# PROTEASE SECRETION BY *TORULA THERMOPHILA* ON CELLULOSE-CONTAINING MEDIA

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF ENGINEERING SCIENCES
OF
THE MIDDLE EAST TECHNICAL UNIVERSITY



BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE
IN
THE DEPARTMENT OF FOOD ENGINEERING

SEPTEMBER 2000

Approval of the Graduate School of Natural and Applied Sciences

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## ABSTRACT

### PROTEASE SECRETION BY *TORULA THERMOPHILA* ON CELLULOSE-CONTAINING MEDIA

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September 2000, 83 pages

In this work, *Torula thermophila* protease production was analyzed in cellulose-containing media and effect on cellulase activity was investigated. In this respect, *T. thermophila* was cultivated on media containing Avicel (microcrystalline cellulose), grass clippings and apple pomace as the carbon source in shake flask cultures at 45°C and 155 rpm, and protease as well as endoglucanase activities were determined. Prior to protease activity measurements, the temperature and pH of enzyme assays were also optimized. Accordingly, proteases produced by *T. thermophila* were active at neutral and alkaline pH and at a temperature range of 37-45°C. As a result, the assay pH and temperature were selected as pH 7.5 and 40°C. On cellulose-containing media, the maximum production of proteases was on the 3rd day of cultivation where endoglucanase activity was low. While, endoglucanase production was maximum on the 6th day where protease activity was declining. The production

of proteases and endoglucanases on different days suggested a possible negative effect of proteases on endoglucanase activity. To test this hypothesis, experiments were carried out by mixing supernatants from the 3rd, 6th and 9th day of cultivations and by measuring changes in protease and endoglucanase activities. It was observed that proteases have little effect on extracellular endoglucanase activities in the sample supernatants, despite an increase in protease levels. Further experiments were made to determine the type of proteases secreted by *T. thermophila* on avicel-containing media. Inhibition experiments were performed using phenyl methyl sulfonyl fluoride (PMSF), p-chloromercury benzoate (pCMB), antipain, ethylene diamine tetraacetate (EDTA) and pepstatin A as inhibitors. Proteases were partially inhibited by the inhibitors PMSF, pCMB and antipain, indicating the presence of both serine and cysteine (thiol) proteases or the presence of thiol-containing serine proteases in the culture filtrates of *T. thermophila*.

Keywords: *Torula thermophila*, cellulase, endoglucanase, protease.

# ÖZ

## SELÜLAZ İÇEREN ORTAMDA *TORULA THERMOPHILA*' NIN PROTEAZ ÜRETİMİ

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Eylül 2000, 83 sayfa

Bu çalışmada, *T. thermophila*'nın selüloz içeren ortamdaki proteaz üretimi ve selülaz aktivitesi üzerindeki etkileri araştırılmıştır. Bu amaçla *T. thermophila,* karbon kaynağı olarak Avicel (mikrokristalin selüloz), çim kırpığı ve elma posası üzerinde, 45°C ve 155 rpm'de büyütülmüş ve proteaz ile endoglukanaz aktivitelerine bakılmıştır. Proteaz aktivitesi ölçümlerinden önce, aktivite ölçüm deneyinin sıcaklık ve pH optimizasyonu yapılmıştır. Buna göre, *T. thermophila* tarafından üretilen proteazlar nötr ve alkali pH'ta ve 37-45°C arasındaki sıcaklıkta aktiftir. Enzim aktivitesi ölçümlerinde kullanılacak pH ve sıcaklık, 7.5 ve 40°C olarak seçilmiştir. Selüloz içeren ortamda maksimum proteaz üretimi, endoglukanaz aktivitesinin düşük olduğu 3. günde görülmüştür. Ayrıca, maksimum endoglukanaz üretimi, proteaz aktivitesinin çok düştüğü 6. günde görülmüştür. Maksimum proteaz ve endoglukanaz üretiminin farklı günlerde olması, iki enzim arasında bir ilişki olabileceğini

göstermektedir. Bu hipotezi test etmek için, büyümenin 3., 6. ve 9. günü alınan kültür ortamları karıştırılarak, proteaz ve endoglukanaz aktiviteleri takip edilmiştir. Buna göre, proteaz aktivitesindeki artışa rağmen, proteazın hücredışı endoglukanaz aktivitesi üzerinde önemli bir etkisinin olmadığı görülmüştür. Bu çalışmalara ek olarak, *T. thermophila* tarafından avicel üzerinde üretilen proteazların türünü belirlemek amacıyla inhibisyon çalışmaları yapılmıştır. Phenyl methyl sulfonyl fluoride (PMSF), p-chloromercury benzoate (pCMB), antipain, ethylene diamine tetraacetate (EDTA) ve pepstatin A, inhibitör olarak denenmiştir. Proteazların, PMSF, pCMB ve antipain tarafından kısmen inhibe edilmesi göz önünde bulundurularak, *T. thermophila*'nın "serine" ve "cysteine (thiol)" proteaz ya da "thiol" grubu içeren "serine" proteaz ürettiği sonucuna varılmıştır.

Anahtar kelimeler: *Torula thermophila*, selülaz, endoglukanaz, proteaz.

To my daughter, Nur

## ACKNOWLEDGEMENTS

I want to express my deepest gratitude and respect to Assoc. Prof. Dr. Zümrüt ÖGEL for her valuable suggestions, advices and support in every step of this study.

I am grateful to Prof. Dr. Faruk BOZOĞLU for his valuable advices and suggestions for my thesis.

I would like to thank Betül SOPACI, Nazan ARİFOĞLU, Halil DÜNDAR and Tamay ŞEKER for their valuable guidance.

The technical assistance of Aytekin GÜLER, Elif DOĞAN and Fatma COŞKUN is gratefully acknowledged.

I also would like to acknowledge Suzan BİRAN, Ayşem BATUR, Şeyda AÇAR, Kıvanç YARANGÜMELİ and Banu EYÜBOĞLU for their help in the laboratory and Didem ZİNCİRKIRAN for her support and patience during the days of hard study.

I would like to thank my husband, Esam IFRIJ and my family for their support, encouragement and patiance. I also thank the babysitter of my daughter, Ayşe YİĞİT for helping me during the days of my hard study.

Finally, I want to thank to everyone for everything they have done for my thesis.

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| | |
|---|---|
| ( | Cellobiohydrolase |
| CMC | Carboxymethylcellulose |
| EDTA | Ethylene diamine tetraacetate |
| EG | Endoglucanase |
| HEC | Hydroxyethylcellulose |
| pCMB | p-chloromercuribenzoate |
| PMSF | Phenylmethylsulfonyl fluoride |
| TCA | Trichloroacetic acid |

# CHAPTER 1

# INTRODUCTION

## 1.1 Structure of cellulose

Chemically, cellulose is a glucose polymer in which 8,000-14,000 individual glucose units are joined by β-1,4 linkages (β-1,4-glucan). Cellulose often contains paracrystalline regions in which the molecules are arranged in fibrils, parallel groups of which form bundles (Fennama, 1976).

## 1.2 Cellulases

Cellulases are hydrolytic enzymes consisting of three general classes:

1.Endoglucanases (1,4-β-D-glucan-4-glucanohydrolase, endocellulase, EG, E.C.3.2.1.4) randomly cleaving β-1,4 glucosidic bonds on cellulose.

2.Exoglucanases (1,4-β-D-glucan-4-cellobiohydrolase, exocellulase, cellobiohydrolase, CBH, E.C. 3.2.1.91) releasing cellobiose units from the non-reducing ends of cellulose.

3.β-glucosidases (β-D-glucosido-glucohydrolase, cellobiase, E.C.3.2.1.21) hydrolysing cellobiose to glucose.

Further, the cellulases of fungi can be classified into two groups: One group consisting of enzymes with a single domain cellulolytic core, and the other group consisting of two-domain structures, in which the catalytic domain is bound to the cellulose binding domain (CBD) by a flexible, glycosylated linker peptide, usually composed of a short amino acid sequence rich in proline and hydroxyamino acids. Most of the fungal cellulases consist of the two-domain structure. The CBDs of the cellulase enzymes are required to target the catalytic domain to cellulose in order to achieve efficient hydrolysis.

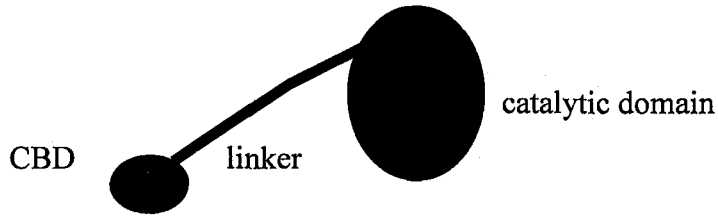


Fig. 1.1 Cellulase with a CBD

### 1.2.1 Endoglucanases

Endoglucanases (EGs) are generally defined as the enzymes that cleave randomly β-1,4-D-glucosidic linkages of the cellulose chain. These enzymes usually can not degrade crystalline cellulose (e.g.: Avicel), but can effectively hydrolyse substituted soluble cellulosic substrates (e.g.: CMC, HEC). Although many studies have been done, the exact role of EG in the degradation of native cellulose is not clearly understood.

The enzymes are generally numbered according to the time when they were first discovered. The International Enzyme Commission has recommended that such enzmes should be categorized according to their isoelectric points. In addition, Kyriacou *et al.* (1987), Henrissat *et al.* (1989) and Schülein (1997) have classified EGs into two groups: One group carrying a

cellulose binding domain (CBD) and the other group lacking the CBD. EGs with CBDs can bind to crystalline cellulose and even some of them can degrade it, while the EGs without CBDs do not show any activity towards crystalline cellulose, but are active on soluble derivatives of cellulose.

### 1.2.2 Exoglucanases

Exoglucanases, also known as cellobiohydrolases (CBHs) release cellobiose units from the non-reducing ends of the cellulose chain. They are active on swollen, partially degraded, amorphous cellulose and show limited or no activity on soluble derivatives of cellulose, such as carboxymethyl cellulose (CMC).

### 1.2.3 β-Glucosidases

β-Glucosidases act on β-linked diglucosides and aryl-β-glucosides. They do not act on cellulose, but they are regarded as components of the cellulase enzyme system because they stimulate the rate and extent of cellulose hydrolysis. They were found to prevent the inhibition of cellulases by cellobiose formed from the action of EGs and CBHs.

## 1.3 Applications of cellulases

Cellulolytic enzymes are used in food, feed, textile, detergent, medical, pharmaceutical, pulp and paper industries, and genetic studies. In food industry, cellulases are mainly used in starch processing, grain alcohol fermentation, malting and brewing, beverage production (clearing of fruit and vegetable juices), peeling of fruits, fruit candying, baking, degumming coffee

extracts, extraction of agar from seaweeds, gelatinization of seaweeds, isolation of proteins from soybean and coconut (Esterbauer *et al.*, 1991, Bhat and Bhat, 1997).

Cellulases are also important in animal feed production. They are used in silage production in order to obtain better hydrolysis of grain for monogastric animals, and thus, increase the digestibility of animal feed (Pentillä *et al.*, 1991).

In biotechnological processes, cellulases are applied to lignocellulosics and saccharification might be achieved. The product might be used in fermentation processes, such as the production of antibiotics. Pure preparations of cellulases are used in genetic engineering studies for the destruction of microbial / plant cell wall for protoplast formation (Esterbauer *et al.*, 1991).

Cellulases (especially EGs) are also used in textile industry for modifying cellulosic materials such as cotton or viscose in order to obtain the desired worn-outlook of the final product. The advantages of enzymatic treatment of textile fabrics against chemical or physical treatments are the strongly reduced tendency for pilling formation, clearer surface structure, improved water absorbancy and better process control (Bazin and Sasserod, 1991).

Cellulases are also used in detergent industry together with lipases and proteases for color brightening, softening and particulate soil removal (Bazin and Sasserod, 1991).

Cellulases are used in paper industry for the acceleration of pulping or for upgrading the pulp properties. Cellulase enzyme complex should be applied in order to avoid saccharification (Rho *et al.*, 1982).

### 1.4 Proteases

All enzymes that hydrolyze the peptide bonds are called as peptidases. Those enzymes that require the presence of an unsubstituted N- or C- terminus in the substrate are called as exopeptidases, and those that do not require such terminis are called endopeptidases. Proteases are equivalent to the term endopeptidase. They are able to hydrolyse macromolecular substrates. The secondary or tertiary structure of protein substrates prevents them from cleavage by exopeptidases. Exopeptidases usually cleave a single aminoacid, a dipeptide or a tripeptide from one terminus (Sterchi & Stöcker, 1999).

#### 1.4.1 Classification of proteases

Endopeptidases (proteases) may be classified in a number of ways such as on the basis of the pH range over which they are active (acid, neutral and alkaline), on the basis of their ability to hydrolyze specific proteins (keratinase, elastase, collagenase, etc.), or on the basis of their similarity to well-characterized proteases like pepsin, trypsin, chymotrypsin, or the mammalian cathepsins. The last classification is misleading if only a restricted number of properties are compared (North, 1982).

The most satisfactory classification is based on the catalytic mechanism. This forms the basis for the Enzyme Commission classification which groups the proteases in four categories according to their catalytic mechanisms. These are serine, cysteine, aspartic and metallo proteases. This division is based on reactivity toward inhibitors that react with specific moieties in the active site region. *Aspergillus* species produce proteases in each of the four divisions (Smith, 1993). In some species, only one type of protease has been reported, but in most species at least two and sometimes three types of proteases are produced, however all may not be produced under the same

culture conditions. Multiple forms of the same kind of protease may also be produced by the same organism. The same type of enzyme from different microorganisms show similarities, however fungal proteases have a broader specificity than equivalent mammalian enzymes (North, 1982).

#### 1.4.1.1 Serine proteases (EC 3.4.21)

Serine proteases form the biggest group of protein hydrolases in microorganisms (North, 1982) and are present in many *Aspergillus* species. They possess a reactive serine moiety in the active site and are inhibited by phenylmethylsulfonyl fluoride (PMSF) or diisopropyl fluorophosphate (DFP) (Smith, 1993). Many of them are also inhibited by some cysteine protease inhibitors such as p-chloromercurybenzoate. This may indicate that a cysteine residue is close to the active site. Binding of a bulky residue to this cysteine group may interfere with substrate binding. However this cysteine residue does not take place in the catalytic mechanism, and in fact many of the fungal serine proteases do not have any cysteine residues (North, 1982). Most of the serine proteases have maximal activity between pH 7.0 and 11.0 and broad substrate specificities and extensive esterase activity (Smith, 1993). The ability to produce serine alkaline proteases has been correlated with growth of fungi at neutral and alkaline pH (Matsushima, *et al.*, 1981).

#### 1.4.1.2 Cysteine (thiol) proteases (EC 3.4.22)

These enzymes are not widely distributed in fungi (North, 1982). An extracellular cysteine protease is secreted by *Aspergillus oryzae.* Most of these enzymes have maximal activity between pH 5.0 and 8.0 (Smith, 1993). They are sensitive to sulphydryl reagents, like p-chloromercuribenzoate (pCMB), however this reagent also inhibits some serine proteases; therefore its sensitivity to additional inhibitors such as iodoacetate should be proved to be

sure that an enzyme is a cysteine protease. Also, its activity can be enhanced by reducing agents such as cysteine and dithiothreitol and possibly EDTA (North, 1982).

### 1.4.1.3 Aspartic proteases (EC 3.4.23)

The second important industrial protease after serine proteases are the rennet enzymes used in cheese-making. They are essentially acid proteases with an aspartic acid moiety as a key catalytic element in the active site. The enzymes have maximal activity between pH 3.0–4.0. They occur widely in fungi and mostly unstable above pH 7.0. They are specific for aromatic amino acid residues on each side of the cleavage point (Smith, 1993). Extracellular pepsin-type aspartic proteases are found in *Aspergillus* species and other molds (North, 1982). They are used in soybean protein hydrolysis in the preparation of soy sauce. *Aspergillus candidus* produces an extracellular rennin-type protease (Smith, 1993).

Fungal aspartic proteases are usually able to hydrolyze a range of native proteins, but most of them have little or no activity on small synthetic substrates. Those types of proteases often acidify the medium in which they are produced. Since many of them are unstable above neutral pH, they are not found in cultures growing at neutral or alkaline pH (North, 1982).

### 1.4.1.4 Metalloproteases (EC 3.4.24)

The maximal activity of metalloproteases occurs between pH 5 and 9, and they all respond to chelating agents such as EDTA but are not sensitive to inhibitors that affect serine proteases or sulphydryl reagents. Zinc, cobalt, calcium, or manganese reactivates EDTA-inactivated enzymes. Most metallo

proteases are zinc containing enzymes, and protein structure is stabilized by the presence of calcium. The most outstanding enzyme in this class is probably thermolysin- the extracellular metalloprotease of *Bacillus thermoproteolyticus* (Smith, 1993). Only a few examples of metalloproteases have been reported in fungi, and most of them have been shown to be zinc-containing enzymes (North, 1982).

### 1.4.2 Regulation of protease synthesis

The effect of different substrates, activating proteins and medium conditions on the production and activity of different proteolytic enzymes has been studied on several microorganisms. The synthesis of most extracellular proteases takes place by two general patterns (Law, 1980). In some cases, amino acids or peptides that are supplied in the medium or generated by proteolytic activity act as an inducer. In these cases, protease may be produced irrespective of the growth stage as soon as the inducer is present. In other cases, growth under suboptimal conditions, such as growth in a nutritionally poor medium or in the post-exponential phase of growth in a rich medium, may induce protease production (Bascarán, *et al.*, 1990). Regulation of protease synthesis and secretion has been studied in many fungi.

#### 1.4.2.1 Effect of carbon source

Protease biosynthesis requires both catabolic derepression and substrate induction (Castro, *et al.*, 1991). Avicel (microcrystalline cellulose) mostly acts as an inducer for protease production, whereas different behaviors are observed in the presence of glucose for different microorganisms.

A 36-kDa protease from *Streptomyces reticuli* was examined for the optimal conditions in which it is produced. Protease activity was highest in the presence of avicel and xylan, whereas no detectable amounts of protease was produced in the presence of glucose or glycerol (Moorman, *et al.*, 1993).

Glucose also repressed the production of proteases by *Clostridium sporogenes*, indicating that protease production is a response to nutrient limitation (Allison & Macfarlane, 1992).

Extracellular carboxyl protease secretion by *Rhizopus oligosporus* is repressed by low molecular mass carbon sources. When the growing cells of *R. oligosporus* were transferred into the carbon-free medium from minimal medium, protease secretion was derepressed and protease secretion started 5 hours after transfer (Farley & Ikasarı, 1992).

The protease production of *Fusarium oxysporum* in five kinds of liquid culture media were compared. It was concluded that the activity of extracellular proteases of *F. oxysporum* involves metabolite repression by glucose, glycerol and lactic acid, but not lactose (Castro, *et al.*, 1991).

In contrast to the findings above, glucose acts as an inducer for protease synthesis by some other microorganisms. The effects of glucose and nitrogen concentrations on the growth and enzyme biosynthesis of immobilized cells of *Humicola lutea* was studied. It was found that the cells require high glucose concentrations for repeated production of proteases and it was indicated that glucose concentration below 30 g/l could reduce or stop enzyme production (Angelova & Petricheva, 1997).

The metabolic requirements of extracellular protease induction by *Candida albicans* were investigated. For that purpose, exponential phase cells were transferred into extracellular protease induction medium without BSA,

without glucose, or lacking both, and incubated for 2 h. Little amount of extracellular protease was detected when either glucose or both BSA and glucose were omitted. This suggests that the presence of glucose in the growth medium also induces extracellular protease by *C. albicans* (Homma, *et al.*, 1993).

### 1.4.2.2 Effect of nitrogen source

The effect of nitrogen concentration on the growth and enzyme biosynthesis of immobilized *Humicola lutea* cells were studied. It was found that the immobilized *H. lutea* cells requires high nitrogen concentrations for repeated acid protease synthesis. Nitrogen-free medium reduces or stops the enzyme production (Angelova & Petricheva, 1997).

The effect of the nature of nitrogen source on the production of extracellular proteases by *Streptomyces clavuligerus* was studied by using free amino acids and different nitrogen sources were examined. It was found that the nature of nitrogen source strongly affects the production of extracellular proteases by *Streptomyces clavuligerus*. Production is stimulated by low concentrations of casamino acids or yeast extract. Addition of ammonium into the culture medium as a sole nitrogen source or together with amino acids interferes with protease production (Bascarán, *et al.*, 1990).

The nutritional determinants of protease secretion by *Clostridium sporogenes* were studied. The protease production by *Clostridium sporogenes* was repressed by ammonia and peptides (Allison & Macfarlane, 1992). This contrasts with the protease production by *Clostridium perfringens*, which is stimulated during growth in the presence of high concentrations of peptone and is not affected by ammonia (Allison & Macfarlane, 1989).

In *Aspergillus nidulans*, protease secretion is induced in the case of limited nitrogen, carbon and sulphur. In *Neurospora crassa* (North, 1982) and *Mucor miehei* (Lasure, 1980), protease secretion requires the lack of nitrogen, carbon or sulphur and is induced by exogenous protein. Protease production by *Agaricus bisporus*, *Coprinus cinereus* and *Volvariella volvacea* was also induced by exogenous protein. In these fungi, induction by protein takes place even in the case of repression by nitrogen, sulphur or carbon (Kalisz *et al.*, 1987). The protease secretion of the fungus *Hymenoscyphus ericae* is neither repressed by ammonium or other nitrogen sources nor dependent on induction by exogenous protein (Leake & Read, 1990). Extracellular carboxyl protease by *Rhizopus oligosporus* is repressed in the presence of low-molecular-mass nitrogen source. The control takes place at the level of transcription. When the growing cells are transferred to the nitrogen-free medium, protease secretion started after two hours of transfer (Farley & Ikasari, 1992).

Effect of free amino acids in the production of extracellular proteases by *Candida albicans* was also studied. Protease production was repressed in a medium containing more than about 12.5 mM amino acids. The concentration of amino acids required for repression is actually lower because some of the amino acids added into the medium are consumed and their concentration decreases during growth. High concentrations of other low-molecular-mass nitrogen sources activated protease production but excess nitrogen source may repress extracellular protease induction (Homma *et al.*, 1993).

Lerner & Goldman (1993) have examined the effect of different macromolecules on protease production by *Candida albicans*, using a chemically defined nitrogen-limited medium. Bovine serum albumin (BSA) and collagen induced protease production. Homopolymers of both poly-L- and poly-D-glutamate also induced protease activity. They have found that protease production is generally induced by peptides of 8 or more residues in length. They suggest that protease induction occurs via a sensory mechanism

which requires both side- and main chain interactions with the inducing polypeptide.

### 1.4.2.3 Effect of pH

Studies on proteases produced by several microorganisms show that pH could be an important factor for the production and secretion of those enzymes. However, little is known about the control mechanism of gene expression by the medium pH.

*Aspergillus niger* extracellular acidic proteases are shown to be regulated by pH at the level of mRNA content (Jarai & Buxton, 1994). In *Aspergillus nidulans*, some acid- and alkaline-expressed genes are regulated by environmental pH with a PacC zinc finger transcription factor (Tilburn *et al*, 1995). In *Candida albicans*, pH has been shown to regulate mRNA levels of a secreted aspartyl protease, SAP2 (Hube *et al*, 1994).

When the pH-regulated expression of the acid and alkaline extracellular proteases of *Yarrowia lipolytica* were analysed, it was found that these proteases are regulated by environmental pH at the level of mRNA content. Acid extracellular protease expression did not take place above pH 7.0, whereas alkaline extracellular protease expression occurred to some extent at all pH values (Glover, *et al.*, 1997).

*Acremonium persicinum* released high levels of protease into the culture fluid during growth at pH 7 or above. The proteolytic inactivation of an extracellular 1,3-β-glucanase produced by the fungus is prevented by growing the fungus at acidic conditions. The highest activities of the protease were detected at high medium pH (Pitson *et al.*, 1996).

The effect of substrate and pH on the proteolytic activity of *Fusarium oxysporum* var. *lini* was determined. With glucose or glycerol and lactic acid as the carbon source, pH 5.0 and 7.0 was optimum respectively. pH 7.0 to 9.0 was leading the maximum proteolytic activity when gelatin and lactose were used. The irreversible inhibition of protease at different pH values was thought to be a consequence of the carbon source used in the medium. Glucose and glycerol causes acidification of the medium, whereas gelatin and lactose causes alkalinization. A direct effect of pH on production and secretion of proteases could not be determined (Castro *et al.*, 1991).

### 1.4.3 Effect of inhibitors on protease activity

Proteases are classified as serine, cysteine, aspartic and metallo proteases. Each class of protease is inhibited by specific inhibitors (Table 1.1). The effect of these inhibitors on proteases are mostly examined to determine the type of protease.

A protease from *Streptomyces reticuli* was inhibited by 1,10-phenanthroline and EDTA, indicating that the protease is a metalloprotease, however no metal ions were detected to be associated with the protein. Alpha-2- macroglobulin also inhibited the proteolytic activity indicating en endo-mode of cleavage (Moorman, *et al.*, 1993).

Extracellular proteases of *Trichomonas vaginalis* were determined to be of cysteine type proteases. They were inhibited by N-ethyl maleimide, $Cu^{2+}$, antipain, N-tosyl-L-phenylalanine chloromethyl ketone, N-tosyl-L-lysine chloromethyl ketone, leupeptin, chymostatin, and iodoacetamide. They were enhanced by cysteine, EDTA, and dithiothreitol, which is also a characteristic of cysteine proteases (Garber & Lemchuk-Favel, 1989).

Table 1.1 Specific inhibitors of proteases

| **Type of protease** | **Inhibitor** |
|---|---|
| Serine proteases | Phenylmethylsulfonyl fluoride (PMSF), diisopropyl fluorophosphate (DFP) |
| Cysteine proteases | p-chloromercuribenzoate, N-ethyl maleimide, leupeptin, antipain, chymostatin, $Cu^{2+}$ |
| Aspartic proteases | Pepstain A |
| Metalloproteases | EDTA, 0-phenanthroline, cysteine |

Six extracellular proteases secreted by *Clostridium sporogenes* were characterized. Protease activity was strongly inhibited by EDTA, indicating that they are metalloproteases. Divalent metal ions and mercaptoethanol stimulated protease activity. Some inhibition was also observed with PMSF and thimerosal, suggesting the presence of serine and cysteine proteases (Allison & Macfarlane, 1992).

Several inhibitors were tested for their effects on microbial proteases. The activity was monitored by the analysis of cleaved peptide bonds in SDS-polyacrylamide gel electrophoresis. Casein was used as a substrate. Fungal protease (type XIX from *Aspergillus sojae*) was inhibited by PMSF, chymostatin, antipain and leupeptin. Casein is a mixture of polypeptides which exhibited three major peptide bands. The fungal proteolysis of the first two uppermost polypeptides of casein was inhibited by PMSF. The proteolysis of the first and third polypeptides of casein was inhibited by chymostatin and antipain. The proteolysis of all three peptides of casein was inhibited by leupeptin. Aprotinin, pepstatin, phosphatidyl serine and phosphatidyl choline were also tested but had no significant effect on fungal proteolysis of casein.

Bacterial protease (type XXVI from *Serratia* species) was inhibited by phosphatidyl glycerol, phosphatidyl inositol and sphingosine (Kuo, *et al.*, 1990).

### 1.4.4 Proteolytic cleavage of cellulases

Proteolytic cleavage of peptide bonds is one of the modification ways of proteins. The ability of proteolytic enzymes to carry out selective modification of proteins by limited cleavage means that some proteases have a regulatory function (North, 1982). Proteolytic enzymes have a high potential in the study of the structural and functional properties of proteins. The processing of cellulases by proteases to enzymatically active truncated forms from several microorganisms has been reported. This indicates that proteolysis might be an important source of multiple enzyme forms which may still be active or completely inactive after cleavage.

The in-vitro cleavage of cellobiohydrolase I (CBH I, 65 kDa) from *Trichoderma reesei* by papain led to a core protein (56 kDa) which is fully active against small, insoluble substrates such as the chromophoric glycosides derived from the cellodextrins and lactose. However, activity against an insoluble substrate, such as Avicel is completely lost and adsorption onto this microcrystalline cellulose is decreased (Tilbeurgh, *et al.*, 1986).

An 82-kDa cellulase, which can degrade Avicel (microcrystalline cellulose), water soluble derivatives like carboxymethylcellulose (CMC) or hydroxyethylcellulose (HEC) , and the cellodextrins p-nitrophenyl-cellobioside and xylan has been purified from *Streptomyces reticuli* to homogeneity. A 42-kDa enzyme with similar enzymatic properties was also identified during later stages of growth. It has been shown that it had been generated from the 82-kDa enzyme in vivo by proteolysis. A 36-kDa protease had been shown to be

responsible for the in vivo and in vitro processing of the 82-kDa cellulase Cel-1 from the microorganism to a 42-kDa truncated enzyme. The *in vitro* cleavage of the large cellulase to a 42-kDa truncated form which retained cellulolytic activity and a 40-kDa enzymatically inactive protein has been demonstrated after the isolation of the 36-kDa protease from the culture filtrate. The 42-kDa cellulase from *S. reticuli* showed higher activity against p-nitrophenyl-β-D cellobioside (Moormann, *et al.*, 1993).

The filamentous fungus *Acremonium persicinum* secretes three 1-3-β-glucanases, a 1-6-β-glucanase and a β-glucosidase. The fungus also produces high levels of protease enzyme during growth at pH 7 or above. This protease inactivates one of the three extracellular 1-3-β-glucanases, but the activities of the other two 1-3-β-glucanases is not affected. Lower proteolytic activity is seen when *A. persicinum* was grown in acidic conditions and no 1-3-β-glucanase inactivation was detected (Pitson, *et al.*, 1996).

Crude preparations of cellulase and xylanase from *Cellulomonas flavigena* stored at 4°C has shown a rapid loss of activity. A variety of protease inhibitors were added to crude enzyme preparations and cellulase and xylanase activity were determined in order to determine if this loss of activity was due to proteolytic activity. The inactivation was reduced with the protease inhibitors aprotinin and α-2 macroglobulin (Whelan, *et al.*, 1989).

A protease-negative *Humicola grisea* var. *thermoidea* mutant was isolated. The protease negative mutant has shown higher endoglucanase activity than the parent strain in mold bran culture at 50°C for 4 days (Hayashida & Mo, 1986).

The total carboxymethyl-cellulase activity of the culture supernatant from *Cellulomonas fimi* remained constant during several months of storage at 4°C, but there were changes in the protein and activity profiles, both process

involving the conversion of slow-moving or fast-moving components. These changes were prevented by the addition of PMSF, inhibitor of serine proteases, which indicates that they were at least partly caused by protease activity (Langsford, *et al.*, 1984).

An extracellular protease from *Cellulomonas fimi* has been shown to cleave an endo-β-1,4-glucanase (Cen A) and an exo-β-1,4-glucanase (Cex) from the same microorganism. The process takes place in vivo and in a highly specific manner. The fragments produced as a result of proteolytic cleavage still retained activity but were less active against microcrystalline cellulose implying that the cellulose binding domain is cleaved by proteases (Gilkes, *et al.*, 1988).

### 1.5 Thermophilic fungi

The commonly accepted definitions of thermophilic and thermotolerent fungi are those of Cooney & Emerson (1964). Thermophilic fungi are those that have a growth temperature maximum at 45-50°C or above and a temperature minimum of 20°C or higher.

Enzymes from thermophilic organisms are supposed to be more thermostable from those produced by mesophilic organisms. Therefore nowadays, there is a trend through studying cellulases from thermophilic fungal species. The fermentation conditions of thermophilic fungi are also more adventageous than those of mesophilic ones. The higher cultivation temperatures reduce the risk of contamination by other microorganims. Since sterilization is not an economic process on an industrial scale, higher cultivation temperatures are adventageous. Therefore, the use of thermophilic fungi, with efficient thermostable enzymes, high rates of hydrolysis and ability

to saccharify under non-aseptic conditions, might make the hydrolysis of cellulose more economic (Latif *et al.*, 1995).

### 1.6 *Torula thermophila (=Scytalidium thermophilum)*

*Torula thermophila* is a thermophilic fungus isolated in 1950 from straw. Isolation of this fungus from mushroom compost was made by Ögel, *et al.* (1998). *Torula thermophila* can grow within a wide temperature range of 23-58°C (Cooney and Emerson, 1964).

In previous studies, an intergradation was thought to exist between the isolates of *Torula* and *Humicola* strains. Straatsma and Samson (1993) examined more than 30 thermophilic isolates in the *Torula-Humicola* complex morphologically in order to clarify the taxonomy and nomenclature. They defined *T. thermophila* as a type 2 culture, *H. grisea* var. *thermoidea* as a type 1 culture and *H. insolens* as an intermediate type culture of *Scytalidium thermophilum*. However, the characterization of type cultures of *S. thermophilum* complex still remains unclear and the isolates are recognized according to the morphological studies (Lyons & Sharma, 1998).

### 1.7 Aim of the study

In previous studies, production of cellulases *by T. thermophila* was followed at 45°C on Avicel (microcrystalline cellulose) as the main carbon source. It was shown that cellulase activity rapidly decreases after the 6$^{th}$ day of cultivation (Arifoğlu, 1999). In other studies, cellulase production of *T. thermophila* on several lignocellulosic substrates were compared and a sharp decrease in cellulase activity was again observed, following maximal levels of activity (Dündar, 1999). This rapid decrease in cellulase activities may be

related to the degradation of the enzymes by extracellular proteases. As opposed to their possible negative effect on cellulase activities, some proteases are also suggested to enhance cellulase activities by, possibly, activating a zymogenic form of the enzymes (Moormann, *et al.*, 1993; Eriksson & Pettersson, 1982). All these studies indicate that proteases have important effects on cellulase activities.

This study was conducted in order to determine the level and time course of protease production by *T. thermophila* on microcrystalline cellulose (Avicel), to analyze the effect of extracellular proteases on cellulase activities, as well as to establish the type of those proteases secreted under the specified conditions.

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1 Materials

*Torula thermophila* strain 3A was isolated from mushroom compost (Ögel et al., 1998). Avicel (Microcrystalline cellulose for column chromotography) (Sigma), CMC (carboxymethyl cellulose) (Sigma), Azoalbumin (Sigma) and all other chemicals were of analytical grade.

## 2.2 Methods

### 2.2.1 Maintenance and cultivation of strains

Stock cultures of *T. thermophila* were grown on YpSs agar slants at 45°C for 2-3 days, and maintained at room temperature. Stock cultures were subcultured every month. The composition of YpSs agar was: Difco powdered yeast extract, 4.0 g; $K_2HPO_4$, 1.0 g; $MgSO_4.7H_2O$, 0.5; soluble starch, 15.0g; agar, 20.0 g in 1 L distilled water (Cooney and Emerson, 1964).

Shake-flask culture experiments were carried out with 1 L of fermentation medium in 2 L Erlenmeyer flasks and 250 ml of fermentation medium in 500 ml Erlenmeyer flasks. The fermentation medium consists of

modified YpSs medium with 2% microcrystalline cellulose (Avicel) as the carbon source instead of starch. When grass clippings and apple pomace were used as the carbon source, starch was replaced with 2% of lignocellulosic compounds. The pH of the fermentation medium was adjusted to the desired values by 0.1 M $H_3PO_4$ or 0.1 M KOH in the initial pH effect experiments. Initial spore load was 3 mg/L main culture. Spores were suspended in sterile distilled water and were used to inoculate 20 ml of preculture medium in 50 ml Erlenmeyer flasks for 1 L cultures and 0.75 mg spores were used to inoculate 5 ml of preculture medium in 50 ml Erlenmeyer flasks for 250 ml cultures. The precultures consisted of YpSs medium containing 1% glucose as the carbon source, instead of starch. Precultures were incubated for 24 hours at 45°C which was determined as the optimum temperature for EG production (Arifoğlu, 1999), and then used to inoculate the main cultures of 1 L or 250 ml. The cultures were incubated in a shaker incubator at 155 rpm at 45°C. The enzyme activity measurements were carried out every 24 hours starting from the $2^{nd}$ until the $7^{th}$ or $9^{th}$ day of incubation.

Samples were removed every 24 hours and centrifuged at 3000 rpm for 20 min. The supernatant obtained was used for the measurement of both protease and EG activities and for pH determination.

### 2.2.2 Cellulase activity measurements

The methods of Somogyi-Nelson based on the measurement of reducing sugar content was used for the determination of endoglucanase and Avicel-adsorbable endoglucanase activities (Wood & Bhat, 1988).

### 2.2.2.1 Endoglucanase activity measurements

Endoglucanase activity was used by using carboxymethyl cellulose (CMC) as the substrate. 1 ml of CMC solution (1 % (w/v) in water) was mixed in the test tubes with 0.5 ml of 25 mM sodium acetate buffer at pH 6.0. The contents of the tubes were heated to 65°C in water bath. Then, 0.5 ml of appropriately diluted supernatant (enzyme solution) was added. Dilutions ranged from 1/2 to 1/ 25. After incubating at 65°C for 30 min., the enzymatic reaction was stopped by the addition of 2 ml of a mixture of Somogyi I and Somogyi II reagents (Appendix B). The tubes were immediately placed into boiling water bath and kept for 15 min. After cooling the tubes to room temperature, 2 ml of Nelson reagent (Appendix B) was added into each tube and the contents were immediately mixed on a Vortex mixer. 4 ml of distilled water was added into the tubes and mixed again. The contents of the tubes were transferred into 50 ml Falcon tubes, and centrifugated at 5000 rpm for 10 min to remove any remaining insolubles. Optical densities were measued at 520 nm against a reagent blank prepared by using buffer instead of the culture supernatant (Wood and Bhat, 1988). The absorbance values were used to calculate the enzyme activities by using a glucose standard curve prepared prior to the experiments (Appendix A). The experiments were carried out in duplicates and the results were taken as the mean of the two data. One unit of cellulase activity was defined as the amount of enzyme required to release 1 μmol glucose equivalents per minute under the specified assay conditions. Therefore, the enzyme activity per ml supernatant was determined by using the following equation:

$$U/ml = (X.D) / (M.t)0.5$$

where X is the amount of reducing sugar in terms of μg of glucose, D the dilution factor, M the molecular weight of glucose (180 g/mol), t the time of

incubation at assay conditions (30 min.) and 0.5 is the amount of culture supernatant (enzyme solution) used in the assay.

#### 2.2.2.2 Avicel-adsorbable endoglucanase activity measurements

The endoglucanases that can bind and adsorb on avicel are called avicel-adsorbable endoglucanases. The activity of avicel-adsorbable endoglucanases are determined indirectly by substracting the activity of the endoglucanases that can not bind to avicel from the total endoglucanase activity. 5 ml of 25 mM pH 6.0 sodium acetate buffer and 1.0 g Avicel were mixed in a 50 ml Falcon tube and cooled to 4°C. 5 ml of culture supernatant was added to the prepared solution, mixed and kept at 4°C for 15 min., the tubes being placed horizontally (Ali et al., 1993). After centrifugation, the reducing sugar content in the supernatant was measured (as explained in Sec. 2.2.3.1) and the activity of Avicel-nonadsorbable endoglucanase was calculated as follows:

$$U/ml = (X.D_1.D_2)/(M.t)0.5$$

where X, M and t are as explained in Sec. 2.2.3.1, $D_1$ is the dilution factor and $D_2$ is the dilution caused by mixing 5 ml sodium acetate buffer and 5 ml of culture supernatant prior to cooling, which is 2. Alternatively, avicel-adsorbable activity can be measured as follows: 5 ml of 25 mM pH 6.0 sodium acetate buffer and 1.0 g Avicel were mixed in a 50 ml Falcon tube and cooled to 4°C. 5 ml of culture supernatant was added to the prepared solution, mixed and kept at 4°C for 15 min., the tubes being placed horizontally. After centrifugation, 1 ml is taken from the supernatant and mixed with 1 ml of CMC solution, the rest of the procedure being the same. In another tube, the same procedure is carried out without the addition of avicel for the determination of total endoglucanase activity.

Avicel-adsorbable endogucanase activity was determined by subtracting the activity of avicel-nonadsorbable endoglucanase from the activity of total endoglucanase. The definition of one unit of enzyme activity was again defined as the amount of enzyme that liberated 1 μmol of glucose equivalents per minute per ml of culture supernatant under the assay conditions.

### 2.2.3 Protease activity measurements

Azoalbumin, a chromogenic, water-soluble substrate for proteases was used for the determination of protease activities. 1.5 ml of 50 mM pH 7.5 phosphate buffer was mixed with 0.5 ml of culture supernatant (enzyme solution) in test-tubes and incubated at 40°C for 5 min. Further, 1 ml of pre-warmed azoalbumin solution (12.5 mg/ml) was added. After incubating the mixture at 40°C for 60 min, 2 ml of 10 % (w/v) trichloroacetic acid (TCA) was added in order to stop the enzymatic reaction. The undigested protein which was precipitated by the addition of TCA was removed by centrifugating the mixture at 5000 rpm for 10 min. Two ml of supernatant was neutralized with an equal volume of 0.5 N NaOH. The released colour was quantified as absorbance at 400 nm.

One unit of protease activity was defined as the dye release of 0.1 OD absorbance per hour. Therefore the protease activity per ml supernatant was determined by using the following equation:

$$U/ml = OD_{400} /(0.1)(0.5)$$

### 2.2.4 Optimization of assay time for protese activity

The incubation time of the culture supernatant, protein substrate (Azoalbumin) mixture was varied from 15 to 120 min., with 15 min. increments. The assay pH and temperature were kept constant at 7.0 and 37°C.

### 2.2.5 Optimization of assay pH for protease activity

In order to find the effect of assay pH on protease activity, pH of assay medium was altered from 4.0 to 9.0 with 1 increments until pH 6, and with 0.5 increments above pH 6. From pH 4.0 to 6.5, 50 mM sodium acetate buffer was used and from 7.0 to 9.0, 50 mM potassium phosphate buffer was used. The assay temperature and the assay incubation time were kept constant at 37°C and 60 min.

### 2.2.6 Optimization of assay temperature for protease activity

In protease assays, incubation temperature was varied from 30 to 55°C, with 5°C increments. Meanwhile, the pH and duration of the assays were kept constant at 7.5 and 60 min, respectively.

Percent relative activity was determined in the experiments for the effects of assay pH, temperature and incubation time by taking the maximum value as 100 % and all other values were calculated as the percentage of the maximum activity of the given set of experimental data.

### 2.2.7 Effect of inhibitors on protease activity

The inhibitors were added into the phosphate buffer used in protease activity assay, except PMSF. PMSF was dissolved in buffer containing 25 % (v/v) ethanol. The control of the assay with PMSF was carried out by using buffer with ethanol at the same concentration, without the addition of PMSF. PMSF, pCMB and EDTA were added in 5 mM concentrations, antipain and pepstatin A were added in 50 μg/ml. The culture supernatant was preincubated with the inhibitors dissolved in the buffer solution, and protease activity was determined.

### 2.2.8 Effect of initial medium pH on protease activity

The initial pH of the medium was varied from 7 to 8.5, with 0.5 increments. The initial pH was adjusted to the desired value by adding 0.1 M KOH or $H_3PO_4$ into the growth medium.

# CHAPTER 3

# RESULTS AND DISCUSSION

The aim of this study was to analyze the extracellular protease production by *T. thermophila* and to investigate its influence on cellulase production, with emphasis on endoglucanase activity. It was further aimed to determine the type of protease(s) secreted into the medium during growth on Avicel (microcrystalline cellulose) as the carbon source. Protease and endoglucanase (EG) activities, together with medium pH were followed with respect to time during the cultivation of the microorganisms. Parameters considered for optimum protease assay conditions were pH, temperature and incubation time. The inhibitors tested for protease activity were phenyl methyl sulphonyl fluoride (PMSF), antipain, p-chloromercuribenzoate (pCMB), ethylene diamine tetraacetate (EDTA) and pepstatin A.

## 3.1 Optimization of protease assay conditions

Proteases produced by different microorganisms differ in their requirements for conditions to show maximum activity. Each type of protease has different pH and temperature optima. The activation and inactivation time also varies for different types of proteases. Therefore, it was required to determine the optimum conditions for the proteases produced by *T. thermophila.*

### 3.1.1 Optimization of assay time

The optimum assay incubation time was determined by growing the fungus at 45°C and 155 rpm on Avicel as the carbon source and carrying out protease assay at different assay incubation times. Assay temperature and pH were kept constant at 37°C and 7.0, respectively. Protease activity was high between the assay incubation times of 60 and 90 min (Fig. 3.1). Optimum assay incubation time was selected as 60 min where 100% relative activity was observed for 3 days, although the differences were very little between 60, 75 and 90 min (Fig. 3.2).

In the literature, data for the optimization of assay incubation time was not shown by any researcher. Nevertheless, the incubation time depends on the type of substrate used in the assay and the capability of the proteases to cleave the substrate in a short time. The incubation times used in the assays for fungal proteases were 4 min for *Rhizopus oligosporus* extracellular carboxyl protease using BSA (Farley & Ikasari, 1992), 15 min for proteases from *Rhizopus* SMC strain using casein (Ramamurthy & Kothari, 1993), 12 hours for proteases from *Fusarium oxysporum* var. *lini* using azocasein (Castro, *et al.*, 1991), 20 min for *Acremonium persicinum* proteases using casein (Pitson, *et al.*, 1996), and 10-30 min for different strains of Trichoderma using azoalbumin, azocasein and azocoll (Mischke, 1996).

Incubation time also may depend on the assay temperature. If the assay temperature is high, proteases or the protease substrate may be denatured if they are incubated for extended periods.

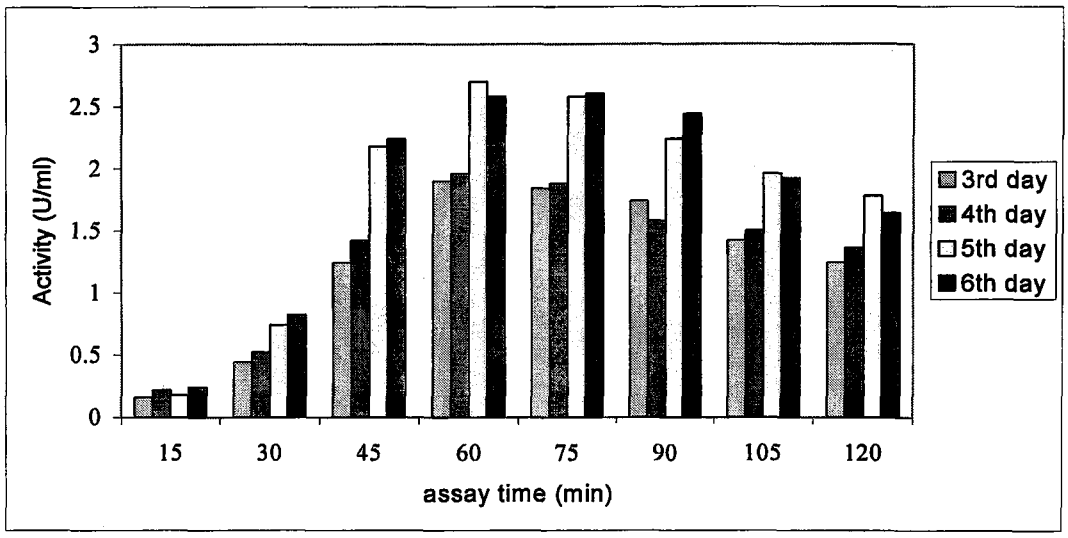


Figure 3.1 Effect of assay time on protease activity of *T. thermophila* at 37°C and pH 7.0.

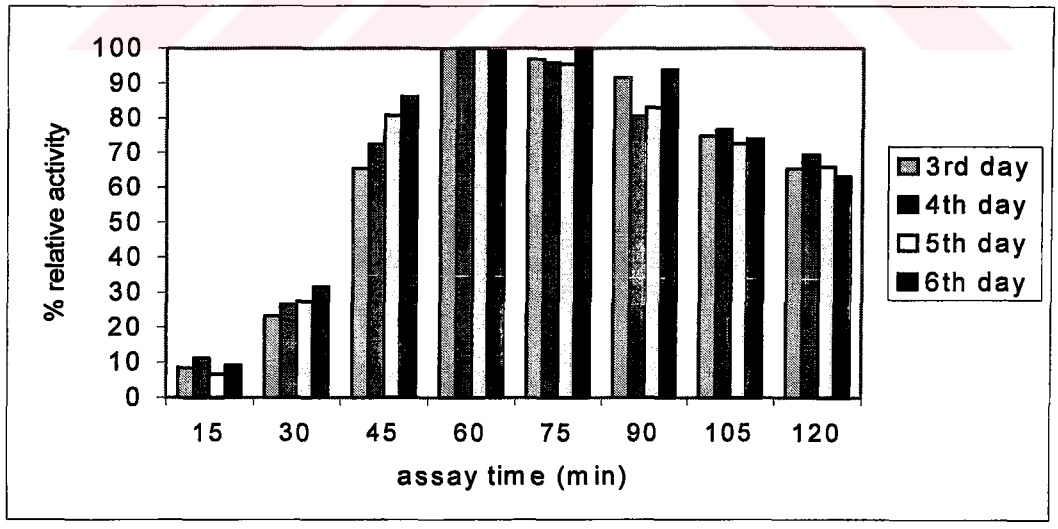


Figure 3.2 Relative effect of assay time on protease activity of *T. thermophila* at 37°C and pH 7.0.

### 3.1.2 Optimization of assay pH

The effect of assay pH on the protease activity of *T. thermophila* was analyzed at the pH range of 4.0-9.0. The enzyme activity was measured at 37°C and the incubation time for the enzymatic reaction was kept constant at 60 min. Like all enzymes, the activity of proteases is greatly affected by pH. In order to determine the pH at which the enzyme shows maximum activity, the fungus was grown at 45°C and 155 rpm on Avicel as the carbon source and the protease activities were determined at different assay pH. Fig. 3.3 and 3.4 indicate that the optimum pH for protease activity is 7.5, however the protease activity is high from pH 6.5 to 9.0. The results indicate that the proteases produced by *T. thermophila,* under the specified growth conditions, are active at neutral and alkaline pH. This may be related to the medium pH which ranges from 6.66 to 7.52 during the cultivation, so that only the neutral or alkaline proteases are produced (Fig. 3.9).

The differences between the % relative activities on different days of incubation might be due to the synthesis of different types of proteases on different days of cultivation. The fungus *Acremonium persicinum* has been found to produce acid proteases with optimum pH 5.0, neutral proteases with optimum pH 7.0 and alkaline proteases with optimum pH 9.0 (Pitson, *et al.*, 1996). *Fusarium oxysporium* var. *lini* also produces both acid and alkaline proteases, that are active at pH 4.0 and 8.0, respectively (Castro, *et al.*, 1991). The proteases of *Yarrowia lipolytica* have been shown to be active at pH 3.2, which are acid proteases, and pH 7.5, which are alkaline proteases (Glover, *et al.*, 1997). *Torula thermophila* seems to produce neutral and alkaline proteases, under the specified growth conditions, with alkaline proteases being dominant mainly on the $5^{th}$ and $6^{th}$ days of incubation.

The assay pH for the other fungal proteases were 3.5 for *Rhizopus* SMC strain (Ramamurthy & Kothari, 1993), 3.0 for *Humicola lutea* 120-5 (Angelova

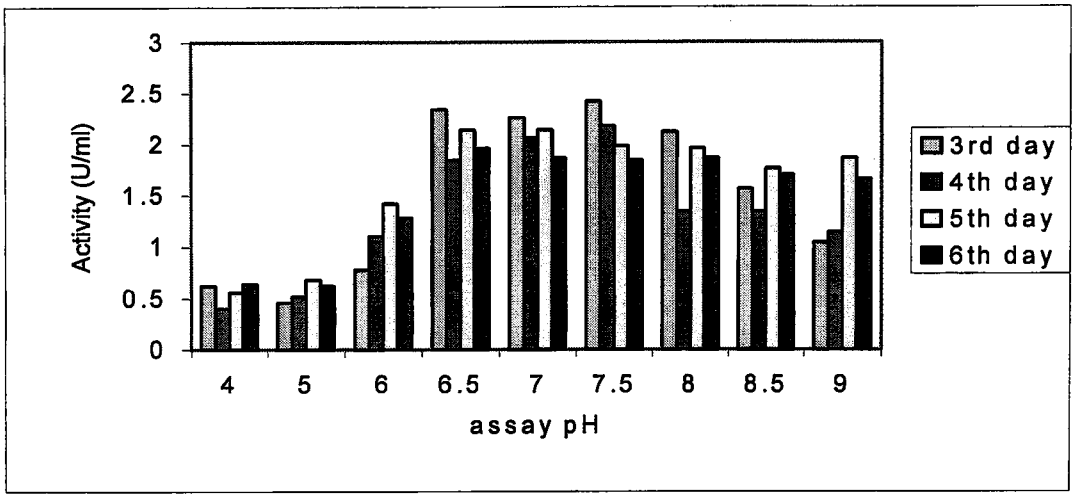


Figure 3.3 Effect of assay pH on protease activity of *T. thermophila* at 37°C and assay incubation time of 60 min.

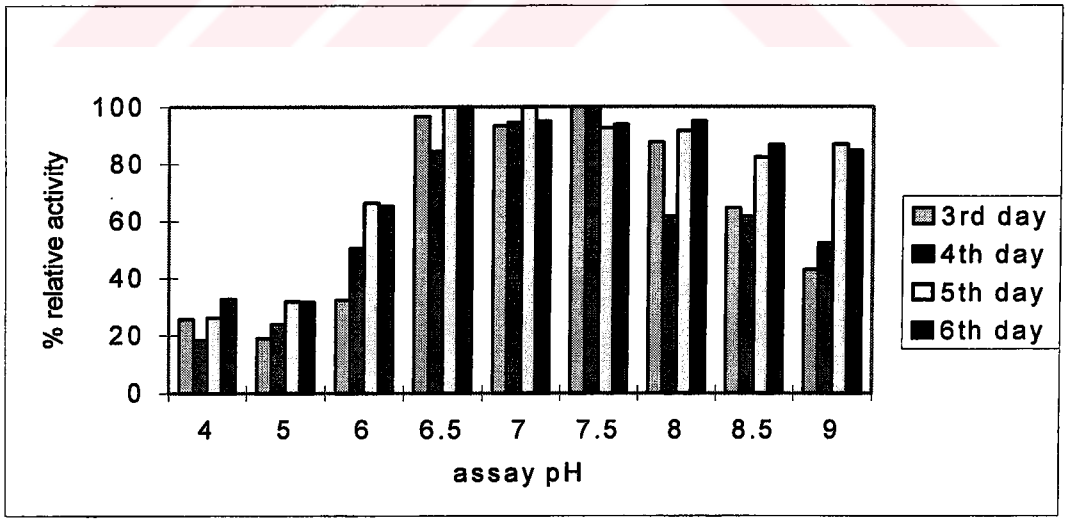


Figure 3.4 Relative effect of assay pH on protease activity of *T. thermophila* at 37°C and 60 min of assay incubation time.

& Petricheva, 1997) and 3.3 for *Rhizopus oligosporus* (Farley & Ikasari, 1992), which are acid proteases. The protease activity of *Trichoderma virens* strain GI-21 was found to be constant at and above pH 7.0, but decreased at lower pH. Protease activity from strains TRI-4 and T-1-R4 from the same fungus was optimum at pH 6.5-7.5 (Mischke, 1996), which are close to the pH optimum of *T. thermophila*. Thus, acid protease production is more common among filamentous fungi, as opposed to what was observed with *Torula thermophila*, whose endoglucanases also have a pH optima close to neutrality (Arifoğlu, 1999). In addition, the pH optimum of EGs from *T. thermophila* were found to be 6.5 (Arifoğlu, 1999), at which proteases are also highly active.

### 3.1.3 Optimization of assay temperature

In order to determine the optimum assay temperature, the fungus was grown at 45°C and 155 rpm on Avicel as the carbon source and the protease activities were determined at different assay temperatures at a temperature range of 30-55°C. The assay pH and the assay incubation time was kept constant at 7.5 and 60 min, respectively. The optimum temperature range at which *T. thermophila* protease showed maximum activity was 37-45°C (Fig. 3.5). Optimum assay temperature to be used in further assays was selected as 40°C because this temperature yields the maximum relative protease activity on the 3$^{rd}$ day at which protease production is maximum, and on the 6$^{th}$ day where endoglucanase production reaches maximum on Avicel as the carbon source (Fig. 3.6).

The optimum temperatures for protease activity of other fungi were found as 37°C for *Rhizopus* SMC strain (Ramamurthy & Kothari, 1993), 40°C for *Humicola lutea* 120-5 (Angelova & Petricheva, 1997) and *Rhizopus oligosporus* (Farley & Ikasari, 1992). Proteases from *Fusarium oxysporum* var.

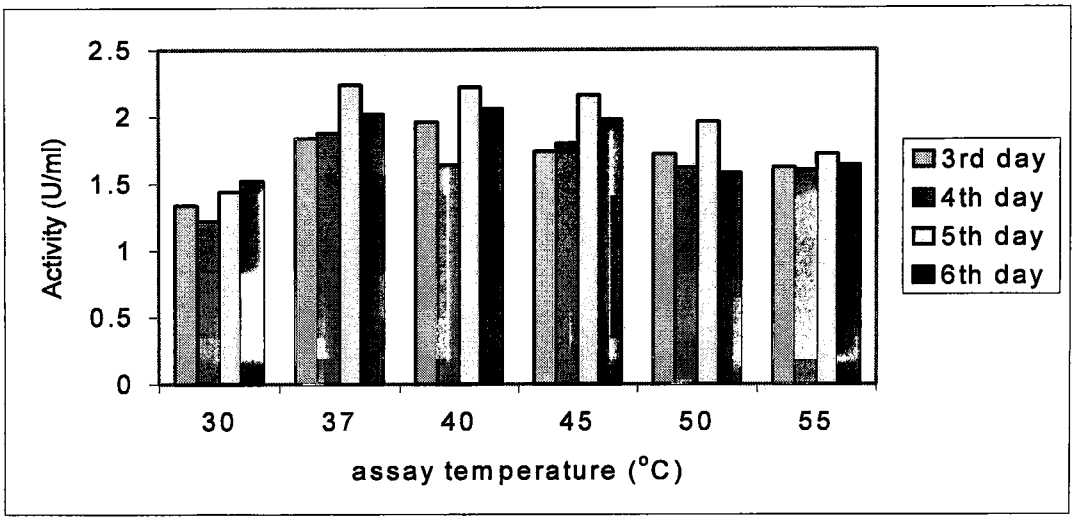


Figure 3.5 Effect of assay temperature on protease activity of *T. thermophila* at pH 7.5 and assay incubation time of 60 min.

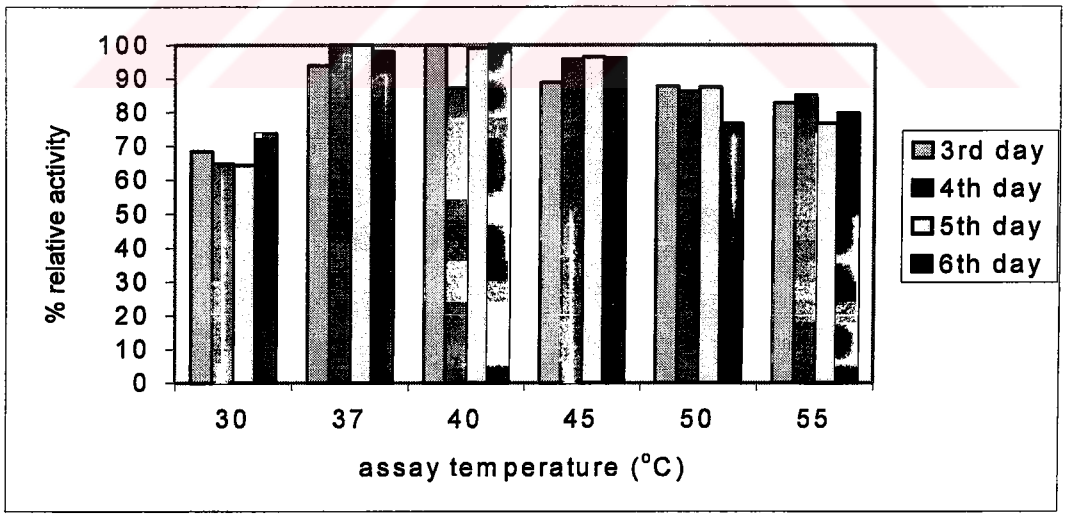


Figure 3.6 Relative effect of assay temperature on protease activity of *T. thermophila* at pH 7.5 and assay incubation time of 60 min.

*lini* were assayed at 37°C (Castro, *et al.*, 1991) and those from *Acremonium persicinum* were assayed at 30°C (Pitson, *et al.*, 1996).

The temperature range at which proteases from *T. thermophila* show high activity indicate that proteases from this microorganism are not as thermostable as cellulases. This can be due to two reasons: The proteases are inactivated or the substrate used for protease assay is denatured at high temperatures. Gould (1975) indicates that an enzyme does not have a true optimum temperature for very short incubation times. Temperatures as high as 70-80°C may not have any effect on protease activity. However, as the incubation time is increased, these high temperatures cause inactivation of the enzyme and greater activity is then found with lower temperatures.

## 3.2 Relationship between the endoglucanase and protease production of *T. thermophila* on different substrates

### 3.2.1 Protease and endoglucanase production by *T. thermophila* on Avicel

In previous studies, endoglucanase (EG) and avicel-adsorbable endoglucanase (AAEG) activities were followed on Avicel as the carbon source and it was seen that the endoglucanase activity decreases after the 6$^{th}$ day of cultivation (Arifoğlu, 1999). It was thought that this decrease in the activity may be due to proteolytic cleavage. In order to determine the time course of protease and cellulase production, protease activities were followed in parallel with EG and AAEG activities during cultivation on Avicel at 45°C in 1 L shake-flask cultures.

Accordingly, protease activity in the culture supernatant reaches maximum on the 3$^{rd}$ day, where EG and AAEG activities seem to be low.

However it was shown in previous studies that about 50 % of cellulose is consumed until the 3rd day (Arifoğlu, 1999). Therefore, cellulases may be produced efficiently within the first 3 days of cultivation but may be partially inactivated by proteases after a certain time. As the protease activity starts to decrease after the 3rd day, EG activity starts to increase and reaches its maximum value on the 6th day, followed by a plateu until the 8th day, and a decrease after the 8th day (Fig.3.7). A possible explanation of this observation may be as follows: Cellulases are produced until the 6th day, but are cleaved by proteases. The activity increases until the 6th day due to a decrease in protease activity. Gene expression stops on the 6th day due to reasons such as nutrient depletion and termination of gene expression, followed by degradation of cellulases by intracellular proteases. It is also possible that, some secondary metabolites are produced during the stationary phase and inhibit the activity of cellulases. Arifoğlu (1999) has followed fungal biomass generation of *T. thermophila* at different growth temperatures (Fig. 3.10). According to the data obtained, *T. thermophila* grown on Avicel under the same conditions indeed, reaches the stationary phase on the 5th-6th days. The microscopic examination of *T. thermophila* culture grown on Avicel also indicate that almost all the cellulose is consumed and sporulation starts after the 6th day (Fig.s 3.11–3.14), which indicates the start of stationary phase. So, it is indeed possible that secondary metabolites are responsible for the inhibition of cellulase activity after the 8th day of incubation.

AAEG activity decreases more sharply than the total EG activity after the 6th day. Adsorbability of EG onto Avicel (microcrystalline cellulose) requires the presence of a cellulose binding domain. Moorman, *et al.* (1993) presumes that the removal of the cellulose-binding domain by proteolytic cleavage leads to the release of the enzyme from crystalline cellulose, which results in accelerated degradation of small, soluble substrates like oligosaccharides generated during cellulose degradation. The same researchers have shown that the proteolytically derived 42-kDa cellulase from

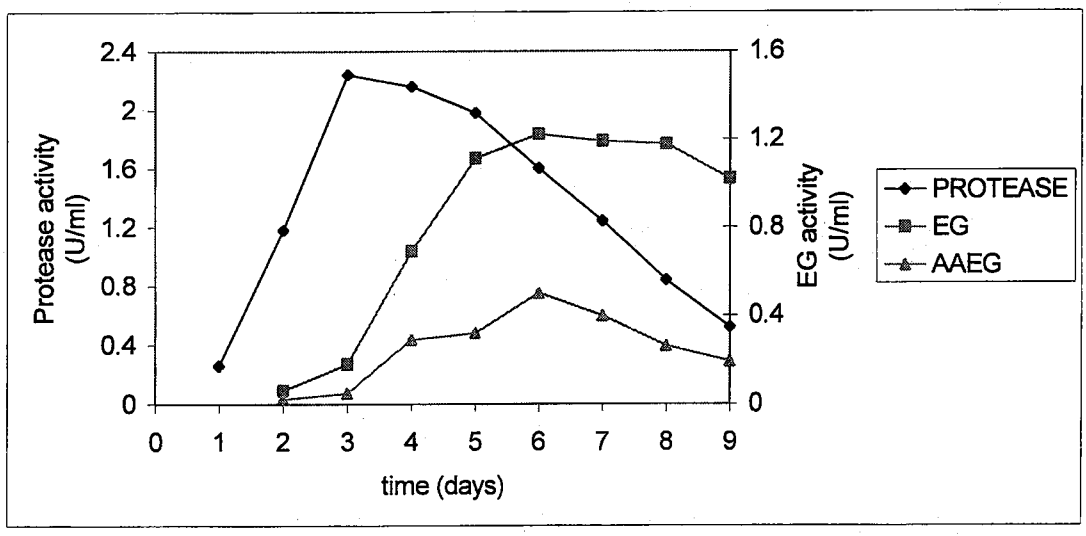


Figure 3.7 Time course of endoglucanase (EG), Avicel-adsorbable endoglucanase (AAEG) and protease production by *T. thermophila* on Avicel, using 1 L shake-flask culture at 45°C and 155 rpm.

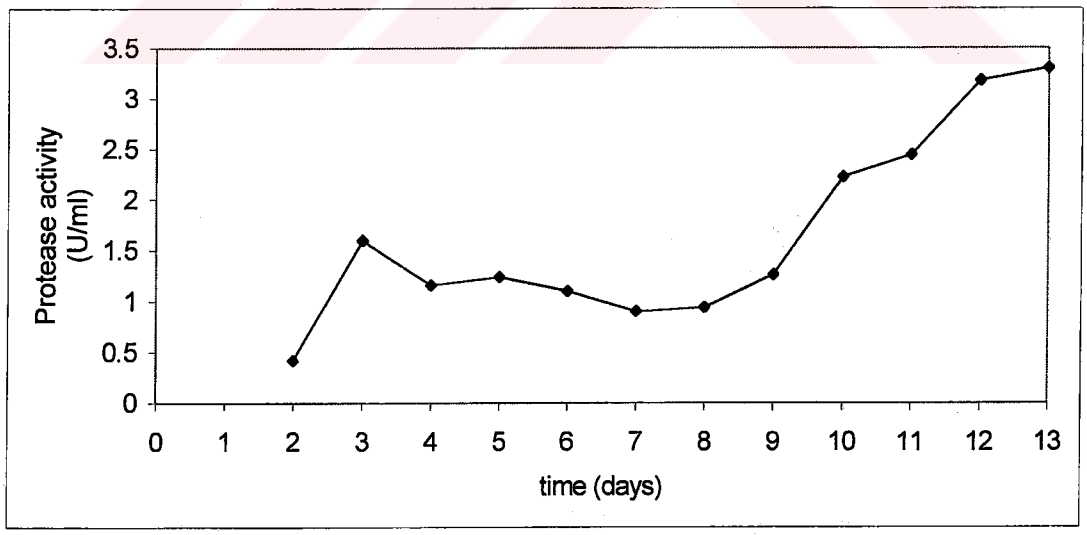


Figure 3.8 Time course of protease production by *T. thermophila* on Avicel using 250 ml shake-flask culture at 45°C and 155 rpm.

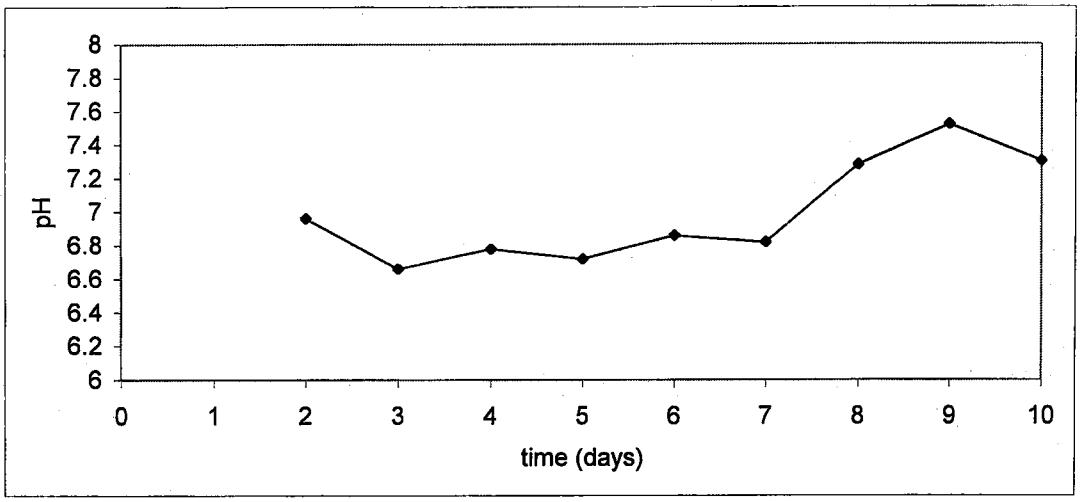


Figure 3.9 Change in pH of the growth medium of *T. thermophila* on Avicel in 1 L medium.

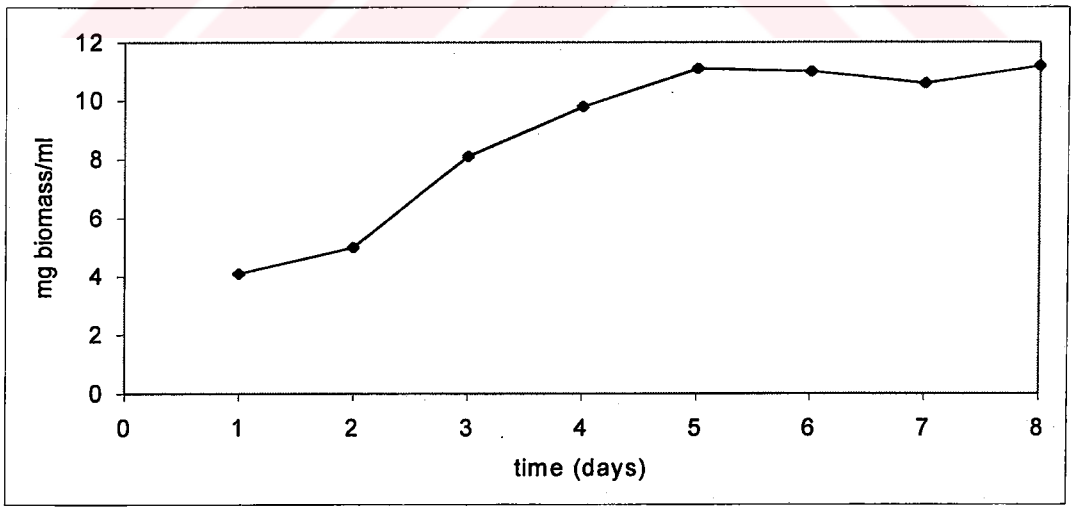


Figure 3.10 Time course of fungal biomass generation of *T. thermophila* at 45°C on Avicel as carbon source (Arifoğlu, 1999).

*Streptomyces reticuli* shows a considerably higher activity against p-nitrophenyl-β-D-cellobioside. When the cell-free culture solution obtained after 4 days cultivation of *Sporotrichum pulverulentum (Phanerochaete chrysosporium)* on 1% cellulose as sole carbon source was treated with two acid proteases purified from the same microorganism, a 10-fold increase in the EG activity was seen. It was suggested that the enzymes are produced in a zymogenic form and are activated by processing by proteases (Eriksson & Pettersson, 1982). Nakayama, *et al.* (1976) argue that processing of cellulases by proteases might lead to the generation of glucanases with new substrate specificities. The sharp decrease in AAEG activity as opposed to EG activity of *T. thermophila* can thus be explained as the proteolytic cleavage of the cellulose binding domain (CBD) which results in a decrease in the AAEG activity but increases the Avicel-nonadsorbable EG activity, allowing total EG activity to remain more or less constant until the 8$^{th}$ day of incubation.

When the fungus is grown on Avicel in 250 ml culture medium over an extended period of time, protease activity starts to increase after the 8$^{th}$ day (Fig. 3.8), which can be explained as the release of intracellular proteases due to cell lysis. In the mean time, pH of the growth medium ranges between 6.66 and 7.52 (Fig. 3.9).

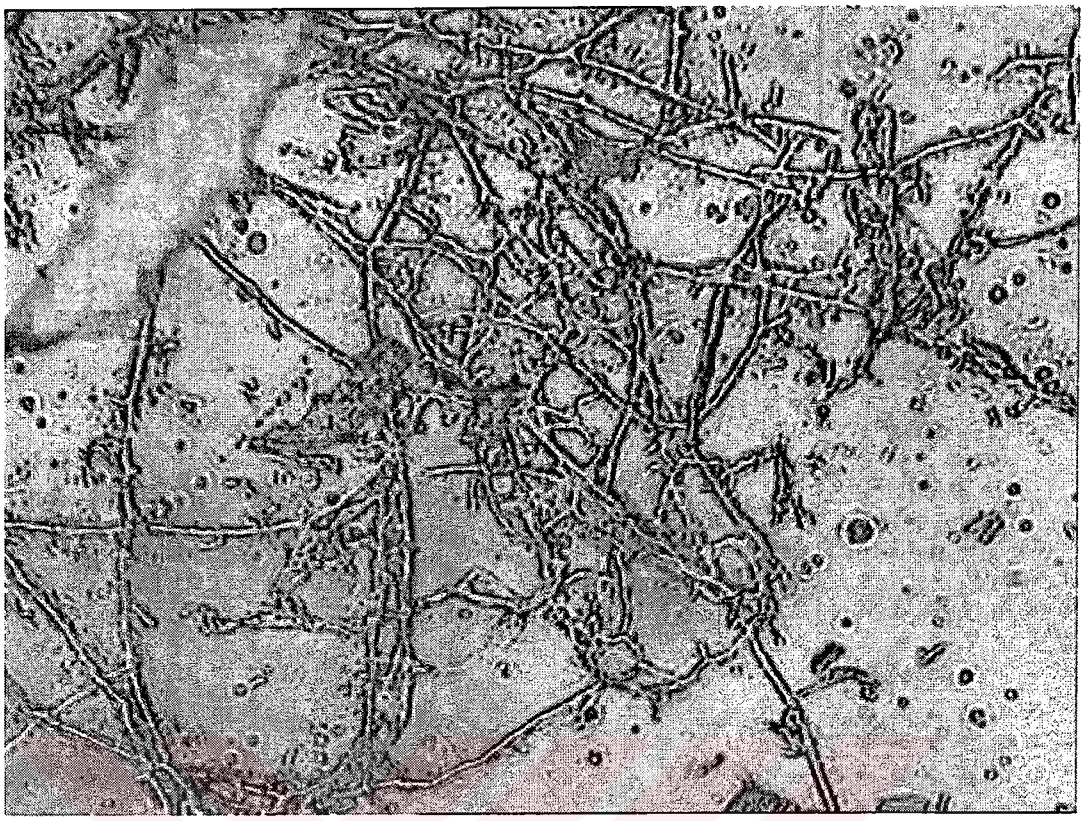

Figure 3.11 3rd day culture of *T. thermophila* under microscope (400 X).

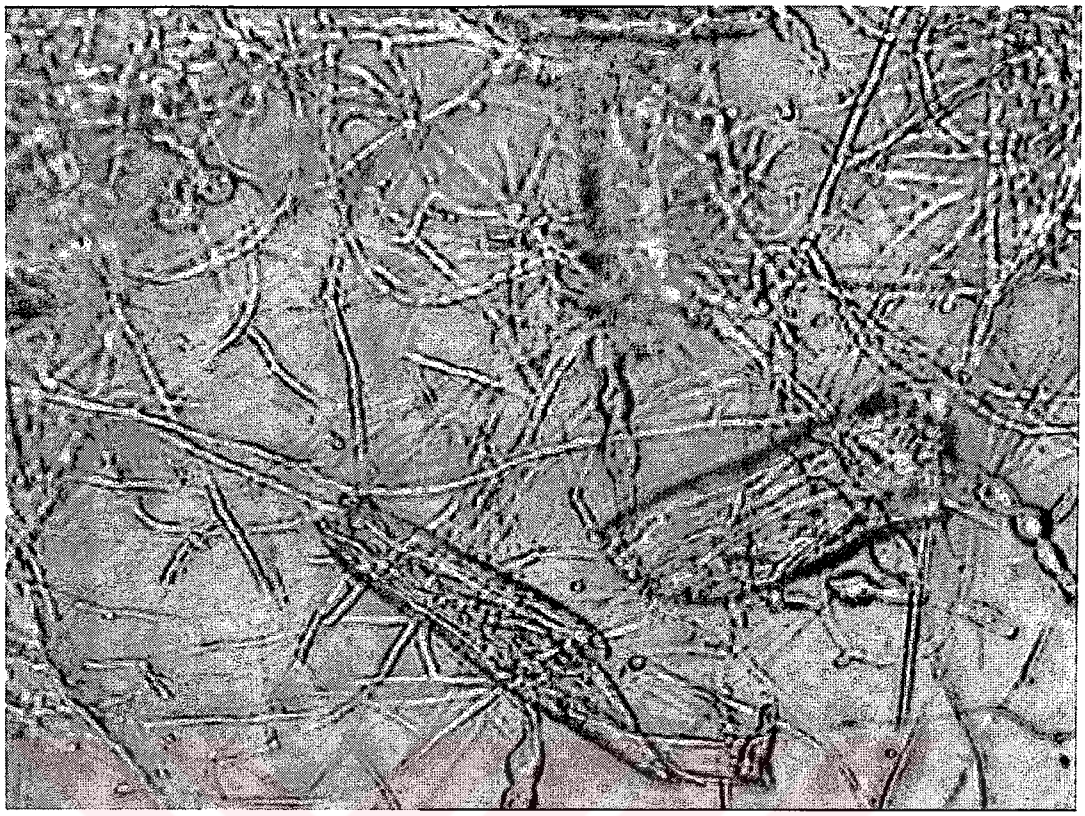

Figure 3.12 4th day culture of *T. thermophila* under microscope (400 X).

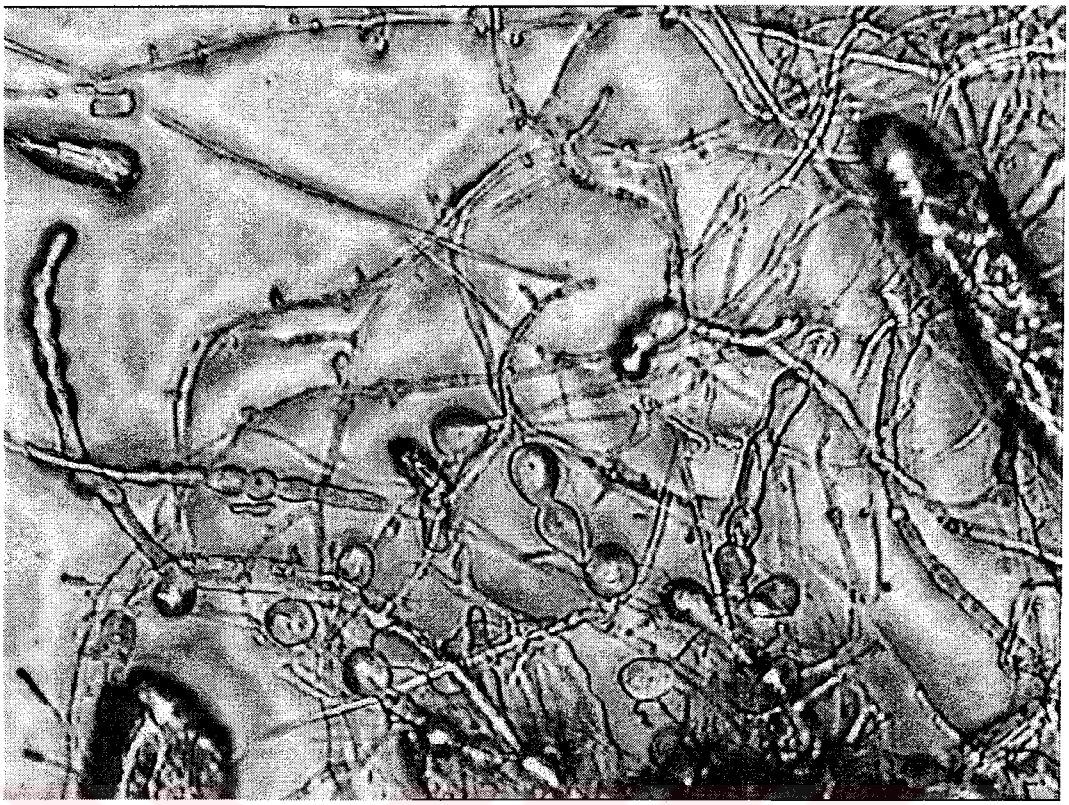

Figure 3.13 6th day culture of *T. thermophila* under microscope (400 X).

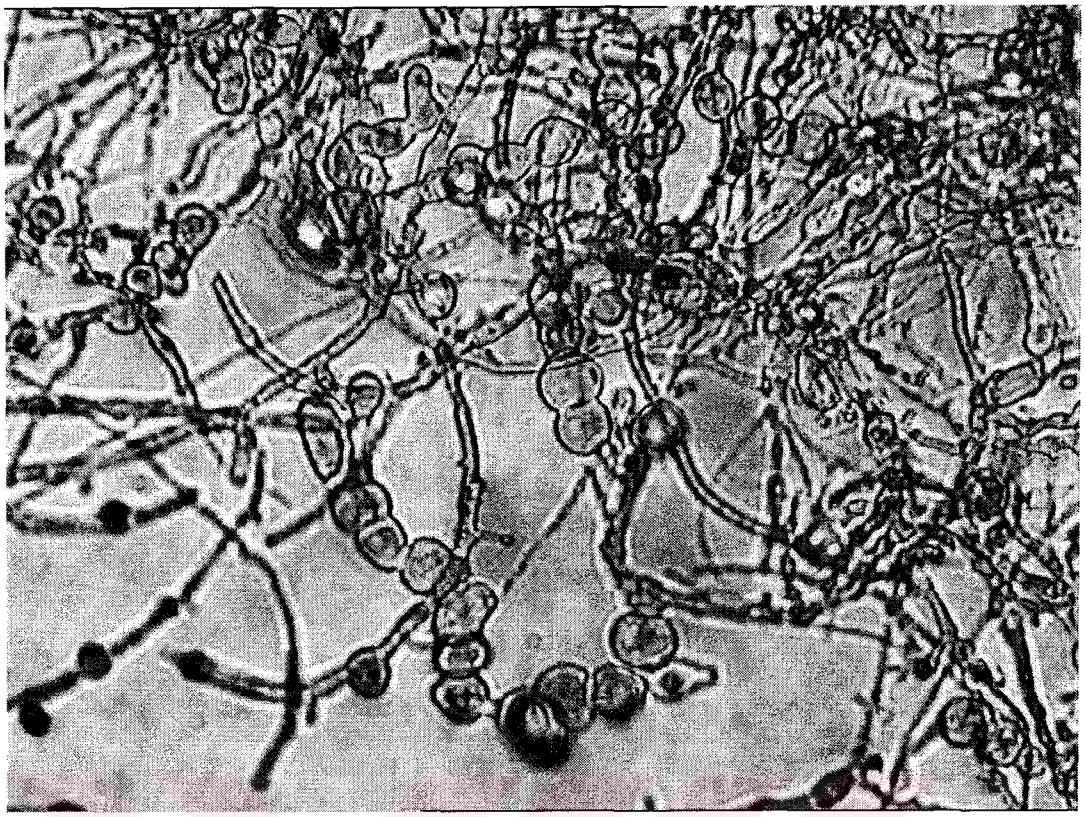

Figure 3.14 9th day culture of *T. thermophila* under microscope (400 X).

### 3.2.2 Protease and endoglucanase production by *T. thermophila* on grass clippings

Time course of EG and protease production by *T. thermophila* on grass clippings was followed in 250 ml shake-flask cultures at 45°C and 155 rpm. In previous studies, EG and AAEG activities were followed on different carbon sources and grass clippings yielded the highest cellulolytic activities when compared to the other lignocellulosic compounds (Dündar, 1999). Decrease in EG and AAEG activities was seen on the 5$^{th}$ and the 4$^{th}$ day, respectively. Again, the decrease in the AAEG activity was sharper than the total EG activity. As seen in Fig. 3.15, EG activity decreases sharply on the 5$^{th}$ day in accordance with the observations of Dündar (1999). Protease activity is again maximum on the 3$^{rd}$ day, and decreases more sharply than the decrease observed on Avicel as the carbon source. The trend is about the same as the relation between EGs and proteases on Avicel, which leads to the same comments made in section 3.2.1.

Maximum protease activity on grass clippings was lower than that on Avicel (Fig 3.8) in 250 ml cultures, corresponding to 1.1 and 1.6 U/ml, respectively. This difference may be related to an accelerated growth of the fungus on Avicel, however, data on biomass generation on grass clippings as the main carbon source, is not available. It is also likely that protease production on grass clippings and Avicel shows difference due to a change in medium composition. There may be free amino acids in grass, that will repress protease production. Homma, *et al* (1993) indicate that extracellular aspartic protease production by *Candida albicans* is repressed in presence of free amino acids. Moormann, *et al.* (1993) indicate that protease activity of *Streptomyces reticuli* is induced only in the presence of Avicel, hydroxyethylcellulose (HEC) and xylan. The reason may be that the microorganism can not efficiently utilise Avicel initially so that it produces proteases to utilise proteins present in the medium for energy production.

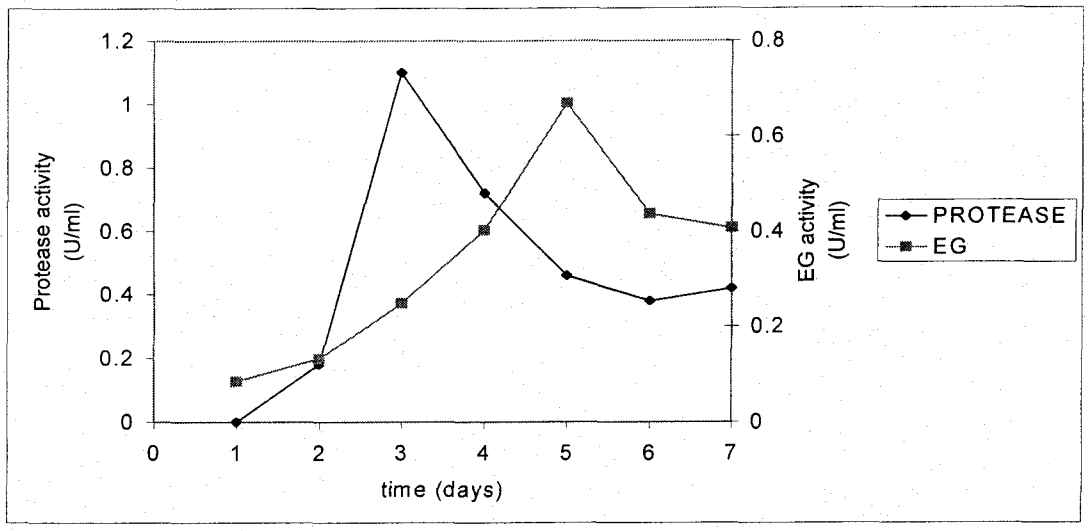


Figure 3.15 Time course of protease and endoglucanase (EG) production by *T. thermophila* on grass clippings at 45°C and 155 rpm.

### 3.2.3 Protease and endoglucanase production by *T. thermophila* on apple pomace

Apple pomace is the remaining part after the extraction of juice from apple. It contains insoluble carbohydrates and in less amounts, protein, minerals and remaining juice containing sugar and other soluble substances (Dündar, 1999). The time course of protease and EG production by *T. thermophila* was followed in 250 ml shake-flask cultures using apple pomace as a carbon source. EG activity on apple pomace was determined in previous studies and a sharp decrease was observed after day 2 (Dündar, 1999). As seen in Figure 3.16, protease activity is low until the 5$^{th}$ day, and EG activity is maximum on the 2$^{nd}$ and the 3$^{rd}$ days. When the protease activity reaches maximum, EG activity is minimum. Protease activity starts to decrease after the 5$^{th}$ day, where EG activity starts to increase again, indicating that EGs are still produced, or the inactivated EGs are re-activated, i.e. the inhibition is reversible.

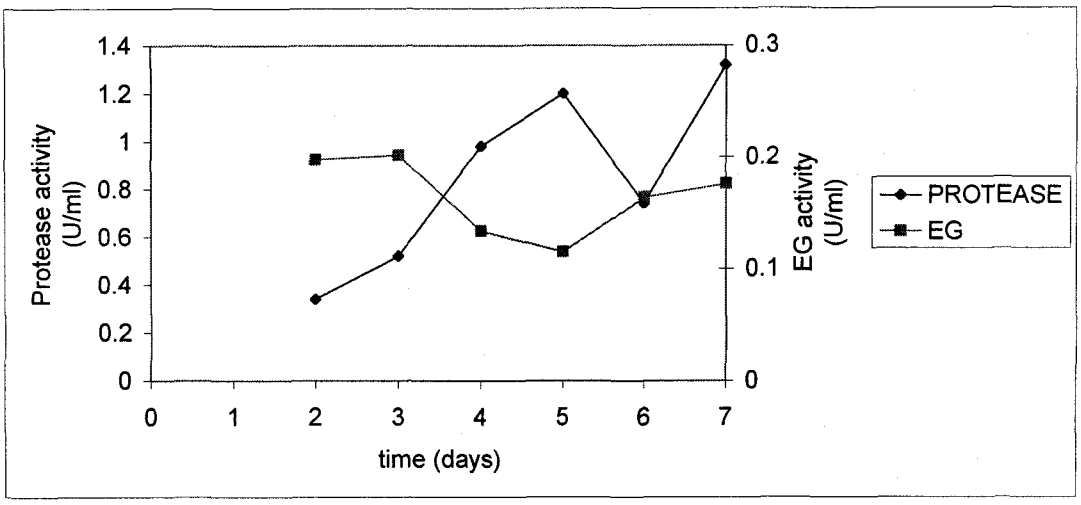


Figure 3.16 Time course of protease and EG production by *T. thermophila* on apple pomace at 45°C and 155 rpm.

The increase in protease activity after the 6th day may be explained as the release of intracellular proteases as a result of cell lysis. In fact, lysis is seen after the 8th day when the fungus was grown on Avicel in 250 ml shake-flask cultures (Fig. 3.8). The protease activity increases about 2-fold after the lysis of the cells, indicating that *T. thermophila* has considerable amounts of intracellular proteases. When the fungus is grown on Avicel in 1 L shake-flask culture, release of intracellular proteases is not observed until the 9th day (Fig. 3.7). This difference may be due to the difference in the volume of flasks.

### 3.3 Analysis of the effect of proteases on endoglucanases by mixing culture supernatants

Regardless of the carbon source used for the cultivation of *T. thermophila*, a different time course is observed for maximal protease and EG production (Section 3.2). Proteases are secreted at an earlier stage of growth where extracellular cellulase activities are low, and visa versa. These results

suggest that there may be a relationship between protease and EG activities. EGs may be efficiently produced at an earlier stage of growth, but activity may be suppressed by the proteases in the culture supernatant. Likewise, when protease activity starts to decrease, due to inactivation or loss of stability, there is less degradation by proteases, leading to an increase in EG activity. Indeed, earlier studies by Arifoğlu (1999) have shown that the cellulose in the medium is rapidly consumed starting from the first day of incubation. This suggests that EGs should also be produced during the early, rather than the late exponential phase.

In order to test this hypothesis, culture supernatants from the $6^{th}$ day (maximum EG activity) were mixed with supernatants in which protease activity is low ($9^{th}$ day supernatant) and high ($3^{rd}$ day supernatant) (Fig. 3.7). Mixtures were prepared in 1:1 ratio and were incubated at 45°C at 155 rpm in order to mimic the growth conditions. Starting from time zero for 10 hours, EG activities in the mixed supernatants were followed every hour. The supernatant from the $6^{th}$ day culture was used as a control.

Results were rather unexpected. EGs were stable over the 10 hours of incubation period, and activities remained almost constant in mixtures prepared by mixing supernatants of the $3^{rd}$ and $6^{th}$ days, and in the $6^{th}$ day control (Fig. 3.17). Surprisingly, EG activity decreased in the mixture of the $9^{th}$ and the $6^{th}$ day supernatant by 19.3 %, where the loss of activity in the $6^{th}$ day control and

Table 3.1 Mixtures of supernatants from cultures of *T. thermophila* at different stages of growth

| | PROTEASE ACTIVITY | EG ACTIVITY |
|---|---|---|
| 3rd day + $6^{th}$ day | Maximum | Low |
| 9th day + $6^{th}$ day | Minimum | High |
| 6th day | Low | Maximum |

the mixture of the 3rd and the 6th day supernatant was 8.8 and 12.2, respectively. These results indicate that extracellular proteases have little, if any, effect on EG activity, at least in terms of inactivating these enzymes on the 6th day of incubation.

On the other hand, inactivation may be caused by secondary metabolites produced during the stationary phase or by low levels of intracellular proteases or metabolites released as a result of cell lysis, such as endotoxins. Alternatively, there may be low levels of specific exocell proteases in the medium on the 9th day, that cause the observed inactivation of EG activity.

The protease activities were also followed in the mixtures of the same culture supernatants for 8 hours (Fig. 3.18). There was not much decrease in the protease activity in any of the supernatant mixtures, which indicates that proteases produced by *T. thermophila* are stable at 45°C for at least 8 hours. The protease activity in the mixture of the 3rd and the 6th day culture supernatant was the highest of all, as expected. However, the protease activity of the mixture containing the 9th and the 6th day culture supernatants was lower than that of the 6th day control supernatant although the 9th day culture shows minimum protease activity. This may be due to the release of low levels of intracellular proteases prior to the collection of supernatants, however, differences are small and may be caused by slight experimental errors in the measurement of absorbances.

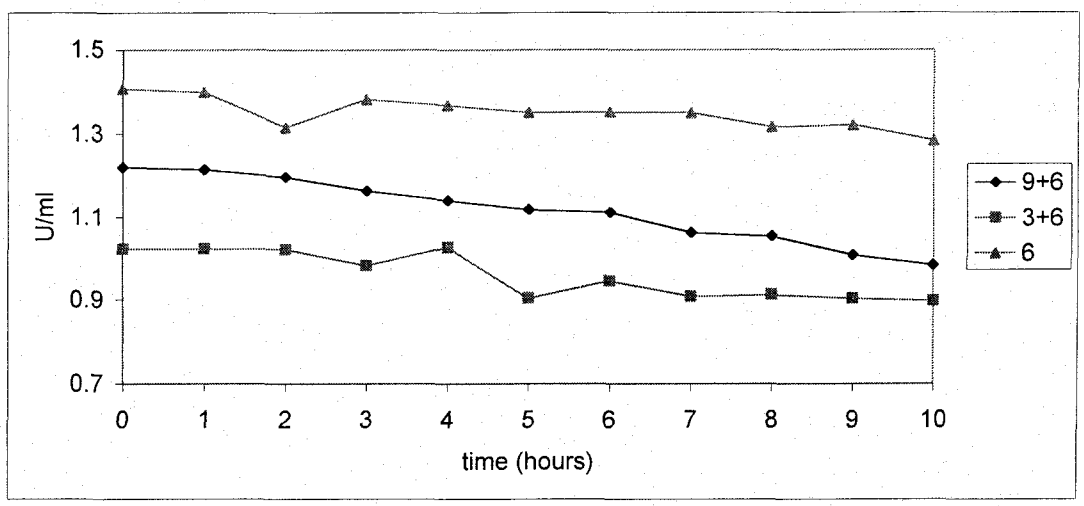


Figure 3.17 Time course of change in EG activity in mixed culture supernatants of *T. thermophila.*

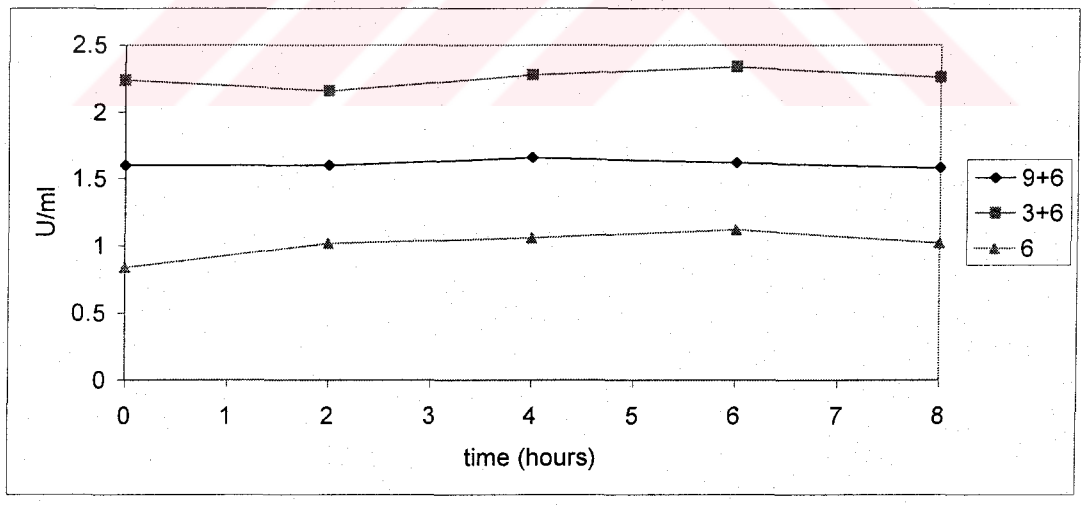


Figure 3.18 Time course of change in protease activity of mixed culture supernatants of *T. thermophila.*

### 3.4 Effect of the initial medium pH on endoglucanase and protease production by *T. thermophila*

Many researchers indicate that pH could be an important factor for the production and secretion of proteases by different microorganisms, which is explained in detail in Sec. 1.4.2.3. Effective pH control in shake-flask cultures by the continuous addition of acid or alkali during the incubations was not possible and buffers were not sufficient in keeping the medium pH constant. Pitson, *et al.* (1996) indicate that buffers for pH control were not used in their study because few can buffer over the entire pH range required (pH 4-9). In addition, several researchers indicate that some buffers are unsuitable since they are often degraded, used as nutrient sources, are directly toxic, or inhibit growth by binding other substances (particularly metal ions) in the medium (Breznak & Costilow, 1994, Côté & Gherna, 1994).

Due to the above reasons, in this study the effect of culture pH on EG and protease production by *T. thermophila* was examined at 45°C and 155 rpm on Avicel as the carbon source. *T. thermophila* did not grow when the medium pH was adjusted to acidic pH (pH 4.0), so neutral and alkaline pH were used to examine the effect of initial pH on EG and protease production.

#### 3.4.1 Protease and endoglucanase production in medium with initial pH 7.0

The pH of the medium before adjustment was 7.28 and was brought to 7.0 by the addition of 0.1M $H_3PO_4$. Medium pH changed between 7.15 and 6.78 during cultivation which may be due to the metabolic activities of the microorganism (Fig.3.20). The protease activity was low until the 4$^{th}$ day, followed by a sudden increase in activity. EG activity also continued to increase until the 5$^{th}$ day, and then decreased sharply. Release of intracellular

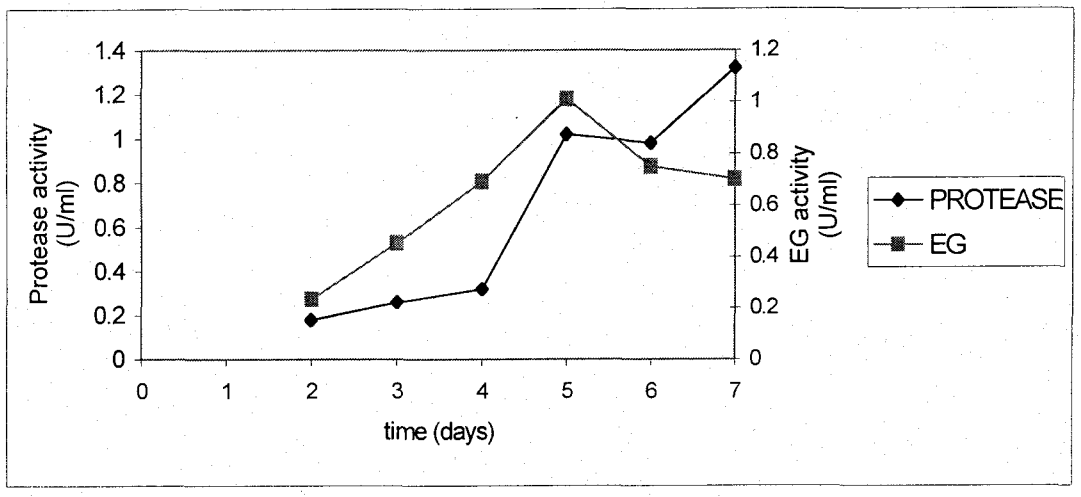


Figure 3.19 Time course of endoglucanase (EG) and protease production by *T. thermophila* on Avicel with an initial pH 7.0.

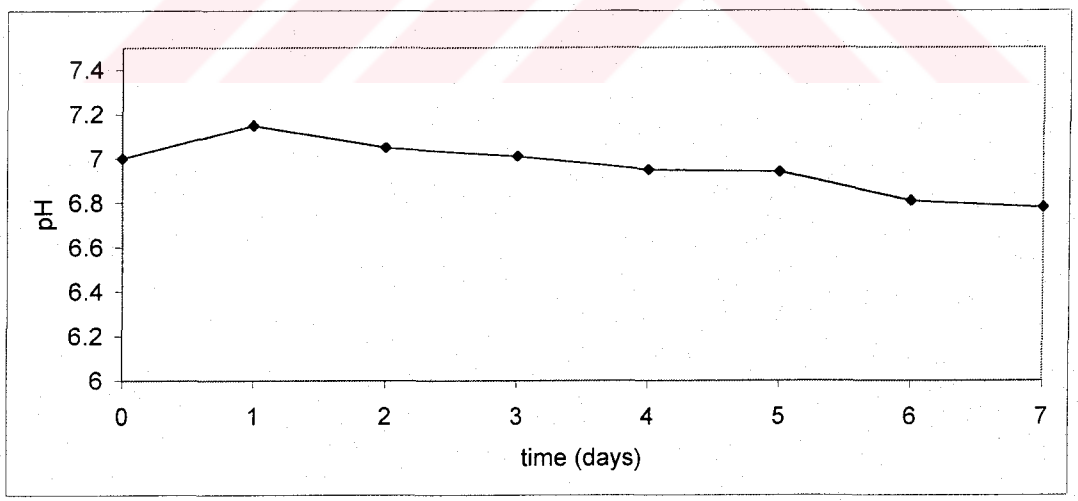


Figure 3.20 Change in medium pH of *T. thermophila* with an initial pH 7.0.

proteases due to lysis is seen after the 6th day which led to an increase in protease activity (Fig. 3.19).

### 3.4.2 Protease and endoglucanase production in medium with initial pH 7.5

The pH of the medium before adjustment was 7.32 and was brought to 7.5 by the addition of 0.1M KOH. The pH of the medium varied between 7.82 and 6.78 (Fig. 3.22). Proteases showed maximum activity on the 5th day and then decreased sharply. The EG activity was low until the 5th day, and showed a sharp increase on the 6th day of incubation (Fig. 3.21). The release of intracellular proteases is not observed until the 7th day of incubation.

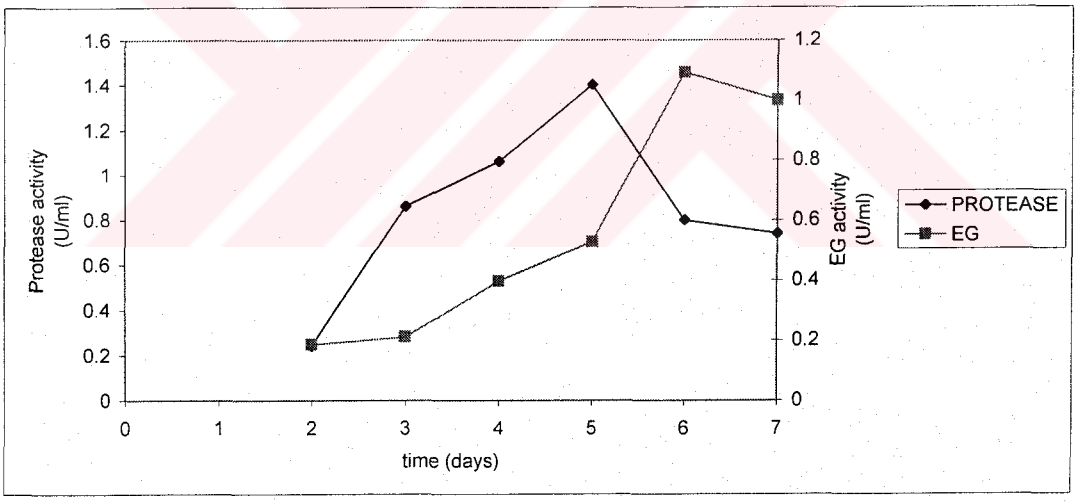


Figure 3.21 Time course of endoglucanase (EG) and protease production by *T. thermophila* on Avicel with an initial pH 7.5.

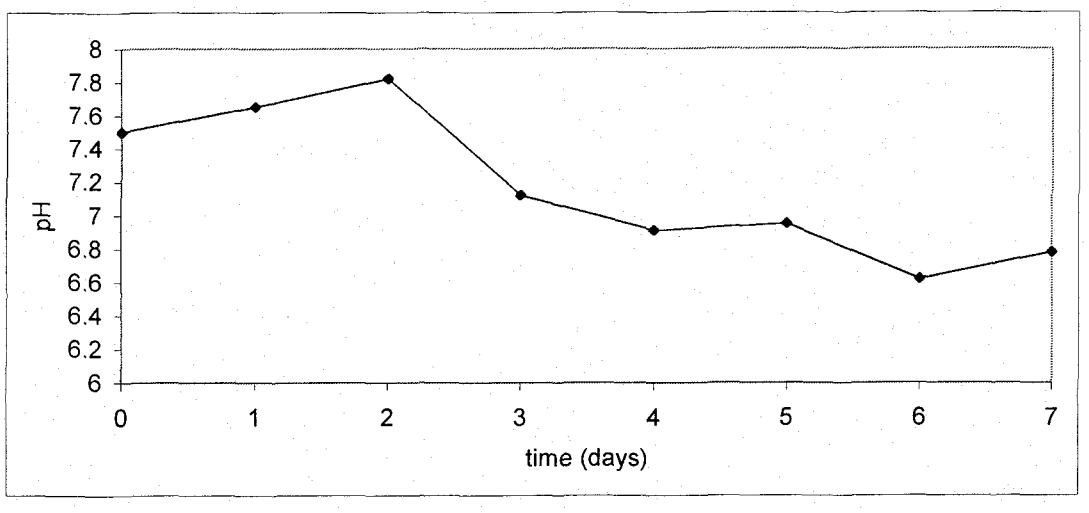


Figure 3.22 Change in pH of the growth medium of *T. thermophila* with initial pH 7.5.

### 3.4.3 Protease and endoglucanase production in medium with initial pH 8.0

The medium pH of 7.38 was adjusted to 8.0 with 0.1M KOH. The pH of the culture varied from 7.65 to 7.18 during cultivation (Fig.3.24). Protease activity reached maximum on the 6$^{th}$ day which is very late when compared to that observed on medium with unadjusted pH, where maximum activity was attained on the 3$^{rd}$ day. The EG activity is high until the 4$^{th}$ day where the protease activity is very low. After the 4$^{th}$ day, there is a sharp increase in protease activity and the EG activity decreases, then increases again on the 6$^{th}$ day (Fig.3.23). As in the case of initial medium pH 7.5, release of intracellular proteases is not apparent until the end of the incubation period. EG activity continues to increase after the 6$^{th}$ day, which is in contrast with the case of unadjusted medium pH on Avicel in 1 L shake-flask cultures.

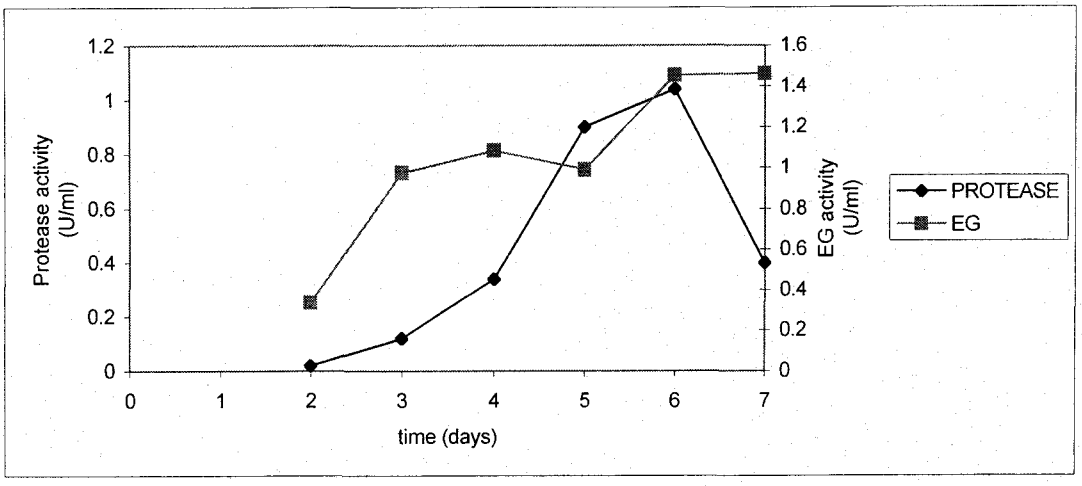


Figure 3.23 Time course of endoglucanase (EG) and protease production by *T. thermophila* on Avicel with an initial pH 8.0.

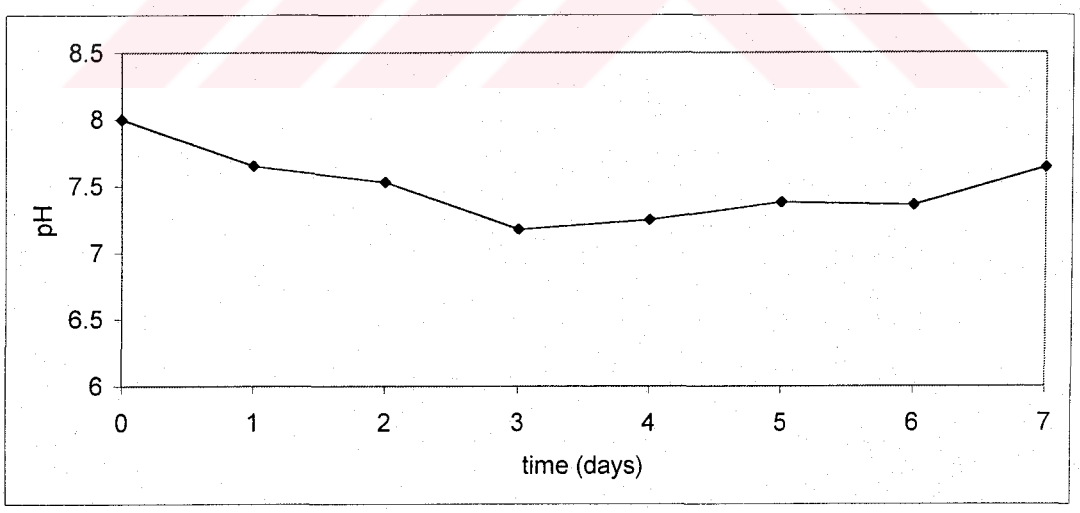


Figure 3.24 Change in pH of the growth medium of *T. thermophila* with initial pH 8.0

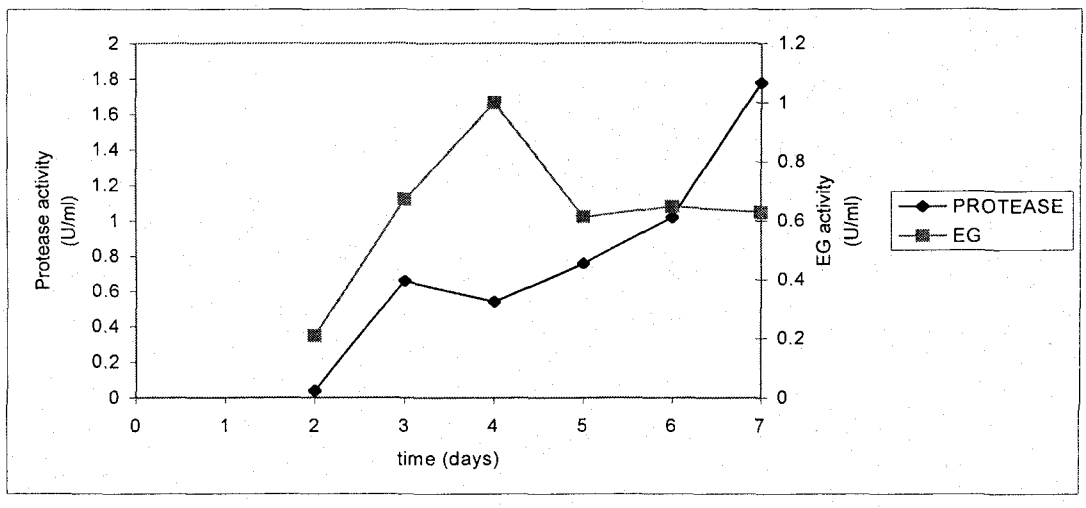


Figure 3.25 Time course of endoglucanase (EG) and protease production by *T. thermophila* on Avicel with an initial pH 8.5.

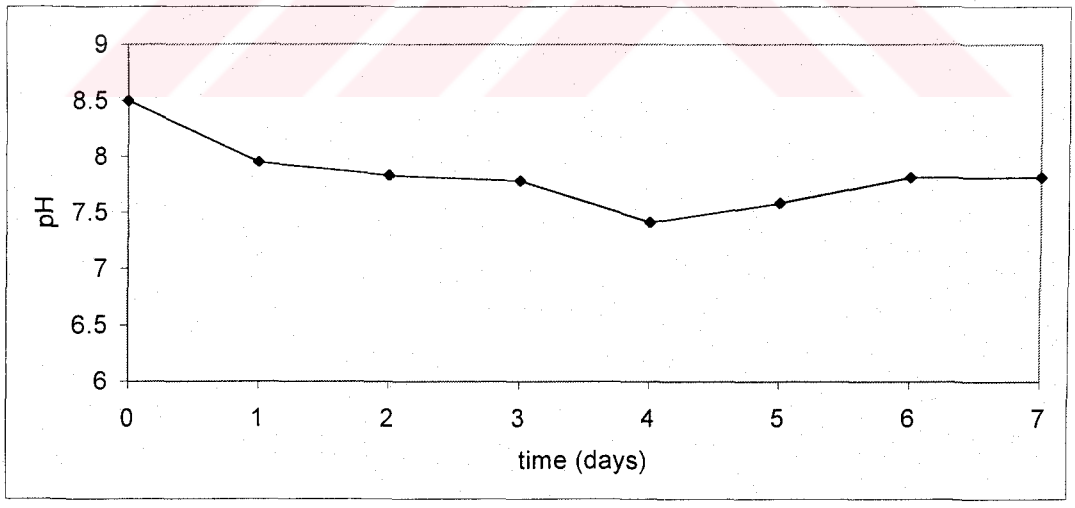


Figure 3.26 Change in pH of the growth medium of *T. thermophila* with initial pH 8.5.

### 3.4.4 Protease and endoglucanase production in medium with initial pH 8.5

The initial pH of the medium was adjusted to 8.5 by addition of 0.1M KOH, which was 7.39 before. The medium pH dropped to 7.95 on the 1st day and changed between 7.95 and 7.41 during cultivation (Fig.3.26). Protease activity continuously increased after a peak on the 3rd day, while EG activity was maximum on the 4th day, where protease activity was low. It's hard to guess the exact day of lysis because protease activity increases continuously throughout cultivation (Fig.3.25).

### 3.4.5 Optimum initial medium pH for endoglucanase and protease production by *T. thermophila*

Maximum activities of proteases and EGs with different initial medium pH values were plotted in order to determine optima for both group of enzymes (Fig.3.27). Accordingly, maximum EG production takes place at pH 8.0 with an activity of 1.46 U/ml, where protease activity is at a minimum with 1.04 U/ml. On the other hand, protease production is maximum at pH 7.5 with the activity of 1.40 U/ml, where EG production is low with 1.09 U/ml activity. The initial medium pH which yields maximum protease production is the same as the optimum pH for protease activity, while the optimum pH for endoglucanase production and activity are different, which are 8.0 and 6.5, respectively. It is interesting that endoglucanase activity is minimum in the medium with initial pH which yields maximum protease activity, and visa versa. The results may suggest the existance of an inverse relationship between the regulation of protease and EG production, however, this is yet to be determined.

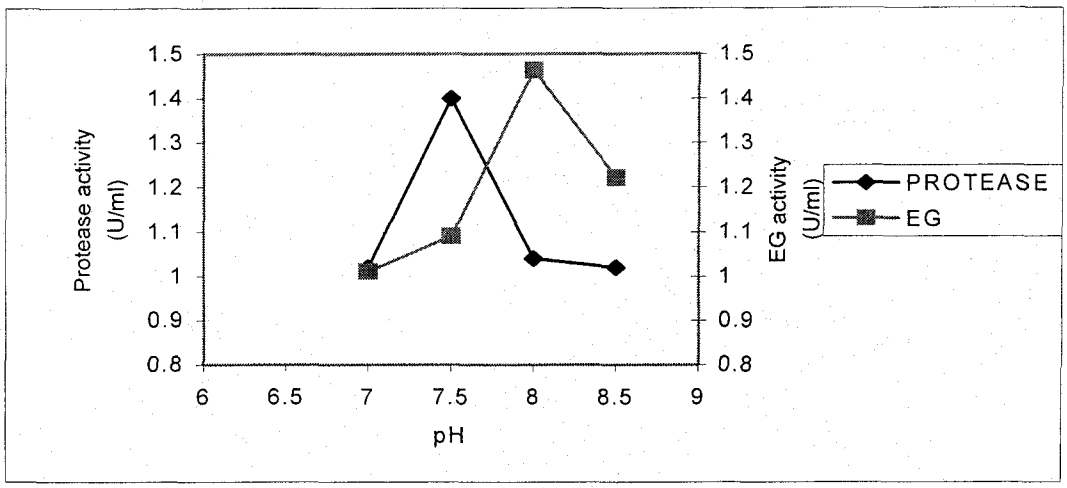


Figure 3.27 Maximum protease and EG production by *T. thermophila* as a function of initial medium pH.

### 3.5 Effect of protease inhibitors on the activity of proteases produced by *T. thermophila*

The effect of protease inhibitors on the protease activity of *T. thermophila* was determined by incubating culture supernatants in the presence and absence of various protease inhibitors, during the pre-incubation time of 30 min, followed by the determination of protease activities. This work was conducted in order to establish the type of proteases secreted by *T. thermophila* on Avicel as the main carbon source.

#### 3.5.1 Effect of pCMB and antipain on protease activity of *T. thermophila*

Antipain and p-chloromercuribenzoate (pCMB) were examined for their effect on proteases produced by *T. thermophila* at concentrations of 50 μg/ml and 1mM, respectively. Proteases produced by *T. thermophila* are

partially inhibited both by pCMB (Fig. 3.28a and 3.28b) and antipain (Fig. 3.30). pCMB was tried first and it was seen that the maximum inhibition is on the 3rd day, which is about 58% (Fig. 3.29a and 3.29b). North (1982) suggests that pCMB may also inhibit some serine proteases. In order to be sure that the inhibition caused by pCMB is due to the presence of cysteine proteases, antipain, another inhibitor of cysteine proteases has been examined for its effect on protease activity of *T. thermophila*. The inhibitory effect of antipain seems to be more than pCMB. 70 % inhibition is seen on the 5th day, which is the maximum inhibition among all days (Fig. 3.31). The results indicate that proteases produced by *T. thermophila* contain cysteine proteases.

### 3.5.2 Effect of PMSF on protease activity of *T. thermophila*

Phenyl methyl sulphonylfluoride (PMSF), an inhibitor of serine proteases, is not stable in a solution without ethanol. So, it has been dissolved in assay buffer containing 25 % ethanol and added to the assay mixture at a final concentration of 3 mM. Addition of ethanol in the assay mixture yields higher protease activities, so the control assay without the addition of PMSF is also made with the addition of ethanol. PMSF also seems to be effective on inhibiting the proteases produced by *T. thermophila*, indicating that serine proteases are also produced by the fungus (Fig. 3.32a and 3.32b). The maximum inhibition is seen on the 4th-5th days. The inhibition on the 5th day is 67 % in a set of experiment (Fig. 3.33a) and 52 % in another set of experiment (Fig. 3.33b). Antipain also shows the highest inhibitory effect on the 5th day with 70 % inhibition. Hasnain, *et al* (1991) have studied on an extracellular thiol-containing serine protease from the thermophilic fungus *Thermomyces lanuginosus*, which is inhibited mostly by PMSF and $Hg^{2+}$ and also by pCMB. The percent remaining activity was 9.3% and 0% after a 24 hour incubation of the enzyme with pCMB and PMSF, respectively. The enzyme was thought to be a cysteine protease previously (Shenolikar and Stevenson, 1982), but

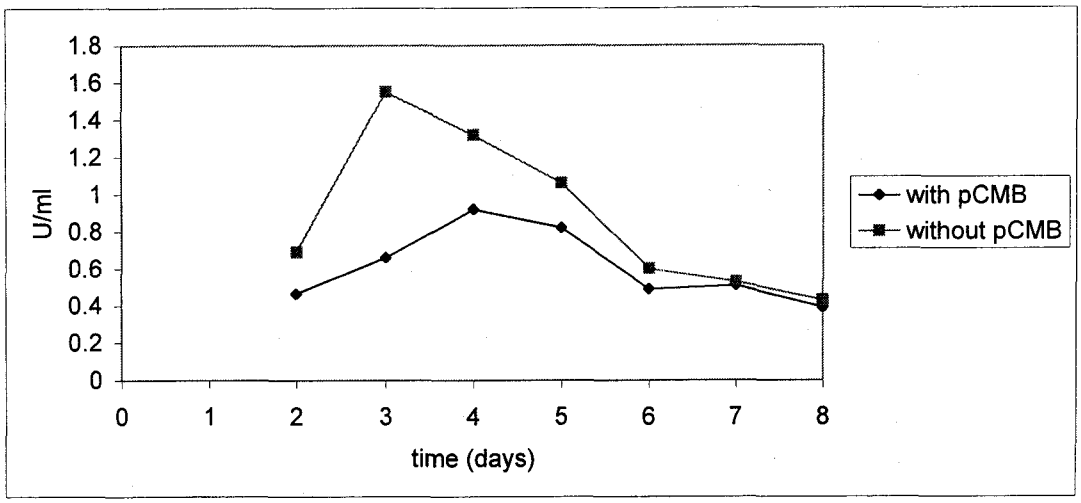


Figure 3.28a Effect of pCMB on the activity of proteases produced by *T. thermophila* on Avicel as the main carbon source.

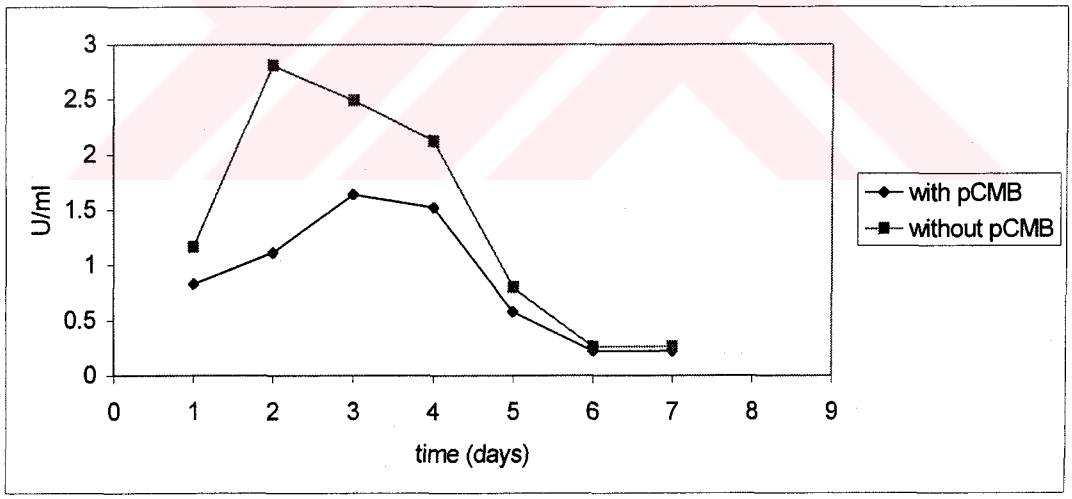


Figure 3.28b Effect of pCMB on the activity of proteases produced by *T. thermophila* on Avicel as the main carbon source.

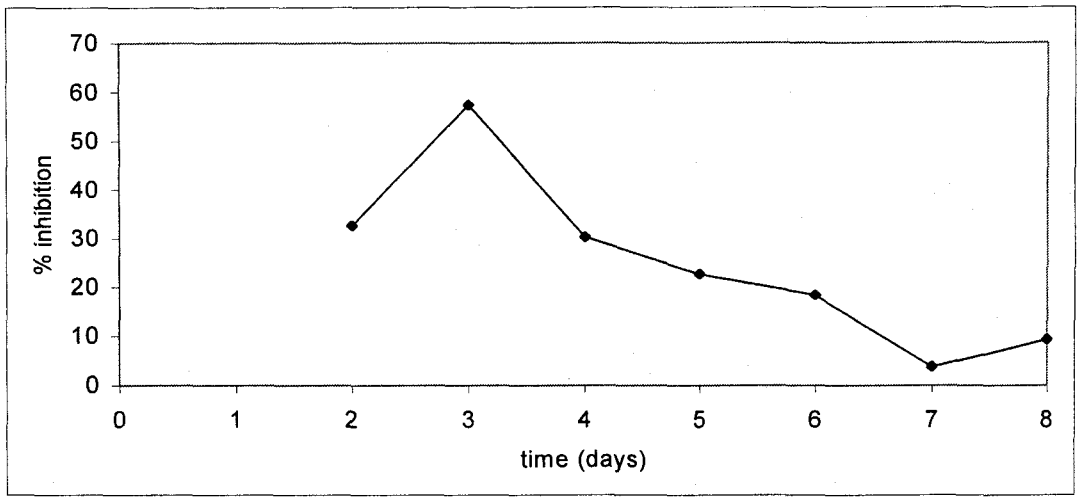


Figure 3.29a Percent inhibition of proteases produced by *T. thermophila* after treatment with pCMB (standard deviations given in Appendix E).

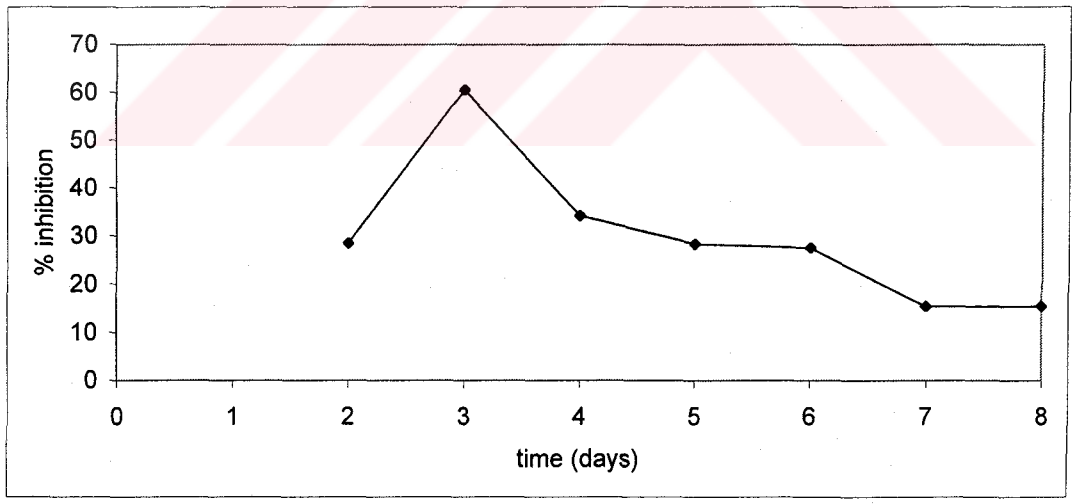


Figure 3.29b Percent inhibition of proteases produced by *T. thermophila* after treatment with pCMB.

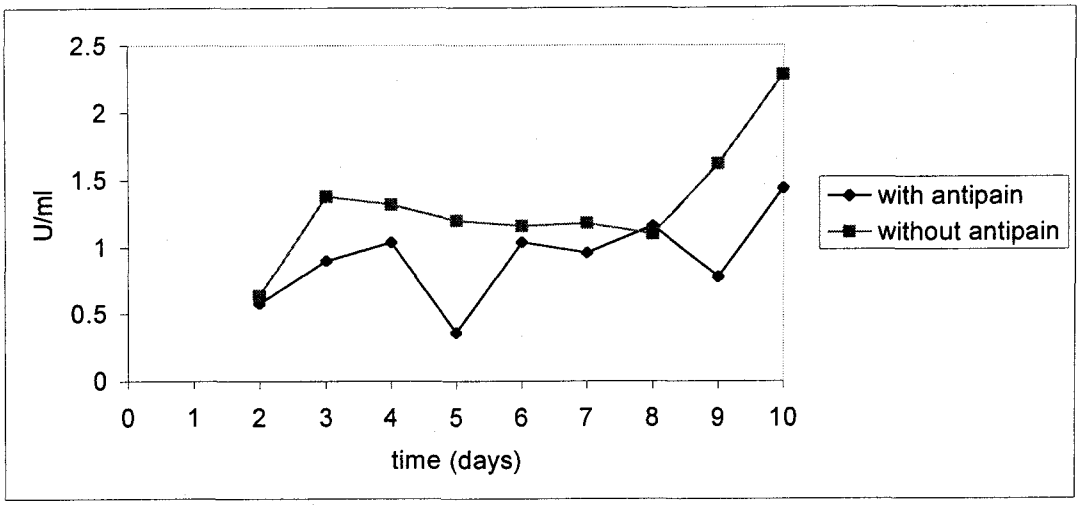


Figure 3.30 Effect of antipain on the activity of proteases produced by *T. thermophila* on Avicel as the main carbon source.

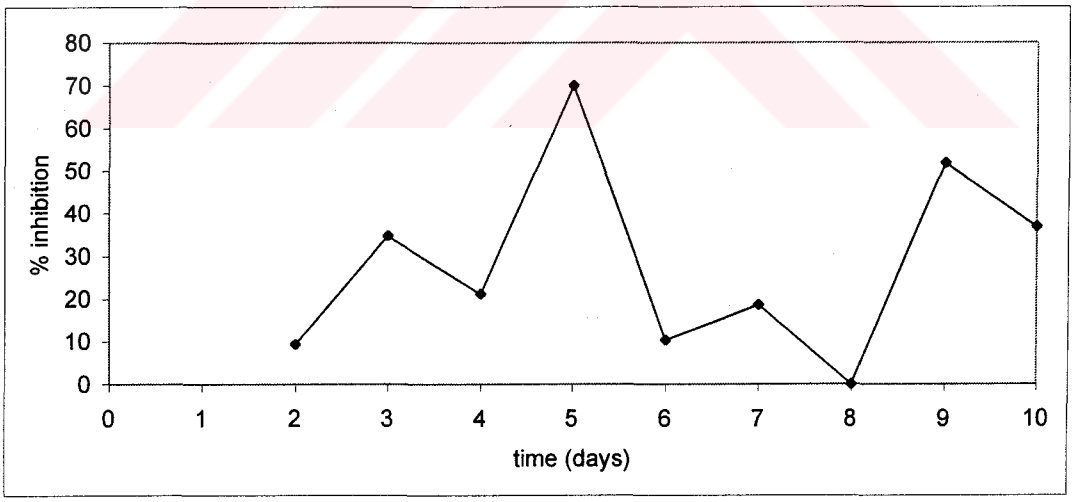


Figure 3.31 Percent inhibition of proteases produced by *T. thermophila* after treatment with antipain.

then it was shown that it is a thiol-containing serine protease and not a cysteine protease as previously thought (Hasnain, *et al*, 1991).

It is possible that proteases produced by *T. thermophila* are thiol-containing serine proteases since they are inhibited both by serine and cysteine protease inhibitors. The assay pH of the proteases from *Thermomyces lanuginosus* is 8.0 (Hasnain, *et al*., 1991), whereas that of *T. thermophila* is 7.5, which are close to each other. Other examples of thiol-containing serine proteases are from *Bacillus thuringiensis* (Epremyan *et al*., 1980; Stepanov *et al*., 1981), *Thermoactinomyces vulgaris* (Stepanov, *et al*., 1981; Meloun, *et al*., 1985), *Streptomyces rectus* var. *proteolyticus* (Mizusawa and Yashida, 1973), and *Tritirachium album* Limber (Epremyan *et al*., 1980; Betzel, *et al*., 1988).

### 3.5.3 Effect of EDTA on protease activity of *T. thermophila*

Ethylene diamine tetraacetate (EDTA), an inhibitor of metalloproteases, was used in 1mM concentration with an adjusted assay pH of 8.0, since it is not stable in any other pH. EDTA had no inhibitory effect until the $10^{th}$ day (Fig. 3.34). This may be due to the release of intracellular proteases on which EDTA has an inhibitory effect. The enhancing effect of EDTA on proteases is seen until the $4^{th}$ day. In the literature, it has been indicated that thiol-containing protease activities (mostly cysteine proteases) are enhanced by EDTA (Garber and Lemchuk-Favel, 1989; North, 1982). The differences observed in this study are however, within the range of experimental errors.

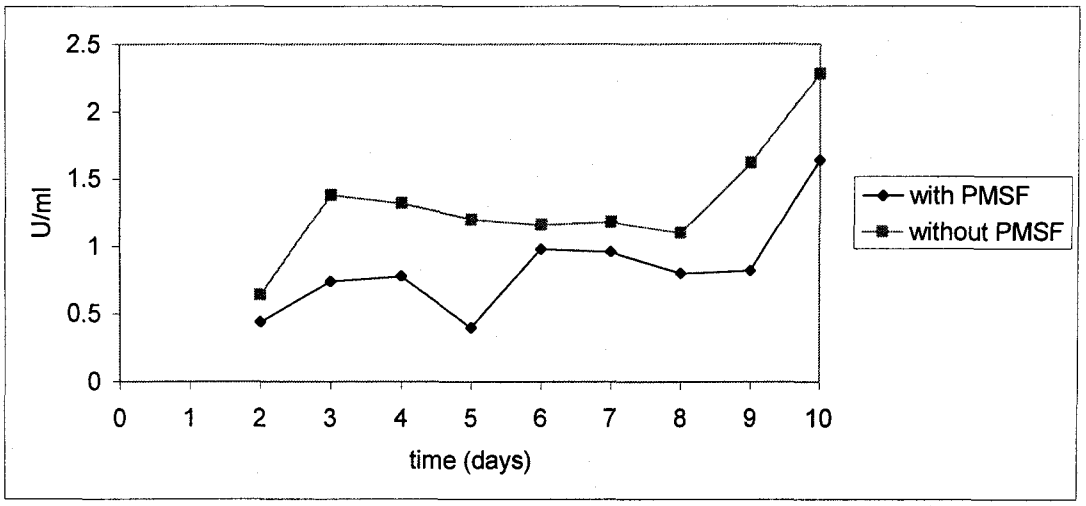


Figure 3.32a Effect of PMSF on the activity of proteases produced by *T. thermophila* on Avicel as the main carbon source.

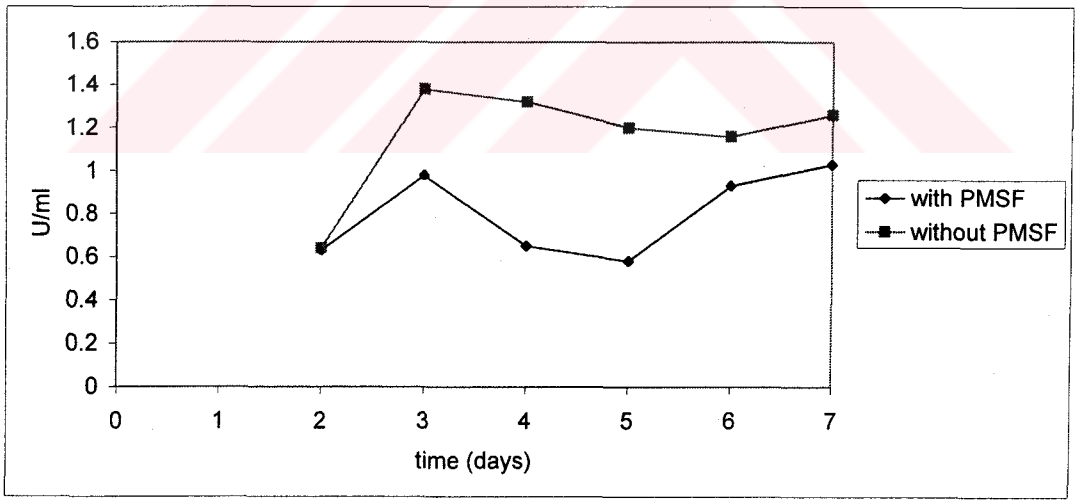


Figure 3.32b Effect of PMSF on the activity of proteases produced by *T. thermophila* on Avicel as the main carbon source.

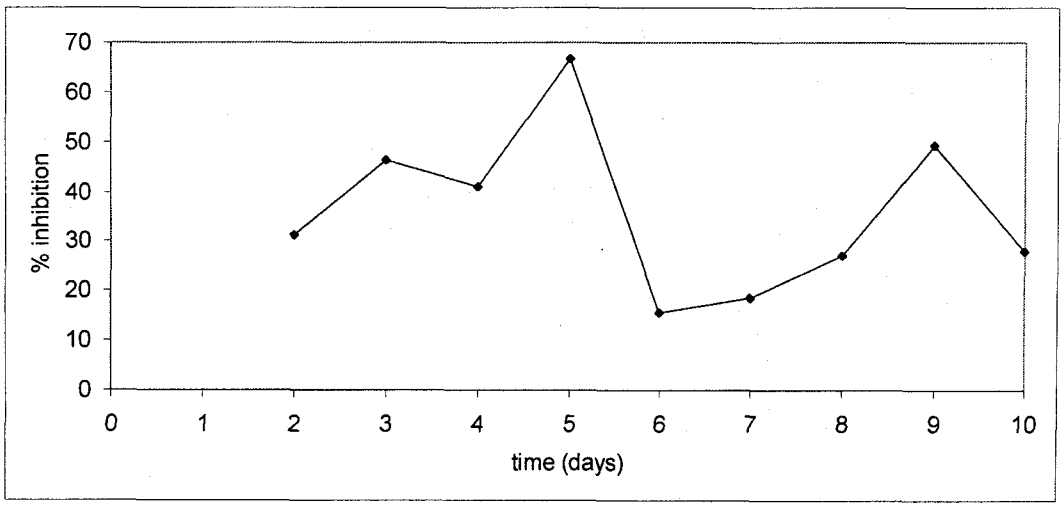


Figure 3.33a Percent inhibition of proteases produced by *T. thermophila* after treatment with PMSF (standard deviations given in Appendix F).

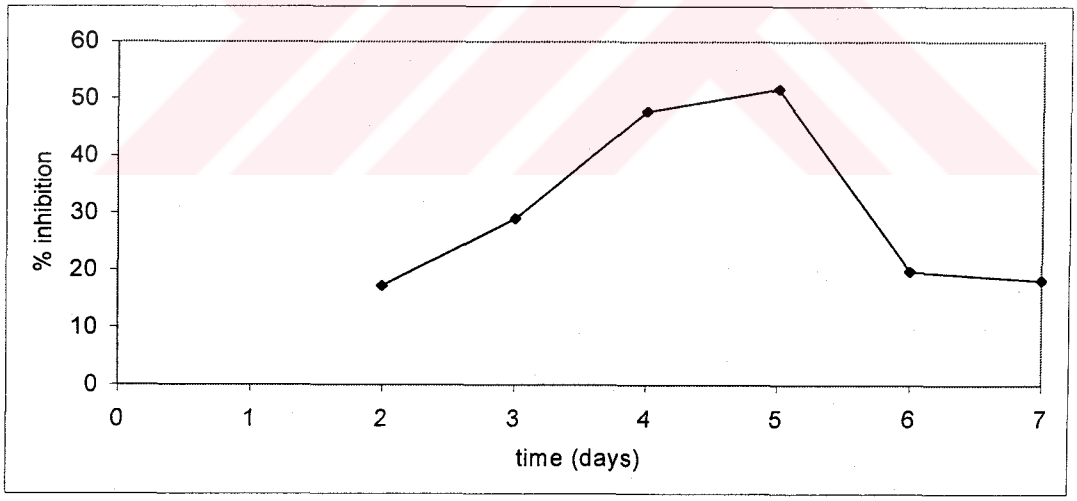


Figure 3.33b Percent inhibition of proteases produced by *T. thermophila* after treatment with PMSF.

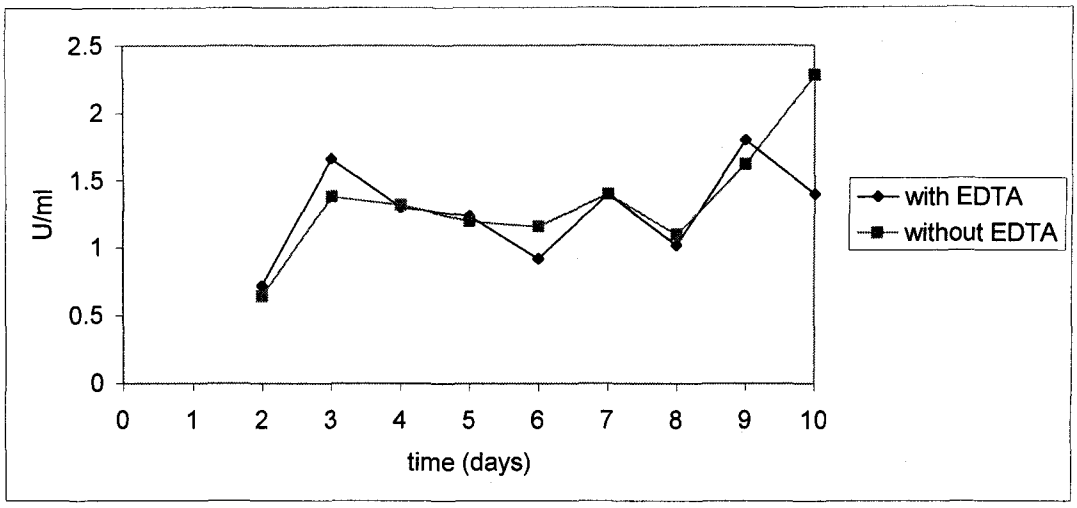


Figure 3.34 Effect of EDTA on the activity of proteases produced by *T. thermophila* on Avicel as the main carbon source.

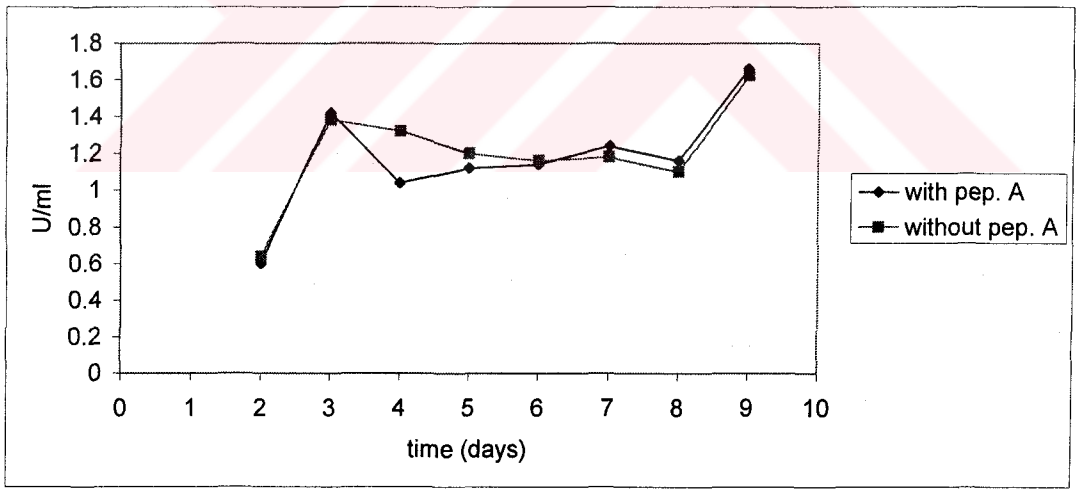


Figure 3.35 Effect of pepstatin A on the activity of proteases produced by *T. thermophila* on Avicel as the main carbon source.

### 3.5.4 Effect of pepstatin A on protease activity of *T. thermophila*

Pepstatin A is an inhibitor of aspartyl proteases, which are mostly produced at acidic pH. The growth medium pH of *T. thermophila* is around neutrality, thus the presence of aspartyl proteases in the medium is unlikely. Pepstatin A was used at a concentration of 50 μg/ml. As expected, it was seen that pepstatin A was not inhibitory on proteases produced by *T. thermophila* except a slight decrease at day 4 which may be due to experimental errors (Fig. 3.35).

# CHAPTER 4

# CONCLUSIONS

In this study, *Torula thermophila* protease production was analyzed in cellulose-containing media and compared with cellulase production at the same growth conditions.

The assay pH and temperature were optimized and proteases produced by *T. thermophila* were found to be most active at neutral and alkaline pH and at a temperature range of 37-45°C.

Time course of protease and endoglucanase production on cellulose-containing media were compared. It was observed that proteases and endoglucanases display a different time course of production suggesting that there may be a negative effect of proteases on extracellular endoglucanase activity.

To test the possible effect of proteases on endoglucanases, mixture of supernatants from different days of cultivation were prepared and changes in protease and endoglucanase activities were measured. According to the results, extracellular proteases do not appear to have a significant effect on the activity of endoglucanases by *T. thermophila*. The results suggest that some secondary metabolites produced in the stationary phase may be effective on inactivation of endoglucanases.

The effect of initial medium pH on protease and endoglucanase secretion was determined. Initial medium pH 7.5 appears to be optimum for protease production and decreases endoglucanase activity. On the other hand, initial medium pH 8.0 is optimum for endoglucanase activity.

As the last step, the type of proetases produced on Avicel was determined. Inhibition of protease activity by pCMB, antipain and PMSF indicates the production of cysteine and serine proteases or thiol-containing serine proteases by *T. thermophila.*

Further studies on protease and cellulases of *T. thermophila* might be carried out. The effect of medium pH on the production and secretion of proteases and cellulases might be studied by working with fermentors. The secondary metabolites that are secreted into the growth medium during the stationary phase and their possible effect on cellulases may be investigated. Additional studies may be conducted in the future, using purified enzyme preparations.

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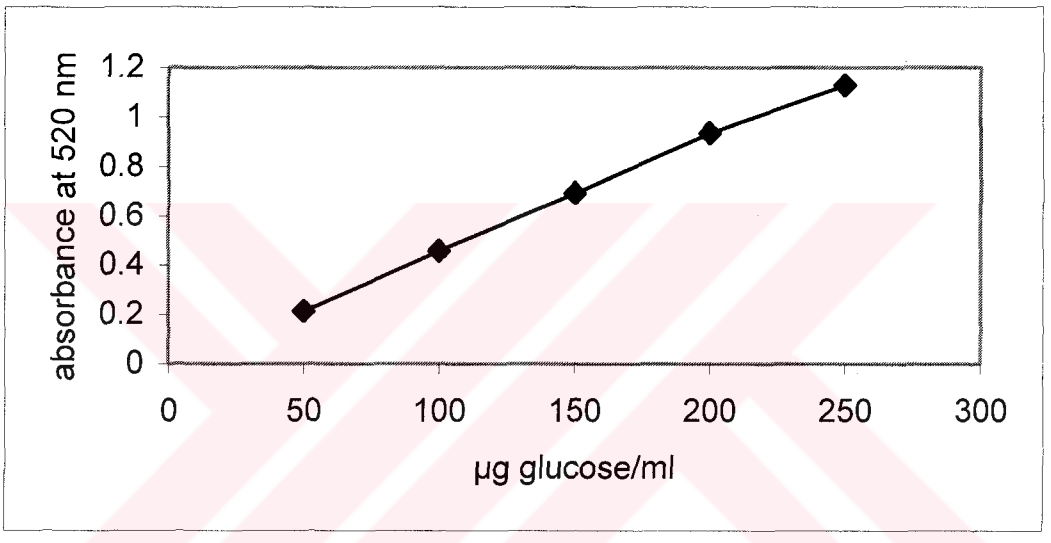
# APPENDIX A

## STANDARD CURVE FOR NELSON-SOMOGYI METHOD



## APPENDIX B

## COMPOSITIONS OF SOMOGYI I, SOMOGYI II AND NELSON REAGENTS

**Somogyi Reagent I:**

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

**Somogyi Reagent II:**

72 g of $Na_2SO_4$ are dissolved in 300 ml boiled distilled water. 8 g of $CuSO_4$ are added, and the solution is diluted to 400 ml with boiled distilled water. The prepared solution is stored at 27°C.

Somogyi reagent I and II are mixed in 4:1 ratio immediately before use.

**Nelson Reagent:**

100 g of $(NH_4)_6Mo_7O_{24}.4H_2O$ is dissolved in 1.8 liters of distilled water and 84 ml of concentrated $H_2SO_4$ is added. Then, 100 ml of $Na_2HAsO_4.7H_2O$ solution is added (12 g of $Na_2HAsO_4.7H_2O$ is dissolved in 100 ml of distilled

water). The solution is stored in brown glass bottle at 37°C for 24-48 hr and then at room temperature.

## APPENDIX C

## STANDARD DEVIATIONS FOR PROTEASE ACTIVITIES IN 1 L CULTURES (FIG. 3.7)

| Time (days) | Protease activity (U/ml) | Standard deviation |
|---|---|---|
| 2 | 1.18 | ± 0.13 |
| 3 | 2.24 | ± 0.44 |
| 4 | 2.16 | ± 0.35 |
| 5 | 1.98 | ± 0.28 |
| 6 | 1.6 | ± 0.34 |
| 7 | 1.24 | ± 0.24 |
| 8 | 0.84 | ± 0.07 |
| 9 | 0.52 | ± 0.52 |

## APPENDIX D

## STANDARD DEVIATIONS FOR EG ACTIVITIES (FIG. 3.7)

| Time (days) | EG activity (U/ml) | Standard deviation |
|---|---|---|
| 2 | 0.062 | ± 0.02 |
| 3 | 0.18 | ± 0.01 |
| 4 | 0.691 | ± 0.001 |
| 5 | 1.112 | ± 0.07 |
| 6 | 1.223 | ± 0.19 |
| 7 | 1.191 | ± 0.19 |

## APPENDIX E

## STANDARD DEVIATIONS FOR PROTEASE ACTIVITIES IN THE PRESENCE OF pCMB (FIG. 3.29a)

| Time (days) | Protease activity (U/ml) - pCMB | Protease activity (U/ml) +pCMB | % inhibition |
|---|---|---|---|
| 2 | 0.69 | 0.465 | 32.6 ± 2.94 |
| 3 | 1.55 | 0.66 | 57.4 ± 2.18 |
| 4 | 1.32 | 0.92 | 30.3 ± 2.71 |
| 5 | 1.06 | 0.82 | 22.6 ± 4.00 |
| 6 | 0.6 | 0.49 | 18.3 ± 6.48 |
| 7 | 0.53 | 0.51 | 3.77 ± 8.21 |
| 8 | 0.43 | 0.39 | 9.3 ± 4.30 |

## APPENDIX F

## STANDARD DEVIATIONS FOR PROTEASE ACTIVITIES IN THE PRESENCE OF PMSF (FIG. 3.33a)

| Time (days) | Protease activity (U/ml) - PMSF | Protease activity (U/ml) +PMSF | % inhibition |
|---|---|---|---|
| 2 | 0.64 | 0.44 | 31.3 ± 9.94 |
| 3 | 1.38 | 0.74 | 46.4 ± 12.29 |
| 4 | 1.32 | 0.78 | 40.9 ± 4.82 |
| 5 | 1.2 | 0.4 | 66.7 ± 10.60 |
| 6 | 1.16 | 0.98 | 15.5 ± 3.04 |
| 7 | 1.18 | 0.96 | 18.6 ± 0.28 |