UTILIZATION OF LIGNOCELLULOSIC COMPOUNDS FOR THE PRODUCTION OF CELLULASES BY *TORULA THERMOPHILA*

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ABSTRACT

UTILIZATION OF LIGNOCELLULOSIC COMPOUNDS FOR THE PRODUCTION OF CELLULASES BY *TORULA THERMOPHILA*

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Cellulolytic microorganisms play an important role in the biosphere by recycling cellulose. All organisms known to degrade cellulose produce a series of cellulases with different specificities, which act together in synergism. The enzymatic degradation of cellulose requires the synergistic activity of at least three enzymes, endoglucanase, cellobiohydrolase, and β-glucosidase that hydrolase cellulose to oligosaccharides and finally glucose. These enzymes remain too expensive to be used on a commercial scale. The major cost factor is carbon source since most cellulase production is based on expensive substrates.

In this work, *T. thermophila* was cultivated on various lignocellulosic compounds, for the production of cellulases in shake flask cultures at 45°C and 155 rpm.

The cellulase components investigated in this study included endoglucanase, avicel-adsorbable endoglucanase, exoglucanase and total cellulase. Of the 14 lignocellulosic compounds, grass clippings gave the highest endoglucanase and avicel-adsorbable endoglucanase activities. Soybean meal and apple pomace followed grass clippings in terms of the stated enzyme activities. In addition to 250 ml cultures, spore induction experiments were carried out on *T. thermophila* in 1 L cultures by cultivating on grass clippings and by inoculating the preculture medium with 1 mg, 3 mg, 5 mg, 10 mg and 20 mg spores. It was shown that cellulase production can be induced by increasing spore concentration up to 10 mg. For the aim of comparison, industrial cellulase producer *Humicola insolens* was cultivated on grass clippings with 10 mg spore in preculture medium and using 1 liter working volume in main culture. Accordingly, *T. thermophila* produced 5 fold more endoglucanase than *H. insolens* under the stated culture conditions. As for avicel adsorbable endoglucanase, *T. thermophila* produced 6 fold more avicel adsorbable endoglucanase activity. With respect to FPA, *T. thermophila* produced 1.7 fold more filter paper activity and 1.14 fold more exoglucanase activity than *H. insolens.*

Keywords: *Torula thermophila, Humicola insolens,* endoglucanase, avicel-adsorbable endoglucanase, exoglucanase.

ÖZ

LİGNOSELLULOZİK MADDELERİN *TORULA THERMOPHILA* TARAFINDAN SELÜLAZ ÜRETİMİ AMACI İLE KULLANILMASI

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Selülolitik mikroorganizmalar selülozu yeniden döngüye katarak biyosferde önemli bir rol oynarlar. Selülozu parçaladığı bilinen tüm organizmalar farklı spesifisitelere sahip, sinerjistik olarak etki eden bir dizi selülaz üretirler.

Selülozun enzimatik olarak parçalanması endoglukanaz, sellobiyohidrolaz ve β-glukosidaz olmak üzere en az üç enzimin sinerjistik aktivitesini gerektirir ve bu enzimler selülozu oligosakkaridlere ve son olarak glukoza parçalarlar. Bu enzimler endüstriyel alanda kullanılmaları bakımından çok pahalıdırlar. Selülaz üretimi çoğunlukla pahalı sübstratlar üzerinde gerçekleştirildiği için, en önemli maliyet faktörünü karbon kaynağı oluşturmaktadır.

Bu çalışmada bir dizi lignoselülozik madde termofilik fungus *Torula thermophila* tarafından selülaz üretimi için incelenmiştir. Çalışılan selülaz komponentleri endoglukanaz, avisel'e tutuklanan endoglukanaz, ekzoglukanaz ve toplam selülaz enzimlerini içermektedir. Çalışılan 14 lignoselülozik madde içinde çim kırpığı en yüksek endoglukanaz ve avisel'e tutuklanan endoglukanaz aktivitelerini vermiştir. Soya unu ve elma posası sözü edilen enzim aktiviteleri bakımından çim kırpığının arkasında yer almaktadır. 250 ml çalkalamalı çalışmalara ilave olarak, ön-kültür besiyerini 1 mg, 3 mg, 5 mg, 10 mg ve 20 mg spor ile aşılayarak ve 1 litre çalışan hacim kullanılarak, çim kırpığı üzerinde büyüyen *T. thermophila* için spor indüksiyon deneyleri gerçekleştirilmiştir. Selülaz üretiminin, spor konsantrasyonunun 10 mg'a kadar arttırılmasıyla aktive edildiği görülmüştür. Karşılaştırma amacı ile endüsdriyel *Humicola insolens* suşu 10 mg spor ve anakültürde 1 litre çalışan hacim kullanarak çim kırpığı üzerinde büyütülmüştür. Buna göre, belirlenen koşullar dahilinde *T. thermophila*'nın 5 kat fazla endoglukanaz, 6 kat fazla avisel'e tutuklanan endoglukanaz, 1.7 kat toplam selülaz aktivitesi ve 1.14 kat fazla eksoglukanaz ürettiği görülmüştür.

Anahtar Kelimeler: *Torula thermophila*, *Humicola insolens*, Endoglukanaz, Avisel'e tutuklanan endoglukanaz, Eksoglukanaz.

To My Parents

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ABBREVIATIONS

AAEG	Avicel-Adsorbable Endoglucanase
CBD	Cellulose Binding Domain
CBH	Cellubiohydrolase
CMC	Carboxymethylcellulose
CMCase	Carboxymethylcellulase
EG	Endoglucanase
EXG	Exoglucanase
FPA	Filter Paper Activity

CHAPTER 1

INTRODUCTION

1.1 Structure of Cellulose

When the structure of cellulose examined, we find an example of a polysaccharide in which nature has arranged monomeric glucose units in a manner that suits its function. Cellulose contains D-glucopyranoside units linked in 1:4 fashion in every long unbranched chains. Unlike starch and glycogen, however the linkages in cellulose are β-glycosidic linkages. This configuration of the anomeric carbon atoms of cellulose makes cellulose chains essentially linear; they do not tend to coil into helical structures as do glucose polymers when linked in an α, 1:4 manner. The linear arrangement of β-linked glucose units in cellulose presents a uniform distribution of OH^- groups on the outside of each chain. When two or more cellulose chains make contact, the hydroxyl groups are ideally situated to zip the chains together by forming hydrogen bonds. Zipping many cellulose chains together in this way gives a highly insoluble, rigid, and fibrous polymer that is ideal as cell-wall material for plants. This special property of cellulose chains is not just as a result of β, 1:4 glycosidic linkages; it is also a consequence of the precise stereochemistry of D-glucose at each stereocenter. If D-galactose or D-allose units linked in a similar fashion, they almost certainly would not give rise to a polymer with properties like cellulose. Each glucose residue is rotated by 180° relative to its neighbors, so that the basic repeating unit is in fact cellobiose. Chain length varies between 100 and 1400 residues.

Cellulose chains form numerous intra and intermolecular hydrogen bonds, which account for the formation of the rigid, insoluble microfibrils. Microfibrils range in lateral dimension from 3-4 nm in higher plants up to 20 nm for the microfibrils of the alga *Valonia macrophysa*, which contain up to several hundred cellulose chains. The chains are oriented in parallel and form highly ordered, crystalline domains interspersed by more disordered, amorphous regions. Raman spectroscopy and solid state C-NMR have shown that crystalline cellulose occurs in various forms (Henrissat, 1985).

The native crystalline form of cellulose has a structure designated as Type 1, which can be converted into type two by alkali treatment. The two types differ in their intrachain hydrogen bonding pattern. Type 2 can not be reconverted to Type 1. Furthermore, most native celluloses are composed of two slightly different forms of Type 1 cellulose, termed I_α and I_β, which differ in their intermolecular hydrogen bonding pattern. The two forms occur in different proportions, depending on the source of the cellulose. In addition to Type 1 and Type 2, there are another two types, namely, Type 3 and Type 4. Type 3 is formed on treatment with liquid ammonia. Type 4 is formed on heating to a high temperature in a polar liquid, and is very similar to Type 1. Depending on origin and pretreatment, the degree of crystallinity of cellulose can vary from 0 % for amorphous, acid-swollen cellulose, to nearly 100 % for the cellulose isolated from *Valonia macrophysa* (Henrissat, 1985). Cellulose from cotton is about 70 % crystalline. (Wood, 1988) and the degree of crystallinity of most commercial celluloses varies between 30 and 70 %.

In contrast to starch, which serves as a storage polymer for glucose, the role of cellulose is exclusively structural. The high tensile strength of cellulose enables plant cells to withstand osmotic pressure and is responsible for the resistance of plants to mechanical stress.

The mechanical strength of cellulose is particularly obvious in the case of wood and textile fibers, which consist of the walls of elongated empty cells. In the secondary wall of plant cells, cellulose forms several sheets in which microfibrils are organized in parallel, each sheet having a different orientation. The microfibrils are usually embedded in a matrix of hemicellulose and lignin. Hemicellulose is composed of complex carbohydrate polymers, with xylans and glucomannans as the main component.

1.2 Cellulolytic Microorganisms

Cellulolytic microorganisms are found among extremely variegated taxonomic groups. Most belong to eubactaria and fungi, but anaerobic, cellulose-degrading protozoa have also been identified in the rumen (Coleman, 1978).

The avocado fruit (Tucker *et al.*, 1987) and the slime mold *Dictyostelium discoideum* (Jones *et al.*, 1979 and Blume *et al.*, 1991) also produce cellulolytic enzymes, but the major function of these enzymes is thought to be related to the maturation of fruits and spores, respectively.

Cellulolytic microorganisms can be found in all biota where cellulosic waste accumulates. They usually occur in mixed populations comprising cellulolytic and non-cellulolytic species, which often interact synergistically. These interactions lead to the complete degradation of cellulose, which is ultimately converted into carbon dioxide and water under aerobic conditions, and into carbon dioxide, methane and water under anaerobic conditions.

1.3 Aerobic Degradation of Cellulose

Topsoil is the most important aerobic biota where cellulosic wastes accumulate. Among these, wood is the component most slowly attacked by cellulolytic microorganisms, due to its high content of lignin. Complete lignin degradation is an oxidative process which is only performed by a few microorganisms such as the white rot fungus *Phanerochaete chrysosporium*. However, a larger variety of organisms, in particular Actinomycetes are capable to effect partial delignification in order to get access to the cellulosic substrate (Mc Charthy, 1987).

The fungus *Trichoderma reesei*, an aerobic and highly celluloytic *Deuteromycete*, is probably the organism whose cellulase system has been most thoroughly investigated.

Among aerobic, cellulolytic soil bacteria, several species belonging to the genera *Cellulomonas, Pseudomonas (Cellvibrio), Thermomonospora* and *Microbispora* have been studied in detail.

Eriksson and coworkers proposed an oxidative mechanism for the initiation of cellulose degradation (Eriksson *et al.*, 1974 and Eriksson, 1981).This concept was based on enhanced degradation by crude or reconstituted mixtures of cellulases under aerobic compared to conditions. Oxidative enzymes (cellobiose quinone oxidoreductase, lactonase and cellobiose oxidase) have since been recognized and, though occurring in relatively minor amounts may be of significant importance. Cellobiose quinone oxidoreductase (CBQOR) was discovered in *Phanerochaete chrysosporium* by Westermark and Eriksson. CBQOR oxidizes cellobiose (or higher oligosaccharides) to cellobiono-δ-lactone .Westermark and Eriksson (1974) suggested that CBQOR facilitated lignin degradation by preventing the repolymerization of quinones and phenoxyradicals, by their reduction with the concomitant oxidation of cellobiose. Morpeth (1985) noted that *P. chrysosporium* CBQOR oxidizes cellulose

(besides xylan, chitin and agarose) to yield the superoxide ion which could participates in ligninolysis.

1.4 Anaerobic Degradation

Anaerobic cellulolytic organisms are the first link of a complex nutritional chain converting polymeric carbohydrates into methane and carbon dioxide. In a first step, glucose derived from cellulose is fermented by cellulolytic and associated saccharolytic organisms, yielding hydrogen, carbon dioxide and various organic compounds including acids and alcohols. In a second step, these compounds are converted into acetate and carbon dioxide. This step is thermodynamically unfavorable, unless the partial pressure of hydrogen is maintained at very low levels. Excess reducing equivalents must therefore be transferred to appropriate acceptors such as sulfate or carbon dioxide. Sulfate is reduced by sulfate reducers into hydrogen sulfide. Carbon dioxide is reduced into acetate by acetogens and into methane by methanogens. As a last step acetate is converted into methane and carbon dioxide by methanogens (Béguin and Aubert, 1994).

From the evolutionary principle of retention of energy efficiency it might be predicted that cellulases from anaerobes will have a higher specific activity than aerobic cellulases since anaerobes have less energy available for protein synthesis (36 ATP compared to 2 ATP/mole sugar in aerobic versus anaerobic metabolism) (Goyal *et al.*, 1991).

1.5 Application of Cellulases and Hemicellulases

From practical point of view, several processes involving cellulases or hemicellulases can be considered. In the short term applications requiring only partial

hydrolysis are the most likely to become economically feasible and some of them are already commercialized.

Enzymatic hydrolysis by microbial cellulases has advantages over the chemical processes, especially for the avoidance of environmental pollution. Cellulolytic enzymes are recommended for a number of applications, as in starch processing, animal feed production, grain alcohol fermentation, malting and brewing, extraction of fruit and vegetable juices, pulp and paper industry, and the textile industry.

1.5.1 Applications in Food Precessing

Cellulases and hemicellulase preparations are already used to clear fruit juices from remaining pulp particles.Cellulases can also help the extraction of juice and oil from fruit and seed pulp.Moreover, several cellulases can hydrolase β -1,3- β- 1,4-glucan,which is present in high amounts in low grade barley and hampers the filtration of beer (Béguin and Aubert, 1994).

1.5.2 Applications in Animal Feed Production

Cellulases and hemicellulases are also expected to have impact on the processing of animal feed. Treatment of silage with cellulases is expected to be beneficial for two reasons. Partial hydrolysis of the plant cell wall should speed up the release of sugars to be fermented by lactic acid bacteria. In addition, cellulases might improve the digestibility of silage. Crude enzyme preparation from *T. reesei* and *Aspergillus niger* are already marketed, but some basic and technological problems remain to be solved (Jorgensen *et al.*, 1989).

Studies are also in progress to improve the digestibility of feed in monogastric animals. One goal is the hydrolysis of glucans and xylans present in low grade feed-stuffs, which increase the viscosity of bowel content, reduce nutrient uptake and lead to the 'sticky faeces' problem in chicken. For example, *T. reesei* was grown on spent

grains from breweries, and the effect of the treatment was assessed with respect to the nutritional value of grains as chicken feed (Oh Chit Soon, 1993).

1.5.3 Applications in Textile Processing

Cellulases added to the laundry powder shave off the microfibrils which tend to stick out of cotton fibers after several washing cycles. This significantly helps restore softness and color brightness to cotton fabrics. Cellulases have also been used to remove excess dye from denim fabric in pre-faded blue jeans ('biostoning') (Béguin and Aubert, 1994).

1.5.4 Applications in Paper Pulp Processing

Several studies indicate that xylanases help reduce the amount of chlorine required to bleach the pulp (Linko, and Poutanen, 1989). This could have important environmental consequences since chlorine combines with phenolics derived from lignin, resulting in pollution of paper mill effluents. Beside reducing enzyme costs, one important goal would be to obtain preparations entirely devoid of cellulase activity, which is detrimental to paper quality. In this respect, enzymes produced by genetic engineering can prove attractive, since they do not need to be purified from the cellulases that are produced by most xylanolytic organisms (Paice *et al.*, 1987).

1.5.5 Applications in the Treatment of Cellulosic Waste

Cellulosic wastes are produced in increasing amounts either as municipal solid waste or as agricultural waste As an alternative, anaerobic conversion into methane and carbon dioxide by bacterial consortia is gaining increasing acceptance as the solution of choice for the treatment of industrial wastes and manure. At present, the

process relies on naturally occuring bacteria, but efficiency could be improved by using better strains, particularly for the two limiting steps, cellulolysis and methanogenesis, which occurs at either end of the metabolic chain (Béguin and Aubert, 1994).

1.5.6 Applications in Protein Fusions

Cellulase systems may also offer interesting spin-offs not directly related to cellulase activity. For example, CBDs could be used as tagging sequences for the affinity purification of recombinant proteins on a cheap cellulose matrix. The possibility was explored for the CBD of *Cellulomonas fimi* Cen A, which was fused by genetic engineering to *E.coli* alkaline phosphatase (Greenwood *et al.*, 1989). In one case, the *Agrobacterium* gene encoding β-glucosidase was fused in frame to the cellulose binding domain of a cellobiohydrolase from *C. fimi*. The translational fusion was expressed in *E. coli*. When immobilized onto cellulose, 42 % of the β-glucosidase activity was retained (Ong *et al.*, 1989). The immobilized enzyme is stable for long periods but can be desorbed at low ionic strengths or at alkaline pH. Although the approach to date has been limited to bacterial cellulases and *E. coli* expression systems, it is clear that the binding domains of fungal cellulases and fungal expression systems could be similarly exploited.

Given the efficiency of heterologous expression in filamentous fungi such as *Trichoderma reesei* and *Aspergillus* spp. relatively higher yields of fusion proteins might be expected.

1.6 Cellulases

Since cellulose can not get into the cells, cellulolytic enzymes are by necessity secreted into the medium or bound to the outside surface of cellulolytic

microorganisms. Furthermore, cellulase systems generally display a set of typical properties. The systems contain a multiplicity of enzyme components showing a marked synergism against crystalline cellulose. These components often posses a substrate-binding site independent from the catalytic site, and are often associated with each other and with the surface of cellulolytic microorganisms. Sequence analysis of cellulase genes and the biochemical characterization 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. Sequence similarities define families of domains having related structures. Whenever investigated, domains of the same family appear to share similar biochemical properties (Claeyssens and Henrissat, 1992).

1.6.1 Catalytic Domains

All cellulolytic microorganisms have evolved a battery of enzymes having different specificities with respect to endo /exo mode of action, activity towards amorphous or crystalline regions, or preference for substrates of different chain length. Such a diversity may be needed, in part, to cope with the physical heterogeneity of the substrate. Moreover, the structure of cellulose changes during the process of degradation, requiring different enzymes at different times (Béguin and Aubert, 1994).

In 1989, Henrissat *et al.* introduced a classification of 21 cellulolytic and xylanolytic enzymes into six families based on structural similarities detected by hydrophobic cluster analysis (HCA). Since then, the classification has been extended to a variety of glycosyl hydrolases comprising 482 sequences grouped in 45 families (Henrissat, 1991).

Several families comprise enzymes produced by a wide range of organisms, spanning different kingdoms. Furthermore, within the same family, the relatedness of

enzymes does not necessarily reflect the phylogenic relatedness of the organisms that produce them. This suggests that cellulase and xylanase genes have spread to a significant extent by horizontal transfer across a wide range of organisms (Nakamura *et al.*, 1986). Since the most of the catalytic domains can be combined with a variety of non-catalytic domains, it is likely that early genes were transferred as modules encoding single domains, which were subsequently recombined to yield genes encoding multidomain proteins. While being very useful, such molecular taxonomy studies point out some of the problems encountered by computer biologists. The first one is due to errors in the databases. New sequences not fitting in any of the above categories, or not matching strictly conserved regions present in enzymes of the same family , should be viewed with some caution (Gibbs *et al.*, 1992). Another problem is to define a threshold of similarity below which proteins should be ordered into different families. Originally , the HCA-based classifications was supposed to define families of proteins sharing the same polypeptide folding pattern. No significant difference in folding pattern has yet been found between the members of the same family. However, even HCA may fail to detect a similar folding in proteins whose sequences are too divergent (Torronen *et al.*, 1993).

A further difficulty lies in proteins carrying more than one catalytic domain . For example, *Prevotella* (formerly *Bacteroides*) *ruminicola* endoglucanase was classified as belonging to a new cellulase family (family I or 26), but in fact , catalytic activity resides within a domain quite clearly belonging to the well-known family A (or 5) (Matsushita *et al.*, 1990).

Three dimensional structures have been determined by X-ray diffraction analysis for *T. reesei* CBH II, *Clostridium thermocellum* endoglucanase CelD, *Bacillus pumilus* xylanase and *A. aculeatus* endoglucanase (family H) (Rouvinen *et al.*, 1991).

The folding pattern of *T. reesei* CBH II consists of a 7-stranded β-barrel. The active site is formed by two extended loops at the C- terminal end of the barrel.

Clostridium thermocellum Cel D comprises on N-terminal domain composed of two β sheets , and a large C-terminal catalytic domain composed of 12 helices forming a α barrel. Three of the loops connecting six of the helices on the same side of the barrel form the active side. The geometry of the active site of the various enzymes provides an elegant explanation of their endo or exo specificity. In the case of endoglucanases and of the xylanase, the substrate comes to lie in an open cleft, which can straddle cellulose or xylan molecules anywhere along the chain, in agreement with the endo mode of action of these enzymes. The exo mode action of CBH II is explained by the fact that the active site forms an almost perfectly enclosed tunnel through which the cellulose chain has to be threaded from the non-reducing end (Béguin and Aubert, 1994).

1.6.2 Reaction Mechanisms

It is generally assumed that the hydrolysis reaction catalyzed by glycosidases, including cellulases and xylanases, proceeds via an acid-base mechanism involving two residues. The first residue acts as a general acid catalysts and protonates the oxygen of the osidic bond. The second residue act as a nucleophile, which either interacts with the oxocarbonium intermediate (for retaining enzymes) or promotes the formation of an OH^- ion from a water molecule (for inverting enzymes). Reactions leading to retention of configuration involve a two-step mechanism, with a double inversion of configuration at the anomeric carbon, and the formation of oxocarbonium intermediate. The paradigm of this type reaction is the mechanism of lysozyme (Kelly *et al.*, 1979). Reactions leading to inversion of configuration proceed via a single nucleophilic substitution (Sinnot, 1990). The sterochemical course of hydrolysis has been determined for 17 β- 1,4 glucanases and xylanases belonging to six glycosyl hydrolase families. Enzymes of family 5, 7, 10 and 11 proceed via retention of configuration. Enzymes of family 6 and 9 proceeds with inversion of configuration (Knowles *et al.*, 1988).

1.6.3 Cellulose Binding Domains

Many cellulolytic enzymes contain non-catalytic cellulose binding domains (CBDs). These are usually located at the NH2 or COOH terminus of the enzymes, and are often separated from the catalytic domains by glycosylated, Pro/Thr/Ser – rich linker segment. Cellulose binding properties have actually been demonstrated for only a fraction of the putative domains that can be identified by sequence similarity. Similar domain structures are also found in enzymes degrading other insoluble carbohydrates such as raw starch, and chitin. Such binding domains improve the binding and facilitate the activity of the catalytic domain on the insoluble but not on soluble substrates. Based on their aminoacid sequence similarities, the CBDs have been divided into several different families. CBDs are typically separated from catalytic domains by relatively long linker peptides which are often heavily glycosylated and thought to adopt extended conformations (Abuja *et al.*, 1988a). In some cellulases multiple CBD copies or internal CBDs have also been identified (Meinke *et al.*, 1991). The catalytic domains of the cellulases in a cellulosome are attached to a scaffolding protein through specific docking regions. The "scaffolding' has a CBD for binding to cellulose, although some of the catalytic domains also have additional CBDs of their own (Lamed *et al.*, 1983). Interestingly, strikingly similar domain structures have been found in several enzymes active on other insoluble carbohydrates such as raw starch and chitin. Glucoamylases, the enzymes active on the insoluble raw starch have distinct starch binding domains (Jespersen *et al.*, 1991) and many different chitinases contain a chitin binding domain (Watanabe *et al.*, 1992). Similar to cellulases, removal of the subtrate-binding domains of the enzymes decreases their activities on insoluble but not on soluble substrates (Iseli *et al.*, 1993). It is thus apparent that such a modular domain structure offers some significant advantages in the degradation of these insoluble substrates.

CBDs are widespread in all types of cellulases and some hemicellulases, mainly produced by many different aerobic and anaerobic bacteria and fungi and have also been found in many different hemicellulases (Tomme *et al.*, 1995a).

Instead of single CBD, some cellulases have two CBDs (Winterhalter *et al.*, 1995). Possible advantages of two successive CBDs include synergistic binding interactions resulting in increased affinity (Linder *et al.*,1996) or a broaden or altered substrate specificity of the enzyme (Coutinho *et al.*,1992).

According to several reports, some CBD also have affinity towards chitin (Goldstein *et al.*, 1993), which reflects the close structural similarity of chitin and cellulose . Although the CBDs generally do not have affinity for xylan or mannan , they have been found in several xylanases , mannanases and other hemicellulases (Gilkes *et al.*,1988).

These enzymes have low or no activity on cellulose, but their CBDs function as in cellulases , binding specifically to cellulose . In some cases xylan-binding domains have been reported (Irwin *et al.*,1994).

There is a strong correlation between the capacity of cellulolytic enzymes to degrade crystalline cellulose and their affinity for cellulose. In addition, all of the cellulases that are active against crystalline cellulose, and whose sequence has been determined, posses a CBD or are associated with a cellulose-binding protein. This suggests that the presence of a CBD enhance the activity of cellulolytic enzymes towards crystalline cellulose. Indeed the presence of an independent CBD should allow cellulases to perform many catalytic cycles while remaining tethered to the substrate. For this, the link between the CBD and the catalytic site must be sufficiently flexible to permit cleavage of more than one bond without desorption of the CBD,

and binding of the CBD to cellulose must be reversible in order to allow movement of the enzyme along the substrate.

For *T. reesei* CBH I and CBH II , removal of the CBD by proteolytic cleavage reduced the activity towards avicel by 85 % and 40-60 %, respectively, whereas activity towards avicel chromogenic oligosaccharides remained unchanged (Tomme *et al.*, 1988).

In the case of *T. reesei* CBH I, the isolated catalytic domain retains some activity on insoluble substrates but the activity seems to be limited to the more easily accessible part of the substrate. At saturation , the catalytic domain of CBH I has been estimated to bind to 10-30 % of the surface covered by the intact CBH I with a fully function CBD (Stahlberg *et al.*, 1991). These results suggest that the catalytic domain and the intact CBH I have different binding sites on the cellulose surface. The presence of CBD clearly enhances the overall efficiency of CBH I on crystalline , cellulose but also leads the enzyme to non-productive binding sites at higher enzyme concentrations (Srisodsuk *et al.*, 1993).

The enzymatic activity of many different cellulases is also affected by shortening or lengthening of the linker region between the CBD and the catalytic domain (Shen *et al.*, 1991). Such data suggest that the two domains act in concert on the cellulose surface during catalysis, and that relatively long linker region with some flexibility are needed to express full cellulolytic activity. The interdomain linker region of many cellulases is sensitive to proteolytic digestion, and consequently enzyme forms both with and without CBDs have been found in the culture supernatants of several cellulolytic organisms (Soloheimo *et al.*, 1988). Some cellulolytic organisms have been shown to produce proteases which specifically remove CBDs (Gilkes *et al.*, 1988) and it has been suggested that the presence or the absence of a CBD could also controlled by differential splicing (Birch *et al.*,1995).

1.7 Fungal Cellulase Model

Fungi colonize diverse ecological niches, yet their cellulases are based on a central theme-exo-and –endo-splitting glucanases acting co-operatively and in some instances synergistically. The characterization of cellulases has proved to be complex. This is due in part to the ill-defined nature of the natural substrates, plus the multiple nature of cellulase components. Cloning studies have shown far less diversity at the genomic level. Cellulolysis is normally considered from an ecological perspective , an industrial application or with regard to the utilization of cellulose for growth. It seems likely that a wide spectrum of fungal cellulases will exist, differing according to their broad ranging roles. The classic fungal system based primarily on *Trichoderma* cellulase includes endoglucanases (1,4-β-D- glucan 4 glucanohydrolase, EC 3.2.1.4 – abbreviated to EG), cellobiohydrolase (1,4- β-D-glucan cellobiohydrolase,- EC3.2.1.91- abbreviated to CBH), and cellobiase (β-D- glucoside glucohydrolase, EC 3.2.1.21-abbreviated to CB). This classification rests mainly on assumed substrate specificity, a theoretical criterion fraught with exceptions. The classical action of cellulase is envisioned as an initial attack by endoglucanases followed by the combined action of cellobiohydrolases and endoglucanases, with final hydrolysis of the small oligosaccharides to glucose by cellobiase.

1.7.1 Cellobiohydrolases

Trichoderma reesei has two distinct cellobiohydrolases, CBH I and CBH II. CBH I is the dominant enzyme forming up to 60 % of the secreted proteins. Purified CBH I from several sources, range in molecular weight from 42 – 72 kDa with carbohydrate content from 1.4-10.4 % and iso-electric points reported from 3.5 to 4.2. The CBH I aminoacid sequence was first determined by Fagerstam *et al.* (1984) and is essentially in agreement with that deduced through cloning and sequencing of the gene which yielded a 496 amino acid protein (52214 Da) (Shoemaker *et al.*, 1983a). Bhikhabhai and Petterson (1984) reported that CBH I has 12 disulfide bridges

and thus there were no free cysteine residues. From the nucleotide sequence, there are four putative N-glycosylation sites (Asn-X-Ser/Thr), probably three of which are glycosylated.

Salovuori *et al.*, (1987) analyzed the glycoprotein nature of CBH I via biosynthesis combined with the use of tunicamycin (an inhibitor of N-glycosylation), and also degradatively using endo H glycosidase (which cleaves N-acetylchitobiose of high mannose type glycans) and via β-elimination of derived glycopeptides plus further degradation using α-mannosidase. The N-glycosylated sites were shown to contain $(man)_9$ $(GNAc)_2$ and $(man)_5$ $(GNAc)_2$ chains. The O-glycosylated sites are also comprised mainly of mannose. CBH II forms roughly 20% of the secreted protein with molecular weights ranging from 50-58kDa, its pI's from 5.0-6.3 and it shows 8-18% glycosylation. CBH II does not act as a truly theoretical cellobiohydrolase for besides releasing cellobiose from amorphous cellulose, it shows low activity towards CMC and causes short fiber formation (Kyriacou *et al.*, 1987). Its classification as a cellobiohydrolase is thus debatable. More importantly, these combined exo- and endo-splitting characteristics lead to a re-interpretation and perhaps an endo-splitting role of CBH II in the exo / endo-synergism found between CBH I and CBH II (Fagerstam and Petterson, 1980). An alternative explanation of synergism due to competition via absorption and resultant high enzyme turnover, is not excluded. (Henrissat *et al.*, 1985). Differences in the action of CBH I and CBH II are illustrated in the ability of CBH II to promote small particle formation from Solka floc, a property not shared with CBH I.

Cloning of the gene coding for CBH I and CBH II in *Saccharomyces cerevisiae* has resulted in greater understanding of the cellulase system (Pentilla *et al.*, 1987). CBH I and II expressed in *S. cerevisiae* were found to be highly glycosylated and consequently of greater molecular weight than the native CBHs. Substrate specificity of the cloned enzymes indicates that CBH II is active towards a barley β-glucan in plate assays (endoactivity, clearing of this glucan by EG), whereas CBH I is inactive in this assay.

1.7.2. Endoglucanases

Endoglucanases preferentially attack internal glycosidic linkages of substrates such as acid-swollen cellulose and soluble barley glucan, besides the modified substrates carboxymethylcellulose and hydroxyethyl cellulose.

Three endoglucanese groupings based on the isoelectric focusing classification scheme, can be recognized. The endoglucanase grouping I (EG I) is fairly well substantiated. EG I is a major cellulase component comprising 6-10% of the secreted protein (54 kDa), pI 4.7 and carbohydrate content of 4% (Shoemaker *et al.*, 1983). A second major endoglucanase grouping 'EG II', appears common to *Trichoderma* spp. EG III is illustrative and has been characterized through cloning and sequencing. It has 397 amino acids(42.5 kDa) plus 15 percent glycosylation to yield a total molecular weight of 49.8 kDa. EG III lacks transferase activity. Saloheimo *et al.* (1988) noted that the amino acid composition resembles that of Endo 4. Though the nucleotide sequence analysis of egl3 is quite distinct from the other *Trichoderma* egl1, cbh1 and cbh2 genes , it does show remarkable homology to that of an endoglucanase gene from *Schizophyllum commune* (Saloheimo *et al.*,1988). The gene is also remarkable for having relatively long introns (123 and 174 base pairs) in comparison to other fungi. EG III alone hydrolyses the a glycone linkage of 4-MU-cellotrioside.

The endoglucanase III grouping is comprised of smaller moleculer weight enzymes (20-23.5 kDa), which being relatively labile, have been harder to characterize, e.g. endo IV (Beldman *et al.*, 1985) .Though apparently showing no attack on crystalline cellulose, EGI acts synergistically with CBH I and CBH II towards this substrate. The other EGs have similar general endo-cleavage properties as EGI towards CMC but each has a unique specificity. EGII also shows synergism with CBHI and CBHII in attack of crystalline cellulose. EGI, II and III all attack CMC and can disaggregate Solka Floc to yield small particles (Kyriacou *et al.*, 1987).

EG II and Eg III also show relaxed substrate specificity, as they also attack the aglycone bond of MUC and MUL.

The proposed theoretical specificity of cellulase components does not always agree with the experimental results. Certain endoglucanases show little activity towards carboxymethyl cellulose, e.g. Avicelase II of Y-94 (Yamenobe *et al.*,1988), EGIb (syn, CBHII) of *T. reesei*. Other EGs show relaxed specificity and are also active on xylans, e.g. *T. reesei* endo IV, V and VI and exo I (as defined by Beldman *et al.*, (1985) all attack xylan), and *T. reesei* EG II and EG III (Kyriacou *et al.*,1987). Whether these dual cellulolytic and xylanolytic activities are due to different active sites has yet to be determined.

1.7.3 β-Glucosidases

The knowledge on *Trichoderma* β-glucosidases is still poor in comparison to cellobiohydrolases and endoglucanases, although its purification has been reported by several researchers. This is in part due to the fact that its gene has only very recently been isolated and its sequencing is still being investigated.

Moreover, β-glucosidase represents only a very low portion (0,5 – 1 %) of the total extracellular protein mixture secreted by *T. reesei*, and considerable difficulties are observed in isolation of an intact, homogenously purified preparation. Chirico and Brown (1987) report that this enzyme contains about 50% hydrophobic amino acids, and a single N-linked sugar antenna. Hofer *et al.* (1989), using monoclonal antibodies, reassessed the origin of multiple forms of *T. reesei* β-glucosidase. They provided evidence that partial proteolysis of the 80 kDa, IP 8.4 β-glucosidase results in the formation of 50 and 35 kDa protein fragments, which are still active and exhibit isoelectric points of 6.1 and 5.7, respectively. It is thus very likely that some of the β-glucosidases are proteolytic fragments of the 80 kDa β-glucosidase. This assumption is strengthened by the fact that several commercial cellulase preparations of *T.reesei,*

contain high protease activity, and lack an intact β-glucosidase protein (Gsur *et al.*, 1991). However, the occurrence of a 100-120 kDa β-glucosidase can not be explained in this way. It is possible that this enzyme is identical to the intracellular β-glucosidase of *T. reesei*.

This enzyme does not react with monoclonal antibodies against the 80 kDa β-glucosidase. The role and intracellular location of this enzyme is still unclear, but it has been proposed to function in the controlling of the accumulation of cellulase inducers. It is possible that this enzyme can be secreted in certain strains or under certain conditions; alternatively, its extracellular occurence may be due to autolysis.

The low amount of β-glucosidase in the culture fluid has been shown to be due to a predominant amount bound to the cell wall. The cell-wall bound enzyme has been solubilized by treatment with *Aspergillus niger* glycosidases, and characterized, showing that the cell-wall bound β-glucosidase activity is due to a single, specific enzyme, resembling the 80 kDa enzyme with respect to size, change and reactivity against monoclonal antibodies (Messner *et al.*, 1990).

T. reesei also contains a plasma-membrane-bound β-glucosidase of 70 kDa and IP 8.4, which reacts with monoclonal antibodies against the 80 kDa enzyme. This enzyme probably plays a role in the mechanism of cellulase induction; however, it may also be an intermediate of the β-glucosidase secretary pathway.

1.8 Regulation of Cellulase Synthesis

Current understandings of cellulase regulation is mostly based on biochemical and physiological studies, as well as on the quantification and characterization of mRNA transcripts using probes derived from cloned genes. Very few organisms are amenable to genetics other than random mutagenesis. No gratuitous inducer has been

described, which makes it difficult to sort out effects due to induction and to catabolite-type repression.

Cellulase synthesis appears to be controlled by two basic mechanisms. All known cellulase systems are repressed in the presence of low molecular weight carbon sources that are more easily metabolized than cellulose. In addition, in many systems cellulase biosynthesis is induced in the presence of cellulose or its degradation products.

1.8.1 Repression by Easily Metabolized Carbon Sources

Addition of easily metabolized substrates blocks the synthesis of cellulase. Addition of easily metabolized substrates blocks the senthesis of cellulase observed with cultures grown in the presence of cellulose alone. In *T. curvata*, the cAMP level under varying growth conditions is correlated with the specific rate of cellulase synthesis and cellulase production in toluenized cells is stimulated 2–3-fold in the presence of cAMP or theophyllin, a phosphodiesterase inhibitor. The authors point out that the relationship between cAMP level and rate of cellulase synthesis is not proportional, since the rate of synthesis can vary by 200-fold while the cAMP level varies only by 10-fold (wood *et al.*, 1984). Mutants insensitive to catabolite-type repression have been isolated in *T. reesei* and *Cellulomonas* sp., either by selection in the presence of cellulose and deoxyglucose, or by plating mutagenized strains on solid media containing cellulose and glucose and selecting mutants producing a large hydrolysis halo. Such mutants have been used in the development of industrial strains of *T. reesei* hyperproducing cellulase.

1.8.2 Induction by Cellulose and Derived Metabolites

Although catabolite-type repression is the only regulatory mechanism known in some systems, cellulase synthesis generally requires the presence of cellulose or its soluble metabolites (Johnson *et al.*, 1985).

In *Cellulomonas* and *T. reesei*, growth on non-repressing substrates, slow feeding of glucose or mutations affecting catabolite-type repression are not sufficient to induce cellulase synthesis (Stewart *et al.*, 1976). This suggests that cellulase synthesis is controlled by a specific induction mechanism beside catabolite-type repression. The generally accepted mechanism for induction by cellulose is that cellulose first undergoes limited hydrolysis by cellulases constitutively produced in low amounts. The soluble hydrolysis products thus generated could then penetrate the cells and cause induction of cellulase synthesis.

In the case of *T. reesei*, several observations support this hypothesis. Cellulose induces cellulase synthesis in germinating conidia, but not in mycelium, (Kubicek, 1987) and this is correlated with the presence on the cell surface of conidia of a set of cellulases capable of digesting crystalline cellulose (Kubicek, 1988). Conidia are the survival form of many fungi, and it is therefore not surprising that in a soil-borne, litter degrading organism such as *Trichoderma*, they contain a whole set of enzymes capable of hydrolyzing a wide range of polysaccharides and related compounds, to enable the fungus to germinate and grow whenever various substrates become available (Dahlberg, 1982). All of these enzymes are found on the conidial surface under various nutrient conditions and are therefore constitutive in the sense that they are formed whenever the organism sporulates, irrespective of the presence of an inducer (Kubicek *et al.*, 1988). With respect to the cellulases, the presence of CBH I and CBH II, but not EG I, was demonstrated with monoclonal antibodies (Messner and Kubicek-Franz, 1991). Despite this deficiency, the conidial cellulase system was able to hydrolyze crystalline cellulose, indicating that degradation occurs by an exo-

exo synergism (Roberts *et al.*, 1989). Another interesting feature of the conidial bound cellulase system is its relative excess of CBH II (Messner *et al.*, 1991). In culture filtrates, about 15-20 % of total secreted protein is CBH II, whereas CBH I accounts for up to 60%. On the conidial surface, however, the amount of CBH II exceeds that of CBH I by twofold. These findings are noteworthy because a) they indicate that there must be a specific signal which preferably enhances expression of CBH II during conidiogenesis; and b) they suggest that an elevated level of CBH II may be beneficial to the initial attack of the crystalline cellulose molecule. The latter assumption is supported by looking at the optimum of synergism between CBH I and CBH II, which clearly occurs at a ratio of 2:1 in favour of CBH II (Henrissat, *et al.*, 1985).

According to the synergistic action of CBH I and CBH II, the major end-product formed by the initial attack is cellobiose. This disaccharide is the first soluble compound formed from cellulose, and its appearance in the cell should specifically indicate the presence of extracellular cellulose. Indeed, there are several microorganisms known in which cellulase formation can be provoked by cellobiose (Canevascini, 1979).

However, the situation in *Trichoderma* appears to be more complicated, and even to be different from species to species: growth on cellobiose or addition of cellobiose to resting mycelia does not lead to the formation of cellulases, and addition of extra cellobiose to *T. reesei* cultures growing on cellulose rather inhibits than stimulates cellulase formation. On the other hand, slow feeding of cellobiose as the only carbon source or inhibition of extracellular hydrolysis of cellobiose by β-glucosidase leads to cellulase formation in equal amounts as on cellulose. Consistent with this assumption is the presence of a β-linked disaccharide permease that is obligatory for the induction process.

This suggests that further metabolism of cellobiose is a critical point determining whether cellobiose acts as a provoker, and it may do so when it can escape hydrolysis by β-glucosidase. The effect of slow feeding of cellobiose is in accordance with this view, since the Km and Vmax of β-glucosidase and the cellobiose permease (2.5 and 0.02 mM; 35 and 0.22 U mg^{-1} mycelium, respectively) favor uptake at low stationary cellobiose concentrations. If this explanation is correct, then substances which resemble poor substrates of β-glucosidase, and hence more likely transported by disaccharide permease, may provoke cellulase induction. Table 1.1 lists some components reputed to induce cellulase formation in *Trichoderma* sp., and also the affinity (Km) and Vmax of β-glucosidase for these substrates.

Table 1.1 Reputed provokers of cellulase formation by *T. reesei* and kinetics of their hydrolysis by β-glucosidase.

Provoker	Km (mM)	Vmax (U mg^{-1})
Cellobiose	2.4	27
Sophorose	3.6	14
Cellotriose	1.8	31
Cellotetraose	0.9	29
Cellopentaose	1.1	27
Cellohexaose	0.55	29
δ-Cellobiono – 1,5-lactone	0.03	4.3

Canevascini (1979)

It provides evidence that in fact all reputed "inducers" of cellulase formation, especially the most prominent , the disaccharide sophorose (β-1,2-glucosyl-glucose), are rather poor substrates for the extracellular *T. reesei* β-glucosidase. Sophorose documents this effect also by the pH dependency of its provoking action, since maximal induction by sophorose is observed only at pH 3, which strongly reduces β-glucosidase activity (Sternberg *et al.*, 1979).

Sophorose has been considered as the natural inducer of cellulase formation for years, because it can be formed by transglycosylation via β-glucosidase or EG I (Toussaint *et al* ., 1990) .

Eriksson and coworkers proposed an oxidative mechanism for the initiation of cellulose degradation (Eriksson *et al.*, 1974; Eriksson, 1981).This concept was based on enhanced degradation by crude and/or reconstituted mixtures of cellulases under aerobic compared to anaerobic conditions. Oxidative enzymes (cellobiose quinone oxidoreductase, lactonase and cellobiose oxidase) have since been recognized and, though occuring in relatively minor amounts, may be of crucial importance.

Cellobiose quinone oxidoreductase (CBQOR) oxidizes cellobiose (or higher oligosaccharides) to cellobiono-δ-lactone. Iyayi *et al.*, (1989) have shown the important role of cellobionolactone (CBL) in inducing in *T. reesei* a distinct set of cellulase components required for the degradation of crystalline cellulose. Cellulase induction was promoted by low concentrations (2.9×10^{-4} M) of CBL, while higher concentrations (8.8×10^{-3} M) inhibited the activity of all components of *T. reesei* celullase. With CBL arising through the action of CBQOR, they postulate that lactonase acts as a "cellulase inducer" through attacking and thereby optimizing the inductive concentration of CBL. Lactonase catalyzes the hydrolysis of glucanolactone and cellobiolactone. Lactonase, besides having an inductive role in cellulase synthesis as suggested above, can also promote cellulolysis by removing lactones which are inhibitors of β-glucasidase.

1.8.3 Regulation of Cellulase Gene Expression and Characterization of Promoters

As described before, the basic conidia-associated levels of CBH I and CBH II bring about a limited degree of cellulose degradation whenever cellulose becomes available. An increase in the rate of cellulose degradation depends on the stimulation of expression of the cellulase genes.

Although earlier studies using inhibitors of protein synthesis had suggested that cellulase formation is regulated at the translational level, today's evidence (based on m RNA assays) documents that the regulation of cellulase formation occurs at a pretranslational level and leads to cellulase gene transcription within 20 minutes after addition of inducer (ElGogary *et al.*, 1989). With the exception of conidiation, all cellulase-inducing conditions have been shown to promote the synthesis of a constant ratio of three major cellulases (CBH I, CBH II, and EG I), indicating that their expression is regulated coordinately. At least the cbh 1, cbh 2, egl 1, and bgl genes are present as single copies in the genome of *T. reesei* (Moravetz *et al.*, 1992). The cbh 1, cbh 2, and egl 3 genes are located on chromosome II, and egl 1 on chromosome VI of the total six chromosomes (Gilly *et al.*, 1991). Whether the individual cellulase genes are strongly or weakly expressed appears to be a function of both the chromosomal locus and the structure of the 5'-non-coding sequences; when the egl 1 structural gene was fused under the cbh 1 promoter, single copy recombinants secreted a higher level of EG I. This indicates that the cbh 1 promoter is stronger than that of egl 1. Furthermore, when multiple copies of egl 1 were introduced into T.reesei, the highest EG I expression was observed in those transformants in which egl 1 had integrated into the cbh 1 locus (Harkki *et al.*, 1991). Cellulase gene promoters tend to resemble other consensus promoters described for the same or related species. For example, the promoters of the *celA* and *celD* genes of *C. thermocellum*, a member of the Bacillaceae, are similar to the σ^A and σ^D promoters described in *Bacillus subtilus* (Beguin *et al.*, 1986). The cen B_{pl} and *cex* promoters of *Cellullomonas fimi*, which

belongs to the Corynebacteria and is related to Actinomycetes, resemble the *Streptomyces erythreus* $ermp_2$ and *Streptomyces azureus* $tsrp_1$ promoters (Greenberg *et al.*, 1987).

Investigation of various gene transcripts within the same species indicates that genes are transcribed to different extents and that transcription is not necessarily strictly coordinated. In addition, transcription of the same gene can start at different promoters according to culture conditions. In *Cellulomonas fimi*, transcription of the *cex* and *cenC* genes was only detected in cells grown in the presence of CMC, whereas *cenA* and *cenB* transcripts were present in cells grown in the presence of glycerol, and even glucase in the case of *cenB* (Greenberg *et al.*, 1987). High level transcription of *cenB* in cells induced by CMC started mainly at the $cenB_{p1}$ promoter, whereas constitutive transcription in the presence of glucose or glycerol started at the $cenB_{p2}$ promoter.

1.9 Thermophilic Fungi in Cellulase Production

With the discovery of cellulase enzymes in various bacteria and fungi, lots of research has been done with respect to producer organisms, genetic improvement and optimization of cultivation conditions. There is a world wide interest in producing cellulase enzymes by thermophilic fungi because of their some preferred characteristics. Thermophilic fungi have advandages over the bacteria for cellulase production in downstream processing. Downstream processing requires expensive separation methods (filtration). Fungal cells are more easily separated in filtration because of its mycelial structure. In contrast to bacteria, the use of anaerobic bacteria requires the oxygen to be evacuated and this process also causes an extra cost.

The cellulase enzymes from thermophilic fungi has been found to be more stable than those produced by mesophilic fungi. The cellulases of thermophilic fungi are more resistant to proteolysis and extreme pH values. Proteolytic and thermal stability is in relation to glycosylation. All cellulases investigated so far contain covalently bound N-linked carbohydrate, and the cellobiohydrolases and endoglucanases also contain O-linked carbohydrate. While there is evidence that N-glycosylation serves to maintain thermal stability and resistance to proteolysis, there is some evidence available that functional O-glycosylation of the cellulases may be indispensible for their secretion (Merivuori *et al.*, 1985).

The use of thermophilic fungi are more advantageous than those of mesophilic fungi in the production of cellulase enzymes on industrial scale. Sterilization being not economic in industry, the use of thermophilic fungi in cellulase production is preferred since the higher temperatures used for cultivation of thermophilic fungi reduce the risk of contamination by other unwanted microorganisms and much more aseptic conditions might be achieved (Arifoğlu, 1999). Also, there are some researches on cellulase production by thermophilic fungi in relation to carbon sources used in media. The major cost factor in cellulase production has been estimated to be the carbon source since most of the cellulase preparations have been based on expensive substrates such as Solka Floc, Cotton, Avicel, carboxymethylcellulose (CMC) and commercial cellulose pulp (Doppelbauer, 1987). An effective approach to lowering the cost factor of the carbon source is to use less expensive substrates, and some lignocellulosic compounds have been tried on for their potential in cellulase production (Chen and Wayman, 1991). Some thermophilic fungi has been investigated recently due to their potential in enzyme production. To examplify, *Humicola insolens*, *Humicola grisea var. thermoidea*, *Thermoascus aurantiacus*, *Sporotrichum thermophile* are among these fungi. *Mucor pusillus*, *Malbranchea pulchella*, *Thielavia terrestris* are among these fungi.

Khandke and his coworkers (1989) studied thermophilic fungus *Thermoascus aurantiacus* for the production of endocellulase, exocellulase, β-glucosidase and xylanase. A strain of thermophilic fungus, *Thermoascus aurantiacus,* was isolated from local soil. From the culture filtrates of the organism grown on blotting paper, xylanase, β-glucosidase, exocellulase and endocellulase were obtained in large amounts in highly purified form by employing ion-exchange and gel-permeation chromatography. The xylanase and endocellulase were stable at 70°C for 8 hours, whereas the β-glucosidase and exocellulase were less stable at 70°C.

Hayashido and his coworkers (1986) performed mutation experiments to decrease the protease productivity of *Humicola grisea var thermoidea YH-78* using UV light and N-methyl-N'-nitro-N-nitrosoguanidin. A protease-negative mutant exhibited higher endoglucanase activity than the parent strain in mold bran culture at 50°C for 4 days. The culture extract rapidly disintegrated filter paper but produced a small amount of reducing sugar. About 30% of total endoglucanase activity in the extract was adsorbed onto Avicel.

The electrophoretically homogeneous preparation of Avicel-adsorbable endoglucanase (molecular weight, 128,000) showed intensive filter paper disintegrating activity but did not release reducing sugar. The preparation also exhibited a highly synergistic effect with the cellulase preparation from *T. reesei* in the hydrolysis of microcrystalline cellulose. This endoglucanase was observed via scanning electron microscopy to disintegrate Avicel fibrils layer by layer from the surface, yielding thin sections with exposed chain ends.

A mutant of *Humicola grisea var. thermoidea* producing higher protease activity and an Avicel-unadsorbable Avicel-nondisintegrating endoglucanase was isolated. The purified enzyme (molecular weight, 63,000) showed no disintegrating activity on filter paper and Avicel and a less synergistic effect with *T. reesei* cellulase

in hydrolysing microcrystalline cellulose than did the former enzyme. Endoglucanase was therefore divided into two types, Avicel disintegrating and Avicel nondisintegrating (Hayashida *et al.*, 1986).

1.10 The Basis of Thermophilism

Microorganisms grow at various temperatures and according to their growth temperature ranges, they are classified into pyschrophilic, mesophilic, thermophilic, psychrotolerant, and thermotolerant. Thermophily, literally, a love of heat, is a characteristic that appears among widely different groups of microorganisms. Besides the algae, bacteria, and Actinomycetes, there is another group of different organisms displaying the property of thermophily. They are members of the true fungi– Eumycetes or Eumycota – with temperature maxima between 50°C and 60°C.

The phylogenetic heterogeneity that characterizes the thermophilic fungi makes it certain that thermophilism arose repeatedly among different genera of Eumycota. It seems likely that the present day thermophiles were derived from mesophilic ancestors. There is no a sharp line between mesophiles and thermophiles. Each group grades very gradually into the other.

It is interesting to observe that the entire range of fungal thermophilism is spanned by the species of *Humicola*, with *Humicola stellata* at the bottom, *H. insolens* and *H. grisea var. thermoidea* about midway, and *H. lanuginosa* at the very top (Cooney and Emerson, 1964). Temperatures of 45°C on 50°C provide an environment that inhibits the vast numbers of mesophilic fungi while favoring and hence, enriching the thermophilic ones.

Setchell (1903), commenting upon the bacteria and blue-green algae that occur at such high temperatures in hot springs, noted that dehydrated proteins are more resistant to coagulation than are fully hydrated ones (Cooney and Emerson, 1964). But he said he had no reason to suppose that the water content of organisms found in hot springs was less than that of other related forms, and he decided that there must be some intrinsic differences between the proteins of mesophiles and those of thermophiles. After a lengthy of study, however, Allen (1950-1953) came to the conclusion that no basic difference between their proteins could be established. Metabolic processes were essentially similar, and there was no sharp dividing line between mesophiles and thermophiles. In fact, the property of thermophily could in certain instances be induced or removed by making a series of culture transfers at successively higher or lower temperatures (Cooney and Emerson, 1964).

From these observations, Allen (1950-1953) was led to support an idea that, whereas the proteins of thermophiles are just as unstable at high temperatures as are those of mesophiles, thermophily depends on a capacity for exceptionally rapid enzyme synthesis and steady replacement. This has become known as the "dynamic" hypothesis of thermophily (Cooney and Emerson, 1964).

Baker *et al.* (1953, 1955) found that thermophiles tend to show an increasing complexity of nutrient requirements with increasing temperatures, and they considered that this supported Allen's dynamic hypothesis because thermophiles at higher temperatures would require more ready-made building blocks to meet the requirement for rapid enzyme replacement (Cooney and Emerson, 1964). However, there is growing evidence to support another point of view namely, that there exist, as Setchell suggested, proteins with special properties; this can be termed the "stable protein" hypothesis. Militzer and his colleagues (1949, 1951) have shown, for example, that certain widespread enzymes, such as malic dehydrogenase, in cell-free systems are heat-labile when obtained from mesophiles but heat-stable when obtained from thermophiles, eventhough apparently essentially identical in other respects

(Cooney and Emerson, 1964). Somewhat contradictory results with other enzymes led them to suggest that in thermophiles some proteins might be intrinsically stable whereas others could have stability conferred upon them by the presence of a special factor. Koffler and others (Koffler, 1957) have demonstrated that the nonenzymic proteins in bacterial flagella – so called flagellins – are much more resistant to heat when derived from thermophiles than they are when derived from mesophiles. These investigators suggest that the greater stability of thermophile flagellins may result from a different degree of hydrogen bonding (Cooney and Emerson, 1964). A third suggestion to account for thermophily is the so-called "converting principle" or "temperature factor" hypothesis. Sie, Sobotka, and Baker (1961) claim that thermophilic bacteria produce a dialyzable, heat-stable substance which, when extracted and incorporated in small amounts into growing cultures of mesophiles, will induce them to become thermophiles (Cooney and Emerson, 1964).

Swartz and his associates (1957) have discussed the evidence for a possible mechanism to explain the high minimum temperatures for thermophiles. If a particular enzyme that is fairly heat stable is blocked by a specific protein inhibitor that is somewhat less heat-stable, activity of the enzyme will not take place until the temperature has risen high enough to destroy the inhibitor. Such systems have been demonstrated in bacteria. The enzymes themselves, freed of their inhibitors, act like any other enzymes. That is, heat does not activate the enzyme molecule directly, it simply removes the inhibitor (Cooney and Emerson, 1964).

1.11 Torula thermophila

Torula thermophila is a thermophilic fungus belonging to subdivision *Fungi Imperfecti (Deuteromycetes),* class *Hyphomycetes,* order *Moniliales.* There is limited research on this organism except taxonomical studies. *Torula thermophila* can grow within a wider temperature range compared to other thermophiles, such as

Chaetomium thermophile and *Humicola stellata*. It is able to grow in the range of 23-58 ^{0}C (Cooney and Emerson, 1964). Four strains of *T. thermophila* have been isolated in our study. Samples from mushroom compost were taken and analyzed for the presence of thermophilic organisms. As a result of a number of subculturings, four fungal strains were isolated. The examination of these strains grown an YpSs agar (given in 2.2.1), by the use of image analysis techniques, has revealed that all the isolates were *Torula thermophila*. It was observed that, within a few days the hyphae were divided by segments called septa, usually covering the surface of the agar. When examined microscopically at this time, spores can be seen to be developing either as chains of intercalary swellings or as terminal swellings on the tips of short lateral branches. Eventually the entire mycelium is transformed into a mass of dark brown spores, giving the colony the appearance of a thin layer of soot (Tütek and Ceylan, 1996; Ögel *et al.*, 1998).

1.12 Humicola insolens

Humicola insolens is a thermophilic soft rot fungus belonging to subdivision *Deuteromycetes*, class *Hyphomycetes*, order *Moniliales*. *Humicala insolens* is a well studied fungus and some strains are used in cellulase production for textile industry.

H. insolens grows readily in self-heating heaps, manure, mushroom composts, guayule pets (Cooney and Emerson, 1964). Hayashida and coworkers (1988) studied *Humicola insolens YH-8* and *Humicola grisea var. thermoidea YH 78* isoleted from manure and compost heaps. The molecular weights of cellobiohydrolase, endoglucanase, and β-glucosidase from *H. insolens YH-8* was estimated as 72,000, 57.000, and 250.000, respectively, by SDS-gel electrophoresis. Cellobiohydrolase, endoglucanase, and Avicel-adsorbable endoglucanase was stable with heat treatment at 65°C for 5 minutes. β-Glucosidase was stable to heating at 60°C for 5 minutes. When 90% of the carbohydrate residues of cellobiohydrolase and endoglucanase was

liberated, cellobiohydrolase was inactivated after heating at 65°C for 5 minutes, and endoglucanase was inactivated after heating at 75°C for 5 minutes. The thermal stabilities of the enzymes hence have a correlation with their carbohydrate moieties. The optimum temperature of the enzymes for activity was 50°C. In addition, it was found that the pH stability behaviors of the enzymes had a correlation with their carbohydrate moieties.

Schülein (1997) studied and characterized seven cellulases from a strain of *Humicola insolens*. Schülein found out that five of these cellulases were endoglucanases and two were cellobiohydrolases. These enzymes represents five families, namely 5, 6, 7, 12, and 45. All cellulases have been cloned and expressed in a non-cellulolytic host *Aspergillus oryzae*. In addition, *Humicola insolens* produces xylanases belonging to families 10 and 11.

EG II is a family 5 endoglucanase with the N-terminal CBD. The amino acid sequence of the EG II-cellulase was found to be homologous with EG II from *T. reesei*. The degree of homology between these two cellulases is approximately 50%.

CBH II is a family 6 cellulase with N-terminal CBD. The sequence of this CBH is more than 65% homologous with *T. reesei* CBH II. It was indicated that the sequence is so homologous that no gaps are required in the sequence alignment. This indicates that the *Humicola* CBH II has the same loops as *T. reesei* CBH II.

The EG VI is a fungal endoglucanase with activity on CMC. The sequence of the core has 60% homology with CBH II. However, in the alignment a gap can be identified at amino acids 405-415 in CBH II corresponding to a loop which covers the active site of CBH II. Other cellulases from this family comprise other fungal CBH II enzymes as well as EGs and CBHs from bacteria.

Family 7 cellulases have only been found in fungi. CBH I has been found in most cellulolytic strains including *Neurospora, Agaricus, Penicillium, Geotrichum, Phanerochaete* and *Trichoderma*. The mechanism was identical with the other family 7 cellulases i.e. retaining. The cellulase is homologous to *Trichoderma* EG I although it does not contain a CBD at the C-terminal end, in contrast to the EG from *Trichoderma.* The structure of this enzyme has been solved. Only the first 398 amino acid residues form an ordered structure. The remaining 17 residues are not required for catalytic activity. A construction with 402 residues has catalytic properties identical to the intact 415 residue long EG I.

The EG III is a family 12 cellulase. The EG V is from family 45. The full sequence of the catalytic core, the O-glycosylated linker and C-terminal CBD are available (Rasmussen *et al.*, 1991). Schülein reported that the presence of a cellulase binding domain (CBD) lowers the apparent Km. The CBD has been recommended to have a dual function, one is to find the substrate and anchor the core close to it, the other is the dispersing effect. Both functions are important when the hydrolysis is done under substrate limiting conditions. In the case of the CBH II, the anchoring effect has slowed down the activity by binding to areas without reducing end. The decreased mobility due to the presence of CBD also reduces the apparent kcat.

1.13 Effect of Medium Composition and Growth Conditions on the Production of Cellulases

The growth and metabolism of microorganisms is greatly dependent on the environment in which they are found. Therefore, the specificity of the growth environment has to be taken into consideration when a certain organism is desired to be grown and made to produce some metabolites

Detailed studies on the production of cellulases have revealed that growth of a specific microorganism and its enzyme production is dependent on nutritional and environmental factors. Changes in the medium composition and the growth conditions, including carbon source, nitrogen source, mineral and vitamins, pH, and water contents have been found to affect growth and cellulase productivity. Therefore, in order to find the optimum conditions for maximum cellulase production, these factors have to be investigated and thus, optimization of these parameters should be done.

1.13.1 Carbon Source

Fungi, in general, can utilize a variety of carbohydrates, including glucose, starch, cellulose and hemicellulose. When considering the success of fermentation for cellulase production, most significant factor is the nature and type of the carbon source used. In laboratory scale, mostly pure celluloses such as Avicel, Solca floc, cellulose powder, cotton or sulfite pulp are used. Usually, these substrates are good inducers. However, because of the high cost of these substrates, they are not suitable for large-scale fermentations. An effective approach to lowering the cost factor of the carbon source is to use less expensive substrates. Recent studies have shown that lignocellulosic compounds such as wheat straw, bagasse and aspenwood are promising substrates for cellulase production (Doppelbauer and Esterbauer, 1987). However, the utilization of these materials is always accompanied by harsh pretreatment conditions using either chemicals such as alkali or physical treatments such as ball milling. Such pretreatment methods increase the cost of these carbon sources.

Kawamori *et al.* (1987) have studied the effect of different carbon sources on the cellulase production of the thermophilic fungus *Thermoascus aurantiacus* A-131 and mesophilic *T. reesei* QM 9414. While for *T. reesei* QM 9414, Avicel was the best carbon source, for *T. aurantiacus* A-131 alkali-treated bagasse, Walseth's cellulose, and xylan yielded higher CMCase activities than Avicel.

In order to increase the cellulase productivity in fermentations with lignocellulosic substrates, pure cellulose might be added in low concentrations to affect the induction of enzyme synthesis. For example, for *Trichoderma* sp. M_7 , endoglucanase production was studied using Micricel (pure microcrystalline cellulose) alone or in combination with lignocellulosic materials such as wheat straw, wheat bran, maize stems. The results indicated that the combined substrates were better for cellulase production than a single substrate (Atev *et al.*, 1987).

Chen and Wayman (1991) investigated waste newspaper (or discarded office paper) for their use in cellulase production by *T. reesei.* Partially enzymatically saccharified newspaper was found to be most effective. This probably resulted from the wide spectrum of its metabolizability. It is much less expensive than pure cellulose and is widely available. Its enzymatic treatments involved no harsh conditions which otherwise contribute to the additional cost of carbon sources for cellulase production.

Doppelbauer and Esterbauer (1987) examined inexpensive lignocellulosic materials for cellulase production by *T. reesei.* Sulfite pulp used a standard substrate yielded 3.7 IU/ml filter paper units (FPU) and 2.15 IU/ml β-glucosidase. Steam treated wheat straw (2%) gave 1.9 FPU/ml, 0.83 IU/ml β-glucosidase, whereas the spent fibers remaining after enzymatic hydrolysis of steamed wheat straw gave 2.4 FPU/ml, 1.55 IU/ml β-glucosidase. A good substrate (3%) was also the combustible fraction of municipal waste treated with NaOH, which gave 2.5 FPU/ml, 0.86 IU/ml β-glucosidase. In shake cultures 5% steamed wheat straw gave 3.8 FPU/ml and 1.95 IU/ml β-glucosidase. Untreated wheat straw gave only low final enzyme titers.

Peitersen (1975) used barley straw pretreated with NaOH for cellulase production; the enzyme titer obtained was rather low (0.28 FPU/ml). Tangnu *et al.* (1981) reported that untreated corn stover yielded an enzyme solution with only 0.28

FPU/ml, whereas alkali treated corn stover gave 2.0 FPU/ml. A relatively high cellulase activity of 3.7 FPU/ml was obtained by Chahal *et al.* (1982) in fermentation of *T. reesei* Rut-C30 with 2.2% cellulose from washed, steam treated poplar wood. High cellulase activity and good yields were also obtained by the solid state fermentation of rice straw and newspaper in the Koji-process (Toyama 1976). Such types of fermentations were again proposed as an alternative to the submerged cultivation by Chahal (1985).

Dueñas *et al.* (1995) examined ammonia treated sugar cane bagasse by using mixed culture solid state fermentations (SSF) with *T. reesei* LM-UC_4 and *Aspergillus phoenicis* QM_{329} in flask or pot fermenters for cellulase production. Significantly higher activities of all the enzymes of the cellulase complex were achieved in four days of mixed culture SSF than in single culture (*T. reesei*) SSF. The highest filter paper cellulase and β-glucosidase activities seen in mixed culture SSF were 18.7 and 38.6 IU/g dry wt, respectively, representing the approximately 3-and 6 fold increases over the activities attained in single culture SSF. The mixed-culture SSF process also converted about 46% of the cellulose and hemicellulose to reducing sugars and enriched the product with 13% fungal protein.

1.13.2 Nitrogen Source

Another nutritional factor which is highly effective on the growth and metabolite production of microorganisms is nitrogen source. The type and kind of nitrogen source might be used for the regulation of the metabolic activities of fungi. Organic and inorganic nitrogen source may have different effect on fungi, namely some may grow well on organic nitrogen, whereas others may grow on inorganic nitrogen. Therefore, the nitrogen source used in the fermentation for the production of cellulases vary from organism to organism.

The production of cellulases by *Trichoderma* spp. S_1 was studied by using organic and inorganic nitrogen sources such as KNO_3, $(NH_4)_2SO_4$, NH_4NO_3, peptone and urea. The results indicated that the production of the cellulase enzyme complex was greatly enhanced by inorganic nitrogen sources, the highest activities being obtained with NH_4NO_3 (Ali and Akhand,1992).

1.13.3 Minerals and Vitamins

For a proper growth, all microorganisms require elements such as phosphorous, sulfur, magnesium, potassium, iron, copper, and so on. In addition to these elements, vitamins are also essential for some organisms. Since metabolic activities and enzyme production of microorganisms are growth-associated, the presence or absence, and the concentration of these compounds also affects the production of cellulolytic enzymes.

Trichoderma spp. S_1 was grown on different inorganic salts such as NaCl, KCl, K_2HPO_4, $CaCl_2.2H_2O$ and $MgSO_4.7H_2O$. The study showed that $MgSO_4$ markedly increased endoglucanase activity but was not effective on exoglucanase and β-glucosidase production (Ali and Akhand, 1992).

1.13.4 Water Content

Water content of the fermentation medium of any microorganism greatly influences its growth and metabolism, since organisms are composed largely of water. According to the water content, fermentations might be divided into submerged, semi solid-state, and solid-state cultivations. Usually,microorganisms grow differently in these media and thus, their metabolic activities are different. Studies on the cellulose

production in submerged and solid-state fermentations have shown that in both media, cellulases are produced, the enzyme activities being different.

1.13.5 pH

The pH of the fermentation medium is another factor which influences microbial growth and thus, cellulase production. Studies showed that different microorganisms grow at a particular pH ranges, and the optimal growth and cellulase production for every cellulolytic microorganism are dependent on specific organism.

The effect of fermentation medium pH on the cellulase production of the aquatic hyphomycetes *Lunulaspora curvula* and *Flagellospora penicillioides* was investigated. The pH was tested in the range of 3.0 to 8.0. The results showed that the pH value of 5.0 favored the production of FPA and CMCase for both fungi (Chandrashekar and Kaveriappa, 1991).

1.13.6 Pretreatment of Lignocellulosic Compounds

Structural cellulose is a crystalline polymer associated in a matrix with lignin and hemicellulose and is highly resistant to enzymatic attack. Pretreatment is therefore necessary, and , not suprisingly, it has been the subject of intensive investigation. The rate of enzymatic conversion of native lignocellulose is slow because of the physical barrier to enzymatic attack resulting from the close association of lignin with cellulose in lignocellulose of the plant cell wall and also from the highly resistant crystalline structure of cellulose itself. This has necessiated the development of various kinds of pretreatment to enhance the susceptibility of lignocellulose to hydrolysis. Such pretreatments can be classified into physical, chemical, and biological ones and their combination depending on the mode of reaction.

There have been numerous reports on the pretreatment with oxidizing agents such as inorganic oxidase, ozone, peracetic acid and hydrogen peroxide, which works effectively for lignin degradation and cellulose disintegration (Toyama *et al.*, 1972). Pretreatment of lignocellulosic materials such as newspaper, rice straw, pulp waste, municipal solid waste with hydrogen peroxide in the presence of manganese compounds greatly enhances their susceptibility to enzymatic saccharification. This pretreatment can be achieved using rather mild conditions with only a minimal decrease in the recovery and little change in composition. Manganase salts in this hydrogen peroxide pretreatment works effectively in particular when the concentration of hydrogen peroxide is relatively low (Takagi, 1986). It was reported that treating wheat straw and corn stover with 1% hydrogen peroxide at pH 11.5, at 25 ^{0}C, and for 18-24 h gave rise to the solubilization of approximately one-half of the lignin and most of the hemicellulose (Chen and Wayman, 1991). The enzymatic hydrolysis of the fraction that remained insolubilized yielded glucose at almost 100% efficiency calculated from the cellulose content of the starting materials.

Steam explosion of poplar wood has been reported to transform the lignin, which shields cellulose and hemicellulose in the native wood, into small droplets. In addition to physical alteration of lignin,steaming may also have effects on the lignocellulosic materials. Such effects affecting enzyme production could be the increase in surface and pore sizes, partial decristallinization of cellulose, removal of acetyl groups from hemicellulose,depolymerization of hemicellulose and others (Fan *et al.*, 1980).

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Torula thermophila strain 3A was isolated from mushroom compost (Tütek and Ceylan, 1996 and Ögel *et al.*, 1998). The industrial strain of *Humicola insolens* was obtained from ORBA Inc.

Avicel (No: 2331, microcrystalline cellulose for column chromatography) for adsorbing Avicel-adsorbable endoglucanase, Avicel (No: 2330, microcrystalline cellulose for thin layer chromatography) for measuring cellobiohydrolase (exoglucanase) activity and CMC (Carboxymethylcellulose) for measuring endoglucanase activity were obtained from Merck Chem Ltd., Germany. Pholin phenol was obtained from Sigma Chem Ltd., USA. All other chemicals used were analytical grade from Merck or Sigma.

2.2 Lignocellulosic Compounds Used as Carbon Source

Lignocellulosic compounds used were razmol, wheat bran, lentil bran, barley grain, wheat fracture, soybean meal, sunflower seed bagasse, cotton seed bagasse, sugar beet bagasse, apple pomace, tomato pomace, grape pomace, wheat straw and grass clippings. Avicel (No: 2331, microcrystalline cellulose for column chromatography) was used as a control to compare other substrates in terms of

cellulase activity. All the lignocellulosic compounds, after being dried, were milled to 1 mm size except for razmol, wheat bran and soybean meal by using Thomas Wiley Laboratory Mill. The aim of milling was to reduce the particle size down to a managable size for the fungus to consume.

2.3 Methods

2.3.1 Maintenance and Cultivation of Strains

Stock cultures of *Torula thermophila* and *Humicola insolens* were grown on YpSs agar slants at 45°C, and maintained at room temperature.

Stock cultures were subcultured every month. The composition of the YpSs agar was as follow; Difco powdered yeast extract, 4.0 g; K_2HPO_4, 1.0 g; $MgSO_4$. 7 H_2O, 0.5 g; soluble starch, 15.0 g; agar, 20.0 g in 1 L distilled water (Cooney and Emerson, 1964).

Shake-flask culture experiments were carried out in 500 ml Erlenmayer flasks with 250 ml of medium, containing modified YpSs medium which contained lignocellulosic compounds as the carbon source, instead of starch or Avicel. The composition of fermentation medium was as follows: Difco powdered yeast extract, 4.0 g; K_2HPO_4, 1.0 g; $MgSO_4$. $7H_2O$, 0.5 g; 20.0 g related lignocellulosic compounds as carbon source in 1 L distilled water.

0.75 mg spores suspended in sterile distilled water were used to inoculate 5 ml of preculture medium in 50 ml erlenmayer flasks containing YpSs with 1 % glucose as carbon source. Spores were not counted on colony counter because of the special structure of *T. thermophila* and *H. insolens* spores. The spores of these organisims are not separated easily. Because these spores form a mass structure that is not suitable for counting. Otherwise, the counting of spores would give rise to

misleading results. Therefore, it has been necessarily weighted and before inoculating the preculture medium the spores in eppendorf tubes containing sterile distilled water were degraded strongly on a vortex mixer for achieving a homogenous mixture in order to take the right amount. Preculture volume was 2 % of the main culture volume. In addition to 250 ml working volume in 500 ml erlenmayer flasks, 1 L working volume in 2 L erlenmayer flasks was used in the optimization of the size of inoculum carried out in the fermentation media with grass clippings. For this purpose, 1 mg, 3 mg, 5 mg, 10 mg and 20 mg of spores for the optimization of the size of inoculum were suspended in 20 ml of preculture medium in 50 ml erlenmayer flasks. Precultures and main cultures were incubated in a shaker incubator at 155 rpm and 45^0C.

In order to measure enzyme activity, total exocell protein, and pH , samples were taken every 24 hr starting from the first day until the 8^{th} day. Samples were placed in 50 ml Falcon tubes and centrifuged at 5000 rpm for 20 min. Culture supernatant was used to determine enzyme activities and the amount of total exocell protein. Medium pH was also measured every 24 hr.

2.3.2 Cellulase Activity Measurements

To determine cellulase activities, Nelson-Somogyi method was used (Wood and Bhat, 1988). Total cellulase, endoglucanase, Avicel-adsorbable endoglucanase and exoglucanase activities were measured.

Total cellulase activity was measured by using filter paper as the substrate. Stripes of filter paper (Whatman No: 1, 1 x 6 cm) ruled as small cylinders were put into the test tubes. Then, 0.5 ml 25 mM sodium acetate buffer pH 6.0 and pH 6.5 for *Torula thermophila* and *Humicola insolens*, respectively was added into the test tubes

and the test tubes were covered with lids. After heating the substrate solutions to the reaction temperatures (65°C and 60°C for *T. thermophila* and *H. insolens*, respectively), 0.5 ml supernatant appropriately diluted in the same buffer, was added to the test tubes. Dilutions ranged from 1 to 1/30. The temperature and pH used in enzyme assays were experimentally determined as optimal by Arifoğlu (1999). The tubes were incubated at the stated temperatures for 30 min. The time of 30 min was determined experimentally as optimum (Appendix G). After incubation at the stated temperature for 30 min, the reaction was stopped by the addition of 2 ml of the mixture of Somogyi I and Somogyi II reagents in 4:1 ratio (Appendix B).

The aliquots were transferred into dry tubes, immediately placed into boiling water bath and kept for 15 min. Next, the tubes were cooled to room temperature and 2 ml of Nelson reagent (Appendix B) was added into tubes, the test tubes were immediately mixed on a vortex mixer. Then, 4 ml of distilled water was added into the tubes and the contents were mixed. The suspension was transferred into 50 ml Falcon tubes, and centrifuged at 5000 rpm for 15 min to remove any remaining residues.

Finally, the absorbances of the supernatants were measured at 520 nm against a substrate blank prepared by using sodium acetate buffer instead of the culture supernatant. After subtracting the value of the substrate blank, the absorbance was converted into unit of enzyme activity determined in terms of glucose equivalent released (Appendix A) per minute (per ml of the culture of supernatant) under the stated assay conditions.

The experiments were carried out in duplicate and the results were taken as the mean of the two data. The enzyme activity per ml supernatant was measured by using the following equation:

$$EU / ml = (X \cdot D) / (M.t.d),$$

Where X is the amount of reducing sugar as μg of glucose, D is the dilution factor, M is the molecular weight of glucose (180 g/mol), t is the time of incubation (30 min) and d is the amount of diluted supernatant (0.5 ml). Specific activities (EU/mg protein) were calculated by dividing the enzyme activity (EU/ml) to the amount of total protein in 1 ml. One unit of total cellulase activity was defined as the amount of enzyme required to release 1 mol glucose equivalents per minute.

Endoglucanase activity was measured by using carboxymethylcellulose (CMC) as the substrate.

1% (w/v) CMC solution was prepared in distilled water. 1.0 ml of CMC solution and 0.5 ml of 5 mM sodium acetate buffer, pH 6.0 and pH 6.5 for *T. thermophila* and *H. insolens*, respectively, were mixed in the test tubes and heated to the reaction temperature (65°C for *Torula thermophila* and 60°C for *Humicola insolens*).

After heating the substrate solution at the stated temperature, 0.5 ml of supernatant diluted appropriately in the same buffer, was added.Dilutions ranged from 1 to 1/30. After incubation at the stated temperature for 30 min, the reaction was stopped by the addition of 2 ml of the mixture of Somogyi I and Somogyi II reagents in 4:1 ratio (Appendix B). Finally, the reducing sugar was measured and the enzyme activity was calculated as stated for total cellulase activity. One unit of endoglucanase activity was defined as the amount of enzyme required to liberate 1 μmol reducing sugar per minute expressed as glucose equivalent under the stated assay conditions.

For the measurement of Avicel-adsorbable endoglucanase activity, Non-adsorbable endoglucanase activity was measured directly and subtracted from total endoglucanase activity. In order to separate Non-adsorbable endoglucanase from Avicel adsorbable endoglucanase, microcrystalline cellulose (Avicel used for column chromatography) was used as adsorbent. 5 ml of 25 mM sodium acetate buffer (pH

6.0 for *Torula thermophila* and 6.5 for *Humicola insolens*) and 1.0 g Avicel were mixed in a 50 ml Falcon tube and kept for 15 min in refrigerator at 4°C. Then, previously cooled 5 ml of culture supernatant was added to the cooled Avicel solution, mixed and kept for 15 min at 4°C. Having centrifuged the mixture at 5000 rpm for 15 min, the supernatant was taken for the measurement of Avicel – nonadsorbable endoglucanase activity, and all the treatments were done as in the case of endoglucanase activity measurement. Avicel – adsorbable endoglucanase activity was measured indirectly by subtracting the activity of Avicel–nonadsorbable endoglucanase from the activity of total endoglucanase. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 μmol glucose equivalents per minute per ml under the stated assay conditions. Dilutions ranged from 1 to 1/8.

Exoglucanase activity was measured by using Avicel (used for thin layer chromatography) as the substrate. Avicel solution (1% w/v) was prepared in 25 mM sodium acetate buffer pH 6.0 and 6.5 for *T. thermophila* and *H. insolens*, respectively.

After heating 0.5 ml of substrate solution to the reaction temperature (65°C for *T. thermophila* and 60°C for *H. insolens*), 0.5 ml culture supernatant diluted appropriately in the same buffer was added to the subtrate solution. Dilutions ranged from 1 to 1/5. Test tubes were incubated for 30 min at the stated temperatures in a water bath. Tubes were mixed on a vortex mixer periodically to maintain Avicel as a suspension. After the incubation period, the reaction was stopped by the addition of 2 ml of the mixture of Somogyi I and Somogyi II reagents in 4:1 ratio (Appendix B). Tubes were boiled in a water bath for 15 min, cooled at room temperature. Then, 2 ml of Nelson reagent (Appendix B) was added into test tubes, and mixed immediately on a vortex mixer. Next, 4 ml of distilled water was added into tubes, the suspension was mixed and centrifuged at 5000 rpm for 15 min.

The absorbance was converted to micrograms of glucose equivalent released using a glucose standard (Appendix A).

One unit of exoglucanase activity was defined as the amount of enzyme required to release 1 μmol glucose equivalents per minute per ml supernatant under the stated assay conditions.

2.3.3 Protein Measurement

The total exocell protein in the culture supernatant was measured by Lowry's method (Lowry, 1951)

75 μl of appropriately diluted (dilutions ranged from 1 to 1/8) culture supernatant was put into Eppendorf tubes. Then, 750 μl of reagent C (Appendix D) was added and the solution was mixed by a vortex mixer and kept for 20 min at room temperature. 75 μl of Folin phenol reagent D (Appendix D) was added and the solution was mixed by a vortex mixer. The solution was left for 1 hr at room temperature and the absorbance was read at 750 nm against a reagent blank obtained by using buffer instead of culture supernatant. To calculate protein concentration, reagent blank was subtracted from the absorbance values of culture supernatant and converted into micrograms of protein per ml by using a BSA (bovine serum albumin) standard (Appendix C). All the experiments were carried out in duplicate and the results were taken as the mean of the two data.

2.3.4 Determination of Cellulose in Lignocellulosic Compounds

The cellulose present in dry matter was analyzed by using the method of Updegraff for the determination of cellulose in biological materials (Updegraff, 1969).

A certain amount of dried matter (0.25-0.50 g) was put into 50 ml Falcon centrifuge tube. 3 ml of acetic – nitric reagent (Appendix F) was added firstly by

adding 1.0 ml, mixing well on a vortex mixer, then adding the remaining 2.0 ml and remixing. The tubes were placed in a boiling water bath for 30 min. Then the tubes were centrifuged at 5000 rpm for 5 min, and the supernatant was discarded.

Next, 10 ml of distilled water was added into tubes by adding 5 ml of distilled water first, mixing on a vortex mixer well, and then adding the remaining 5 ml of distilled water and remixing. Next, the tubes were centrifuged at 5000 rpm for 5 min and having discarded the supernatant, 10 ml of 67% H_2SO_4 (V/V) was added into tubes by adding 5 ml of H_2SO_4 first, mixing on a vortex mixer well, and then adding the remaining 5 ml of H_2SO_4 and remixing. Then, the tubes were left 1 hr at room temperature. After 1 hr, the mixtures were diluted in the ratio of 1/100 by adding 100 µl of this mixture to 9900 µl of distilled water. 1 ml of this mixture was placed in a screw-cap type culture tube and mixed by adding 4 ml of distilled water. The tubes were placed in an ice bath to cool. Next, 10.0 ml of cold anthrone reagent (Appendix F) was added by layering with a pipette, and mixed well on a vortex mixer. The tubes were placed in an ice bath until all tubes were mixed. Then, the tubes were placed in a boiling water bath for 16 min, cooled on ice for 2-3 min, and then kept at room temperature for 5-10 min.

Absorbances were measured at 620 nm against a reagent blank. The absorbances of the mixtures read from the spectrophotometer were used to determine the amount of cellulose by using a cellulose standard curve (Appendix E). The cellulose concentration (mg cellulose / ml) was determined by using the dilution factors.All the experiments were carried out in duplicate and the results were taken as the mean of the two data.

2.4 Selection of the Medium for Cellulase Production

In previous studies modified YpSs and Czapek's media were compared (Arifoğlu, 1999) for cellulase production. Both *T. thermophila* and *H. insolens* were grown on 250 ml of YpSs and Czapek's media at 40°C, the EG and FPA activities were measured. Growth temperature of 40°C was selected since in previous studies it has been found that *T. thermophila* and *H. insolens* grow most rapidly at about 40°C (Cooney and Emerson, 1964). In the measurement of cellulase activities, assays were carried out at pH 6.0 and 55°C. These assay conditions were provisionally selected, as they have proved to be suitable for the determination of cellulase activities of *H. insolens* (M. Batum, Personal Communication).

Consequently, it has been found that both FPA and EG activities of *T. thermophila* and *H. insolens* were much higher in the modified YpSs than in the modified Czapek's medium. Therefore, it was concluded that the modified YpSs medium was better for the production of cellulases by *T. thermophila* and *H. insolens*.

2.5 Determination of Assay pH

The effect of the assay pH on the activity of EG of *T. thermophila* and *H. insolens* was analyzed. The pH of the buffer used in the assay was varied from 4.5 to 9.0, with 0.5 increments. From 4.5 to 6.5, 25 mM sodium acetate buffer, while from 7.0 to 9.0, 25 mM sodium phosphate buffer was used. The enzyme activity was measured at 55°C and time of incubation for enzymatic reaction was kept constant as 30 min. It was concluded that the pH optima of EGs of *T. thermophila* and *H. insolens* are 6.0 and 6.5, respectively (Arifoğlu, 1999).

2.6 Determination of Assay Temperature

The endoglucanase activity was assayed at different temperatures in the range of 35 to 75°C, with 5°C increments, and with a constant assay pH of 6.0 for *T. thermophila* and 6.5 for *H. insolens*, while the time of incubation for the enzymatic reaction was 30 min. Accordingly, the optimum temperature at which *T. thermophila* EG showed maximum activity was 65°C, while for *H. insolens* EG the temperature optimum was found as 60°C (Arifoğlu, 1999).

2.7 Determination of Reaction Period

The reaction period was selected by using Avicel as carbon source and measuring endoglucanase activity at 6^{th} and 7^{th} day of incubation at 65°C in a water bath. Endoglucanase activity was measured by taking the reaction mixture with 5 minutes intervals starting from 5^{th} min up to 50^{th} min. Consequently, an exponential phase in reaction rate was found between 20^{th} and 30^{th} min and the reaction period was selected as 30 min for all cellulolytic activity measurements (Appendix G).

2.8 Determination of Growth Temperature for Cellulase Production by *T. thermophila* and *H. insolens*

In order to establish the effect of growth temperature on cellulase, and specifically endoglucanase by *T. thermophila* and *H. insolens*, the FPA, EG and AAEG activities were determined at different growth temperatures ranging from 35 to 55°C. Primarily the EG activity was considered, since this enzyme gains significance in food technology.

The results indicated that cellulolytic enzymes were produced by *T. thermophila* in the range of 35-55°C, and 40-50°C appeared to favor cellulase production. The maximum FPA and EG production was at 45°C, and maximum AAEG was at 50°C, although the values at 45 and 50°C were nearly identical. Therefore, it has been concluded that maximum EG and AAEG were produced in the range of 45-50°C under the specified cultivation conditions.

H. insolens also produced cellulolytic enzymes in the range of 35-55°C, and again 40-50° seemed to be optimal for cellulase production. The maximum FPA and AAEG was obtained at 50°C, although activities of AAEG were found to be similar within the range of 40-50°C. Maximum levels of EG were produced at 45°C. As a consequence of these findings, 45°C growth temperature was selected as optimum for *T. thermophila and H. insolens* (Arifoğlu, 1999).

CHAPTER 3

RESULTS AND DISCUSSION

The aim of this study was to analyze the production of cellulases by the thermophilic fungus *T. thermophila* on lignocellulosic compounds. The strain 3A was used for cellulase production in all experiments and endoglucanase (EG), avicel-adsorbable endoglucanase (AAEG), total cellulase (FPA) and exoglucanase (EXG) activities were measured. In addition to cellulase enzyme activities, total exocell protein and medium pH were measured as a function of time during cultivation of *T. thermophila*. Finally, spore induction experiments were carried out by cultivating *T. thermophila* on grass clippings with increasing spore concentrations. *Torula thermophila* was compared with an industrial thermophilic fungus, namely *Humicola insolens* for cellulase production on grass clippings as the carbon-source.

3.1 Lignocellulosic Compounds Used for the Cultivation of *Torula thermophila*

The most important factor with respect to cellulase production is the carbon source and most of the cellulase production has been based on expensive substrates such as Solca Floc, cotton, Avicel, carboxymethylcellulose (CMC) and commercial cellulose pulp. An effective way to decrease the cost of the carbon source is to use less expensive substrates. Other approaches to reduce enzyme costs are manyfold and include recovery of cellulase, more effective pretreatment of raw materials, production of cellulases with higher specific activities by means of genetic improvements, and optimization of fermentation parameters with respect to oxygen supply, productivity and final enzyme titers. According to Dunlop (1980), the costs

of cellulose from waste materials such as crop residues or municipal refuse without pretreatment is about 10 times lower than the cost of primary cellulosics such as cellulose pulp. Some investigations reported that natural lignocellulosic compounds such as wheat straw, bagasse and aspen wood are promising substrates for cellulase production (Doppelbauer *et al.*, 1987). On the other hand, the utilization of these substrates is accompanied by harsh pretreatment conditions using either chemicals such as alkali, ozone, acid, sodium hydroxide (NaOH), ammonia (NH_3), hydrogen peroxide (H_2O_2) or physical treatments such as ball milling, hammer milling, roll milling, irradiation and pyrolysis (Chen *et.al.*, 1991). Pretreatment methods remove the lignin and hemicellulose without significantly affecting the cellulose component. The resulting pretreated material is more accessible and susceptible to enzymatic attack, however such applications further increase the cost of the operation (Chen *et al.*, 1991).

The lignocellulosic compounds used in this study except for grass clippings are waste materials originating from the processing of crops. These complex compounds may be flour industry wastes (razmol, wheat bran, wheat fracture), beer and wine industry wastes (grape pomace, apple pomace, barley grain), sugar industry wastes (sugar beet bagasse), vegetable oil industry wastes (sunflower seed bagasse and cotton seed bagasse), food wastes (soybean meal, lentil bran, tomato pomace). Grass is naturally available from parks and gardens. Wheat straw are used as animal feed and to obtain cellulose required for the production of paper. These complex compounds were investigated in terms of their potential use in the production of cellulases in Turkey by solid-state cultivation method (K. Yarangümeli, Personal Communication). It was reported that the most easily and cheaply obtainable raw materials are those used in animal feed industry. Wheat bran was reported to be suitable among these raw materials. Grape pomace, sugar beet bagasse, soybean meal, grass clippings, sunflower seed bagasse, cotton seed bagasse and soybean bagasse are used as animal feed also. Cotton seed bagasse, sunflower seed bagasse and soybean bagasse are especially important as animal feed since these compounds are rich in protein 28–34 %, 26 – 32 %, 43 – 46 %, respectively (K. Yarangümeli, Personal Communication). The other factors with respect to lignocellulosic

compounds which have to be taken into consideration are that whether they are seasonal or not, if seasonal, storable or not, unit price of raw materials, the site where raw materials are produced, and pretreated or not. For example, wheat straw, grape pomace, sugar beet bagasse production is seasonal and it is possible to store these compounds for cellulase production. On the other hand, since the production of wheat bran and razmol continue throughout the year, it is possible to take advantage of obtaining these compounds freshly. If the substrate is cropped seasonally, then unless it is capable of being stored, the operation of enzyme production will also be seasonal. In addition, the production site needs to be close to the source of the substrate to minimize transportation costs. In this respect, Table 3.1 shows the unit prices and annual production of some lignocellulosic compounds in Ankara in 1994.

Table 3.1 The unit prices and annual production of some lignocellulosic compounds in 1994.

Compounds	Unit price (TL/kg)	Production (1000 ton/year)
razmol	18.500	
wheat bran	17.000	2.474
soybean bagasse	61.000	72
Sunflower seed bagasse	27.500	437
cotton seed bagasse	28.500	412
wheat straw	8.000	40
grape pomace	Free	0.15
grass clippings	Free	?

Flour Industrialists Association, 1997.

Different lignocellulosic compounds contain different amounts of cellulose, lignin, hemicellulose, starch, other carbohydrates, proteins, fats, and minerals. In this study, however, only the amount of cellulose was determined for the purpose of

analysing the relationship between cellulase secretion and the amount of cellulose in the substrate.

Table 3.2 A comparison between the lignocellulosic compounds used in this study with respect to % total solids, % moisture, % cellulose in total solids, and % cellulose as (w/w).

	% total solids	% moisture	% cellulose (in total solids)	% (w/w) cellulose
Razmol	89	11	8.46	7.52
Wheat Bran	92	8	26.52	24.39
Lentil Bran	90	10	12.12	10.90
Barley Grain	95.5	4.5	9.20	8.78
Fragmented Wheat Fracture	90	10	9.36	8.42
Soybean Meal	97.6	2.4	9.60	9.36
Sunflower Seed Bagasse	90	10	4.56	4.10
Cotton Seed Bagasse	92	8	19.95	12.53
Grape Pomace	36	64	10.8	3.88
Sugar Beet Bagasse	14	86	15.2	2.12
Apple Pomace	28	72	12.4	3.47
Tomato Pomace	9	91	12.8	1.15
Wheat Straw	93	7	3	2.79
Grass Clippings	92	8	16.32	15.01

The percentage of cellulose of the lignocellulosic compounds used in our study are shown in Table 3.2. As can be seen from Table 3.2, wheat bran represents the highest cellulose content (26.52%). It is followed by cotton seed bagasse (19.95%). Grass clippings have a cellulose content of 16.32%, which is close to that

of sugar beet bagasse (15 %). All other lignocellulosic compounds shows lower cellulose content when compared to those mentioned above. Wheat straw represent the lowest cellulose content (3%).

3.2 Cultivation of *Torula thermophila* on Lignocellulosic Compounds

Figure 3.1 illustrates the production of EG, AAEG, FPA and EXG by *T. thermophila* on razmol as the carbon source. Razmol, like wheat bran, is a by-product formed during flour making, however the starch content of razmol is higher (K. Yarangümeli, personal communication, 1997). On the other hand, their protein contents are similar, although, ash, lipid, and pentosan content is higher in razmol. As illustrated in Figure 3.1, maximum EG, AAEG, FPA and EXG levels were all seen on the second day of incubation and decreased after the second day up to fermentation end. On the second day, 0.170 U/ml, 0.102 U/ml, 0.168 U/ml and 0.088 U/ml enzyme activities were reached for EG, AAEG, FPA and EXG, respectively. The protein content in the supernatant (mg/ml) was maximum on the first day of incubation and decreased until the fourth day remaining more or less constant up to fermentation end (Figure 3.2.).

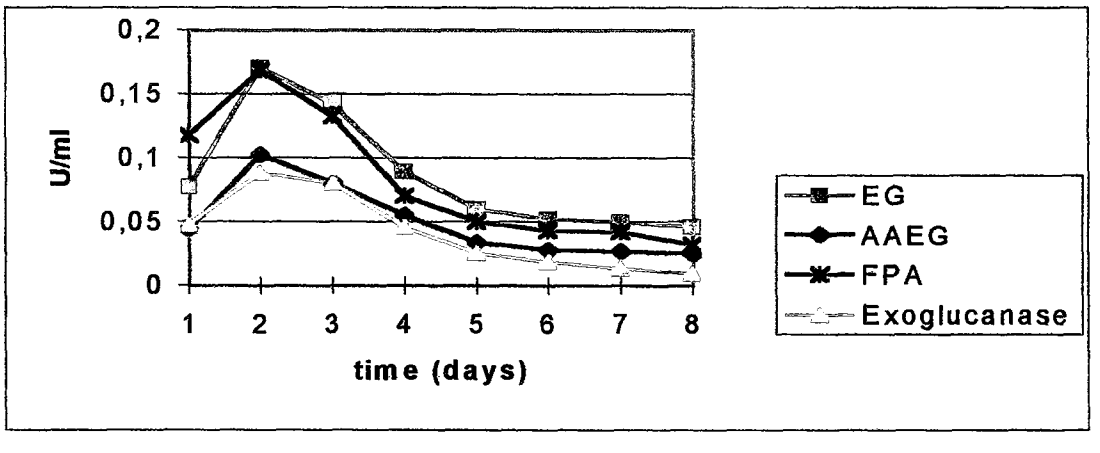

Fig. 3.1 Time course of cellulase production by *T. thermophila* on razmol.

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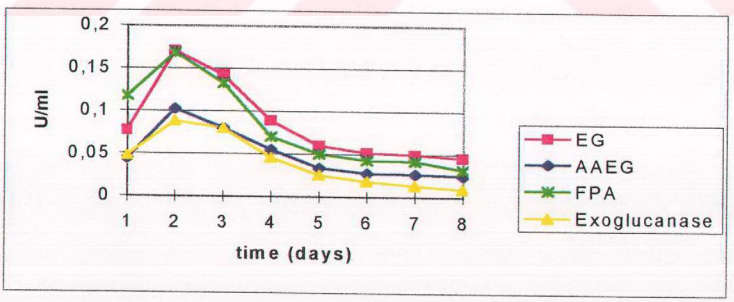

Fig. 3.1 Time course of cellulase production by *T. thermophila* on razmol.

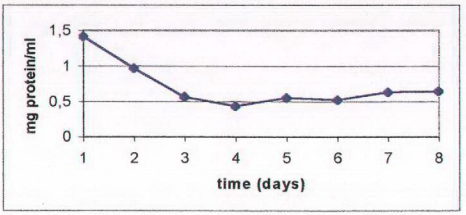

Fig. 3.2 Change in total exocell protein content (mg/ml) in the supernatant of *T. thermophila* cultivated on razmol.

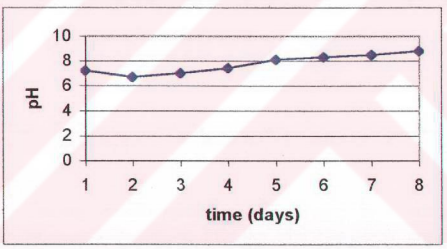

Fig. 3.3 Changes in medium pH during cultivation of *T. thermophila* on razmol.

Due to this unexpected decrease in the amount of total exocell protein, specific cellulase activities (U/mg protein) have shown an optima on the 3rd day of incubation (Table H.1, Appendix H). The initial medium pH changed from 7.17 for

the first day to 8.77 at the end of incubation period (Figure 3.3). The pH of a culture may change in response to microbial metabolic activities due to several reasons. The most obvious is the secretion of organic acids such as acetic or lactic acid, which cause the pH to decrease. On the other hand, the assimilation of organic acids which may be present in media leads to an increase in pH.

Microbial utilization of the nitrogen source can also cause the pH to change (Cooney, 1981). With ammonium salts the pH will usually decrease during microbial growth. Below pH 9 ammonia in solution exists as NH_4^+; it is incorporated into the microbial cell as $R\text{-}NH_3^+$ (where R denotes a carbon skeleton) so that a hydrogen ion is generated during ammonium uptake. Conversely, when nitrate serves as a nitrogen source, hydrogen ions from the medium are consumed to reduce the nitrate ions to $R\text{-}NH_3^+$, causing the pH to rise. The pH also tends to increase if organic amino compounds are deaminated when used for growth. When urea is used in a medium, the pH may rise due to its decomposition, the degree of pH increase depending on the urea concentration (Mitchell *et.al.*, 1988b). The substrate itself can also give rise to a change in pH.

The decrease in the cellulolytic activities on razmol may be caused by the repression of gene expression, due to high starch content or an increase in pH, degradation of cellulases by proteases, substrate depletion or enzyme instability. A significant influence of pH is, however, unrealistic, as can be concluded from the overall results.

As illustrated in Figure 3.4, cellulase activities on wheat bran reached maxima on the second day of incubation and 0.127 U/ml, 0.100 U/ml, 0.138 U/ml, 0.077 U/ml enzyme activities were obtained for EG, AAEG, FPA and EXG, respectively. After the second day of incubation, all the cellulase activities decreased gradually until the 8^{th} day. Although wheat bran has a higher cellulose content (26.52%) than razmol (8.46 %), razmol gave higher EG, FPA and EXG activities, although maximum AAEG activities of *T. thermophila* with both substrate were similar, with 0.102 U/ml for razmol and 0.100 U/ml for wheat bran, respectively.

Total exocell protein (mg/ml) was maximum on the first day and decreased until the 3rd day (Figure 3.5). After the 3rd day, total exocell protein in supernatant again started to increase until the 8th day. Table H.2 (Appendix H) shows the specific cellulase activities (U/mg protein) on wheat bran. Results are similar to those on razmol. The decrease in cellulolytic activities might be due to protease production since cellulolytic activities decreased contrary to total exocell protein increase in culture fluid. The reason for the decrease in enzymatic activities may be similar to those discussed for razmol.

Medium pH changed from 6.48 for the first day to 8.90 for the last day of the incubation period (Figure 3.6). The time course of pH on wheat bran is similar to that of razmol and similar pH values were seen on both substrate.

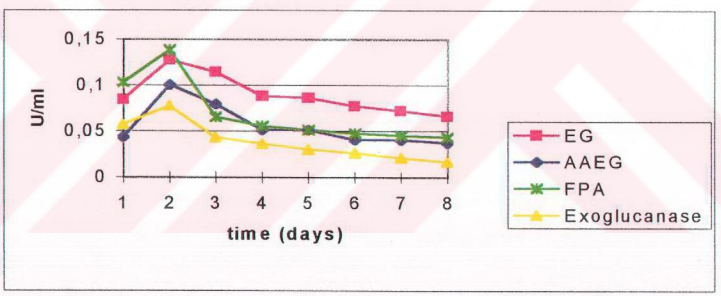

Fig. 3.4 Time course of cellulase production by *T. thermophila* on wheat bran.

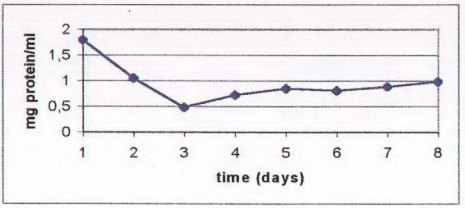

Fig. 3.5 Change in total exocell protein content (mg/ml) in the supernatant of *T. thermophila* cultivated on wheat bran.

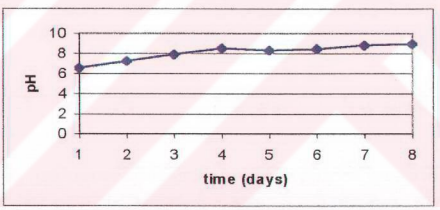

Fig. 3.6 Changes in medium pH during cultivation of *T. thermophila* on wheat bran.

Cellulase activities on lentil bran reached their maximum values on the fourth day of incubation and 0.320 U/ml, 0.246 U/ml, 0.234 U/ml, 0.054 U/ml enzyme activities were obtained for EG, AAEG, FPA and EXG, respectively. After the fourth day, all the cellulolytic activities decreased gradually up to 8^{th} day (Figure 3.7). In the case of lentil bran as the carbon source, total exocell protein (mg/ml) has

increased slightly until 5th day, with a slight decrease until the 8th day of the incubation (Figure 3.8). Medium pH changed from 6.65 for the first day to 8.65 at the end of the incubation period (Figure 3.9).

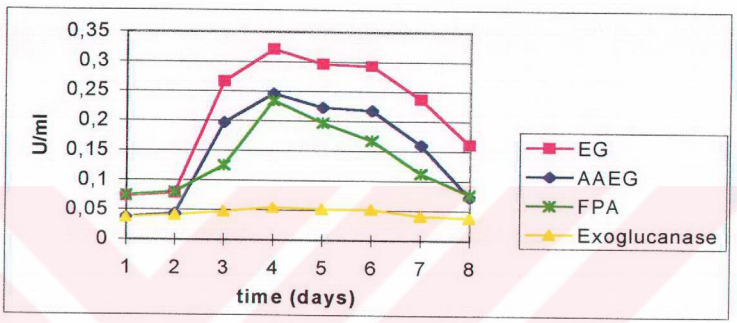

Fig. 3.7 Time course of cellulase production by *T. thermophila* on lentil bran.

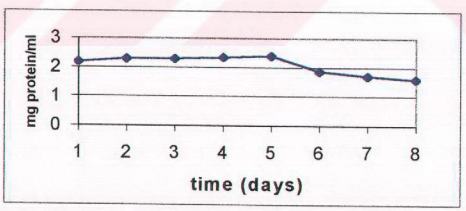

Fig. 3.8 Change in total exocell protein content (mg/ml) in the supernatant of *T. thermophila* cultivated on lentil bran.

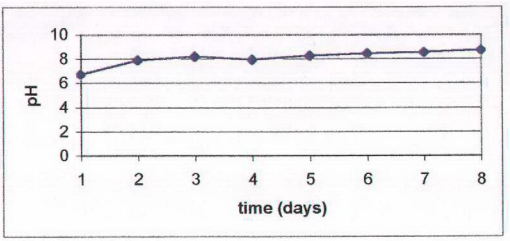

Fig. 3.9 Changes in medium pH during cultivation of *T. thermophila* on lentil bran.

EXG activities were very small when compared to EG, AAEG, and FPA. The reason for this low value of EXG activities might be related to the substrate, as the type of cellulase system required for hydrolysis is dependent upon the nature of the substrate. Another approach to the low level of EXG activity might be due to substrate specificity of the cellulase enzymes. Endoglucanases hydrolyse CMC, alkali-swollen (amorphous) cellulose, but crystalline cellulose such as cotton fiber or Avicel is not attacked to a significant extent. Endoglucanases hydrolyses amorphous regions in cellulose and creates chain ends for EXG attack. EXGs remove glucose and cellobiose from the nonreducing end of the chain. Most EXG appears to release small amounts of glucose from cellulose. CMC and cellobiose are not substrates for EXG. Cellobiose inhibits endoglucanase and EXGs and its hydrolysis to glucose by β-glucosidases is necessary. For this reason, cellulase systems containing low levels of β-glucosidase have poor saccharifying power. If there were enough EXG synthesis, cellulose would not be degraded to glucose unless β-glucosidase exists (Hayashida *et al.*, 1988).

Further explanation of the low level of EXG activity may be that a part of the adsorbed enzyme molecules were not bound at their catalytic site or these enzymes did indeed adsorbed at their catalytic site but were not able to express their hydrolytic action (Beldman *et al.*, 1986).

All the cellulase activities on barley grain reached their maximum values on the third day of incubation (Figure 3.10). After the third day, EG, AAEG, FPA and EXG activities showed a sharp decrease. As illustrated in Figure 3.10, *T. thermophila* yielded 0.180 U/ml EG, 0.100 U/ml AAEG, 0.161 U/ml FPA and 0.099 U/ml EXG activity on the third day. Total exocell protein has increased slighty until the second day, with a sharp decrease on the third day, remaining more or less constant up to fermentation end (Figure 3.11). The change in medium pH was found to be similar to that observed on other carbon sources (Figure 3.12).

Unlike razmol, wheat bran, lentil bran and barley grain, the production of cellulases by *Torula thermophila* on wheat fracture as substrate, showed their maxima on the fifth day, and 0.099 U/ml, 0.066 U/ml, 0.077 U/ml, 0.064 U/ml enzyme activities were obtained for EG, AAEG, FPA and EXG, respectively. All the cellulolytic activities decreased after fifth day (Figure 3.13). In accordance to cellulase activities, total exocell protein (Figure 3.14) increased from the first day until the fifth day and decreased after the fifth day. Medium pH shifted from 5.95 on the first day to 8.60 on the last day of incubation (Figure 3.15).

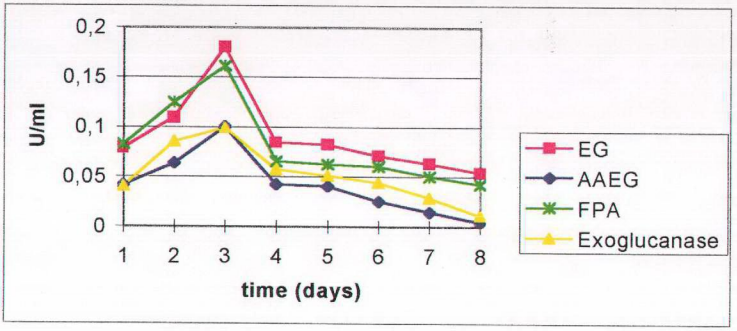

Fig. 3.10 Time course of cellulase production by *T. thermophila* on barley grain.

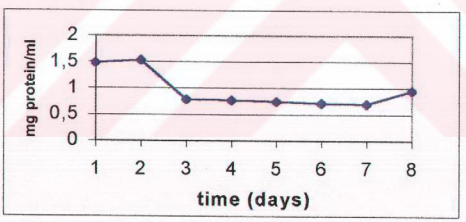

Fig. 3.11 Change in total exocell protein content (mg/ml) in the supernatant of *T. thermophila* cultivated on barley grain.

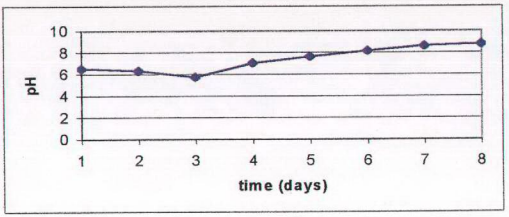

Fig. 3.12 Changes in medium pH during cultivation of *T. thermophila* on barley grain.

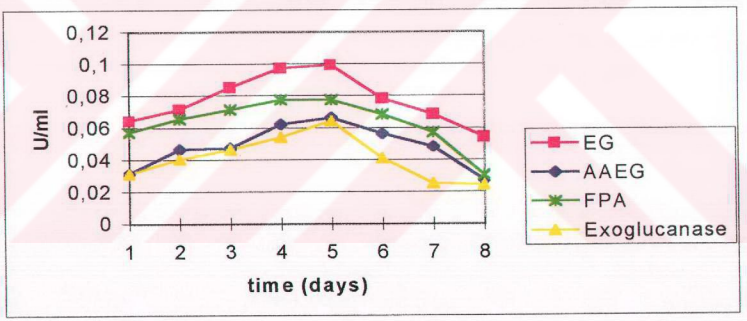

Fig. 3.13 Time course of cellulase production by *T. thermophila* on wheat fracture.

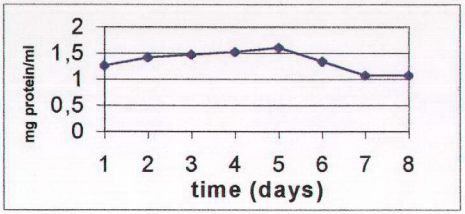

Fig. 3.14 Change in total exocell protein content (mg/ml) in the supernatant of *T.* thermophila cultivated on wheat fracture.

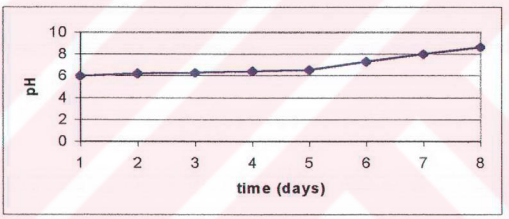

Fig. 3.15 Changes in medium pH during cultivation of *T. thermophila* on wheat fracture.

The relatively lower enzyme activities observed on wheat fracture and slow increase of activities until the 5th day may be due to starch or the inaccessibility of cellulose. Lignin has been shown to restrict enzymatic and microbial access to the cellulose and cellulose crystallinity restricts both the rate and completeness of the enzymatic hydrolysis of the cellulose as well. For achieving good yields of cellulase activity, pretreatment methods may be necessary (Doppelbauer *et al.*, 1987).

However, in this study, milling was applied on the wheat fracture and yet the activities were still lower than those observed on most other substrates.

Figure 3.16 illustrates the time course of EG, AAEG, FPA and EXG activity yielded by *T. thermophila* on soybean meal as the carbon source. The maximum values of EG, AAEG, FPA and EXG activities were 0.376 U/ml, 0.316 U/ml, 0.382 U/ml and 0.191 U/ml, respectively. The enzymes showed their maximum activity on the second day of cultivation. Having reached their maxima, all the cellulolytic activities decreased rapidly after the second day. Total exocell protein reached its maxima on the second day and decreased gradually until the end of incubation period (Figure 3.17). The medium pH changed from 6.52 at the first day to 9.50 for the last day of incubation (Figure 3.18).

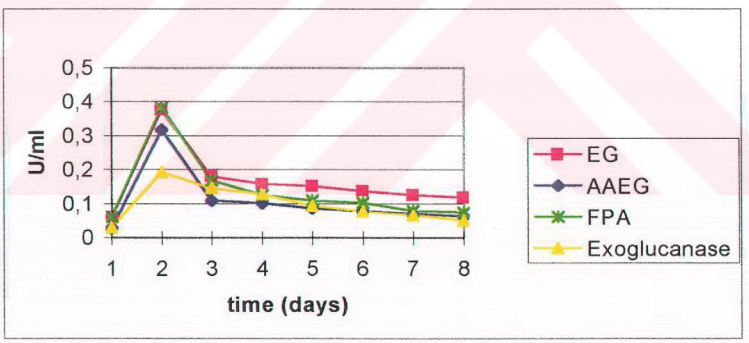

Fig. 3.16 Time course of cellulase production by *T. thermophila* on soybean meal.

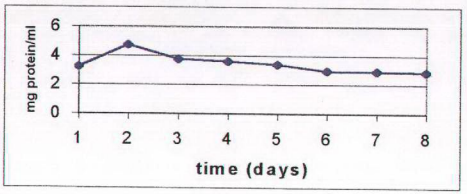

Fig. 3.17 Change in total exocell protein content (mg/ml) in the supernatant of *T. thermophila* cultivated on soybean meal.

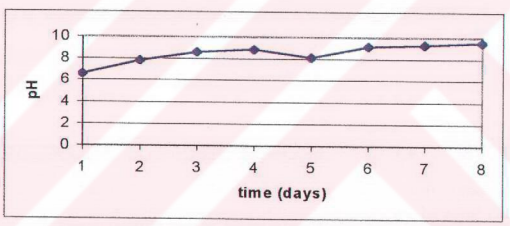

Fig. 3.18 Changes in medium pH during cultivation of *T. thermophila* on soybean meal.

Although higher activities were obtained with soybean meal as compared to most other substrates, the specific activities of these enzymes were lower compared to others (Appendix H, Table H.6). This can be explained by the high protein content of soybean meal or proteases produced in response to this high protein content. Soybean meal contains about 45 % protein (K. Yarangümeli, Personal

Communication, 1997). Nevertheless, the fact that relatively higher activities obtained on soybean meal is interesting, considering its high protein content. It can be suggested that the presence of protein in the substrate is less detrimental on cellulase production as compared to the presence of starch. It is known that filamentous fungi are effective in terms of the secretion of proteases, including alkaline and neutral proteases (Hayashida *et al.*, 1986). Although such proteases may find useful commercial applications (Pitson and Seviour, 1996), in the case of cellulase production, proteases often cause problems, associated to the proteolytic degradation of cellulolytic enzymes. Protease negative mutants have generated higher cellulase activities (Hayashida *et al.*, 1986), and such studies may also be conducted for *Torula thermophila.* Preliminary studies on *T. thermophila* grown on Avicel has shown the production of proteases, starting form the 2nd day of incubation, with activities remaining constant throughout cultivations (observed until the 11th day of incubation) (I. Ifrij, Personel Communication, 1999). It appears that *Torula thermophila* is indeed an effective protease producer even on Avicel as the sole carbon source. Furthermore, these enzymes seem to be rather stable.

As for sunflower seed bagasse as the carbon source, cellulase activities showed their maximum values on the fourth day of cultivation (Figure 3.19). The maximum values of EG, AAEG, FPA and EXG activities were 0.265 U/ml, 0.202 U/ml, 0.205 U/ml and 0.087 U/ml, respectively. After the fourth day, the cellulolytic activities again started to decrease, although this decrease was not as sharp as others, such as that on soybean meal. The total exocell protein reached its maximum level on the fourth day, in accordance to cellulase production (Figure 3.20).

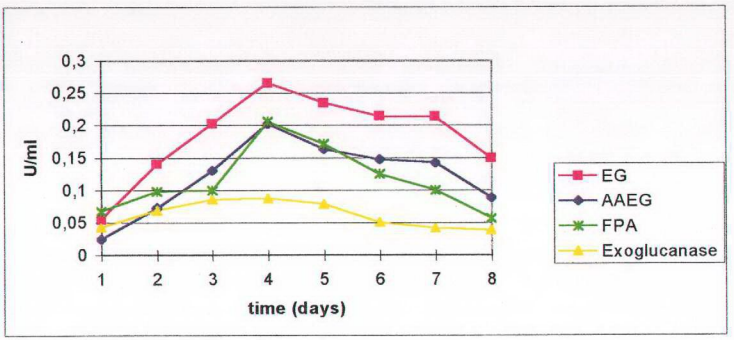

Fig. 3.19 Time course of cellulase production by *T. thermophila* on sunflower seed bagasse.

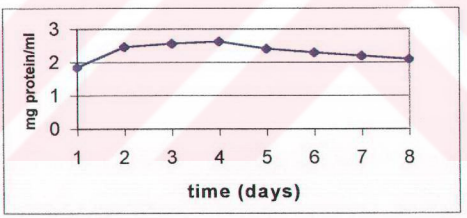

Fig. 3.20 Change in total exocell protein content (mg/ml) in the supernatant of *T. thermophila* cultivated on sunflower seed bagasse.

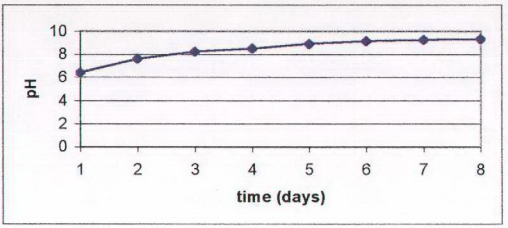

Fig. 3.21 Changes in medium pH during cultivation of *T. thermophila* on sunflower seed bagasse.

The medium pH changed from 6.38 on the first day to 9.28 at the end of fermentation (Figure 3.21). Although endoglucanases produced by *T. thermophila* are more active at pH close to neutrality (Arifoğlu, 1999), activities start to decrease above pH 7.0 and there is only 40% relative activity left at pH 9.0 (Arifoğlu, 1999). This indicates that alkaline pH negatively affects the endoglucanase activities. The fact that the medium pH increases during cultivations to such high values suggests that a pH control of the cultivation medium is essential. In future studies, it is aimed to work on fermentors with pH control, to optimize the pH and analyse its influence on cellulase secretion and activity.

Sunflower seed bagasse used in this study contained 4.56 % cellulose. There does not seem to be a correlation, between the level of cellulase secretion and the cellulose content of the substrate, since the organism yielded higher cellulolytic activities as compared to some other substrates with a higher cellulose content (also see section 3.4). Like soybean meal, sunflower seed bagasse is also rich in terms of protein, with 30 – 35 %. However, the trend of cellulase activities is not similar on the two substrates (Figure 3.16 and 3.19).

On cotton seed bagasse, EG, AAEG, FPA and EXG activities reached their maximum values on the 3rd day of incubation (Figure 3.22). Total exocell protein also reached its maximum value on the 3rd day of incubation as illustrated in Figure 3.23. The medium pH changed from 6.80 to 9.15 (Figure 3.24).

Although cotton seed bagasse contains 19.95 % cellulose in dry matter, which is higher than the other substrates used in this study except wheat bran, the productivity of cotton seed bagasse in terms of U/ml is less when compared to other substrates. The lower cellulolytic activities obtained on cotton seed bagasse might be explained by adsorption phenomena.

The amount of adsorbed cellulase per unit mass of cellulose depends on the crystallinity index and the specific surface area. Proper joining of the CBD to the core enzyme can affect the adsorption / desorption behaviour of the enzyme and its catalytic activity. Cotton seed bagasse may also contain lignin. Lignin is highly resistant to biodegradation and protects cellulose and hemicellulose against enzymatic hydrolysis.

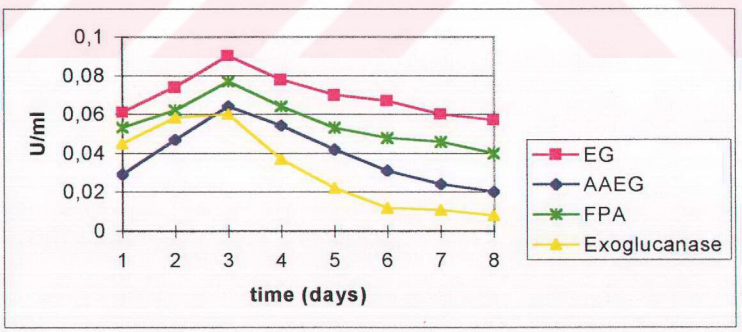

Fig. 3.22 Time course of cellulase production by *T. thermophila* on cotton seed bagasse.

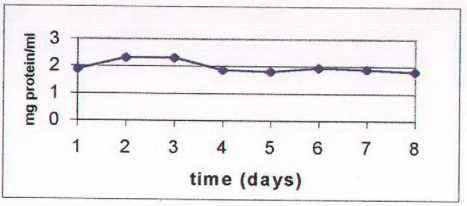

Fig. 3.23 Change in total exocell protein content (mg/ml) in the supernatant of *T. thermophila* cultivated on cotton seed bagasse.

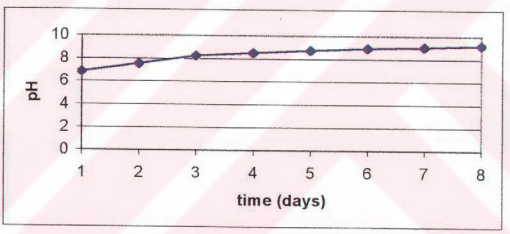

Fig. 3.24 Changes in medium pH during cultivation of *T. thermophila* on cotton seed bagasse.

When *T. thermophila* was grown on grape pomace all the enzymatic activities decreased slightly starting from day 2 until day 4 (Figure 3.25). On the 5th day of incubation, EG, AAEG, FPA and EXG activities started to rise sharply and reached their maximum values on the 7th day of incubation. After the 7th day, enzymatic

activities remain more ore less unchanged. Interestingly, a similar trend was observed for total exocell protein (Figure 3.26). However, as seen in Table H.9 (Appendix H), contrary to activities per ml, specific activities of EG (U/mg protein) did not changed significantly. The medium pH shifted from 5.0 for the first day, to 7.65 for the last day of incubation (Figure 3.27).

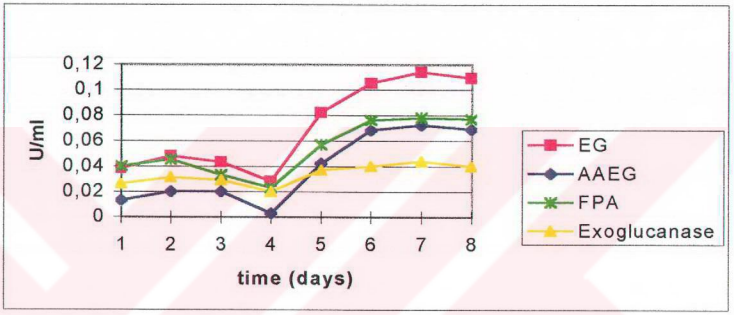

Fig. 3.25 Time course of cellulase production by *T. thermophila* on grape pomace.

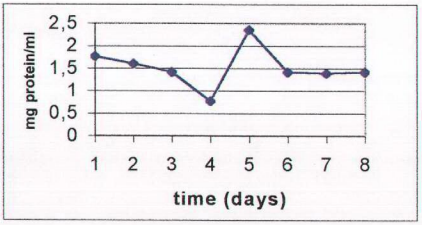

Fig. 3.26 Change in total exocell protein content (mg/ml) in the supernatant of *T. thermophila* cultivated on grape pomace.

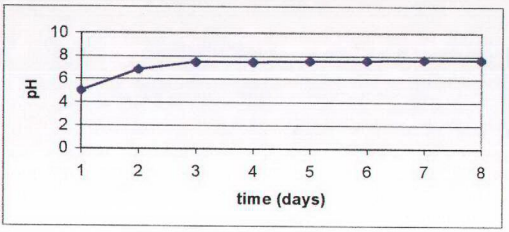

Fig. 3.27 Changes in medium pH during cultivation of *T. thermophila* on grape pomace.

Grape pomace is rich in polyphenols and dietary fiber. The grape industry provides different processed products such as wines juices, jams, jellies, raisins, canned grapes, and by-products including grape skins and seeds, which constitute grape pomace. Grapes are usually crushed to obtain the juice, leaving fresh pomace as residue. Grape pomace constitutes a by-product of great interest to the feed industry since the available carbohydrate fraction can be used to provide syrups and citric acid (Valiente *et al.*, 1995).

Valiente and coworkers (1995) studied the composition of grape pomace and according to this study, cellulose, lignin, neutral sugars, uronic acids, protein and ash were determined as the constituents of grape pomace. The profile of neutral sugars revealed glucose as the major sugar while other neutral sugars found in lower concentrations were arabinose, xylose, galactose and mannose, which are present in similar proportions. Uronic acid content was low, which indicates that small amounts of pectic substances were present in grape pomace, cellulose and hemicellulose being predominant polysaccharides. With respect to amino acids, glutamic acid + glutamine were the most abundant amino acids while cysteine and methionine were lowest. In our study, grape pomace presented 10.8% cellulose in dry material. As

seen in Figure 3.25, during the first 4th day, cellulase activities were less than those of the remaining 4 days. During the first 4 days, the organism may have used glucose or other easily metabolizable carbohydrates as carbon source and cellulase synthesis may have been repressed. The biosynthesis and secretion of cellulases by *T. reesei* are known to be adaptive, ie., inducible by the presence of cellulose, but repressed by easily metabolizable carbon sources, eg., glucose or glycerol. The low amount of cellulases may be due to conidial cellulase system. When easily metabolizable carbohydrates are exhausted, *T. thermophile* may have started to synthesize and secrete cellulases (Chahal *et al.*, 1992).

Since cellulose is normally composed of an amorphous portion (easily accessible to enzymatic attack) and crystalline portion (not accessible to enzymatic attack), it can be assumed that during the second phase (exponential phase) the amorphous portion of the cellulose was consumed, however in the fourth phase (second exponential phase), the crystalline portion of the cellulose was consumed.

In the first declining phase, the organism may have required some time for adaptation to utilize crystalline cellulose and hence, there was no cellulase synthesis during this phase (the first declining phase). In addition, since the cellulase production was slow or due to some proteases destroying the accumulated cellulases, a declining phase was observed.

The EG, AAEG, FPA and EXG production by *Torula thermophila* with sugar beet bagasse as carbon source are given in Figure 3.28. EG, AAEG, FPA and EXG yielded their maximum values at the 2nd day and 0.100 U/ml, 0.046 U/ml, 0.112 U/ml, 0.120 U/ml enzyme activities were obtained for EG, AAEG, FPA and EXG, respectively. In this study, sugar beet bagasse was found to represent 15.2 % cellulose in dry material.

As illustrated in Figure 3.28, all the cellulase activities decreased steadily after 2nd day until 8th day. Total exocell protein (Figure 3.29) has increased slightly

until 6th day and then decreased slightly. Medium pH (Figure 3.30) has started to rise after 2nd day and reached a pH value of 9.0 at the end of incubation.

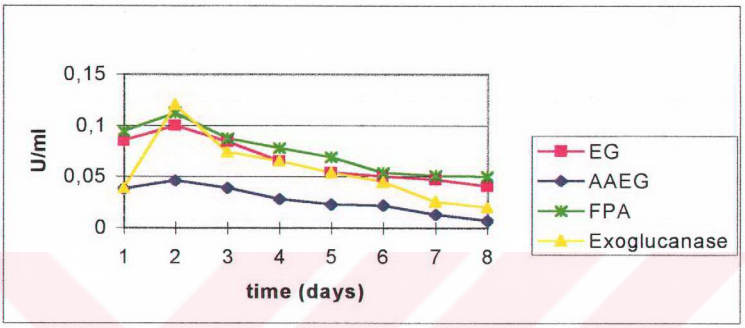

Fig. 3.28 Time course of cellulase production by *T. thermophila* on sugar beet bagasse.

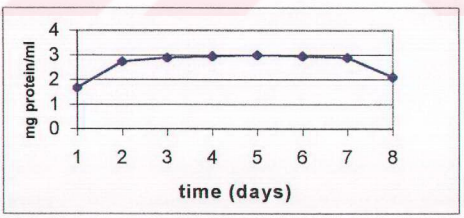

Fig. 3.29 Change in total exocell protein content (mg/ml) in the supernatant of *T. thermophila* cultivated on sugar beet bagasse.

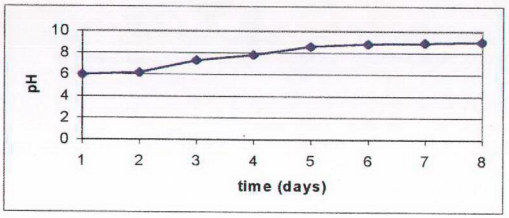

Fig. 3.30 Changes in medium pH during cultivation of *T. thermophila* on sugar beet bagasse.

As mentioned before, the medium pH is an important factor influencing microbial growth and cellulase production. Different organisms have different pH optimum for optimal growth and cellulase production. The optimum pH is also dependent upon substrate. Extracellular proteinases also may affect cellulolytic enzyme activities. Extracellular proteinases are produced by many species of fungi. Proteinases are divided into four groups according to their catalytic mechanisms. This division is based on reactivity toward inhibitors that react with specific moieties in the active site region. The four types of proteinases are serine proteases (maximal activity between pH 7.0 and 11.0), cysteine proteinases (maximal activity between pH 5.0 and 8.0), aspartic proteinases (maximal activity between pH 3.0 and 4.0), and metalloproteinases (maximal activity between pH 5.0 and 9.0).

Pitson and Seviour (1996) showed the proteolytic inactivation of an extracellular (1 → 3) β-glucanase from the fungus *Acremonium persicinum*.

The filamentous fungus *Acremonium persicinum* released high levels of proteolytic enzyme activity into the culture fluid during growth at pH 7 or above. Total inhibition of crude activity by phenylmethyl - sulfonyl fluoride suggested that it was mainly due to the presence of a serine protease. This protease inactivated one

of three extracellular (1 → 3)- β-glucanases produced by this fungus, although the activities of the remaining two (1 → 3)- β-glucanases did not appear to be affected (Pitson and Seviour, 1996).

Growth of *A. persicinum* in acidic conditions resulted in the presence of much lower extracellular proteolytic activity and no (1 → 3)- β-glucanase inactivation.

Sugar beet bagasse constitutes the remaining parts of sugar beet used in sugar production and on some regions of the world sugar-cane is also used in sugar production. It has been reported that in Peru 1,8 million tonnes of sugar-cane bagasse are produced annually, and this abundant but low-value resource contains about 75% hydrolysable cellulose and hemicellulose (Gutierrez–Correa *et al.*, 1995).

Gutierrez – Correa and his coworkers (1995) studied ammonia-treated sugar-cane bagasse by solid-substrate fermentation (SSF) with *T. reesei* LM-UC_4 and *Aspergillus phoenicis* QM329 mixed-culture. Mixed-culture SSF yielded significantly higher activities of all the enzymes of the cellulase complex than in single culture *T. reesei* SSF.

The EG, AAEG, FPA and EXG production by *Torula thermophila* with apple pomace as the substrate are given in figure 3.31.

All the cellulase components reached their maximum values on the 2nd day of incubation. After the 2nd day, EG, AAEG, FPA and EXG activities started to decrease. On the other hand, amount of total exocell protein increased until 4th day, and remained approximately constant after a slight decrease on the 5th day of incubation (Figure 3.32). Considering these and the previous results, there does not seem to be a strict correlation between the amount of total exocell protein (mg/ml) and the cellulase activities (U/ml). There are several possibilities, for example, cellulase activity may be lost due to some slight proteolytic cleavage, which would not alter the protein content. In general, however, the lack of a correlation between enzyme activity and the total protein content in the supernatant, can be explained by

the presence of other enzymes and proteins secreted by the cells, or released due to cell lysis. The medium pH changed from 6.05 for the first day, to 8.50 for the 8th day of incubation period (Figure 3.33).

Apple pomace is the residue that remains following extraction of juice from apple. It consists of insoluble carbohydrates with lesser amounts of protein, minerals and some remaining juice with sugars and other soluble substances. Several uses have been reported for pomace: fuels, animal feeds, citric acid and pectin. In our study apple pomace was selected for use in cellulase production and yielded higher cellulolytic activity than grass clippings (section 3.3) in terms of FPA and EXG activity. Apple pomace yielded 0.385 U/ml FPA and 0.322 U/ml EXG activity whereas grass clippings yielded 0.294 U/ml FPA and 0.213 U/ml EXG activity (Figure 3.50 and 3.52). Conversely, grass clippings produced 0.429 U/ml endoglucanase and 0.327 U/ml AAEG activity, compared to 0.340 U/ml and 0.216 U/ml for the endoglucanase and AAEG activities with apple pomace (Figure 3.46 and 3.48).

As seen in Figure 3.31, FPA is higher than endoglucanase and the amount of endoglucanase activity and FPA varies in different substrates. It should be noted that the overall cellulolytic activity of culture filtrate is not reflected by its FPA. It is well known that the level of β-glucosidase in an enzyme preparation may affect the result of cellulase assays, in particular the assay of FPA. β-glucosidase has no action on cellulosic substrates, such as Avicel and CMC, but enzyme attacks cellobiose and β-glucoside (Hayashida *et al.*, 1988). The assays of EG, AAEG, FPA and EXG are all based on the some principle of estimating a fixed amount of glucose from the relevant substrate.

There are many cellulases known that can hydrolyse the amorphous cellulose but are unable to hydrolyze the crystalline component. These cellulases are characterized by a fast initial rate of reaction on filter paper and then cessation of the release of soluble sugars. Because the FPA assay is non-linear, extrapolation of FPA which is based on initial rate of reaction, would be very misleading.

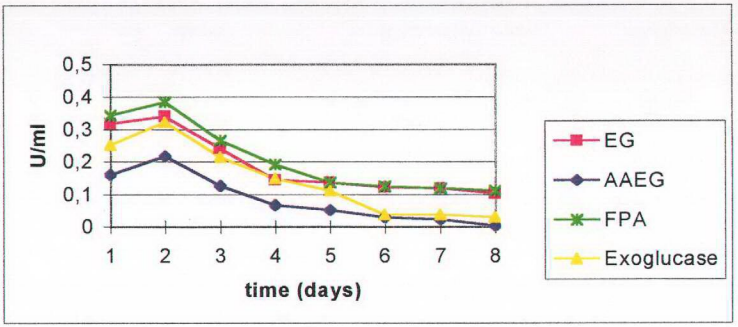

Fig. 3.31 Time course of cellulase production by *T. thermophila* on apple pomace.

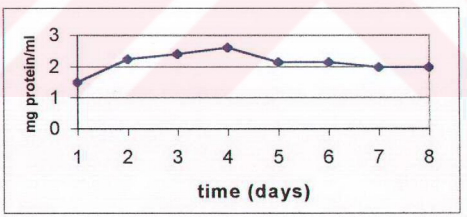

Fig. 3.32 Change in total exocell protein content (mg/ml) in the supernatant of *T. thermophila* cultivated on apple pomace.

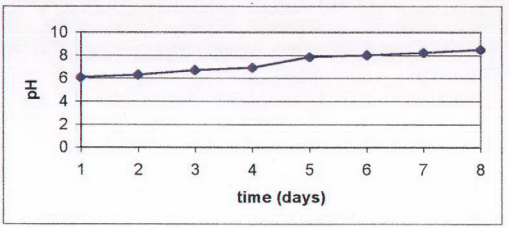

Fig. 3.33 Changes in medium pH during cultivation of *T. thermophila* on apple pomace.

As can be seen in Figure 3.31, unlike other substances EXG activity is also very high. A reason for this high amount of EXG may be that the type of cellulase system required for hydrolysis is dependent on the nature of the substrate which is used to evaluate its hydrolytic potential. Moreover, an approach to high FPA may be the synergism between endoglucanase and EXG in the attack of crystalline cellulose.

Figure 3.34 illustrates the time course of EG, AAEG, FPA and EXG activities by *T. thermophila* on tomato pomace as carbon source. EG, AAEG, FPA and EXG enzymes showed their maximum cellulolytic activities at the fourth day with the values of 0.096 U/ml, 0.060 U/ml, 0.100 U/ml and 0.071 U/ml, respectively. After the fourth day cellulolytic activities decreased until the end of incubation. Extracellular total protein released into culture fluid increased slightly and reached its maximum value at the sixth day and then decreased (Figure 3.35). The specific activities of cellulases (U/mg protein) on first day had the highest values and decreased along with the increase in cellulolytic activities in terms of U/ml gradually up to the last day of incubation (Table H.12, Appendix H). The medium pH was 6.60 on the first day and increased to 9.05 on the fourth day, remaining more or less constant until the end of the fermentation (Figure 3.36).

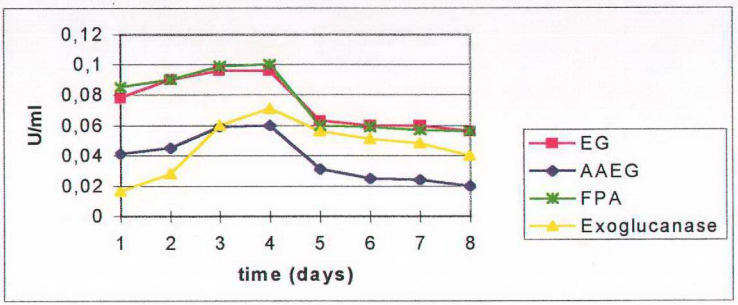

Fig. 3.34 Time course of cellulase production by *T. thermophila* on tomato pomace.

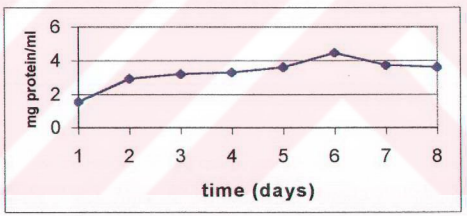

Fig. 3.35 Change in total exocell protein content (mg/ml) in the supernatant of *T. thermophila* cultivated on tomato pomace.

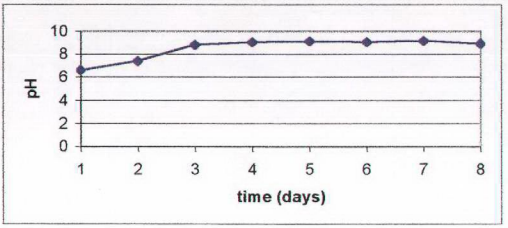

Fig. 3.36 Changes in medium pH during cultivation of *T. thermophila* on tomato pomace.

Tomato pomace used in our study represented 12% cellulose. Although some lignocellulosic wastes having a similar or lower cellulose content gave higher cellulolytic activities, *T. thermophila*, with tomato pomace as substrate yielded lower cellulolytic activities when compared to other substrates. To examplify, apple pomace has a cellulose content of 12.4 %, which is similar to that of tomato pomace (12.8 %), however, apple pomace yielded higher cellulolytic activities.

The different level of cellulolytic activity on both substrate may be due to proteolytic enzymes. *Torula thermophila* may synthesize more protease when cultivated on tomato pomace because of the higher protein content of tomato pomace. Tomato pomace is reported to contain about 10 – 20 % protein whereas apple pomace about 1 – 5 % (K. Yarangümeli, Personal Communication, 1999). The high protein content of tomato pomace may thus be responsible for more protease production in the case of tomato pomace. The cellulases produced on apple pomace had a higher specific activity than those of tomato pomace, possibly indicating higher extracellular protein production on tomato pomace (Table H.11 and H.12, respectively, Appendix H). Therefore, a large portion of extracellular protein may be proteases in the case of tomato pomace. It is also likely that the proteins in tomato

pomace are converted to soluble peptides by slight proteolytic cleavage, which would also yield an increase in the level of total exocell protein.

Protease production is governed by derepression in the absence of sulfur, nitrogen or carbon and substrate induction. On the other hand, studies on phosphatases in *Neurospora crasse* showed that pH could also be an important factor for enzyme production and secretion (Nahas *et al.*, 1982). With respect to proteases it was shown that limited hydrolysis of CBH I (65 kD) from *T. reesei* by papain yielded a core protein which was fully active against small, soluble substrates such as the chromophoric glycosides derived from the cellodextrins and lactose. Activity against an insoluble substrate, such as Avicel was however completely lost and decreased adsorption onto this microcrystalline cellulose was observed (Van Tilbeurgh *et al.*, 1986). Another possibility of lower cellulolytic activities may be the adsorption phenomena. Lignin and hemicellulose, if there are, can prevent cellulases from attacking on cellulose.

Carvalheiro and his coworkers (1994) used tomato pomace for the production of cellulases. The hydrolysis of tomato pomace by *T. reesei* and *Sporotrichum* sp. pure solid state cultures resulted in 36 % and 67 % cellulose conversion and 73 % and 41.5 % hemicellulose degradation, respectively. In consortium these organisms revealed 56.5 % cellulose and 36.2 % hemicellulose conversion yields. The *T. reesei* culture displayed a four-fold specific enzyme activity as compared to the *Sporotrichum* sp. and the mixed culture.

Figure 3.37 illustrates the production of EG, AAEG, FPA and EXG on wheat straw as carbon source. EG, AAEG, FPA and EXG yielded their maximum values on the 2nd day and 0.031 U/ml, 0.015 U/ml, 0.037 U/ml, 0.031 U/ml enzyme activities has been reached for EG, AAEG, FPA and EXG, respectively. Total exocell protein increased from the first day to second day. After the second day, it started to decrease until the 4th day and remained constant between 4th and 5th days. After the 5th day, it started to increase and remain more or less constant (Figure 3.38). The medium pH has changed from 7.85 on the first day to 9.20 at the end of incubation period (Figure

3.39). With wheat straw as a carbon source, the pH value of the medium strongly influences the production of cellulase and xylanase as well as that of β-glucosidase and the relative amounts of these enzymes (Esterbauer *et al.*, 1983). Since it is difficult to regulate pH in shaken flask cultures, it is necessary to employ a nutrient medium with high buffer capacity.

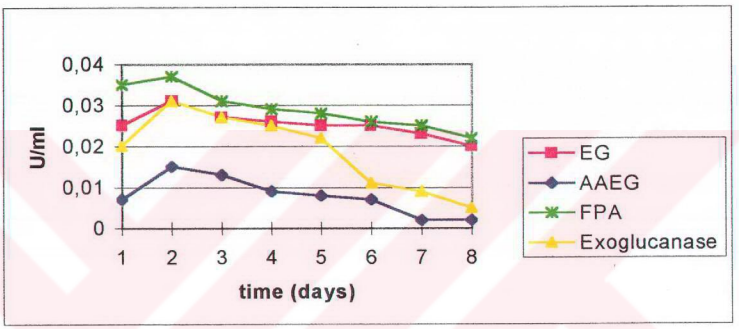

Fig. 3.37 Time course of cellulase production by *T. thermophila* on wheat straw.

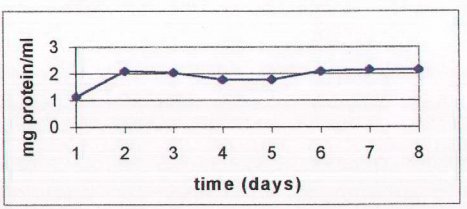

Fig. 3.38 Change in total excocellular protein content (mg/ml) in the supernatant of *Torula thermophila* cultivated on wheat staw.

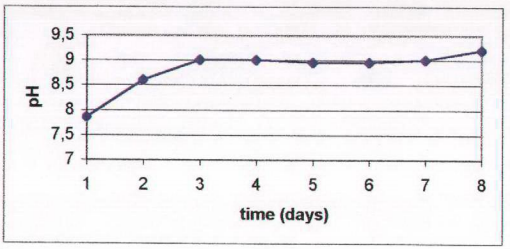

Fig. 3.39 Changes in medium pH during cultivation of *T. thermophila* on wheat straw.

Doppelbauer and Esterbauer (1987) examined the composition of untreated and treated wheat straw. They found that the percentage of the total polymeric carbohydrate content of substrates investigated varied from 44% to 93%. In addition the distribution of glucan, mannan, xylan, arabinan and lignin was remarkable. They found that steam treated wheat straw gave 1.9 FPU/ml, the spent fibres remaining after enzymatic hydrolysis of steamed wheat straw gave 2.4 FPU/ml. Untreated wheat straw gave only low final enzyme titers.

It seems reasonable to assume that steaming leads to an alteration of the physical nature of lignin. Steam explosion of poplar wood has been reported to transform the lignin into smaller droplets. In addition to physical alteration of lignin, steaming may also have effects on the lignocellulosic materials. Such effects could be the increase of surface and pore sizes, partial decrystallinization of cellulose, removal of acetyl groups from hemicellulose.

In the study of Doppelbauer and Esterbauer (1987) with steam pretreated wheat straw, the best productivities were obtained when pH value was maintained at 6.0 throughout the fermentation. The reason why higher pH values are in general

better when lignocellulosic substrates are used may be due to adsorption phenomena caused by lignin. In the case of wheat straw fermentations, it was observed, for example, that a fraction of the lignin became soluble when the pH value was increased above 5.5 at the end of the fermentation. Such a pH increase also led to a rapid increase of the enzyme activity in culture broth.

The time course of EG, AAEG, FPA and EXG production of *Torula thermophila* on grass clippings as substrate are given in Figure 3.40.

All the cellulolytic activities reached their maximum values on the fourth day of incubation. The maximum EG, AAEG, FPA and EXG activities obtained were 0.429 U/ml, 0.327 U/ml, 0.294 U/ml and 0.213 U/ml, respectively. After the fourth day, all the cellulase activities decreased gradually. The total exocell protein reached its maximum value on the fifth day (Figure 3.41), and the maximum specific activities (U/ml protein) of EG, AAEG, FPA and EXG were obtained also on the fourth day of cultivation (Table H.14, Appendix H).

The medium pH showed a similar trend as those of the other complex lignocellulosic compounds, with pH starting from 6.44 and reaching 6.75 at fermentation end (Figure 3.42).

Grass clippings used in this study contained 16.32 % cellulose and yielded the highest cellulolytic activities when compared to other lignocellulosic compounds. Latif (1995) studied seven thermophilic fungi for the production of cellulases when grown on Leptachloa (kallar grass) straw. These thermophilic fungi were as follow: *Aspergillus fumigatus, Sporotrichum thermophile, Chactemium thermophile, Humicola grisea, Torula thermophila, Malbranchea pulchella, Mucor pusillus. Aspergillus fumigatus* and *C. thermophile* gave similar activities of CMCase (2.5 U/ml), and xylanase (0.40 U/ml and 0.32 U/ml, respectively). *Aspergillus fumigatus* also produced the highest FPase and β-xylosidase activities. *Sporotrichum thermophile* produced the highest activity of β-glucosidase after 5 days. *Humicola grisea* produced 0.28 U/ml FPase and 1.6 U/ml CMCase. *Torula thermophila* gave

0.28 U/ml FPase and 1.4 U/ml CMCase activity. *M. pulchella* and *M. pucillus* exhibited low activities.

In previous studies, in optimizing cultivation temperature, avicel was used as a substrate (Arifoğlu, 1999) In this study, Avicel was used for the aim of comparison with complex carbon sources. Avicel is essentially an aggregate of microcrystals. It is prepared from wood α-cellulose by an acid treatment to remove the amorphous component and a blending treatment which fragments the crystallites into colloidal particles and causes them to coalesce (Wood *et al.*, 1988).

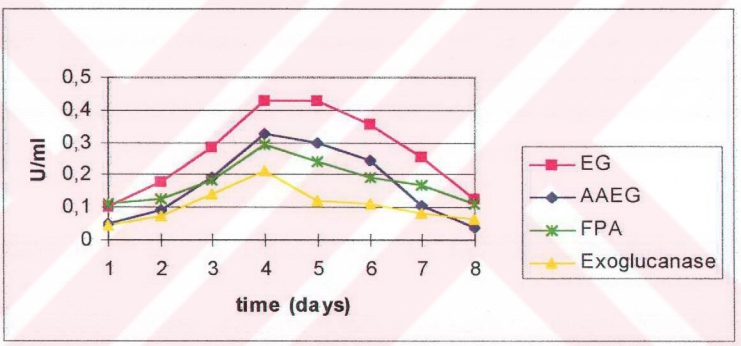

Fig. 3.40 Time course of cellulase production by T. thermophila on grass clippings.

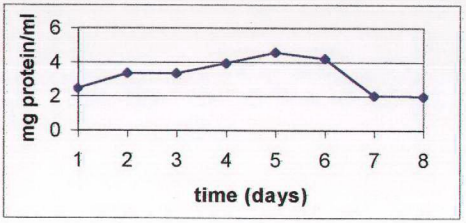

Fig. 3.41 Change in total exocell protein content (mg/ml) in the supernatant of *T. thermophila* cultivated on grass clippings.

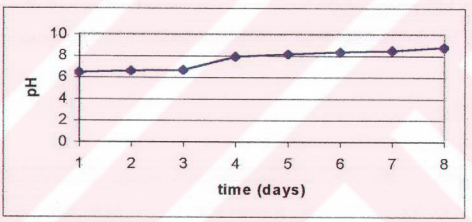

Fig. 3.42 Changes in medium pH during cultivation of *T. thermophila* on grass clippings.

The time course of EG, AAEG, FPA and EXG activities produced by *T. thermophila* on Avicel as substrate is given in Figure 3.43. The maximum cellulolytic activities were obtained on the seventh day of incubation and were 0.681 U/ml, 0.387 U/ml, 0.207 U/ml, 0.296 U/ml for EG, AAEG, FPA, EXG, respectively.

The total exocell protein increased until the 8th day (Figure 3.44). Medium pH showed a different trend as compared to complex lignocellulosic compounds (Figure

3.45). Although an increase in pH was observed until the 3rd day, pH decreased afterwards and reached (7.40) more or less the initial value observed on the first day (7.75).

As mentioned previously, the pH of a culture may change in response to microbial metabolic activities due to some reasons. Organic acids, such as lactic acid and acetic acid cause the pH to decrease. However, the assimilation of organic acids which may be present in media leads to an increase in pH. Moreover, microbial utilization of the nitrogen source can cause the pH to change (Wang *et al.*, 1979). The pH tends to increase if organic amino compounds are deaminated when used for growth. The utilization of ammonium nitrate can initially cause an acid drift during assimilation of the ammonium when nitrate assimilation is repressed, with a subsequent alkaline drift when the nitrate is used as an alternative nitrogen source after the ammonium has been exhausted (Morton and MacMillan, 1954). The substrate itself can also cause a change in pH. Mudget *et al.* (1980) reported that during growth of *A. oryzae* on rice, the pH initially decreased from 6.6 to pH 5.2 but subsequently increased to pH 6.3. The latter increase was believed to be due to deamination of the rice protein.

As can be seen in Figure 3.43 there is a lag phase of 6 days for achieving high yields of cellulolytic activities. This might have been caused by the crystalline structure of Avicel, which is made of about 95 % crystalline cellulose. Crystallinity of cellulose was shown to be the main parameter that determines its biodegradability. The crystallinity index and the specific surface area also effects the amount of adsorbed cellulase per unit mass of cellulose (Beldman *et al.*, 1987).

The long lag phase for high enzymatic activities therefore possibly depend on the crystallinity of Avicel. Avicel-absorbable endoglucanase (AAEG) is observed via scanning electron microscopy to disintegrate Avicel fibrils layer by layer from the surface yielding thin sections with exposed chains; endoglucanase only attacks the surface of Avicel (Hayashida *et al.*, 1986).

In this study Avicel in the modified YpSs medium was that used for column chromatography. Avicel was selected as standard substrate to evaluate other lignocellulosic wastes for the production of cellulases only. Avicel can not be taken as substrate for large-scale production of cellulases since it is not an economical source.

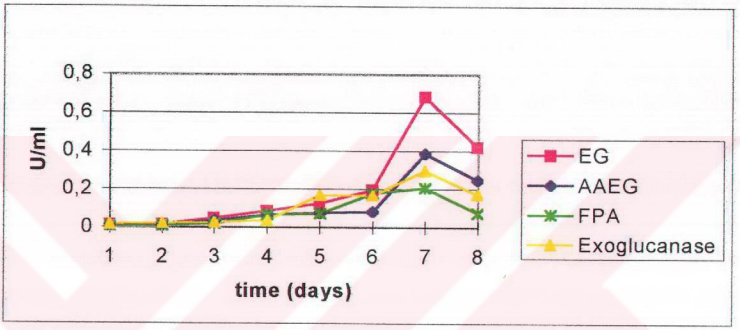

Fig. 3.43 Time course of cellulase production by *T. thermophila* on Avicel.

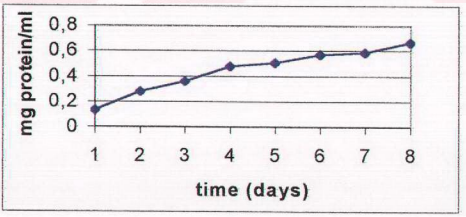

Fig. 3.44 Change in total exocell protein content (mg/ml) in the supernatant of *T. thermophila* cultivated on Avicel.

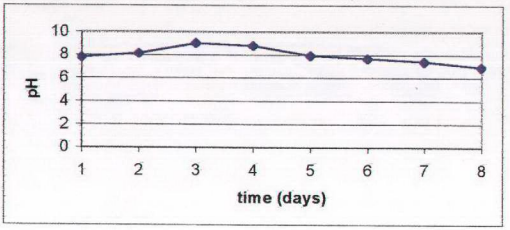

Fig. 3.45 Changes in medium pH during cultivation of *T. thermophila* on Avicel.

3.3 Comparison of Lignocellulosic Compounds as a Carbon Source for Cellulase Secretion

3.3.1 Endoglucanase Production

As illustrated in Figure 3.46, grass clippings yielded maximum EG activity (U/ml) among all the lignocellulosic compounds. Soybean meal followed grass clippings in terms of EG activity and next apple pomace gave the highest activity. In this study, Avicel was used for the aim of comparison with complex carbon sources and Avicel gave higher EG activity than grass clippings did.

Wheat Straw	A	Barley Grain	I
Cotton Seed Bagasse	B	Sunflower Seed Bagasse	J
Tomato Pomace	C	Lentil Bran	K
Wheat Fracture	D	Apple Pomace	L
Sugar Beet Bagasse	E	Soybean Meal	M
Grape Pomace	F	Grass Clippings	N
Wheat Bran	G	Avicel (Control)	O
Razmol	H		

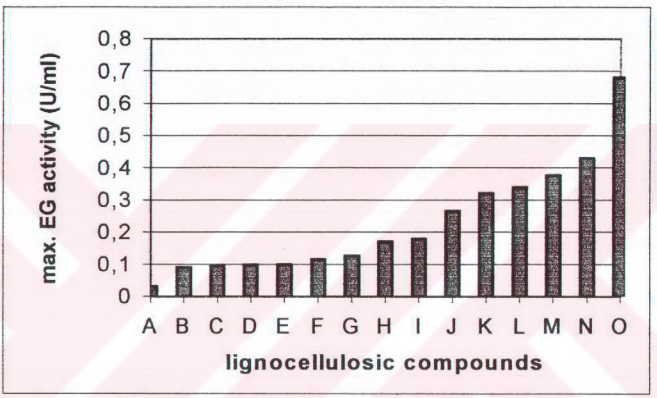

Fig. 3.46 Maximum EG activities in terms of U/ml with respect to lignocellulosic compounds.

In Figure 3.47 lignocellulosic compounds were compared to each other in terms of specific activities (U/mg protein). As can be seen, razmol gave the highest specific activity among lignocellulosic compounds and wheat bran is the second compound in this manner. Barley grain and apple pomace are the other compounds which gave high specific activities (U/mg protein). Although grass clippings yielded the highest EG activity in terms of U/ml, its specific activity rather low when compared to razmol and wheat Bran. Avicel as a control gave much more higher activity than lignocellulosic compounds.

Wheat Straw	A	Grass Clippings	I
Cotton Seed Bagasse	B	Lentil Bran	J
Tomato Pomace	C	Apple Pomace	K
Sugar Beet Bagasse	D	Barley Grain	L
Wheat Fracture	E	Wheat Bran	M
Soybean Meal	F	Razmol	N
Grape Pomace	G	Avicel (Control)	O
Sunflower Seed Bagasse	H		

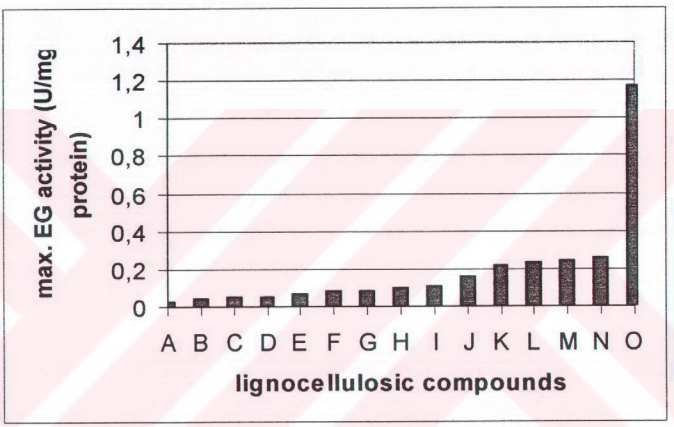

Fig. 3.47 Maximum EG activities in terms of U/mg protein with respect to lignocellulosic compounds.

3.3.2 Avicel-Adsorbable Endoglucanase (AAEG) Production

Figure 3.48 shows the maximum AAEG activities (U/ml). As shown, grass clippings gave the highest AAEG activity (U/ml). Soybean meal followed grass clippings, the activities are nearly similar. Lentil bran and apple pomace are other substrates which yielded higher activities (U/ml).

Wheat Straw	A	Razmol	I
Sugar Beet Bagasse	B	Sunflower Seed Bagasse	J
Tomato Pomace	C	Apple Pomace	K
Cotton Seed Bagasse	D	Lentil Bran	L
Wheat Fracture	E	Soybean Meal	M
Grape Pomace	F	Grass Clippings	N
Wheat Bran	G	Avicel (Control)	O
Barley Grain	H		

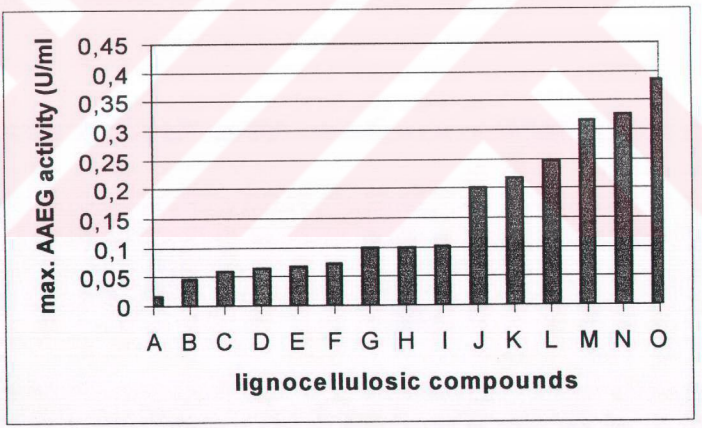

Fig. 3.48 Maximum AAEG activities in terms of U/ml with respect to lignocellulosic compound.

Figure 3.49 shows the maximum specific AAEG activities (U/mg protein). Wheat bran shows the highest values among lignocellulosic compounds and is followed by razmol, their activities are nearly similar. Grass clippings gave rather low specific AAEG activity (U/mg protein) when compared to razmol and wheat bran. Avicel gave higher activities than lignocellulosic compounds.

Wheat Straw	A	Grass Clippings	I
Sugar Beet Bagasse	B	Apple Pomace	J
Tomato Pomace	C	Lentil Bran	K
Cotton Seed Bagasse	D	Barley Grain	L
Wheat Fracture	E	Razmol	M
Grape Pomace	F	Wheat Bran	N
Soybean Meal	G	Avicel (Control)	O
Sunflower Seed Bagasse	H		

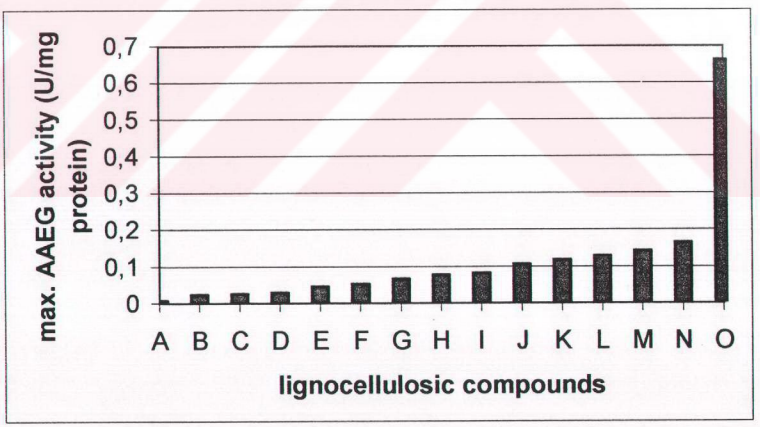

Fig. 3.49 Maximum AAEG activities in terms of U/mg protein with respect to lignocelluloic compounds.

3.3.3 Filter Paper Activity (FPA) Production

Figure 3.50 shows the maximum FPA (U/ml). As illustrated, soybean meal and apple pomace gave the highest filter paper activities, which are being very similar, 0.385 U/ml and 0.382 U/ml, respectively. Grass clippings followed apple pomace and soybean meal in terms of filter paper activities (U/ml), followed by lentil bran. In contrast to EG and AAEG activities (U/ml), Avicel yielded low filter paper activity (0.207 U/ml).

Wheat Straw	A	Razmol	I
Wheat Fracture	B	Sunflower Seed Bagasse	J
Cotton Seed Bagasse	C	Avicel (Control)	K
Grape Pomace	D	Lentil Bran	L
Tomato Pomace	E	Grass Clippings	M
Sugar Beet Bagasse	F	Soybean Meal	N
Wheat Bran	G	Apple Pomace	O
Barley Grain	H		

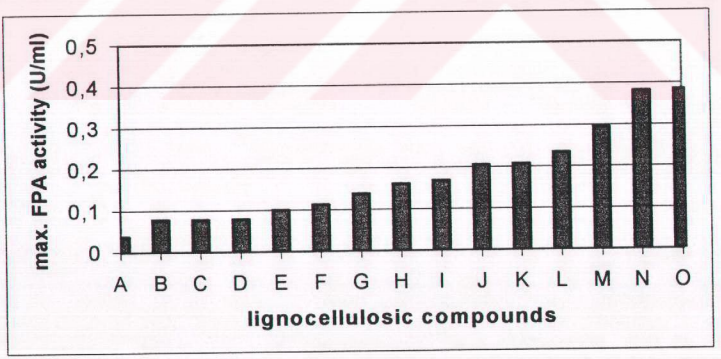

Fig. 3.50 Maximum FPA in terms of U/ml with respect to lignocellulosic compounds.

As for maximum enzyme activities as U/mg protein, razmol and apple pomace gave the highest activities among the lignocellulosic compounds. Avicel (control) gave much higher activity than lignocellulosic compounds (Figure 3.51).

Wheat Straw	A	Grass Clippings	I
Cotton Seed Bagasse	B	Lentil Bran	J
Wheat Fracture	C	Wheat Bran	K
Tomato Pomace	D	Barley Grain	L
Grape Pomace	E	Apple Pomace	M
Sugar Beet Bagasse	F	Razmol	N
Sunflower Seed Bagasse	G	Avicel (Control)	O
Soybean Meal	H		

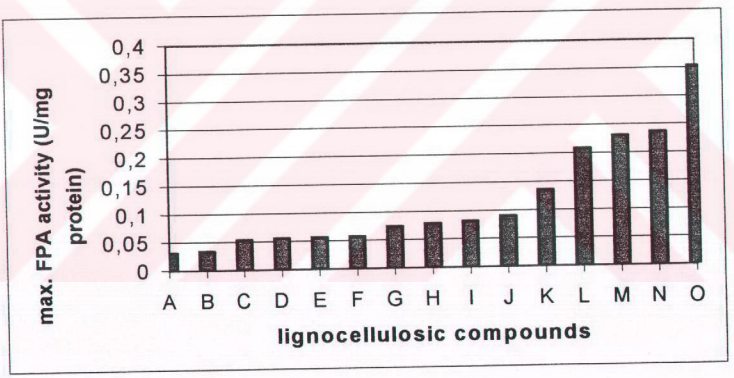

Fig. 3.51 Maximum FPA in terms of U/mg protein with respect to lignocellulosic compounds.

3.3.4 Exoglucanase (EXG) Production

As illustrated in Figure 3.52, apple pomace gave the highest EXG activity (U/ml) and was followed by grass clippings. The maximum EXG activity (U/ml) of avicel (control) was lower than that of apple pomace but higher than that of grass clippings.

Wheat Straw	A	Razmol	I
Grape Pomace	B	Barley Grain	J
Lentil Bran	C	Sugar Beet Bagasse	K
Cotton Seed Bagasse	D	Soybean Meal	L
Wheat Fracture	E	Grass Clippings	M
Tomato Pomace	F	Avicel (Control)	N
Wheat Bran	G	Apple Pomace	O
Sunflower Seed Bagasse	H		

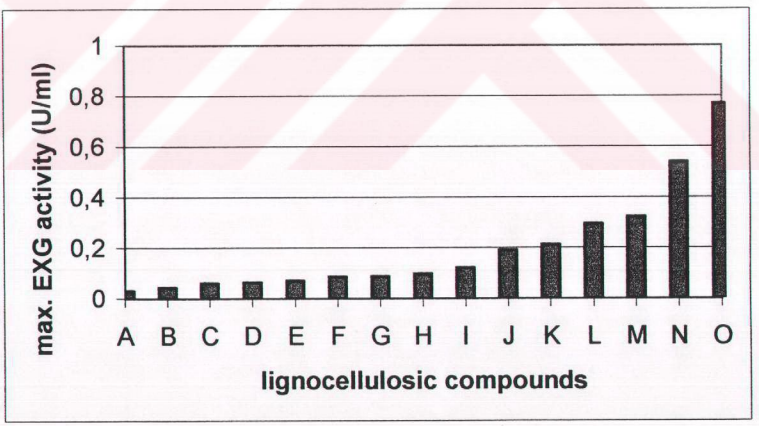

Fig. 3.52 Maximum EXG activities in terms of U/ml with respect to lignocellulosic compounds.

As for the maximum specific EXG activities (U/mg protein), apple pomace gave the highest activity among the lignocellulosic compounds. However, again Avicel gave higher specific activity when compared to lignocellulosic compounds (Figure 3.53).

Wheat Straw	A	Wheat Fracture	I
Tomato Pomace	B	Grass Clippings	J
Cotton Seed Bagasse	C	Wheat Bran	K
Lentil Bran	D	Barley Grain	L
Grape Pomace	E	Razmol	M
Sunflower Seed Bagasse	F	Apple Pomace	N
Soybean Meal	G	Avicel (Control)	O
Sugar Beet Bagasse	H		

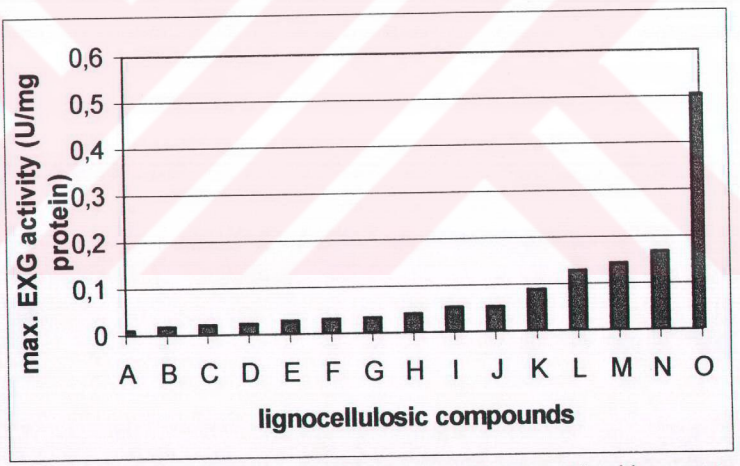

Fig. 3.53 Maximum EXG activities in terms of U/mg protein with respect to lignocellulosic compounds.

3.4 Influence of the Cellulose Content of Lignocellulosic Compounds on Cellulase Production by *T. thermophila*

Figure 3.54 shows the EG activities (U/ml) of lignocellulosic compounds with respect to their % cellulose content. As can be seen from Figure 3.54, although sunflower seed bagasse has a cellulose content (4.56 %), which is lower than that of razmol (8.46%), barley grain (9.20 %) and wheat fracture (9.36 %), *Torula thermophila* gave higher EG activity on sunflower seed bagasse than did on razmol (0.170 U/ml), barley grain (0.180 U/ml), and wheat fracture (0.099 U/ml).

Grape pomace has a cellulose content of 10.8 %, which is slightly higher than that of soybean meal (9.60 %). However, soybean meal yielded 0.376 U/ml EG activity and grape pomace yielded 0.114 U/ml EG activity. Similarly, the cellulose content of lentil bran is higher than that of soybean meal. However, the maximum EG produced on soybean meal is higher than that produced on lentil bran. As seen in Figure 3.54, sugar beet bagasse and grass clippings contain 15.2% and 16.32 % cellulose, respectively. Surprisingly, *T. thermophila* gave much more EG activity on grass clippings (0.429 U/ml) than did on sugar beet bagasse (0.100 U/ml). Also as illustrated in Figure 3.46, *T. thermophila* gave the highest EG activity on grass clippings when compared other lignocellulosic compounds. Cotton seed bagasse and wheat bran contain 19.95 % and 26.52 % cellulose, respectively, which values are higher according to obtained for other substrates. On the other hand, *T. thermophila* cultivated on both substrates showed less EG activity in comparison to other compounds. Wheat bran and cotton seed bagasse yielded 0.127 U/ml and 0.090 U/ml EG activity, respectively.

Finally, *T. thermophila* on wheat straw gave the lowest EG activity among all substrates (0.031 U/ml) when compared to other lignocellulosic compounds. As a conclusion, there is no direct relationship between cellulose content and cellulase production. On the other hand, it is worthy to note that lignocellulosic compounds

compounds have a complex structure, containing lignin and hemicellulose in addition to cellulose. Cellulose is embedded in a matrix composed of lignin and hemicellulose, and enzymatic attack of cellulase is prevented by lignin and hemicellulose. In order to increase the action of cellulases on cellulose, further pretreatment methods may be necessary.

Lignocellulosic compounds	% cellulose content	Lignocellulosic compounds	% cellulose content
wheat straw	3.00	Lentil bran	12.12
Sunflower seed bagasse	4.56	apple pomace	12.40
Razmol	8.46	Tomato pomace	12.80
Barley grain	9.20	sugar beet bagasse	15.20
Wheat fracture	9.36	grass clippings	16.32
Soybean meal	9.60	cotton seed bagasse	19.95
Grape pomace	10.38	wheat bran	26.52

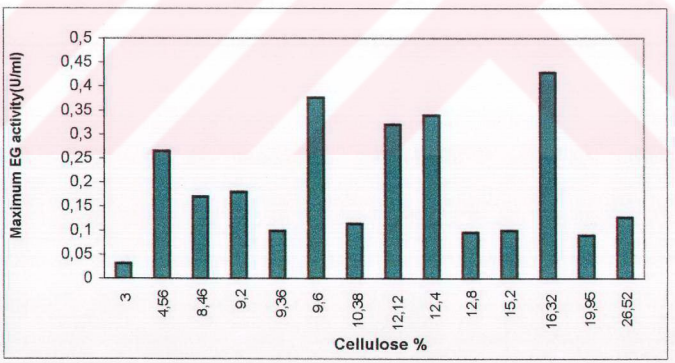

Fig. 3.54 Maximum EG activities (U/ml) of lignocellulosic compounds with respect to their % cellulose content.

3.5 Spore Induction Studies for *Torula Thermophila*

Spore induction experiments were carried out by inoculating the preculture medium with 1 mg, 3 mg, 5 mg, 10 mg and 20 mg spore concentrations. Grass clippings was used as carbon source since it gave maximum cellulolytic activities. Unlike other experiments, in spore induction experiments 1 liter working volume was used in 2 liter Erlenmeyer flasks.

When analysed seperately, each of the different inocula gave rather similar trends in terms of total exocell protein and pH, as well as the celullase activities which showed maxima mainly at days 5-6. The figure 3.55 illustrates the time course of cellulase production by *T. thermophila* with 1 mg spore in preculture medium. As can be seen in from Figure 3.55, maximum cellulolytic activities were obtained on the sixth day. Maximum yields of EG, AAEG, FPA and EXG were 0.358 U/ml, 0.285 U/ml, 0.231 U/ml, 0.108 U/ml, respectively. Total exocell protein in the supernatant has increased slightly until the 6^{th} day, with a slight decrease until the 8^{th} day of the incubation (Figure 3.56). Medium pH changed from 6.60 for the first day to 8.30 at the end of the incubation period (Figure 3.57).

The time course of cellulase production by *T. thermophila* with 3 mg spore in preculture medium gave maximum cellulolytic activities on the fifth day (Figure 3.58). Maximum yields of EG, AAEG, FPA and EXG were 0.527 U/ml, 0.388 U/ml, 0.334 U/ml, 0.120 U/ml, respectively. Total exocell protein has increased slightly until 6^{th} day, with a slight decrease during the first three days, and after the 6^{th} day remained more or less constant (Figure 3.59). The medium pH shifted from 6.55 for the first day to 8.20 at the end of the incubation period (Figure 3.60).

Figure 3.61 illustrates the time course of cellulase production by *T. thermophila* with 5 mg spore in preculture medium. The maximum yields of EG, AAEG, FPA and EXG activities were obtained on the 6^{th} day and 1.07 U/ml, 0.950 U/ml, 0.830 U/ml, 0.195 U/ml enzyme activities were measured, respectively. Total

exocell protein in the supernatant has increased slightly until the 6th day, with a slight decrease during the first three days, and after the 6th day remained more or less constant (Figure 3.62). The medium pH changed from 6.50 for the first day to 8.40 at the end of the incubation period (Figure 3.63).

Figure 3.64 illustrates the time course of cellulase production by *T. thermophila* with 10 mg spore in preculture medium. The maximum yields of EG, AAEG, FPA and EXG activities were obtained on the sixth day and 1.82 U/ml, 1.72 U/ml, 1.03 U/ml, 0.203 U/ml enzyme activities were measured, respectively. Total exocell protein has increased steadily until the 5th day, and decreased slightly until the 8th day (Figure 3.65). The medium pH changed from 6.25 for the first day to 8.60 at the end of the incubation period (Figure 3.66).

With 20 mg spores EG, AAEG, FPA and EXG yielded their maximum activities on the 6th day and 1.148 U/ml, 0.906 U/ml, 0.879 U/ml, 0.137 U/ml enzyme activities were obtained, respectively (Figure 3.67). Total exocell protein has increased until the end of incubation period (Figure 3.68). The medium pH has increased from 7.60 for the first day to 9.0 at the end of incubation period (Figure 3.69).

As can be seen from the Figure 3.70, higher levels of cellulase activities achieved within an increasing amount of spores inoculated into preculture medium. Higher cellulolytic activities with high spore concentrations must have been due to a high amount of germinated cells. However, with 20 mg spore inoculum, cellulolytic activities were lower than those of 10 mg spore inoculum. Organism could have produced more cells with 20 mg spore and this may have caused competition among the cells for the use of cellulose and other sources. In addition, organism may produce much more toxic metabolites which influences the enzyme production.

The increase in cellulolytic activities was reflected also by the increase in total exocell protein. The maximum total exocell protein was 3.63 mg/ml, 3.78 mg/ml, 3.87 mg/ml, 4.52 mg/ml for 1 mg, 3 mg, 5 mg and 10 mg spores, respectively. With 20 mg spore inoculation, total exocell protein was found to be 4.27 mg/ml.

A final observation can be made with respect to the production of AAEG, the organism produced much more AAEG on media inoculated with 5 mg and 10 mg spore concentrations with 89.7 % and 94.5 %, respectively (Table I.18 and 19, Appendix I).

Results indicate that the production of cellulases and especially AAEG was enhanced by an increase in the spore load of preculture media. Best results were obtained with 10 mg of spores, germinated in preculture media and used to inoculate 1 liter of fermentation medium. Since the amount of total exocell protein was not significantly altered by different spore inocula, an increase in specific activities was also observed (Table H.16-17-18-19, Appendix H).

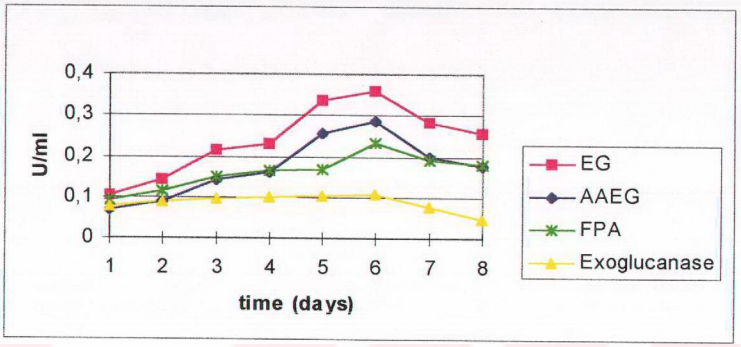

Fig. 3.55 Effect of spore induction on cellulase production with 1 mg spore inoculation in preculture medium by *T. thermophila* cultivating on grass clippings in 1 liter working volume.

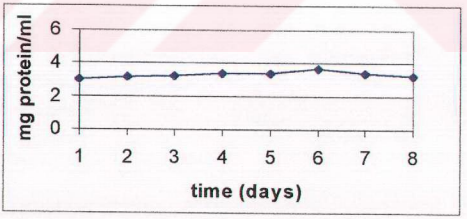

Fig. 3.56 Change in total exocell protein content (mg/ml) in the supernatant of *T. thermophila* cultivated on grass clippings with 1 mg spore inoculation.

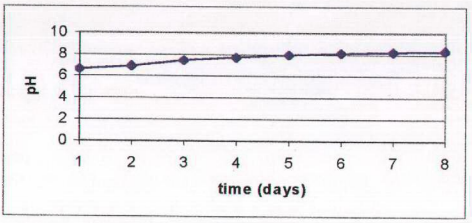

Fig. 3.57 Changes in medium pH during cultivation of *T. thermophila* on grass clippings with 1 mg spore inoculation..

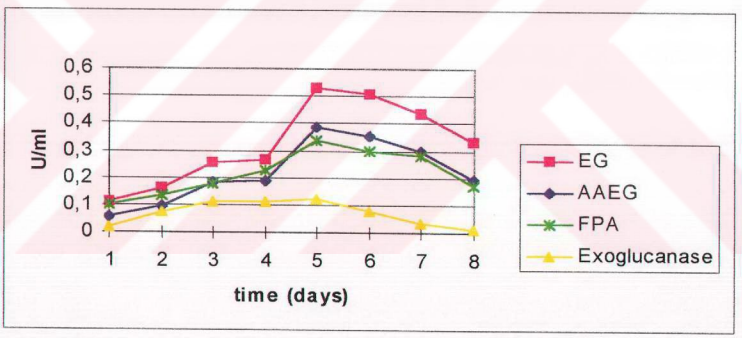

Fig. 3.58 Effect of spore induction on cellulase production with 3 mg spore inoculation in preculture medium by *T. thermophila* cultivating on grass clippings in 1 liter working volume.

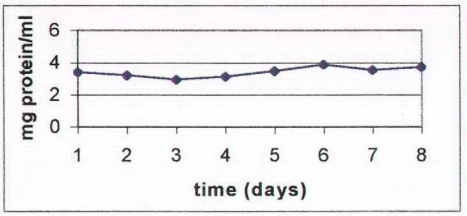

Fig. 3.59 Change in total exocell protein content (mg/ml) in the supernatant of *T. thermophila* cultivated on grass clippings with 3 mg spore inoculation.

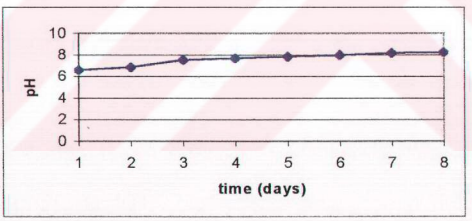

Fig. 3.60 Changes in medium pH during cultivation of *T. thermophila* on grass clippings with 3 mg spore inoculation.

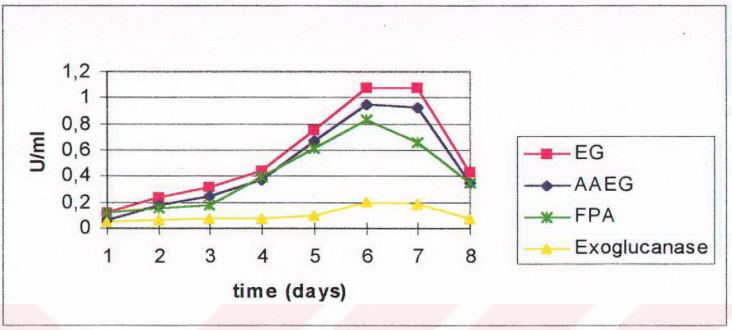

Fig. 3.61 Effect of spore induction on cellulase production with 5 mg spore inoculation in preculture medium by *T. thermophila* cultivating on grass clippings in 1 liter working volume.

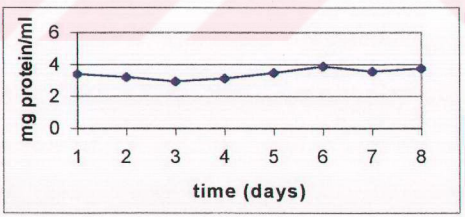

Fig. 3.62 Change in total exocell protein content (mg/ml) in the supernatant of *T. thermophila* cultivated on grass clippings with 5 mg spore inoculation.

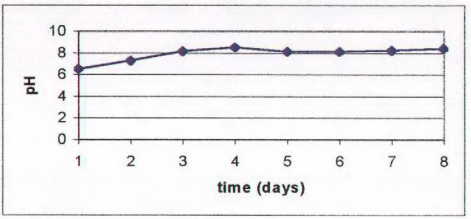

Fig. 3.63 Changes in medium pH during cultivation of *T. thermophila* on grass clippings with 5 mg spore inoculation.

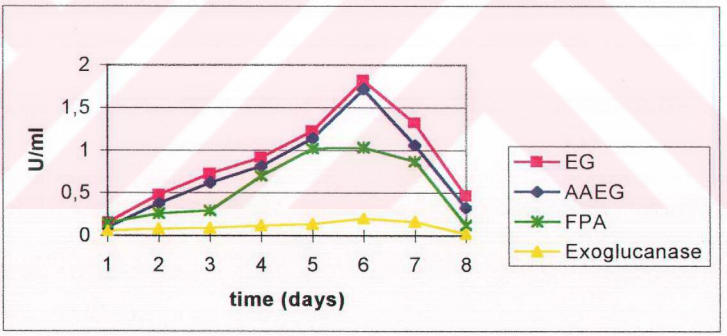

Fig. 3.64 Effect of spore induction on cellulase production with 10 mg spore inoculation in preculture medium by *T. thermophila* cultivating on grass clippings in 1 liter working volume.

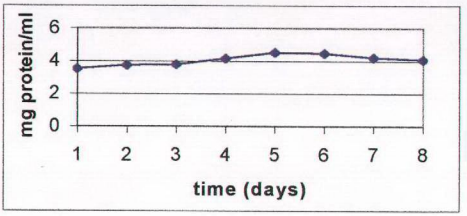

Fig. 3.65 Change in total exocell protein content (mg/ml) in the supernatant of *T. thermophila* cultivated on grass clippings with 10 mg spore inoculation.

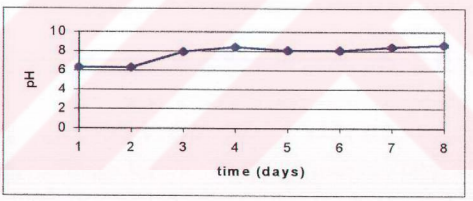

Fig. 3.66 Changes in medium pH during cultivation of *T. thermophila* on grass clippings with 10 mg spore inoculation.

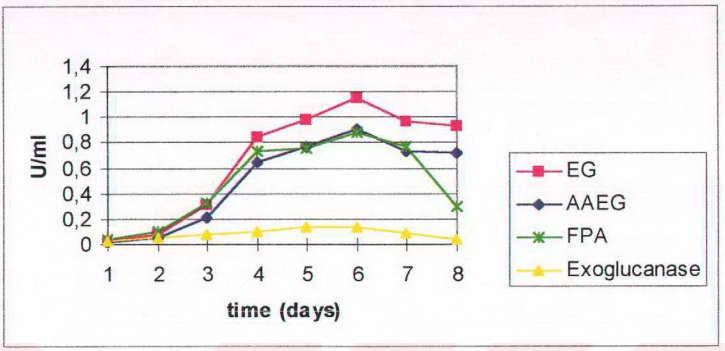

Fig. 3.67 Effect of spore induction on cellulase production with 20 mg spore inoculation in preculture medium by *T. thermophila* cultivating on grass clippings in 1 liter working volume.

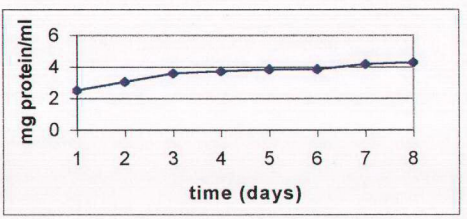

Fig. 3.68 Change in total exocell protein content (mg/ml) in the supernatant of *T. thermophila* cultivated on grass clippings with 20 mg spore inoculation.

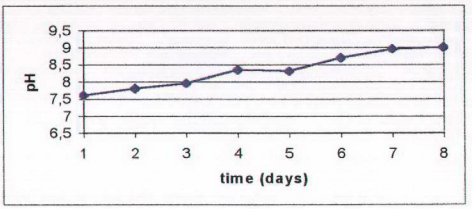

Fig. *3.69* Changes in medium pH during cultivation of *T. thermophila* on grass clippings with 20 mg spore inoculation.

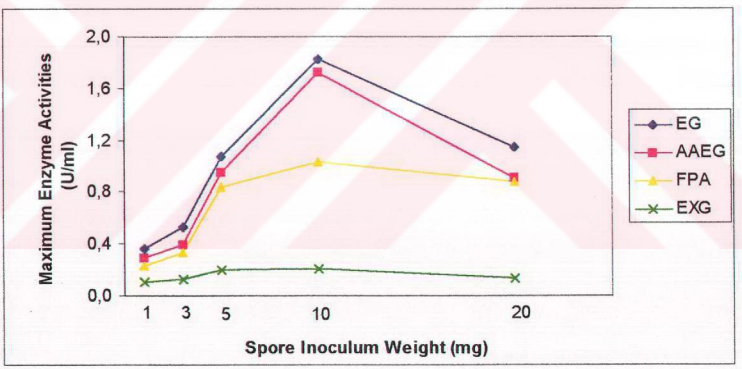

Fig. 3.70 The effect of spore concentration on cellulase production by *T. thermophila* cultivating on grass clippings.

3.6 Comparison of Cellulose Production by *T. thermophila* and *H. insolens* on Grass Clippings as the Carbon Source

An industrial strain of *Humicola insolens* was compared with *Torula thermophila* for its efficiency in growth and cellulase production on grass clippings as substrate with 10 mg spore inoculation in 1 liter working volume.

As can be seen in Figure 3.71, *H. insolens* produced maximum cellulolytic activities at the third day. Maximum values of EG, AAEG, FPA and EXG activities were 0.397 U/ml, 0.280 U/ml, 0.612 U/ml and 0.178 U/ml respectively (Appendix K). After the third day, all the cellulolytic activities decreased.

In addition, total exocell protein reached its maximum level at the third day (Figure 3.72). The rapid decrease in cellulolytic activities may be due to the pH change. Since the composition of grass clippings is complex, the two organisms gave different profiles with respect to cellulolytic activities, total exocell protein and the change in medium pH. The medium pH changed from 7.40 for the first day to 9.40 for the 8^{th} day of incubation (Figure 3.73).

The comparison of *T. thermophila* and *H. insolens* for endoglucanase production on grass clippings with the same amount of spore concentration (10 mg) is illustrated in Figure 3.74. As can be seen in figure , *Torula thermophila* yielded higher EG activities than *H. insolens*. Besides, EG, AAEG, FPA and EXG activities are higher than those of *Humicola insolens* (Figure 3.75, 76 and 77).

In addition, specific activities (U/mg protein) of *T. thermophila* cellulases were higher than those of *H. insolens* as illustrated in Table H.19 and H.21 (Appendix H).

In an effort to compare *T. thermophila* and *H. insolens* in terms of cellulolytic activities on Avicel containing modified YpSs medium by using 3 mg spore and 1 liter working volume, it was found that *T. thermophila* yielded 1.407 U/ml EG and

0.069 U/ml FPA whereas *H. insolens* yielded 1.389 U/ml EG and 0.129 U/ml FPA (Arifoğlu, 1999). EG activities of *T. thermophila* and *H. insolens* were almost similar on Avicel, whereas FPA of *H. insolens* was higher.

In this study, *T. thermophila* gave 1.820 U/ml EG, 1.720 U/ml AAEG, 1.030 U/ml FPA and 0.203 U/ml EXG activity on grass clippings with 10 mg spore inoculum. It was shown that *T. thermophila* gave higher cellulolytic activities than *H. insolens* (Appendix J and K).

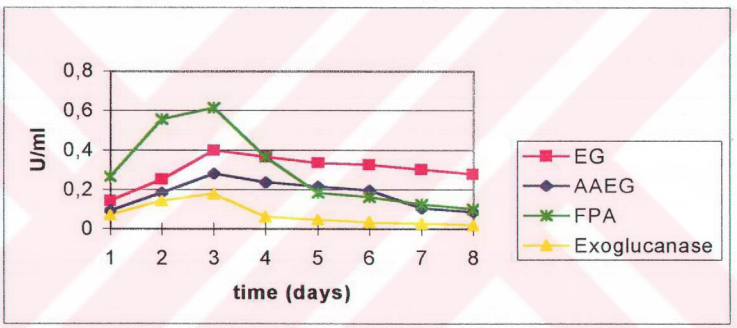

Fig. 3.71 Time course of cellulase production by *Humicola insolens* cultivating on grass clippings with 10 mg spore inoculation in preculture medium by 1 liter working volume.

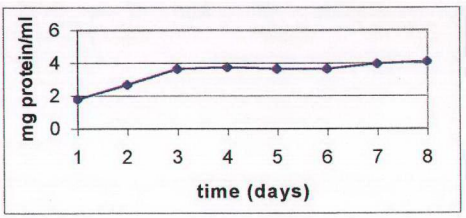

Fig. 3.72 Change in total exocell protein content (mg/ml) in the supernatant of *Humicola insolens* cultivated on grass clippings with 10 mg spore inoculation.

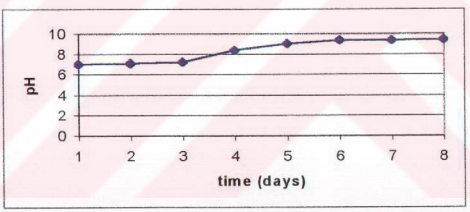

Fig. 3.73 Changes in medium pH during cultivation of *Humicola insolens* on grass clippings with 10 mg spore inoculation.

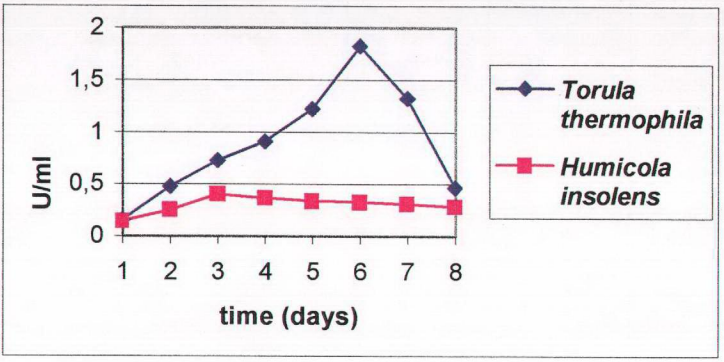

Fig. 3.74 Comparison of EG activity of *T. thermophila* and *H. insolens.*

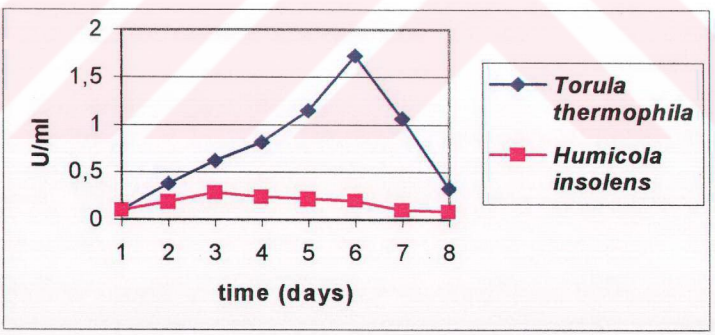

Fig. 3.75 Comparison of AAEG activity of *T. thermophila* and *H. insolens*

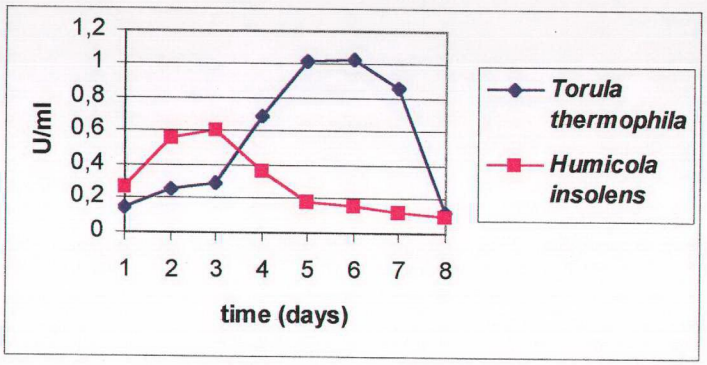

Fig. 3.76 Comparison of FPA of *T. thermophila* and *H. insolens*

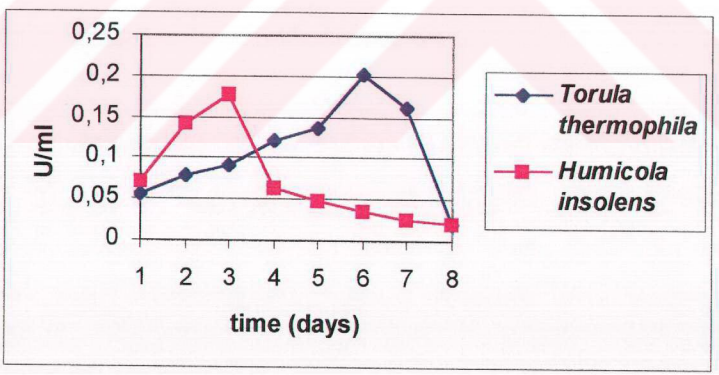

Fig. 3.77 Comparison of EXG activity of *T. thermophila* and *H. insolens*

CHAPTER 4

CONCLUSIONS

In this study, the cellulase activity of *T. thermophila* was investigated on complex carbon sources by using 14 different lignocellulosic compounds, namely, razmol, wheat bran, lentil bran, barley grain, wheat fracture, soybean meal, sunflower seed bagasse, cotton seed bagasse, grape pomace, sugar beet bagasse, apple pomace, tomato pomace, wheat straw, grass clippings.

The EG, AAEG, FPA and EXG activities, extracellular total protein and the medium pH were measured during the cultivation of *T. thermophila.* Moreover, the cellulose contents of the lignocellulosic compounds have been measured.

T. thermophila gave different cellulolytic activities on different substrates. Among the lignocellulosic compounds tested grass clippings was found to be the best carbon source for EG, AAEG, FPA and EXG production. Soybean meal, apple pomace, lentil bran also have proved to be suitable for cellulase production.

It was observed that there was no a correlation between cellulose level and cellulase production. Some lignocellulosic compounds produced more enzymes although their cellulose content was moderate. Whereas some lignocellulosic compounds gave low enzymatic activities although they contained more cellulose as compared to other substrates.

Although some substrates gave low cellulase concentrations, their specific activities (U/mg protein) were higher. For example, the specific activities of

cellulases on razmol, wheat bran and barley grain were higher (Figure 3.47, 49, 51, 53). *Torula thermophila* was also cultivated on grass clippings by increasing spore concentrations in preculture medium. Accordingly, 10 mg of spores was optimal for cellulase production in 1 liter of fermentation media in shake flasks at 155 rpm.

T. thermophila and *H. insolens* were compared on grass clippings with the same amount of spore and it was found that *T. thermophila* is more productive than *H. insolens* for cellulase production. *T. thermophila* yielded 4 fold more endoglucanase, 6 times more AAEG, and 3 times more FPA.

To optimize the production of cellulases by *T. thermophila*, further studies on *T. thermophila* might be done. The cellulase enzymes of *T. thermophila* may be purified and characterized. The proteases and their hydrolytic effect on cellulase enzymes might be investigated. Especially, the effect of pH might be studied in fermentors.

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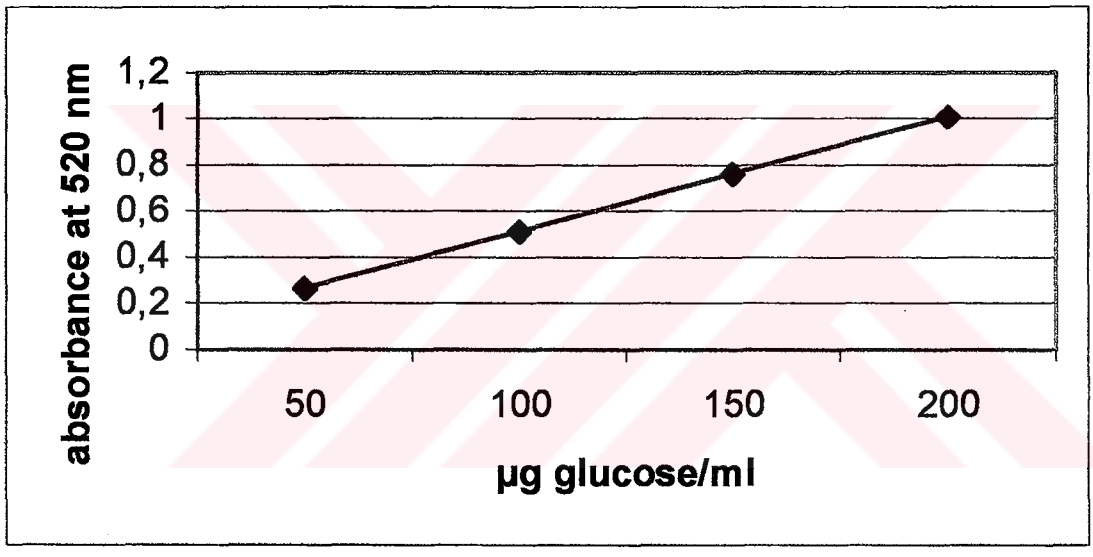
APPENDIX A

STANDARD CURVE FOR NELSON-SOMOGYI METHOD



APPENDIX B

COMPOSITION OF NELSON SOMOGYI REAGENTS

Somogyi Reagent I:

288 g of Na_2SO_4 (anhydrous) is dissolved in 1 liter of boiled distilled water, followed by 24 g of $C_4H_4KNaO_6 \cdot 4H_2O$, 48 g of Na_2CO_3, and 32 g of $NaHCO_3$. The solution is diluted to 1600 ml with boiled distilled water and stored at 27°C.

Somogyi Reagent II:

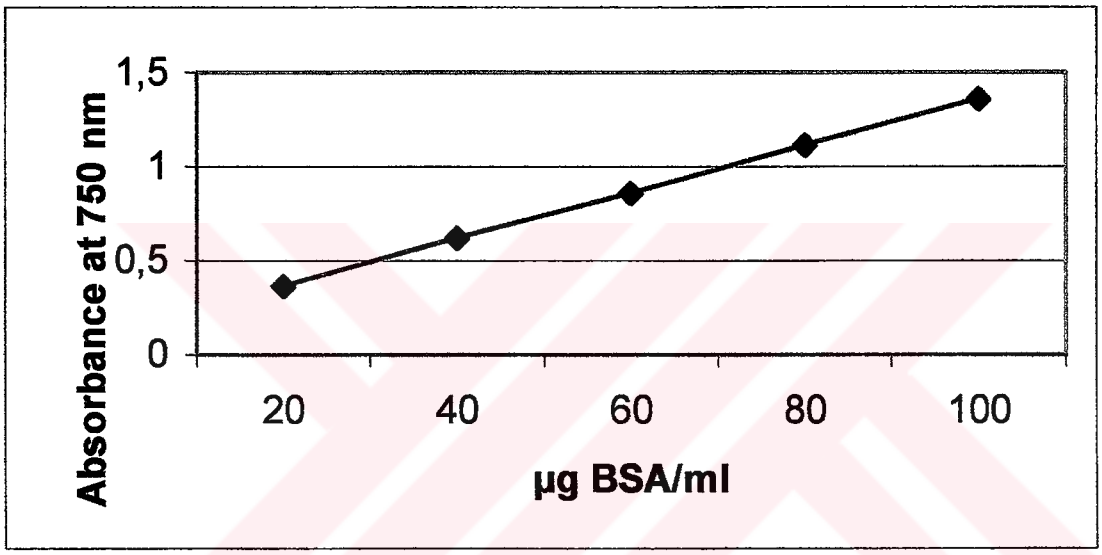
72 g of Na_2SO_4 (anhydrous) is dissolved in 300 ml of boiled distilled water. 8 g of $CuSO_4 \cdot 5H_2O$ is added, and the solution is diluted to 400 ml with boiled distilled water. The solution is stored at 27°C. Somogyi reagent I and II are mixed in 4:1 ratio immediately prior to use.

Nelson Reagent:

100 g of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ is dissolved in 1.8 liters of distilled water and 84 ml of concntrated H_2SO_4 is added to the solution. Next, 100 ml of $Na_2HasO_4 \cdot 7H_2O$ solution is added. (12 g of $Na_2HAsO_4 \cdot 7H_2O$ is dissolved in 100 ml of distilled water). The reagent is stored in brown glass bottle at 37°C for 24 – 48 hours and then at room temperature.

APPENDIX C

STANDARD CURVE FOR PROTEIN MEASUREMENT



APPENDIX D

COMPOSITIONS OF REAGENTS A, B, C, D

Reagent A:

2.0 g of NaOH, 10.0 g of Na_2CO_3 and 0,1 g of $C_4H_4KNaO_6 \cdot 5H_2O$ are dissolved in 500 ml of distilled water.

Reagent B:

0,5 g of $CuSO_4 \cdot 5H_2O$ is dissolved in 100 ml of distilled water.

Reagent C:

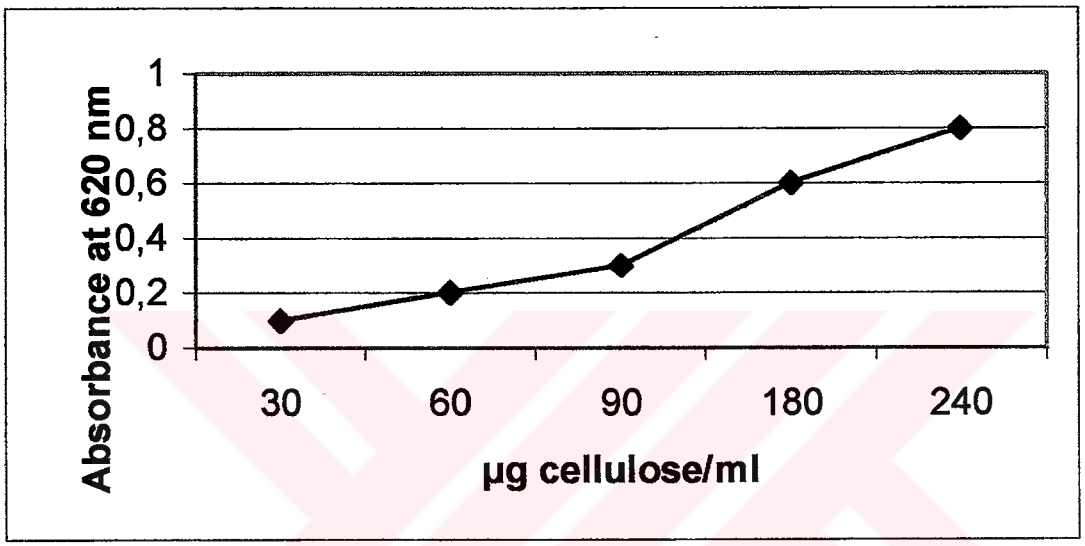
10 ml of Reagent A and 0,2 ml of Reagent B are mixed immediately prior to use.

Reagent D:

Folin phenol (2N) is mixed with distilled water in 1:1 ratio immediately prior to use.

APPENDIX E

STANDARD CURVE FOR CELLULOSE DETERMINATION BY UPPDEGRAFF'S METHOD



APPENDIX F

COMPOSITIONS OF ACETIC – NITRIC AND ANTHRONE REAGENTS

Acetic – Nitric Reagent:

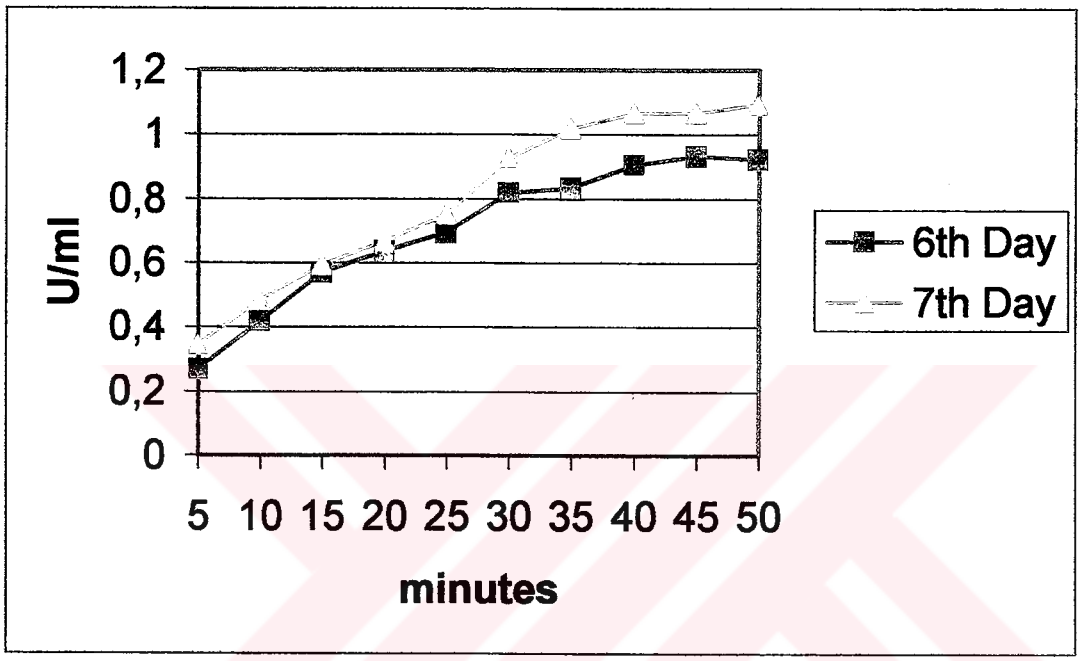
150 ml of 80% acetic acid is mixed with 15 ml of concentrated HNO_3.

Anthrone Reagent:

0,2 g of anthrone is added to 100 ml of concentrated H_2SO_4. The reagent is prepared fresh daily, and chilled about 2 hours in refrigerator prior to use.

APPENDIX G

DETERMINATION OF REACTION PERIOD FOR ENDOGLUCANASE ACTIVITY



APPENDIX H

SPECIFIC ACTIVITIES OF EG, AAEG, FPA AND EXG ENZYMES

Table H.1 Specific activities of the cellulases of *T. thermophila* on razmol.

DAY	EG (U/mg protein)	AAEG (U/mg protein)	FPA (U/mg protein)	EXG (U/mg protein)
1	0.054	0.030	0.082	0.033
2	0.177	0.106	0.175	0.091
3	0.254	0.143	0.236	0.143
4	0.208	0.129	0.164	0.105
5	0.109	0.060	0.089	0.045
6	0.100	0.052	0.080	0.034
7	0.078	0.041	0.065	0.020
8	0.070	0.037	0.048	0.014

Table H.2 Specific activities of the cellulases of *T. thermophila* on wheat bran.

DAY	EG (U/mg protein)	AAEG (U/mg protein)	FPA (U/mg protein)	EXG (U/mg protein)
1	0.047	0.024	0.057	0.031
2	0.122	0.096	0.132	0.074
3	0.237	0.164	0.135	0.089
4	0.122	0.070	0.076	0.050
5	0.102	0.060	0.060	0.035
6	0.096	0.050	0.058	0.032
7	0.081	0.045	0.051	0.023
8	0.067	0.038	0.044	0.017

Table H.3 Specific activities of the cellulases of *T. thermophila* on lentil bran.

DAY	EG (U/mg protein)	AAEG (U/mg protein)	FPA (U/mg protein)	EXG (U/mg protein)
1	0.033	0.017	0.034	0.017
2	0.034	0.18	0.034	0.018
3	0.117	0.086	0.055	0.020
4	0.137	0.106	0.100	0.023
5	0.124	0.093	0.083	0.021
6	0.159	0.117	0.090	0.028
7	0.141	0.095	0.066	0.024
8	0.102	0.045	0.048	0.024

Table H.4 Specific activities of the cellulases of *T. thermophila* on barley grain.

DAY	EG (U/mg protein)	AAEG (U/mg protein)	FPA (U/mg protein)	EXG (U/mg protein)
1	0.053	0.027	0.055	0.027
2	0.071	0.041	0.081	0.055
3	0.232	0.129	0.208	0.128
4	0.110	0.055	0.085	0.075
5	0.111	0.054	0.084	0.069
6	0.102	0.039	0.086	0.063
7	0.092	0.020	0.073	0.042
8	0.057	$4.28 . 10^{-3}$	0.045	0.011

Table H.5 Specific activities of the cellulases of *T. thermophila* on wheat fracture.

DAY	EG (U/mg protein)	AAEG (U/mg protein)	FPA (U/mg protein)	EXG (U/mg protein)
1	0.051	0.024	0.045	0.024
2	0.050	0.032	0.046	0.028
3	0.057	0.032	0.048	0.031
4	0.063	0.040	0.050	0.035
5	0.061	0.041	0.048	0.040
6	0.058	0.042	0.051	0.030
7	0.063	0.045	0.053	0.053
8	0.050	0.025	0.028	0.022

Table H.6 Specific activities of the cellulases of *T. thermophila* on soybean meal.

DAY	EG (U/mg protein)	AAEG (U/mg protein)	FPA (U/mg protein)	EXG (U/mg protein)
1	0.018	$8.44 . 10^{-3}$	0.018	$8.75 . 10^{-3}$
2	0.080	0.067	0.081	0.040
3	0.048	0.028	0.044	0.038
4	0.043	0.028	0.034	0.035
5	0.044	0.025	0.032	0.027
6	0.046	0.026	0.035	0.026
7	0.043	0.024	0.027	0.022
8	0.041	0.022	0.026	0.017

Table H.7 Specific activities of the cellulases of *T. thermophila* on sunflower seed bagasse.

DAY	EG (U/mg protein)	AAEG (U/mg protein)	FPA (U/mg protein)	EXG (U/mg protein)
1	0.029	0.013	0.036	0.022
2	0.057	0.029	0.039	0.027
3	0.078	0.050	0.038	0.033
4	0.101	0.077	0.078	0.033
5	0.097	0.067	0.071	0.032
6	0.093	0.064	0.054	0.021
7	0.097	0.064	0.045	0.018
8	0.071	0.042	0.026	0.018

Table H.8 Specific activities of the cellulases of *T. thermophila* on cotton seed bagasse.

DAY	EG (U/mg protein)	AAEG (U/mg protein)	FPA (U/mg protein)	EXG (U/mg protein)
1	0.032	0.015	0.028	0.024
2	0.032	0.020	0.027	0.025
3	0.039	0.027	0.033	0.026
4	0.042	0.029	0.034	0.020
5	0.039	0.023	0.029	0.012
6	0.034	0.016	0.025	$6.25 . 10^{-3}$
7	0.032	0.012	0.024	$5.89 . 10^{-3}$
8	0.031	0.011	0.022	$4.47 . 10^{-3}$

Table H.9 Specific activities of the cellulases of *T. thermophila* on grape bagasse.

DAY	EG (U/mg protein)	AAEG (U/mg protein)	FPA (U/mg protein)	EXG (U/mg protein)
1	0.021	$7.4 . 10^{-3}$	0.022	0.014
2	0.030	0.012	0.028	0.019
3	0.030	0.014	0.023	0.020
4	0.036	$3.89 . 10^{-3}$	0.029	0.025
5	0.035	0.017	0.024	0.015
6	0.074	0.048	0.053	0.028
7	0.082	0.052	0.056	0.031
8	0.077	0.048	0.054	0.028

Table H.10 Specific activities of the cellulases of *T. thermophila* on sugar beet bagasse.

DAY	EG (U/mg protein)	AAEG (U/mg protein)	FPA (U/mg protein)	EXG (U/mg protein)
1	0.051	0.023	0.057	0.023
2	0.036	0.016	0.041	0.044
3	0.029	0.013	0.030	0.025
4	0.022	$9.56 . 10^{-3}$	0.026	0.022
5	0.018	$7.7 . 10^{-3}$	0.023	0.018
6	0.017	$7.51 . 10^{-3}$	0.018	0.015
7	0.016	$4.52 . 10^{-3}$	0.017	$9.05 . 10^{-3}$
8	0.019	$3.37 . 10^{-3}$	0.024	$9.64 . 10^{-3}$

Table H.11 Specific activities of the cellulases of *T. thermophila* on apple pomace.

DAY	EG (U/mg protein)	AAEG (U/mg protein)	FPA (U/mg protein)	EXG (U/mg protein)
1	0.212	0.107	0.230	0.168
2	0.152	0.096	0.172	0.144
3	0.100	0.052	0.111	0.089
4	0.055	0.025	0.073	0.056
5	0.064	0.024	0.064	0.052
6	0.057	0.014	0.058	0.017
7	0.059	0.011	0.059	0.018
8	0.052	$1.52 . 10^{-3}$	0.056	0.014

Table H.12 Specific activities of the cellulases of *T. thermophila* on tomato pomace.

DAY	EG (U/mg protein)	AAEG (U/mg protein)	FPA (U/mg protein)	EXG (U/mg protein)
1	0.050	0.026	0.055	0.010
2	0.030	0.015	0.030	$9.56 . 10^{-3}$
3	0.030	0.018	0.031	0.018
4	0.029	0.018	0.030	0.021
5	0.017	$8.57 . 10^{-3}$	0.016	0.015
6	0.013	$5.59 . 10^{-3}$	0.013	0.011
7	0.016	$6.44 . 10^{-3}$	0.015	0.012
8	0.015	$5.52 . 10^{-3}$	0.015	0.011

Table H.13 Specific activities of the cellulases of *T. thermophila* on wheat straw.

DAY	EG (U/mg protein)	AAEG (U/mg protein)	FPA (U/mg protein)	EXG (U/mg protein)
1	0.022	$6.26 . 10^{-3}$	0.031	0.017
2	0.014	$7.23 . 10^{-3}$	0.017	0.014
3	0.013	$6.43 . 10^{-3}$	0.015	0.013
4	0.014	$5.12 . 10^{-3}$	0.016	0.014
5	0.014	$4.55 . 10^{-3}$	0.015	0.012
6	0.012	$3.37 . 10^{-3}$	0.012	$5.3 . 10^{-3}$
7	0.010	$9.39 . 10^{-4}$	0.011	$4.22 . 10^{-3}$
8	$9.39 . 10^{-3}$	$9.39 . 10^{-4}$	0.010	$2.34 . 10^{-3}$

Table H.14 Specific activities of the cellulases of *T. thermophila* on grass clippings.

DAY	EG (U/mg protein)	AAEG (U/mg protein)	FPA (U/mg protein)	EXG (U/mg protein)
1	0.040	0.019	0.045	0.017
2	0.052	0.027	0.037	0.021
3	0.084	0.056	0.054	0.041
4	0.108	0.082	0.074	0.053
5	0.093	0.064	0.052	0.026
6	0.084	0.057	0.045	0.025
7	0.125	0.052	0.083	0.039
8	0.063	0.018	0.055	0.031

Table H.15 Specific activities of the cellulases of *T. thermophila* on avicel.

DAY	EG (U/mg protein)	AAEG (U/mg protein)	FPA (U/mg protein)	EXG (U/mg protein)
1	0.082	0.022	0.022	0.120
2	0.046	0.025	0.025	0.071
3	0.130	0.097	0.047	0.066
4	0.177	0.135	0.135	0.071
5	0.247	0.148	0.142	0.330
6	0.341	0.147	0.313	0.294
7	1.164	0.661	0.353	0.505
8	0.630	0.374	0.111	0.261

Table H.16 Specific activities of the cellulases of *T. thermophila* on grass clippings with 1 mg spore inoculation.

DAY	EG (U/mg protein)	AAEG (U/mg protein)	FPA (U/mg protein)	EXG (U/mg protein)
1	0.033	0.023	0.030	0.024
2	0.045	0.027	0.036	0.027
3	0.065	0.043	0.045	0.029
4	0.068	0.047	0.048	0.029
5	0.099	0.076	0.050	0.030
6	0.098	0.078	0.063	0.029
7	0.083	0.058	0.057	0.022
8	0.080	0.055	0.055	0.013

Table H.17 Specific activities of the cellulases of *T. thermophila* on grass clippings with 3 mg spore inoculation.

DAY	EG (U/mg protein)	AAEG (U/mg protein)	FPA (U/mg protein)	EXG (U/mg protein)
1	0.035	0.017	0.032	$5.18 . 10^{-3}$
2	0.049	0.029	0.041	0.023
3	0.076	0.054	0.053	0.033
4	0.076	0.053	0.065	0.032
5	0.150	0.110	0.095	0.034
6	0.134	0.093	0.078	0.020
7	0.127	0.086	0.081	0.010
8	0.097	0.056	0.049	$3.23 . 10^{-3}$

Table H.18 Specific activities of the cellulases of *T. thermophila* on grass clippings with 5 mg spore inoculation.

DAY	EG (U/mg protein)	AAEG (U/mg protein)	FPA (U/mg protein)	EXG (U/mg protein)
1	0.035	0.016	0.034	0.014
2	0.071	0.055	0.046	0.016
3	0.104	0.082	0.060	0.023
4	0.140	0.120	0.124	0.023
5	0.216	0.194	0.175	0.027
6	0.276	0.245	0.214	0.050
7	0.303	0.262	0.187	0.053
8	0.113	0.092	0.093	0.018

Table H.19 Specific activities of the cellulases of *T. thermophila* on grass clippings with 10 mg spore inoculation.

DAY	EG (U/mg protein)	AAEG (U/mg protein)	FPA (U/mg protein)	EXG (U/mg protein)
1	0.043	0.027	0.041	0.015
2	0.128	0.101	0.069	0.021
3	0.190	0.163	0.077	0.024
4	0.220	0.196	0.168	0.029
5	0.269	0.252	0.225	0.030
6	0.408	0.386	0.231	0.045
7	0.315	0.253	0.206	0.038
8	0.114	0.079	0.030	$4.93 . 10^{-3}$

Table H.20 Specific activities of the cellulases of *T. thermophila* on grass clippings with 20 mg spore inoculation.

DAY	EG (U/mg protein)	AAEG (U/mg protein)	FPA (U/mg protein)	EXG (U/mg protein)
1	0.011	$5.58 . 10^{-3}$	0.013	$7.58 . 10^{-3}$
2	0.026	0.014	0.032	0.017
3	0.087	0.058	0.091	0.020
4	0.226	0.173	0.196	0.025
5	0.255	0.201	0.197	0.034
6	0.298	0.235	0.228	0.035
7	0.231	0.176	0.184	0.021
8	0.219	0.167	0.069	$9.84 . 10^{-3}$

Table H.21 Specific activities of the cellulases of *Humicola Insolens* on grass clippings with 10 mg spore inoculation.

DAY	EG (U/mg protein)	AAEG (U/mg protein)	FPA (U/mg protein)	EXG (U/mg protein)
1	0.079	0.052	0.151	0.039
2	0.093	0.068	0.208	0.053
3	0.109	0.077	0.168	0.049
4	0.097	0.063	0.097	0.016
5	0.092	0.058	0.050	0.012
6	0.089	0.053	0.044	$9.65 . 10^{-3}$
7	0.076	0.026	0.031	$6.58 . 10^{-3}$
8	0.068	0.020	0.024	$4.93 . 10^{-3}$

APPENDIX I

THE PERCENTAGES OF AVICEL ADSORBABLE AND NON-ADSORBABLE ENDOGLUCANASE

Table I.1 Percent Avicel-adsorbable endoglucanase and Non-adsorbable endoglucanase of *Torula thermophila* on razmol.

DAY	% Avicel Adsorbable Endoglucanase	% Non-Adsorbable Endoglucanase
1	55.80	44.20
2	60.00	40.00
3	56.30	43.70
4	61.70	38.30
5	55.00	45.00
6	51.90	48.10
7	53.06	46.94
8	53.30	46.70

Table I.2 Percent Avicel-adsorbable endoglucanase and Non-adsorbable endoglucanase of *Torula thermophila* on wheat bran.

DAY	% Avicel Adsorbable Endoglucanase	% Non-Adsorbable Endoglucanase
1	51.19	48.81
2	78.74	21.26
3	69.29	30.71
4	57.95	42.05
5	59.30	40.70
6	51.94	48.06
7	55.55	44.45
8	56.06	43.94

Table I.3 Percent Avicel-adsorbable endoglucanase and Non-adsorbable endoglucanase of *Torula thermophila* on lentil bran.

DAY	% Avicel Adsorbable Endoglucanase	% Non-Adsorbable Endoglucanase
1	52.77	47.23
2	55.12	44.88
3	74.06	25.94
4	76.87	23.13
5	75.33	24.67
6	74.06	25.94
7	67.51	32.49
8	44.72	55.28

Table I.4 Percent Avicel-adsorbable endoglucanase and Non-adsorbable endoglucanase of *Torula thermophila* on barley grain.

DAY	% Avicel Adsorbable Endoglucanase	% Non-Adsorbable Endoglucanase
1	52.56	47.44
2	57.79	42.21
3	55.55	44.45
4	50.00	50.00
5	48.78	51.22
6	35.21	64.79
7	22.22	77.78
8	7.40	92.60

Table I.5 Percent Avicel-adsorbable endoglucanase and Non-adsorbable endoglucanase of *Torula thermophila* on wheat fracture.

DAY	% Avicel Adsorbable Endoglucanase	% Non-Adsorbable Endoglucanase
1	48.43	51.57
2	64.78	35.22
3	55.29	44.71
4	63.94	36.09
5	66.66	33.34
6	71.79	28.21
7	70.58	29.42
8	50	50.00

Table I.6 Percent Avicel-adsorbable endoglucanase and Non-adsorbable endoglucanase of *Torula thermophila* on soybean meal.

DAY	% Avicel Adsorbable Endoglucanase	% Non-Adsorbable Endoglucanase
1	45.00	55.00
2	84.04	15.96
3	60.00	40.00
4	64.33	35.67
5	56.95	43.05
6	57.66	42.34
7	56.00	44.00
8	53.84	46.16

Table I.7 Percent Avicel-adsorbable endoglucanase and Non-adsorbable endoglucanase of *Torula thermophila* on sunflower seed bagasse.

DAY	% Avicel Adsorbable Endoglucanase	% Non-Adsorbable Endoglucanase
1	44.44	55.56
2	51.42	48.58
3	64.35	35.65
4	76.22	23.78
5	69.65	30.45
6	68.69	31.31
7	66.66	33.34
8	59.06	40.94

Table I.8 Percent Avicel-adsorbable endoglucanase and Non-adsorbable endoglucanase of *Torula thermophila* on cotton seed bagasse.

DAY	% Avicel Adsorbable Endoglucanase	% Non-Adsorbable Endoglucanase
1	47.54	52.46
2	63.51	36.49
3	71.11	28.89
4	69.23	30.77
5	60.00	40.00
6	46.26	53.74
7	40.00	60.00
8	35.08	64.92

Table I.9 Percent Avicel-adsorbable endoglucanase and Non-adsorbable endoglucanase of *Torula thermophila* on grape pomace.

DAY	% Avicel Adsorbable Endoglucanase	% Non-Adsorbable Endoglucanase
1	34.21	65.79
2	41.66	58.34
3	46.51	53.49
4	10.71	89.29
5	51.21	48.79
6	64.76	35.24
7	63.15	36.85
8	63.30	36.70

Table I.10 Percent Avicel-adsorbable endoglucanase and Non-adsorbable endoglucanase of *Torula thermophila* on sugar beet bagasse.

DAY	% Avicel Adsorbable Endoglucanase	% Non-Adsorbable Endoglucanase
1	44.70	34.75
2	46.00	54.00
3	46.42	53.58
4	43.07	56.93
5	45.59	54.41
6	44.00	56.00
7	27.65	72.35
8	17.07	82.93

Table I.11 Percent Avicel-adsorbable endoglucanase and Non-adsorbable endoglucanase of *Torula thermophila* on apple pomace.

DAY	% Avicel Adsorbable Endoglucanase	% Non-Adsorbable Endoglucanase
1	50.47	49.53
2	63.52	36.48
3	52.50	47.50
4	46.52	53.48
5	37.95	62.05
6	24.59	75.41
7	18.64	81.36
8	2.91	97.09

Table I.12 Percent Avicel-adsorbable endoglucanase and Non-adsorbable endoglucanase of *Torula thermophila* on tomato pomace.

DAY	% Avicel Adsorbable Endoglucanase	% Non-Adsorbable Endoglucanase
1	52.56	47.44
2	50.00	50.00
3	61.45	38.55
4	62.50	37.50
5	49.20	50.80
6	41.66	58.34
7	40.00	60.00
8	35.71	64.29

Table I.13 Percent Avicel-adsorbable endoglucanase and Non-adsorbable endoglucanase of *Torula thermophila* on wheat straw.

DAY	% Avicel Adsorbable Endoglucanase	% Non-Adsorbable Endoglucanase
1	28.00	72.00
2	48.38	51.69
3	48.14	51.86
4	34.61	65.39
5	32.00	68.00
6	28.00	72.00
7	8.69	91.31
8	10.00	90.00

Table I.14 Percent Avicel-adsorbable endoglucanase and Non-adsorbable endoglucanase of *Torula thermophila* on grass clippings.

DAY	% Avicel Adsorbable Endoglucanase	% Non-Adsorbable Endoglucanase
1	49.00	51.00
2	52.27	47.73
3	66.90	33.10
4	76.22	23.78
5	68.99	31.01
6	68.15	31.85
7	41.73	58.27
8	29.60	70.40

Table I.15 Percent Avicel-adsorbable endoglucanase and Non-adsorbable endoglucanase of *Torula thermophila* on avicel.

DAY	% Avicel Adsorbable Endoglucanase	% Non-Adsorbable Endoglucanase
1	27.27	72.73
2	53.84	46.16
3	74.46	25.54
4	76.47	23.53
5	60.00	40.00
6	43.07	56.93
7	56.82	43.18
8	59.42	40.58

Table I.16 Percent Avicel-adsorbable endoglucanase and Non-adsorbable endoglucanase of *Torula thermophila* on grass clippings with 1 mg spore.

DAY	% Avicel Adsorbable Endoglucanase	% Non-Adsorbable Endoglucanase
1	68.62	31.38
2	60.56	39.44
3	66.66	33.34
4	69.43	30.57
5	76.94	23.06
6	79.60	20.40
7	70.46	29.54
8	68.75	31.25

Table I.17 Percent Avicel-adsorbable endoglucanase and Non-adsorbable endoglucanase of *Torula thermophila* on grass clippings with 3 mg spore.

DAY	% Avicel Adsorbable Endoglucanase	% Non-Adsorbable Endoglucanase
1	50.92	49.08
2	60.50	39.50
3	71.71	28.29
4	69.54	30.46
5	73.62	26.38
6	69.54	30.46
7	67.81	32.19
8	58.61	41.39

Table I.18 Percent Avicel-adsorbable endoglucanase and Non-adsorbable endoglucanase of *Torula thermophila* on grass clippings with 5 mg spore.

DAY	% Avicel Adsorbable Endoglucanase	% Non-Adsorbable Endoglucanase
1	47.50	52.50
2	76.52	23.48
3	78.50	21.50
4	85.22	14.78
5	89.70	10.30
6	88.78	11.22
7	86.54	13.46
8	81.75	18.25

Table I.19 Percent Avicel-adsorbable endoglucanase and Non-adsorbable endoglucanase of *Torula thermophila* on grass clippings with 10 mg spore.

DAY	% Avicel Adsorbable Endoglucanase	% Non-Adsorbable Endoglucanase
1	62.74	37.26
2	79.03	20.97
3	85.73	14.27
4	88.91	11.09
5	93.44	6.56
6	94.50	5.50
7	80.30	19.70
8	69.52	30.48

Table I.20 Percent Avicel-adsorbable endoglucanase and Non-adsorbable endoglucanase of *Torula thermophila* on grass clippings with 20 mg spore.

DAY	% Avicel Adsorbable Endoglucanase	% Non-Adsorbable Endoglucanase
1	50.00	50.00
2	55.00	45.00
3	66.87	33.13
4	76.62	23.38
5	78.89	21.11
6	78.91	21.09
7	76.29	23.71
8	76.57	23.43

Table I.21 Percent Avicel-adsorbable endoglucanase and Non-adsorbable endoglucanase of *Humicola Insolens* on grass clippings with 10 mg spore.

DAY	% Avicel Adsorbable Endoglucanase	% Non-Adsorbable Endoglucanase
1	66.42	33.58
2	73.20	26.80
3	70.52	29.48
4	64.65	35.35
5	63.77	36.33
6	59.45	40.55
7	34.43	65.57
8	30.21	69.79

APPENDIX J

SPORE INDUCTION STUDIES FOR *TORULA THERMOPHILA*

Spore inoculum	EG (U/ml)	AAEG (U/ml)	FPA (U/ml)	EXG (U/ml)
1 mg	0.358	0.285	0.231	0.108
3 mg	0.527	0.388	0.334	0.120
5 mg	1.070	0.950	0.830	0.195
10 mg	1.820	1.720	1.030	0.203
20 mg	1.148	0.906	0.879	0.137

Table J.1 The maximum enzymatic activities of *T. thermophila* cultivating on grass clippings in 1 Liter working volume.

APPENDIX K

MAXIMUM ENZYMATIC ACTIVITIES OF *HUMICOLA INSOLENS* FOR CELLULASE PRODUCTION

Spore inoculum	CMCase (U/ml)	AAEG (U/ml)	FPA (U/ml)	EXG (U/ml)
10 mg	0.397	0.280	0.612	0.178

Table K.1 The maximum enzymatic activities of *Humicola insolens* cultivating on grass clippings with 10 mg spore inoculation in preculture medium and 1 liter working volume.