°C was applied to heterologous hybridisation.

2.2.8.6 Color detection

Detection protocol supplied by Boehringer Mannheim (DIG Nucleic Acid Detection Kit) was followed without modification. Results were documented by photography.

CHAPTER III

RESULTS AND DISCUSSION

3.1 Isolation of Genomic DNA from Torula thermophila and Trichoderma reesei

Genomic DNA was isolated from *T. reesei* according to the procedure mentioned in section 2.2.3. The $A_{260}:A_{280}$ ratio of isolated DNAs were determined as 1.8-1.9. Amount of the genomic DNA isolated by this method was usually in a range of 100-300μg per 5g wet mycelia. The genomic DNA of *T. thermophila,* isolated from the mycelia cultivated in YEPD (Appendix B) medium was not sufficient with respect to its yield and the purity for genomic restriction and Southern analysis. This was probably the result of extensive black pigmentation indicating the presence of large amounts of melanin in rigid cell wall structure of this thermophilic fungus. To eliminate the pigmentation problem, the modified YEPD medium containing 0.5% glycerol was used to cultivate *T. thermophila* for genomic DNA isolation. This medium was used because in this study it was discovered that glycerol inhibits pigmentation. The results show the increased yield of genomic DNA , which is estimated as 100-300μg per 5g wet mycelia. The ratio of $A_{260}:A_{280}$ was quantified as 1.8 – 1.9 indicating the sufficient purity for successive genomic restriction and Southern analysis.

Genomic DNAs isolated from *T. reesei* and *T. thermophila* were also analyzed by agarose gel electrophoresis, and are shown in the Figure 3.1.

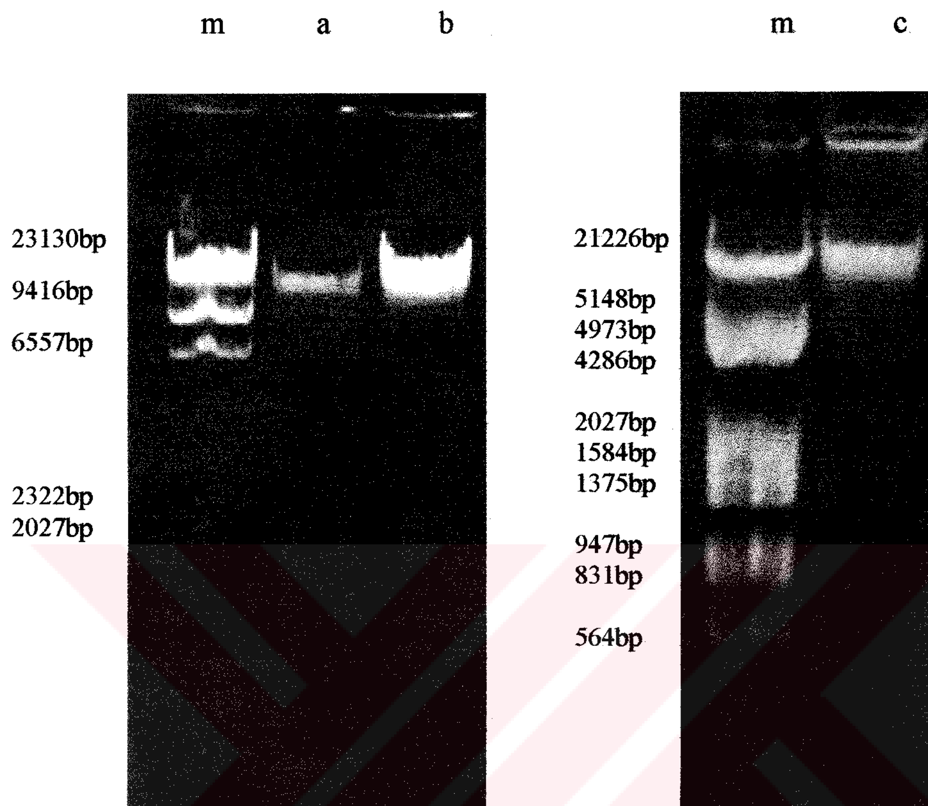

Figure 3.1. DNA isolation results by agarose(0.8%(w/v) gel electrophoresis. **m**; λ DNA/Eco*RI*+Hind*III* marker(0.5μg/μl),**a**; *T. thermophila* genomic DNA isolated from mycelia grown in YEPD, **b**; *T. thermophila* genomic DNA isolated from mycelia grown in YEPD containing 0.5% glycerol, **c**; *T. reesei* genomic DNA.

3.2 RNA Isolation

Total RNA was isolated from *T. thermophila* grown on media containing microcrystalline cellulose (Avicel) and on YEPD medium containing glucose as carbon source (see section 2.2.6). The ratio of A_{260}:A_{280} was determined in the range of 2-2.2. This result shows that RNA samples are sufficiently pure to perform successive dot blot analysis. Absence of RNA degradation and contamination by genomic DNA was confirmed by agarose gel electrophoresis (data not shown).

3.3 PCR Amplification of Probe DNA

In previous studies a 100bp PCR fragment was amplified from *T. thermophila* genomic DNA, using degenerate primers, designed according to a region in the cellulase binding domain (CBD) of cellulases, together with conserved regions in the endoglucanase genes of *Humicola insolens* and *T. reesei* (Appendix F) (Öztürk, 1999, Öztürk and Ögel, 2000). Because of the strategy used in the design of the primers, the 100bp probe is expected to be more specific to endoglucanase genes rather than exoglucanase genes. Nevertheless, the conserved region in the CBD is also present in exoglucanase genes, if not the flanking regions.

In this study, it was of interest to test the specificity of the 100bp probe to endoglucanase genes, and also to find out the multiplicity of endoglucanase genes in *T. thermophila*. Probe specificity to the expressed regions of the genome, was also tested by RNA dot blot analysis (section 3.4.3). Southern experiments with 100bp probe were repeated with *T. reesei* endoglucanase (*egl1*) gene, which would both

confirm results obtained with the 100bp probe, and at the same time, provide further data on the multiplicity of endoglucanase genes of *T. thermophila*.

Because of the reasons mentioned above, both the 100bp probe of *T. thermophila* and the *egl1* gene of *T. reesei* were obtained in this study, by PCR amplifications.

3.3.1 Amplification of *T. thermophila* 100bp probe with Degenerate Primers TEG-deg1 and TEG-deg2

The results of amplification with *T. thermophila* genomic DNA with degenerate primers TEG-deg1 and TEG-deg2 (see section 2.2.5.3) is given in Figure 3.2. A specific 100bp fragment was obtained from this amplification in accordance with the amplification data of another thermophilic cellulolytic fungi *Humicola insolens* with degenerate primers TEG-deg1 and TEG-deg2 (Öztürk, 1999). In fungal cellulases the 100bp region between amplified with the degenerate primers corresponds to the 36-38 aa residue representing most of the CBD (Kraulis *et.al.*, 1989).

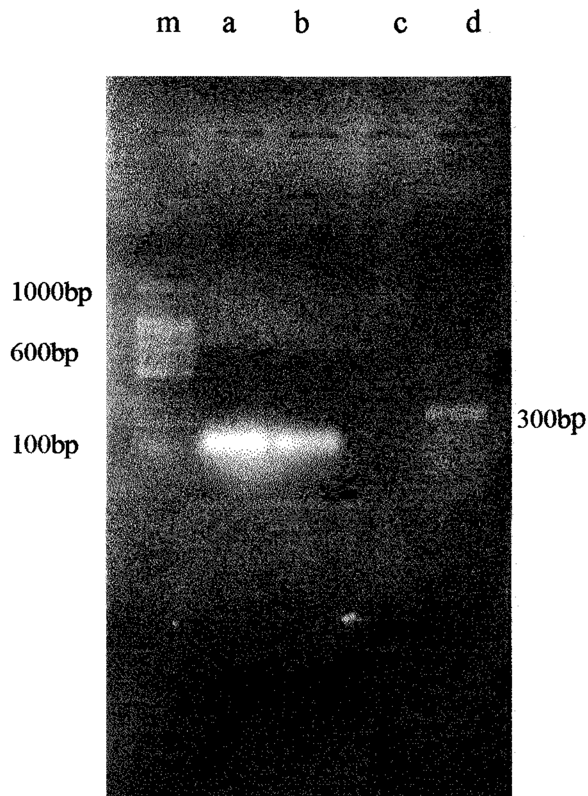

Figure 3.2. Agarose gel electrophoresis (1% (w/v) agarose gel) analysis of the PCR amplification with *T. thermophila* genome with TEGdeg-1 and TEGdeg-2. **m**; 100bp DNA ladder, **a**, **b**; PCR product of *T. thermophila* genomic DNA amplified by TEGdeg-1 and TEGdeg-2, **c**; TEGdeg-1 and TEGdeg-2 (No DNA control), **d**; pBluescript amplified with 315 and 316 gene specific primers (Positive control).

3.3.2 Amplification of *T. reesei* Genomic DNA with *egl1*-specific Primers

Amplification results using *T. reesei* genomic DNA and gene specific primers EG-1A and EG-1B (see section 2.2.5.2) are given in Figure 3.4. A single specific amplification product of 1.3-1.4 kb was obtained. The length between the gene

specific primers EG-1A and EG-1B on the egl1 gene of *T. reesei* is also reported as 1.3kb (Appendix F) (Saloheimo *et.al.*, 1988), thus confirming the result obtained in this study.

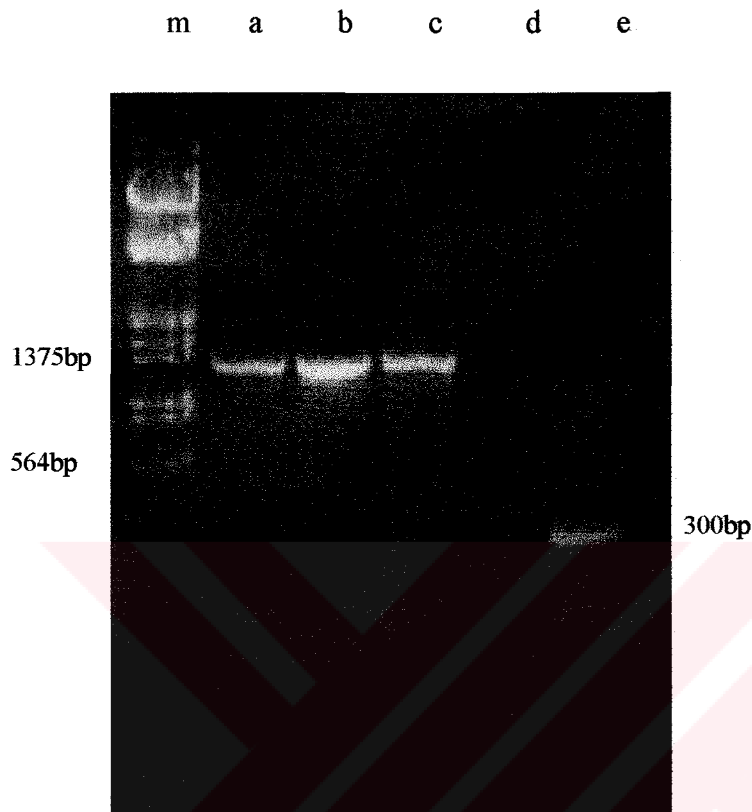

Figure 3.3. Agarose gel electrophoresis (1% (w/v) agarose) analysis of the PCR results of *T. reesei* genomic DNA amplified with EG-1A and EG-1B. **m**; λDNA/Eco*RI*+Hind*III* digest , **a**, **b**, **c**; PCR product of *T. reesei* genomic DNA by EG-1A and EG-1B, **d**; EG-1A and EG-1B (No DNA control), **e**; pBluescript amplified by 315 and 316 gene-specific primers (Positive control).

3.4 Southern Hybridisation Analysis

As mentioned before in this study, it was aimed at analyzing the specificity of the 100bp probe to endoglucanase genes, and to find out the multiplicity of endoglucanase genes of *T. thermophila*. For this purpose, Southern blotting and hybridisation analysis was performed on restriction digests of genomic DNA from both *T. thermophila* and *T. reesei* against the100bp and *egl1* probes amplified by PCR.

For Southern hybridisation analysis and probe labeling, a non-radioactive system namely DIG (digoxygenin) labeling and detection system was employed (Section2.2.8.2). Prior to labeling, probe DNA was isolated from PCR mixtures (Section 2.2.8.1).

3.4.1 Probe Preparation

The 100bp PCR product of *T. thermophila* and the endoglucanase I gene of *T. reesei,* amplified with gene specific primers were isolated from the gel with a method described in section 2.2.8.1.This was a modified method which gave a higher yield of at least 50% more of probe than the method suggested by the suppliers of Promega Wizard DNA Purification kit and than the standard freeze-squeeze method. This method is based on the direct centrifugation of DNA, to elute it from the gel by using minicolumns, which are supplied by Promega DNA purification system. Centrifugation of the gel slices containing the DNA with minicolumns, without prior mixing with purification resin eliminates any loss of DNA. By immersing gel slices

into distilled water prior to centrifugation, allowed the elimination of EtBr and salts present in the gel running buffer. This method was also found easy and safe to handle because of the elimination of EtBr at the beginning of the procedure.

3.4.2 Homologous and Heterologous Genomic Hybridisation of *T. thermophila*

T. thermophila single and double digest genomic DNA was homologously hybridised with DIG labeled 100bp probe to analyze the number and size of the genomic fragments the probe would hybridize (see section 2.2.8.5). Hybridisation was performed at 68 °C to ensure the specificity of probe binding to DNA. According to the results given in the Figure 3.4 the large number of bands in the single and double digested genomic DNA suggest either the multiplicity of cellulase genes or some non-specific hybridizations. It is also likely that 100bp probe has hybridised to the cellobiohydrolase genes in addition to endoglucanase genes.

As can be seen in the Figure 3.4, on each lane around 8 hybridisation bands were observed of which 5 are strong and 3-4 are less intense and diffuse bands. The only exception was the observation of three bands on the Eco*RI* genomic digest, which can be explained by the insufficient digestion of genomic DNA. The hybridisation pattern is given in Figure 3.4 and the size of the hybridisation bands are given in table 3.1. The relatively specific bands may correspond to the endoglucanase genes of *T. thermophila*, because of the higher possibility of the 100bp probe to hybridize to endoglucanase genes while the less specific, weak bands are likely to be derived by the exoglucanase genes.

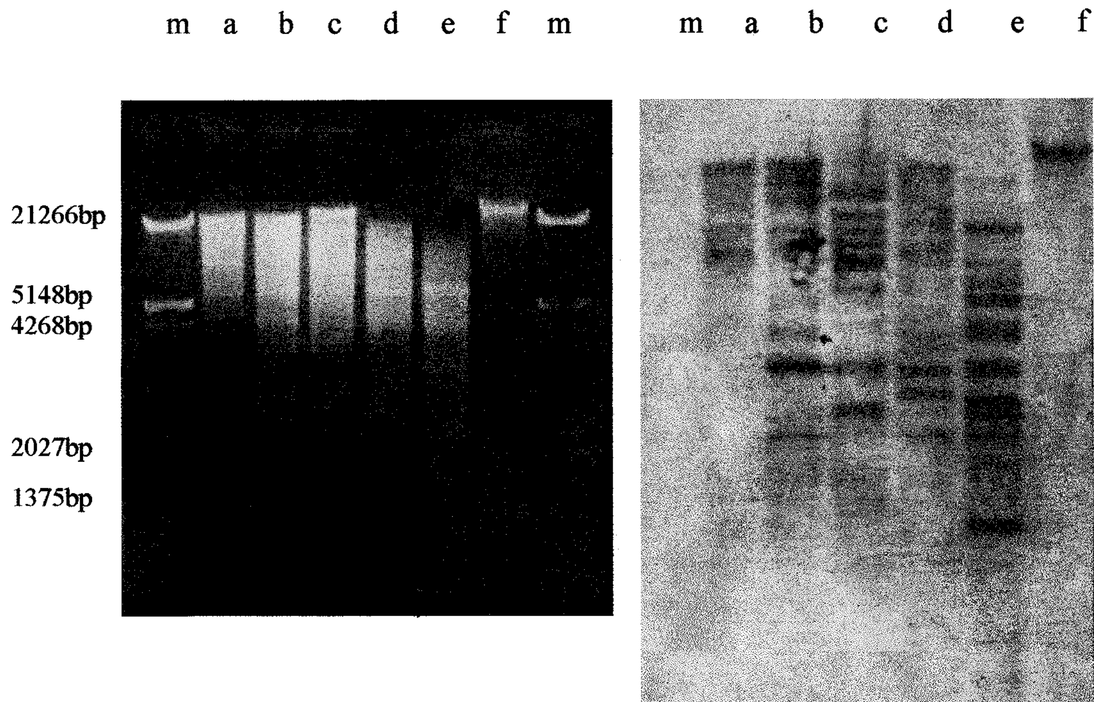

Figure 3.4. Agarose gel (1% agarose) electrophoresis analysis of *T. thermophila* genomic digest and homologous hybridisation results of *T. thermophila* restriction fragments and 100bp probe . **m**; λDNA/ Eco*RI*+Hind*III* digest, **a**; Eco*RI* genomic digest, **b**; Hind*III* genomic digest, **c**; Sma*I* genomic digest **d**; Hind*III*/Eco*RI* genomic digest, **e**; Hind*III*/Sma*I* genomic digest, **f**; *T. thermophila* genomic DNA The size of the marker DNA and identical bands in heterologous and homologous hybridisation of *T. thermophila* genomic fragments are given in terms of base pairs.

Table 3.1. List of strong and diffuse bands of homologous hybridisation with 100bp probe. Estimation of size of the bands, corresponding to the signals, was done by using the semi-log relation between the length of the bands observed and their distance from the well.

	Strong Bands (kb)	Diffuse Bands (kb)
Eco*RI*	**20**, **11**, **7**	
Hind*III*	**2.6**, 1.3, **1.5**, 20,10	2, 3.5, 7.5,
Sma*I*	**2**, **2.6**, 5.2, 11, **10**	13, 13.8, 6.4
Hind*III*/Eco*RI*	**1.5**, **2.2**, **5.4**, 11, 12	13, 19,
Hind*III*/Sma*I*	**1.3**, **2.4**, 2.7, 3.5, 4.4, 10	2, 6, 5.2

In this study double digested genomic DNA fragments were subjected to hybridisation analysis, as well as single digests, with the possibility of obtaining a restriction map of the cellulase genes. While smaller fragments were indeed obtained with Hind*III*/Sma*I* digested DNA the large number of the bands observed in each lane did not allow such an interpretation to be made. Usually the number of hybridised bands is expected to increase in double digests, however this was not the case. This result may be explained by the low probability of having a restriction site within the corresponding region of *T. thermophila*100bp probe.

In accordance with the results given above, from *H. insolens*, which is closely related to *T. thermophila*, five distinct endoglucanase and two cellobiohydrolases have been identified and their genes have been cloned (Schulein, 1997). *T. reesei*,

which is accepted as a model organism for cellulolytic organisms was also found to have five endoglucanase genes (Ilmen *et. al.*, 1997). In this respect, it is likely that *T. thermophila* contains multiple cellulase genes, which could explain the large number of bands observed by hybridisation analysis. Based on the results, *T. thermophila* also appears to have at least 5-7 cellulase genes.

Heterologous hybridisation with single and double digested *T. thermophila* genome versus *T. reesei egl1* gene was also performed, to determine the identity of the hybridisation bands in homologous and heterologous hybridisation. This identity would help to be more conclusive about the number and size of endoglucanase genes of *T. thermophila.* Hybridisation temperature was chosen as 55 °C in this experiment. Results are given in Figure 3.5. Observation of more than 10 hybridisation bands in each lane and signal from the lane including λ DNA/Eco*RI*+Hind*III* marker indicates that *egl1* probe also hybridised to *T. thermophila* genomic digest non-specifically. This may be a result of the relatively low hybridisation temperature. However, some identical bands indicating the endoglucanase genes of *T. thermophila* could still be observed. Sizes of these bands are indicated as bold characters in Table 3.1. To be more conclusive about results, it is necessary to perform heterologous hybridisation with *egl1* gene under more specific conditions, namely the use of a higher hybridisation temperature and more stringent post-hybridisation washings.

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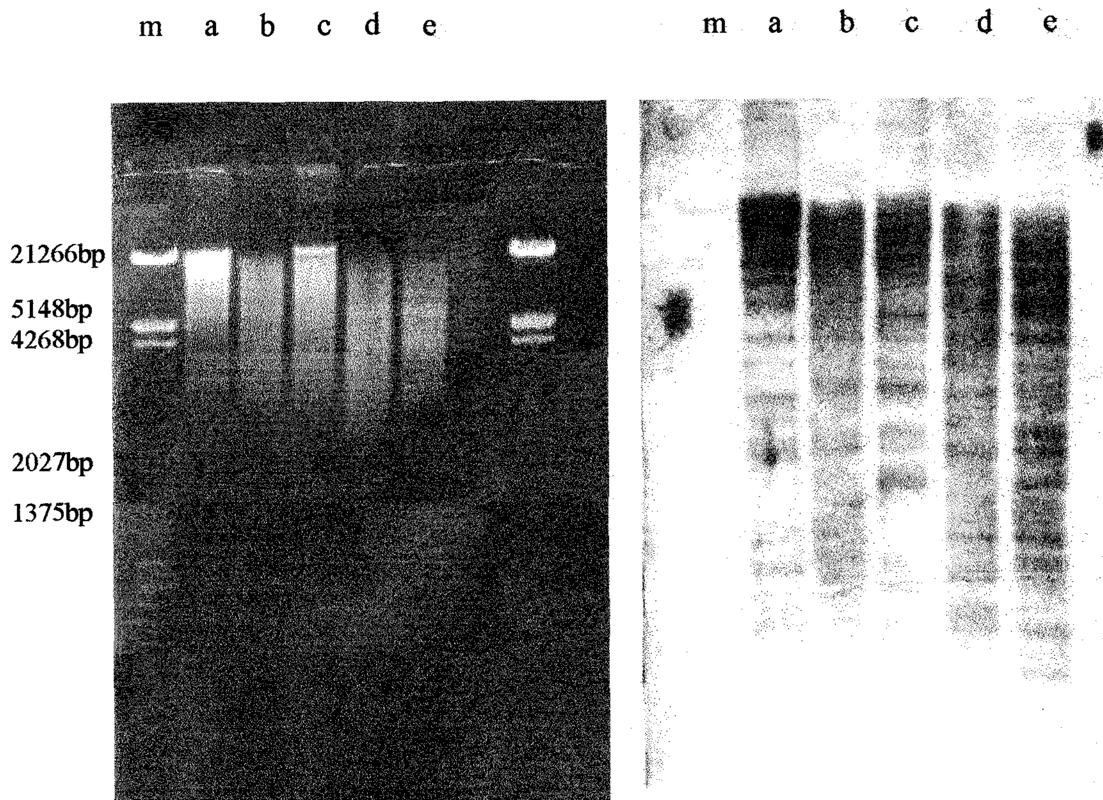

Figure 3.5. Agarose gel electrophoresis (1% agarose) analysis of *T. thermophila* genomic digests and heterologous hybridisation results of *T. thermophila* genomic digest and egl1 gene. **m**; λDNA/Eco*RI*+Hind*III* digest **a**; Eco*RI* genomic digest, **b**; Hind*III* genomic digest, **c**; Sma*I* genomic digest, **d**; Hind*III*/Eco*RI* genomic digest, **e**; Hind*III*/Sma*I* genomic digest. The size of the bands which are identical in homologous and heterologous hybridisation of *T. thermophila* and the size of the marker DNA are given in terms of base pairs.

3.4.3 Heterologous and Homologous Hybridisation with *T. reesei* Genomic Restriction Fragments

To confirm the specificity of 100bp probe to endoglucanase genes, *T. reesei* genomic DNA digests were also subjected to Southern hybridisation analysis, both using *T. thermophila* 100bp probe and the *egl1* probe of *T. reesei*. Hybridisation temperature was 55 °C in heterologous hybridisation with 100bp probe. At this temperature non-specific hybridizations were likely to be largely eliminated.

From heterologous genomic hybridisation of *T. reesei* with *T. thermophila* 100bp DIG labeled probe (see section 2.2.8.5), and homologous hybridisation with *egl1*, almost the same hybridisation pattern was obtained (Figure 3.6- 3.7). While the100bp probe gave signals in addition to the identical bands of homologous and heterologous hybridisation, the small number of bands obtained, do not seem to support the suggestion that the probe recognizes all endoglucanase and cellobiohydrolase genes. This may be explained by the relatively high hybridisation temperature of 55 °C, which may lead to a more specific hybridisation.

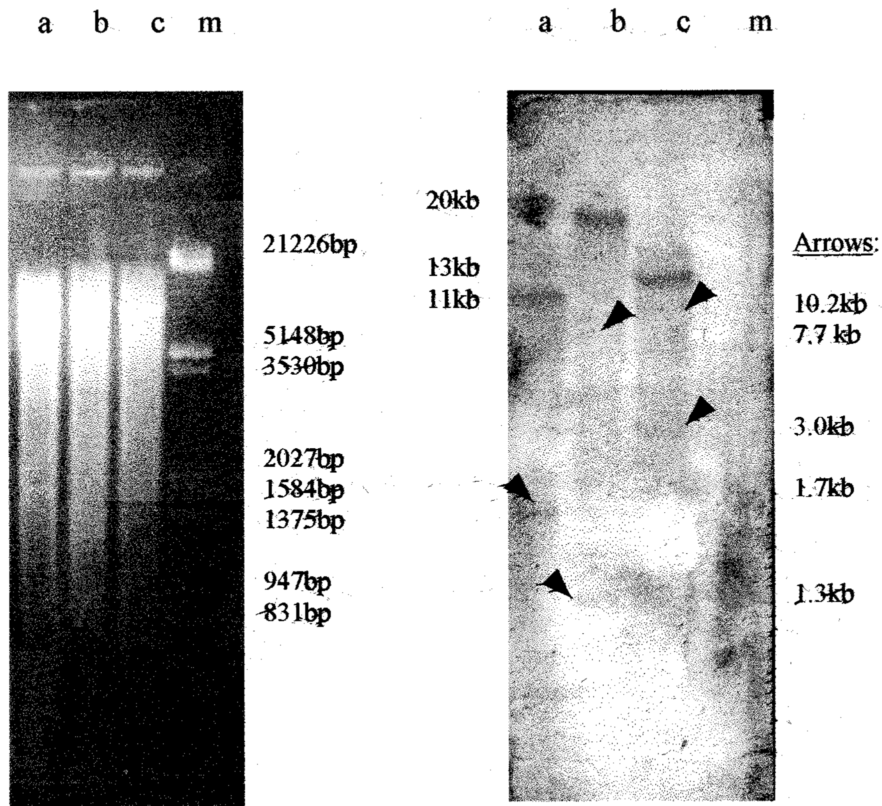

Figure 3.6. Agarose gel electrophoresis (1% agarose) analysis of *T. reesei* genomic digest and heterologous hybridisation results of *T. reesei* genomic digest with the 100bp probe of *T. thermophila* **m**; λDNA/Eco*RI*+Hind*III* digest, **a**; Eco*RI* genomic digest, **b**; Hind*III* genomic digest, **c**; PstI genomic digest. The size of the marker DNA and hybridisation bands are given in terms of base pairs. ,

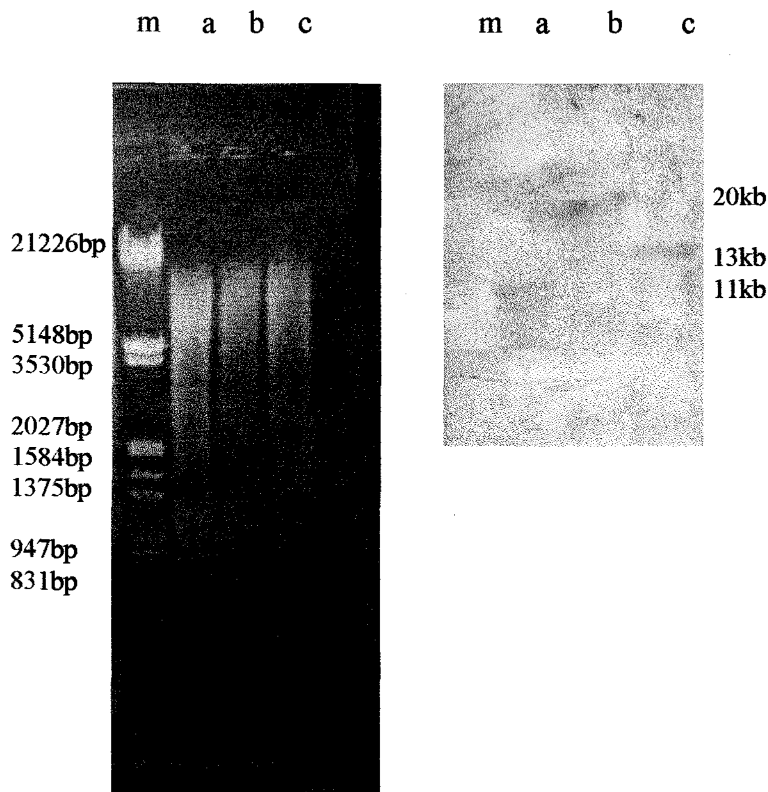

Figure 3.7. Agarose gel electrophoresis (1% agarose) analysis of *T. reesei* genomic digest and homologous hybridisation results of *T. reesei* genomic digest with *egl1* probe. **m**; λDNA/Eco*RI*+Hind*III* digest, **a**; Eco*RI* genomic digest, **b**; Hind*III* genomic digest, **c**; PstI genomic digest. The size of the marker DNA and hybridisation bands are given in terms of base pairs.

In these experiments absence of any non-specific hybridisation signal was demonstrated by using λDNA/Eco*RI*+Hind*III* digest as the negative control (Figure 3.4, 3.5, 3.6, 3.7).

One difficulty encountered in these experiments, was the formation of a strong background, causing difficulty in the analysis of hybridisation signals. It can

be suggested that this problem was a result of observing the signals from the membrane itself, which is the main principle of the color detection system. In order to eliminate this problem chemiluminescent detection system is being widely used even for single copy gene detection. In chemiluminescent detection system the signals in the membrane are reflected on an X-ray film. Thus only the signals, which are strong, can be observed on the film, eliminating the background, which comes from the membrane itself.

3.4.4 Total RNA Dot Blot Hybridisation Analysis

In order to further confirm the specificity of the 100bp probe to an expressed region of the genome and to cellulase genes, RNA dot blot analysis with total RNA of *T. thermophila* was also performed (See section 2.2.8.4.). Total RNA extracted from mycelium, harvested from glucose containing media was used as a negative control, because in the presence of glucose the expression of cellulase genes are repressed. Actually constitutive expression takes place under all conditions but the cellulase transcripts from the non-induced cells are 1100 fold less than that of the induced cells and are undetectable (Carle-Urioste *et.al.*, 1997). In order to detect cellulase gene transcripts mycelia was harvested from Avicel (microcrystalline cellulose) containing media, in which cellulase production is induced.

Under specific hybridisation conditions, signals were obtained from the 3rd, 4th, 5th, 6thand 7th day total RNAs (Figure 3.8), in accordance with the data for the endoglucanase activity profile of *T. thermophila* (Figure 3.9) (Arifoglu, 1999). As can be seen in Figure 3.9, there was no signal from the RNA sample that was

harvested from cultures grown in glucose containing medium. These results support the specificity of the 100bp *T. thermophila* probe to cellulase genes. Moreover they also show that the 100bp probe corresponds to expressed regions of the genome.

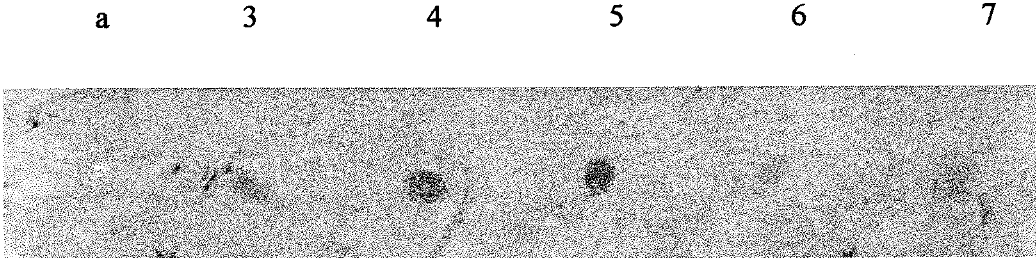

Figure 3.8. The results of dot blot analysis of *T. thermophila* total RNA **a**; total RNA from glucose containing medium, **3 – 7**; total RNA from 3rd to 7th day.

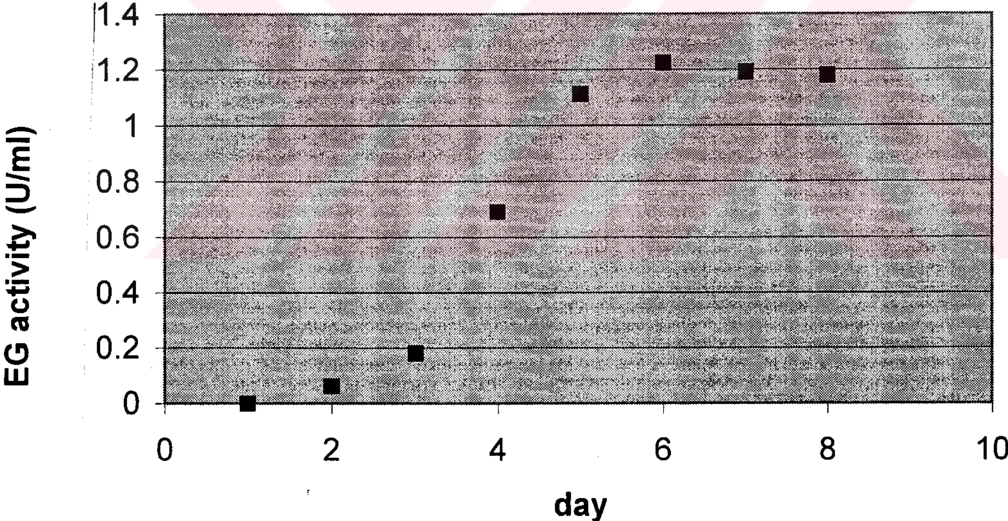

Figure3.9. Endoglucanase activity of *T. thermophila* (Arifoğlu, 1999)

CHAPTER IV

CONCLUSIONS

The aim of this study was to determine the multiplicity of cellulase genes of *T. thermophila* using the 100bp cellulase gene probe, while confirming the endoglucanase specificity of this 100 bp PCR fragment derived from *T. thermophila* genomic DNA.

The probe was amplified with degenerate primers based on conserved regions of the cellulose binding domains of fungal cellulases. The cellulose-binding domain in *T. reesei* shows a 70% homology between cloned cellulase genes. Besides it is also a common region for fungal hemicellulases and shows homology between cellulases and hemicellulases for a certain fungal strain. Thus, it should be expected that the number of genes which can be detected with the *T. thermophila* 100 bp probe may include exoglucanase and xylanase genes in addition to the endoglucanase genes. Actually the homologous hybridisation results, between the 100bp probe and *T. thermophila* genomic restriction fragment support this expectation.

The certainty of the probe specificity was the most important point for the detection of the cellulase genes. Specificity has been demonstrated by the

observation that the 100bp *T. thermophila* probe and the *egl1* gene *of T. reesei* against *T. reesei* genomic digest displayed the same pattern of intense bonds.

Since *T. thermophila* has potential for industrial usage due to the fact that it secretes endoglucanases at levels comparable to that of *H. insolens,* cloning of cellulase genes is required for going to take place in the strain improvement studies. As there is only little information about *T. thermophila* cellulases in literature, having a cellulase specific probe can be estimated as a valuable progress for cloning these genes. In the further steps of cloning studies of *T. thermophila* cellulases, this cellulase specific probe can be used for screening the genomic or cDNA library of *T. thermophila* providing the detection of whole cellulase genes in the genome.

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APPENDIX A

CHEMICALS, ENZYMES AND THEIR SUPPLIERS

Chemical or Enzyme	Supplier
λDNA Hind*III*/Eco*RI* Digest	MBI Fermentas
β-Mercaptoethanol	Merck
100 bp DNA Ladder	MBI Fermentas
Acetic Acid	Merck
Agar	Oxoid
Agarose	Merck
Bacteriological Peptone	Difco Laboratories
Bovine Serum Albumin	MBI Fermentas
Calf Thymus DNA	Sigma
Chloroform	Merck
DNTPs	MBI Fermentas
EDTA	Merck
Ethanol	Birpa
Formamide	Sigma
Glucose	Merck

Glycerol	Merck
Hydrochloric Acid	Merck
Isoamylalcohol	Merck
Isopropanol	Delta
Maleic Acid	Merck
Microcrystalline Cellulose	Merck
Mineral Oil	Sigma
PDA	Oxoid
Phenol	Applichem
Primers EG-1A and EG-1B	MWG Biotech Germany
Primers TEG-deg1 and TEG-deg2	MWG Biotech Germany
Restriction Enzymes and Buffers	MBI Fermentas
RNase	Boehringer Manheim
SDS	Merck
Sodium Acetate	Merck
Sodium Citrate	Merck
Sodium Hydroxide	Merck
Taq DNA Polymerase	MBI Fermentas
Tris	Merck
Yeast Extract	Merck

APPENDIX B

PREPERATIONS OF CULTURE MEDIA, BUFFERS AND SOLUTIONS

1. YpSs Agar

4.0 g/l Yeast extract

1.0 g/l K_2HPO_4

5.0 g/l $MgSO_4 .7H_2O$

15.0 g/l Soluble Starch

20.0 g/l Agar

2. PDA

39 grams of potato dextrose agar is dissolved in 1 liter of distilled water and boiled to dissolve completely. It is sterilized by autoclaving.

3. YEPD Broth

2% (w/v) Glucose

2% (w/v) Yeast extract

0.1% (w/v) Bacteriological peptone

In modified YEPD 0.5% (w/v) was added to this media.

4. PD Broth

200 grams of potatoes is sliced into small pieces and boiled in 1 liter of distilled water. After boiling, it is kept at boiling temperature for 30 minutes. The solution is then filtered and diluted to 1 liter with distilled water. After the addition of 20 grams of glucose, it is sterilized by autoclaving.

5.YpSs Broth with Avicel

4.0 g/l Yeast extract

1.0 g/l K_2HPO_4

5.0 g/l $MgSO_4 .7H_2O$

20.0 g/l Avicel

6. Lysis Solution

100mM Tris-HCl (pH 7.5)

50mM EDTA

2% SDS

1% 2-Mercaptoethanol

7. 5M NaCl

292.2 g NaCl was dissolved in 800 ml H_2O. The volume was adjusted to1 liter and sterilized by autoclaving.

8. 10% CTAB

10 g CTAB is slowly added to the solution containing 4.1 g NaCl while stirring and heating to 65°C. The volume is adjusted to 100 ml and the solution is sterilized by autoclaving.

9. Phenol:Chloroform:Isoamylalcohol (25:24:1)

1 ml of isoamyl alcohol and 48 ml of chloroform is mixed, and then 50 ml of phenol is added. The solution is used after phase separation and stored at 4C.

10. DNase Free RNase

RNase A is dissolved in 0.01 M Sodium acetate (pH 5.2) to give a final concentration 10 mg/ml. The solution is heated to 100C for 15 minutes in a boiling water bath for the in activation of DNase. It is cooled slowly to room temperature. 0.1X volume of 1M Tris-HCl (pH 7.4) is added until the pH of the solution is adjusted to 7.0. The solution is dispensed into aliquots and stored at –20°C.

11. Tris-HCl

121.1 g of Tris base is dissolved in 800 ml of distilled water. The pH adjustment to the desired value is done with concentrated hydrochloric acid. The solution is cooled to room temperature before making final adjustment to pH. The volume of the solution is then adjusted to 1 liter with distilled water. It is dispensed into aliquots. After sterilization by, autoclaving, the solution is stored at 4°C.

12. EDTA (0.5 M, pH: 8, 1 liter)

186.1 grams of disodiumethylenediaminetetraacetate.$2H_2O$ is added to 800 ml of distilled water. It is stirred vigorously on a magnetic stirrer while the pH is adjusted to 8 with sodium hydroxide pellets. It is dispensed into aliquots and sterilized by autoclaving. The solution is stored at 4°C.

13. Sodium acetate (pH 5.5, 3M)

408.1 g of sodium acetate is dissolved in 800 ml of distilled water. The pH of the solution to is adjusted to 5.2 with glacial acetic acid. The volume is adjusted to 1 liter with distilled water. The solution is dispensed into aliquots. After sterilization by autoclaving, it is stored at 4°C.

14. Tris-EDTA (TE) Buffer

10mM Tris (pH: 8)

1mM EDTA (pH: 8)

15. Agarose

The desired amount agarose is added in 1X TAE buffer and dissolved by heating and stirring.

16. 50X TAE Buffer (1liter)

242 grams of Tris base is dissolved in 600 ml of distilled water. The pH is adjusted to 8.0 with approximately 57 ml of glacial acetic acid. After pH adjustment 100 ml of 0.5 M EDTA (pH: 8) is added. The solution is diluted to 1 liter with distilled water.

17. Denaturation Solution (500 ml)

150 ml of 5 M NaCl and 50 ml of 5 M NaOH solutions are mixed and diluted to 500 ml with sterile distilled water.

18. Neutralization solution (500 ml)

150 ml of 5 M NaCl and 250 ml of Tris-HCl (pH: 7.2) are mixed diluted to 500 ml with sterile distilled water.

19. Sodium hydroxide

200 grams of sodium hydroxide pellets is dissolved in 800 ml of distilled water. The solution is diluted to 1 liter with distilled water. It is sterilized by autoclaving and stored at room temperature.

20. 20X SSC (1 liter)

175.3 grams of NaCl and 88.2 grams of sodium citrate are dissolved in 800 ml of distilled water. The pH is adjusted to 7.0 with 10 N NaOH solution. The solution is diluted to 1 liter with distilled water. It is sterilized by autoclaving and stored at room temperature.

APPENDIX C

RECOGNITION SITES OF RESTRICTION ENZYMES USED AND REACTION CONDITIONS

Table C.1. Restriction enzymes used and their recognition and cutting sequences.

Enzyme	Recognition and Cutting Site	Reaction Temperature
Eco*RI*	G↓AATTC	37°C
Hind*III*	A↓AGCTT	37°C
Sma*I*	CCC↓GGG	30°C
PstI	CTGCA↓G	37°C

Table C.2. Double digest reaction conditions

Enzymes	Double Digest Buffer Concentration
HindIII/EcoRI	2X Y^{+}Double Digestion Buffer (Fermentas)
HindIII/SmaI	1X Y^{+}Double Digestion Buffer (Fermentas)

APPENDIX D

LABELING AND DETECTION WITH DIG SYSTEM

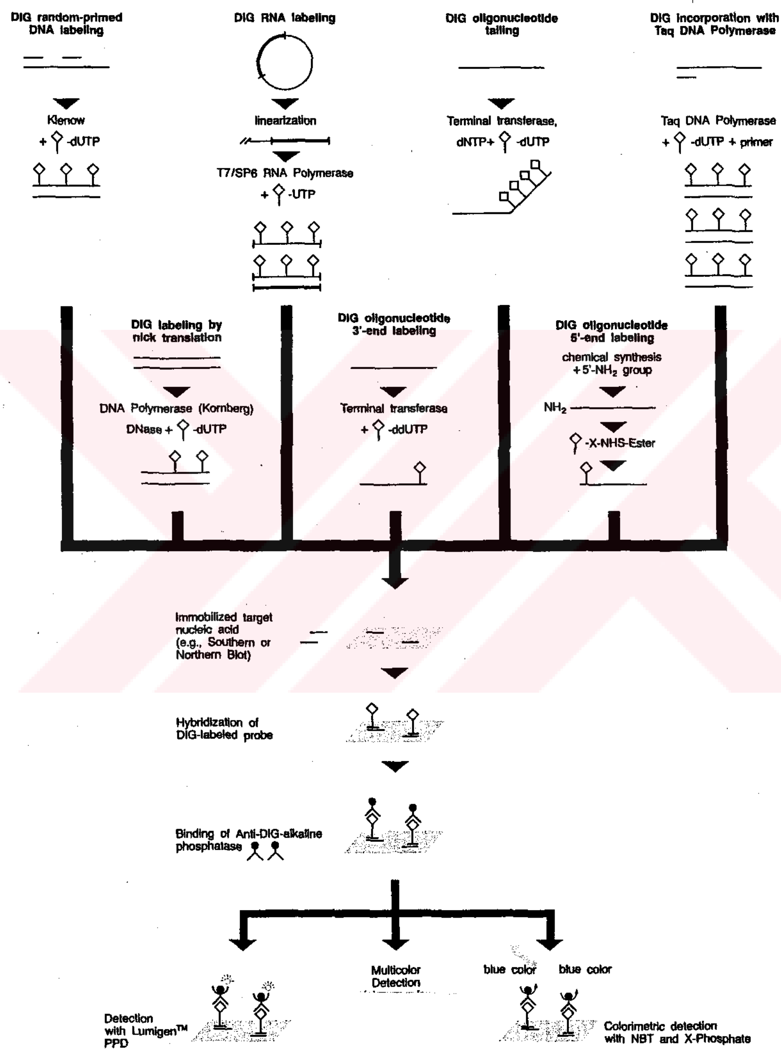

Figure D.1. The labeling and detection alternatives offered by DIG system.

APPENDIX E

GENE SPECIFIC AND DEGENERATE PRIMERS USED

Table E.1.Sequences of gene specific and degenerate primers used in this study

	Primer Name	Sequence (5' to 3')
Forward	EG-1A	CCC CCG AGG TCC ATC C
Primers	TEGdeg1	G(G/A)I (AG)(GC)I TGG (GC)(AGC)I CAG TGC GG
Reverse	EG-1B	CAA TGC CAC CGC ACT G
Primers	TEGdeg2	G(AG)C A(CT)T GI(GT) (AG)(AG)T AI(CT) A(AG)T C(AG)T

	N									C
H. insolens CMC3		**G**	**G**	**A**	**W**	**Q**	**Q**	**C**	**G**	**G**
H. insolens EG		**A**	**E**	**R**	**W**	**A**	**Q**	**C**	**G**	**G**
T. longibrachiatum EGI		**Q**	**T**	**H**	**W**	**G**	**Q**	**C**	**G**	**G**
T. reesei EGI		**Q**	**T**	**H**	**W**	**G**	**Q**	**C**	**G**	**G**
T. reesei EGIII		**Q**	**T**	**V**	**W**	**G**	**Q**	**C**	**G**	**G**

Figure E.1. The region of amino acids used in the forward degenerate primer

N	-	G	G	A	W	Q	Q	C	G	G	-	C
		A	E	R		A						
						G						

Figure E.2. The amino acid possibilities used in the design of the forward degenerate primer.

	N								C
H. insolens CMC3		N	D	W	Y	S	Q	C	Q
H. insolens EG		N	D	W	Y	H	Q	C	L
T. longibrachiatum EGI		N	D	Y	Y	S	Q	C	L
T. reesei EGI		N	D	Y	Y	S	Q	C	L
T. reesei EGIII		N	P	Y	Y	A	Q	C	I

Figure E.3. The region of amino acids used in the design of the reverse degenerate primer.

N	**-**	**N**	**D**	**W**	**Y**	**S**	**Q**	**C**	**L**	**-**	**C**
				Y		**H**			**Q**		

Figure E.4. The amino acids used in the design of the reverse degenerate primer.

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