PRODUCTION OF THERMOSTABLE BETA-GALACTOSIDASE FROM THERMOPHILIC FUNGI FOR USE IN LOW LACTOSE MILK PRODUCTION

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

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

AUGUST 2006

Approval of the Graduate School of Natural and Applied Sciences

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I certify this thesis satisfies all the requirements as a thesis for the degree of Master of Science.

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This is to certify that we have read this thesis and that in our department it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

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ABSTRACT

PRODUCTION OF THERMOSTABLE BETA-GALACTOSIDASE FROM THERMOPHILIC FUNGI FOR USE IN LOW LACTOSE MILK PRODUCTION

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August 2006, 89 pages

The aim of this research was the production of beta-galactosidase from thermophilic fungi for use in low lactose milk production or other possible applications. For this purpose, three thermophilic fungi *Humicola insolens*, *Torula thermophila* and *Thermomyces lanuginosus* were screened for lactase production. Highest lactase activity was observed in *Thermomyces lanuginosus*. The carbon source inducing highest extracellular lactase production in *Thermomyces lanuginosus* was determined as arabinose. When grown on arabinose *T. lanuginosus* produced two major lactase activity peaks, one being at day 4 (beta-galactosidase-A) and second starting following the initiation of biomass degradation at day 3 suggesting the existence of a cell wall-bound beta-galactosidase (beta-galactosidase-B). Maximum activity of the second enzyme was at day 10. Crude enzyme stored at 4ºC and -20ºC was stable over a period of one month. Optimum pH and temperature of crude enzyme were found as pH 6.8 and 65ºC. For concentration of extracellular enzyme, fractional ammonium sulfate

precipitation with 60-85% salt was applied. Comparisons with commercial lactase obtained from *Kluyveromyces lactis* revealed that partially purified lactase from *Thermomyces lanuginosus* was 1.3 times more efficient in hydrolysis of lactose even at 30ºC which is optimum for *Kluyveromyces lactis*. Lactose hydrolysis was enhanced at higher temperatures and reached maximum at 50-60ºC giving 4.7 fold higher hydrolysis than *Kluyveromyces lactis* beta-galactosidase. Molecular weight of the second enzyme was determined as 156 kDa by gel filtration. Being an extracellular enzyme with optimum pH suitable for dairy processes, high thermotolerance and stability, this enzyme has a potential for commercial use.

Keywords: Beta-galactosidase, Thermophilic fungi, *Thermomyces lanuginosus*, *Kluyveromyces lactis*

ÖZ

DÜŞÜK LAKTOZLU SÜT ÜRETİMİ AMACI İLE TERMOFİLİK KÜFLERDEN BETA-GALAKTOZİDAZ ÜRETİMİ

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Ağustos 2006, 89 pages

Bu araştırmanın amacı düşük laktozlu süt üretimi veya olası diğer kullanım alanları için termofilik küflerden beta-galaktosidaz üretimi ve kısmi saflaştırılmasıdır. Bu amaçla üç termofilik küf, *Humicola insolens*, *Torula thermophila* ve *Thermomyces lanuginosus* laktaz üretimi için taranmış. *Thermomyces lanuginosus*'un en iyi aktiviteyi gösterdiği belirlenmiştir. Hücre dışı laktaz üretimini en çok indükleyen karbon kaynağının arabinoz olduğu bulunmuştur. Arabinozda büyütüldüğünde, *T. lanuginosus* laktaz aktivitesinde iki tepe noktası göstermiştir. Bunlardan ilki (beta-galactosidase-A) 4. günde maksimum aktivite gösterirken, hücre-duvarına bağlı olduğu düşünülen ikinci enzimin (beta-galactosidase-B) biyokütle değerindeki düşüşü takiben aktivite gösterdiği ve 10. günde maksimum aktivitede olduğu gözlemlenmiştir. Enzimin 4ºC ve -20ºC'de depolandığında bir ayın sonunda hala aktivitesini koruduğu görülmüştür. Optimum pHsı 6.8, optimum sıcaklığı ise 65ºC olarak bulunmuştur. Hücredışı enzimin konsantrasyonu için 60-85% tuz kullanılarak fraksiyonel amonyum sülfat çöktürmesi kullanılmıştır. *Kluyveromyces lactis'*den elde edilmiş ticari enzimle kıyaslama yapıldığında, *Thermomyces lanuginosus*'dan elde

edilmiş kısmi saflaştırılmış enzimin *K. lactis*'in daha aktif olduğu 30ºC'de dahi laktoz hidrolizinde en az 1.3 daha verimli olduğu saptanmıştır. Daha yüksek sıcaklıklarda enzimin performansı artmış, 50-60ºC arasında maksimum aktiviteyi göstermiş ve 50ºC'de ticari enzimden 4.7 kat daha verimli olduğu bulunmuştur. Enzimin moleküler ağırlığı ise jel filtrastonu ile 156 kDa olarak belirlenmiştir. Enzimin hücre dışı olması, optimum pH değerinin süt pHsına uygun olması, ısıya dayanıklılığı, uzun sure stabil kalabilmesi endüstide kullanılma potansiyeli olduğunu göstermektedir.

Anahtar Sözcükler: Beta-galaktozidaz, Termofilik küf, *Thermomyces lanuginosus*, *Kluyveromyces lactis*

ACKNOWLEDGEMENTS

I would like to express my gratitude to all those who gave me the possibility to complete this thesis. I am deeply indebted to my supervisor Prof. Dr. Zümrüt B. Ögel whose help, stimulating suggestions and encouragement helped me in all the time of research. Her trust, continuous support and scientific excitement inspired me in the most important moments of making right decisions.

I am grateful to my co-supervisor Prof. Dr. Haluk Hamamcı for his guidance, encouragement and support throughout the research.

I extent my sincere appreciation to Dr. Şeyda Açar for her motivation, assistance and advices, especially during protein purification experiments.

I warmly thank to Burçak Gencer and Yonca Yüzügüllü for their guidance in conduction of SDS-PAGE analysis and enzyme assay.

The discussions during weekly seminars of our research group inspired and motivated me throughout the research. I would like to thank everyone in our research group, Sümeyra Gürkök, Gülden Koçlar, Burçak Gencer, Yonca Yüzügüllü, Banu Yalçındağ, Betül Söyler, Elife Ö. Karabacak, Nansilma Amarsaikhan, Alper Söyler, Gökhan Duruksu, Abduvali Valiyev …, for their valuable support and contribution to provide such a warm and stimulating environment.

I would like to express my special thanks to my family and Ruhan Altan, Vuslat Oğuz, Ceylan Şahin, Yeşim Kantaş, H. Mecit Öztop, Elife Ö. Karabacak, Hulusi Acar, Elif Turabi, Ertuğrul Şencan for their friendship, support and love. They never left me alone at difficult times of my life.

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ABBREVIATIONS

A4-β-Gal	*Thermus thermophilus* A4 β-galactosidase
CM	Carboxymethyl
DEAE	Diethylaminoethyl
EC-β-Gal	*E. coli* β-galactosidase
FPLC	Fast Performance Liquid Chromatography
IPTG	Isopropylbetathiogalactoside
KL	*Kluyveromyces lactis*
K_m	Michaelis-Menten constant
ONP	*o*-nitrophenol
o-NPG	*o*-nitrophenyl- β-D-galactopyranoside
Psp-β-Gal	*Penicillium* sp. β-galactosidase
RT	Room Temperature
TL	*Thermomyces lanuginosus* CBS
V_{max}	Maximum velocity
X-Gal	5-bromo 4-chloro 3-indolyl β-D galactoside

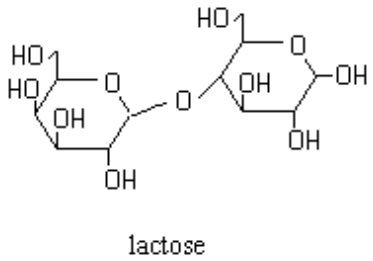
CHAPTER 1

INTRODUCTION

1.1. Lactose and Lactose Intolerance

Dairy products are an important source of many nutrients including calcium, high quality protein, potassium, phosphorus, and riboflavin. In fact, dairy foods provide 73% of the calcium available in the US food supply. This information reveals that it is difficult to meet calcium needs if milk or milk products are not included in the diet. It is known that an adequate intake of calcium throughout life helps to reduce the risk of osteoporosis, hypertension, and possibly some type of cancer like colon cancer (McBean L. D., Miller G. D., 1998; Miller *et al*, 2000).

Lactose in figure 1.1 is a disaccharide formed by β-1,4 glycosidic bond between glucose and galactose. It is the principal carbohydrate in human and animal milk at a percentage of 7 and 4.8, respectively. Thus, it is the most important source of energy during the first year of a human's life, providing almost half of the total energy requirement of infants (Vesa T. H. *et al*, 2000).



lactose

Figure 1.1 Chemical structure of lactose

Lactose has also several applications in the food industry. It is used, for instance, in sweets, confectionery, bread and sausages because of its physiological properties: lactose provides good texture and binds water and color. Lactose is only about one third as sweet as saccharose and less than half as sweet as glucose (Vesa T. H. *et al*, 2000).

Dietary lactose is not digested directly; it needs to be hydrolyzed to its components, glucose and galactose by lactase found at the tip of intestinal vili in the jejunum. There is also lactase-phlorizin hydrolase encoded by the gene encoding lactase in the small intestine. This enzyme is suggested to split β-glycosides with a large hydrophobic alkyl chain as in glycosyl ceramides which are also found in milk (Troelsen J. T., 2005)

In most mammals, lactase enzyme reaches maximal activity soon after birth then decreases gradually, reaching a low level after weaning. Researchers have concluded that human adult-onset lactase decline is controlled by a single autosomal recessive gene. Researchers using the sucrase/lactase ratio and the lactase/maltase ratio measured in intestinal biopsies, found a trimodal distribution (low, intermediate, and high) of lactase expression. Subjects with a homozygous recessive inheritance pattern had low levels of lactase expression; those who were heterozygous had intermediate levels; those who had a homozygous dominant pattern had high lactase activity (Miller *et al*, 2000). Actually, the decline in lactase activity, so-called lactose non-persistence or lactose maldigestion or primary lactase deficiency is not a disease but rather the normal physiologic pattern. In contrast, maintenance of high lactase levels, so-called lactase persistence, occurs in only a few populations of specific ethnicity like those from norhern Europe, central Africa and the Middle East (McBean L. D, Miller G. D, 1998). There is a hypothesis for lactase persistence such that it is commonly observed in areas with long traditions of dairy farming since after the beginning of dairy farming, there would have been an advantage for those individuals who had high lactase in their intestine. Resulting from increased survival, high intestinal lactase would have become typical of such a group (Vesa T. H. *et al*, 2000).

1.1.1 Types of Lactose Intolerance

There are mainly three types of lactase deficiency: congenital, secondary, and primary. Among them, the most severe one is congenital lactase deficiency (CLD) in other words alactasia. It is extremely rare condition in which detectable lactase levels are absent or severely reduced at birth and remains abnormal throughout the life. These infants need to follow a lactose free diet for the rest of their lives; they can not tolerate even small amounts of lactose. Otherwise, severe diarrheal illness is observed beginning a few days after birth (Miller *et al*, 2000).

As defined above primary lactase deficiency-most common type is the decline of lactase activity after weaning between 2 and 20 years of age, depending on race/ethicity. The onset of decline is probably determined genetically. Main symptoms are dehydration, poor calcium absorption, diarrhea, flatulence, bloating, belching and cramps, and in severe cases, even watery explosive diarrhea depending on level of tolerance of the individual and the amount of lactose consumed (Shukla *et al*., 1975, McBean L. D, Miller G. D, 1998). Secondary lactase deficiency results from small intestine resections, gastrectomy and diseases that damage the intestinal epithelium, for example untreated celiac disease or intestinal inflammation. It is reversible, when the epithelium heals, the activity of lactase returns. However, it does not lead to severe symptoms of intolerance (Vesa T. H., 2000).

Sometimes lactose intolerance is confused with milk allergy in which one or more milk proteins stimulate the immune system of the body. Table 1.1 indicates the differences between these two diseases.

1.1.2 Diagnosis

As indicated in Table 1.1, lactose intolerance can simply be diagnosed by breath hydrogen test since when lactose is incompletely digested; the remaining lactose is fermented by the colonic bacteria in the intestine forming hydrogen gas. A portion of hydrogen gas is absorbed to portal circulation and subsequently

expired in the breath. Lactose intolerance can also be diagnosed by measurement of lactase activity in small intestinal biopsy samples. This measured lactase activity is compared with the activity of another small intestine enzyme, sucrase. Breath hydrogen test is appropriate for primary screening of the intolerance however it is not as reliable as the technique based on comparison of enzymatic activities in the small intestine. There are also genetic studies to find a reliable and fast method for diagnosis of the disease (Troelsen J. T., 2005). In order to increase reliability of breath hydrogen test, it is also possible to measure breath $^{13}CO_2$ after ^{13}C-lactose ingestion. Measurement of urinary galactose using an enzyme strip is another method for diagnosis (Vesa T. H. *et al*, 2000).

Table 1.1 Comparison of milk allergy and lactose intolerance (Miller *et al*, 2000)

	Lactose Intolerance	**Milk Allergy**
Cause	Low intestinal levels of lactase	Abnormal immune response to ingestion of cow's milk protein
Age of onset	Early/late childhood	Usually in infancy
Symptoms	Abdominal gas, bloating, cramps, diarrhea	Abdominal pain, vomitting, diarrhea, nasal congestion, skin rash
Diagnosis	Breath hydrogen test	Food elimination and challenge, RAST blood test
Dairy Food Use/ Avoidance	No need to eliminate dairy foods, only adjust the dose of lactose consumed	Eliminate cow's milk from the diet for a time

1.1.3 Prevalence of Lactose Intolerance

Age and ethnicity affects the distribution and level of lactose intolerance. For instance, in Blacks and Asians, disease usually manifests itself in early childhood, whereas in whites, it seems to occur later in childhood or in adolescence. The prevalence is above 50% in South America, Africa, and Asia, reaching almost 100% in some Asian countries. In the United States, the prevalence is 15% among whites, 53% among Mexican- Americans and 80% in

the Black population. In Europe it varies from around 2% in Scandinavia to about 70% in Sicily (Figure 1.2). Although there is not enough research about gender effect on the disease, the results gained so far indicates that there is no gender effect on the disease (Vesa T. H. *et al*, 2000).

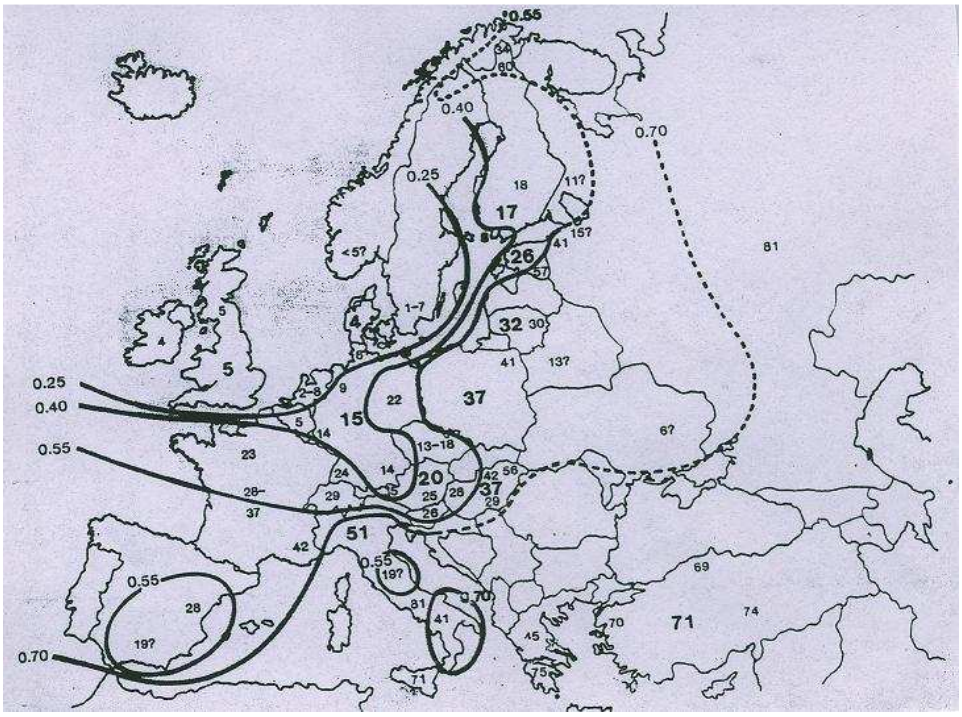


Figure 1.2 Prevalence of adult-type hypolactasia in different European countries and populations (small number = prevalence of a population, large number = average prevalence of the country) and the hypothetical isograms for the frequencies of the lactase non-persistence gene. (Vesa T. H. *et al*, 2000)

1.1.4 Strategies to Struggle with The Disease

Several strategies are available to struggle with primary lactose maldigestion - the most common type. Most of the patients can tolerate 240 ml serving of milk in a day. The amount of lactose to be tolerated can increase when lactose-containing food are consumed with other solid foods since gastric emptying is delayed, so reducing and delaying peak hydrogen levels. Delayed

gastric emptying allows more time for lactase present to digest dietary lactose. It also reduces the amount of undigested lactose that reaches the colon at any one time (Miller *et al*, 2000). Moreover, toleration to lactose changes according to type of dairy food consumed. For instance, many cheeses and ice-cream, surprisingly chocolate milk are better tolerated than milk. In cheese production, most of the lactose is left with whey. Fermented dairy foods are also well tolerated due to consistency of yoghurt resulting in slowing in gastric emptying and release of lactase from bacterial cultures used to make yoghurt. Pasteurization of yoghurt reduces bacterial effect on lactose digestion, so it is important to consume yoghurt with active cultures. There are also studies showing that the level to lactose tolerance can improve by following a diet strategy based on adaptation of the body to lactose by gradual increase of lactose consumed daily. This results from enhancement of colonic bacteria in the intestine to metabolize lactose. Elimination of lactose from the diet may actually worsen the intolerance to lactose (McBean L. D., Miller G. D., 1998).

Lactose maldigesters can also consume low lactose or lactose-free dairy foods which are commercially available. Lactose reduced milk, cheese, ice-cream are available in the market. Oral enzyme tablets containing lactase withstanding stomach acidity is another way to improve tolerance to lactose. However, allergic reactions were seen in some cases (Vesa T. H. *et al*, 2000).

1.2. β-galactosidase

β-galactosidase (E.C. 3. 2. 1. 23) (β-D-galactohydrolase, β-D-galactoside galactohydrolase, galactosyltransferase and lactase), can catalyze both hydrolytic and transfer reactions. The enzyme hydrolyzes β-1,4 linkage between glucose and galactose in lactose (Figure 1.3). This enzyme also cleaves *o*-glycosidic bond of other β-D galactopyranosides.

The resulting sugars –glucose & galactose- are sweeter, more soluble, more readily fermented, and absorbed directly from the intestine. Thus, β-galactosidase increases sweetness, solubility and digestibility of the final product. Another difference observed is higher reactivity of these monosaccharides compared to lactose. Consequently color, flavor, and taste may deteriorate more during heat treatment because of the browning and caramellization reaction (Zarate *et al*, 1990).

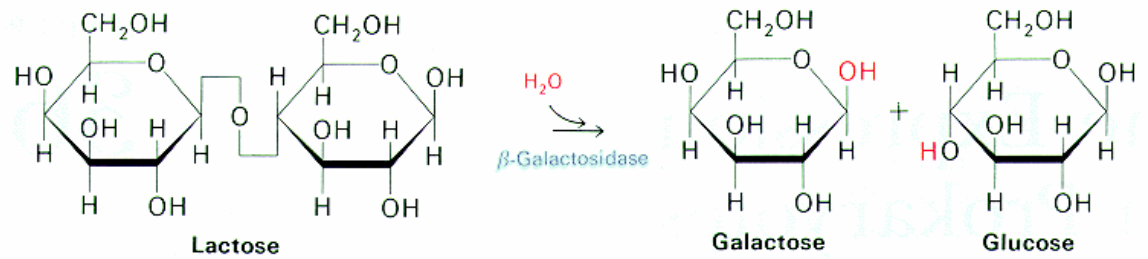


Figure 1.3 Hydrolysis of lactose via β-galactosidase

The mechanism of lactose hydrolysis was first described by Wallenfels and Malhotra (1960) who used the lactase from *E. coli*. The important step is the formation of the enzyme galactosyl complex with simultaneous glucose liberation. According to Wallenfels, only D-pyranoside ring will be hydrolyzed, whereas the β-glucosidic bond stays intact. However, it can block the active side of the enzyme (Prenosil *et al*, 1987).

Galactose is generally considered to be a competitive inhibitor where it competes with lactose for the enzyme active sites. Flaschel and co workers (1982) found α-galactose to be a significantly stronger inhibitor than the β form.

It is suggested that a minimum of three steps were involved, the last of which allows for hydrolysis or transferase activity:

enzyme + lactose ⟶ enzyme-lactose (1)

enzyme-lactose ⟶ galactosyl-enzyme + glucose (2)

galactosyl-enzyme + acceptor ⟶ galactosyl- acceptor + enzyme (3)

1.2.1 Transferase Ability of β-galactosidase

Thus, β-galactosidase has a second role, transferase activity. Actually, its hydrolytic and transferase activity occur together since enzymatic lactose hydrolysis is a complex process involving a multitude of sequential reactions with saccharides as intermediate products. The transferase reactivity by which the enzyme produces and subsequently hydrolyses a series of oligosaccharides containing galactose was reported in 1950s (Aranson *et al*, 1952; Pazur *et al*, 1953). Enzyme transfers galactose moiety to an acceptor containing a hydroxyl group as it is shown in the second part of the Figure 1.4. When this acceptor is water, galactose is formed. However, other sugars present in the solution, can also serve as acceptors, and in this way new oligosaccharides are formed. According to recent studies, the mechanism is analogous to that of lysozyme, i.e. there is a group acting as a general acid which donates a proton to the glycosidic oxygen and another negatively charged group which stabilizes a positively charged carbonium galactosyl intermediate, probably by forming a transient covalent bond (Sinnott *et al*, 1978, Mahoney *et al*, 1998). The general reaction mechanism can then be depicted as shown in Figure 1.4.

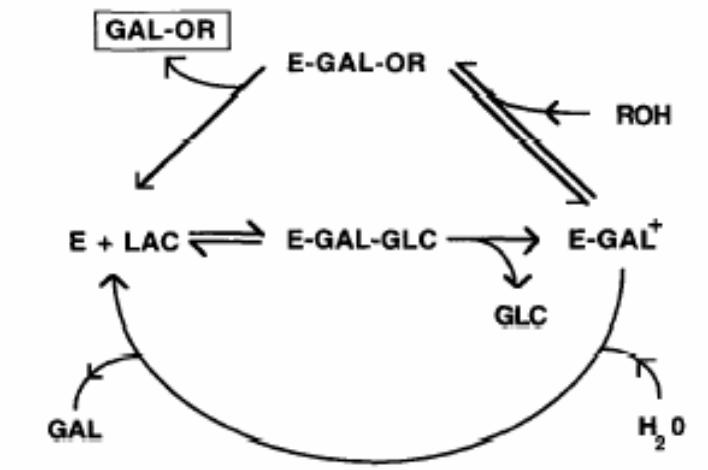


Figure 1.4 A proposed reaction mechanism for the action of β-galactosidase on lactose. E: enzyme, LAC: lactose, GAL, galactose, GAL+: carbonium transition state, GLC: glucose, ROH: acceptor sugar, GAL-OR: galactosyl sugar (oligosaccharide). (Cupples *et al*, 1990, Mahoney *et al*, 1998)

The type and amount of oligosaccharide in the course of lactose hydrolysis depends on; source of the enzyme, nature and concentration of substrate, type of process (free or immobilized enzyme), salts, temperature and pH, degree of lactose conversion (Zarate and Lopez Leiva *et al*, 1990). Peak level of oligosaccharides increases with increased starting lactose levels (Mahoney *et al* 1998, Prenosil *et al*, 1987). For lactose hydrolysis, under industrial conditions, a conversion between 75-85% is considered as being optimal (Prenosil *et al*, 1987, part 2). For oligosaccharide production, highest levels are associated with neutral pH enzymes from bacteria and yeast rather than acid pH enzymes from moulds. Moreover, it would seem that galactose can be transferred to any of the hydroxyl groups on acceptor sugars, except for C1 hydroxyl. Additionally, peak levels follow the order, di> tri> tetra and higher oligosaccharides and the linkages synthesized are β(1-6)>β(1-4)>β(1-3)>β(1-2) (Prenosil *et al*, 1987 part2, Mahoney *et al*, 1998, Toba and Adachi *et al*, 1978, Smart, 1990).

1.2.2 Structural Properties of β-galactosidase

Glycosidases may be classified on the basis of two independent criteria, either via their EC numbers (derived from the chemistry of the catalyzed reaction) or on the basis of their evolutionary relationships. The second criterion, based on sequence homology, divides glycosyl hydrolases into 90 families (GHFs) of which the known β-galactosidases belong to GHF-1, GHF-2, GHF-35 and GHF-42. These are all members of a superfamily (or clan) termed GH-A, which comprises families 1, 2, 5, 10, 17, 26, 30, 35, 39, 42, 51 and 53. GH-A enzymes all cleave glycosidic bonds via a retaining mechanism and possess a catalytic domain which is based on a TIM barrel fold. Two glutamic acid residues act as proton donor and nucleophile14 and eminate from strands 4 and 7 of the barrel and for this reason this clan is sometimes referred to as the 4/7 superfamily (Rojas *et al*, 2004).

The β-galactosidase of *Escherichia coli* (EC-β-Gal) belongs to GH-2 family and was instrumental in the development of the operon model. It is homotetramer with 222 point symmetry and 465,412 kDa molecular weight. Its monomer is composed of five compact domains plus ~ 50 residues at the N-terminus that are relatively extended and contribute to the activating interface, which corresponds to the α-complementation peptide. The first domain corresponds to a jelly-roll β-barrel while the second domain has a fibronectin-type III fold. The third domain forms a distorted TIM barrel and contains the catalytic site. The forth domain, residues 628-736, is topologically identical to the second domain. The core of fifth domain consists of a novel 18-stranded, antiparallel sandwich. The residues Glu461, Met502, Tyr-03 and Glu537 have been shown to be important for catalytic function, or near the active site. (Jacopson *et al*, 1994)

The *Thermus thermophilus* A4 β-galactosidase (A4-β-Gal) consists of 645 amino acid residues and belongs to GH-42. Today, 29 β-galactosidases are placed in GH-42. These enzymes consist of 600–700 amino acid residues, and exhibit no sequence similarity with GH-2 EC-β-Gal. catalytic residues of A4-β-Gal were inferred to be Glu141 and Glu312. A4-β-Gal has a trimeric structure and three monomers are crystallographically identical. An A4-β-Gal monomer consists of three domains: domain A, a TIM barrel fold domain (residues 1–389); domain B, an α/β fold domain (390–589); and domain C, a β fold domain (590–644). Domain A of A4-β-Gal contains a metal-binding site. Cys106, Cys150, Cys152, and Cys155, which are highly conserved in GH-42 enzymes, form a metal-binding cluster. A4-β-Gal monomers interact with each other tightly. It has been reported that native A4-β-Gal is monomeric, because its molecular mass was estimated to be approximately 75 kDa and 80 kDa by SDS-PAGE and gel-filtration, respectively. However, that of recombinant A4-β-Gal was estimated to be 170 kDa by gel-filtration, indicating that the recombinant A4-β-Gal is oligomeric (dimeric or trimeric). Actually, A4-β-Gal is trimeric in the crystal structure, and probably exists as a trimer in Thermus cells, because the trimeric form shows a tight interaction. It seems that this discrepancy was caused by

differences in the purification methods. For example; hydrophobic column reduces the specific activity severely. (Hidaka *et al*, 2002)

Penicillium sp. β-galactosidase (Psp-β-Gal) belongs to family GH-35. Psp-β-Gal is monomeric with 120 kDa molecular weight. Like EC-β-Gal, the structure of Psp-β-Gal can be divided into five domains. However, in EC-β-Gal the catalytic site is in the third domain while in Psp-β-Gal it is in the first domain which is a distorted TIM barrel comprising 355 aminoacid residues. Active site residues of Psp-β-Gal are Glu200 and Glu299. (Rojas *et al*, 2004). Figure 1.5 represents the stereo view of the structural superposition of Psp-β-gal, EC-β-gal and A4-β-gal.

The molecular mass of *Rhizomucor* β-galactosidase was found to be 250 000 by gel filtration and 120 000 by SDS-PAGE indicating that the enzyme was a dimer of identical subunits (Shaikh *et al*, 1999). *Thermomyces lanuginosus* β-galactosidase was also suggested to have a dimeric structure with identical subunits of 75-80 kDa. The molecular mass of native β-galactosidase was estimated by size exclusion chromatography at an apparent 200-220 kDa during gel-filtration. This value is not twice of molecular weight of each subunit as 75-80 kDa possibly due to the fact that the enzyme is glycosylated and not globular in its dimeric configuration (Fisher *et al*, 1995). Molecular mass of native β-galactosidases from different *Aspergillus* species, *A. oryzae*, *A. foetidus*, *A. fonsecaeus* were found as 90 000, 126 000, 126 000, respectively (Park *et al*, 1979; Borglum and Sternberg, 1972; Gonzalez and Monsan, 1991).

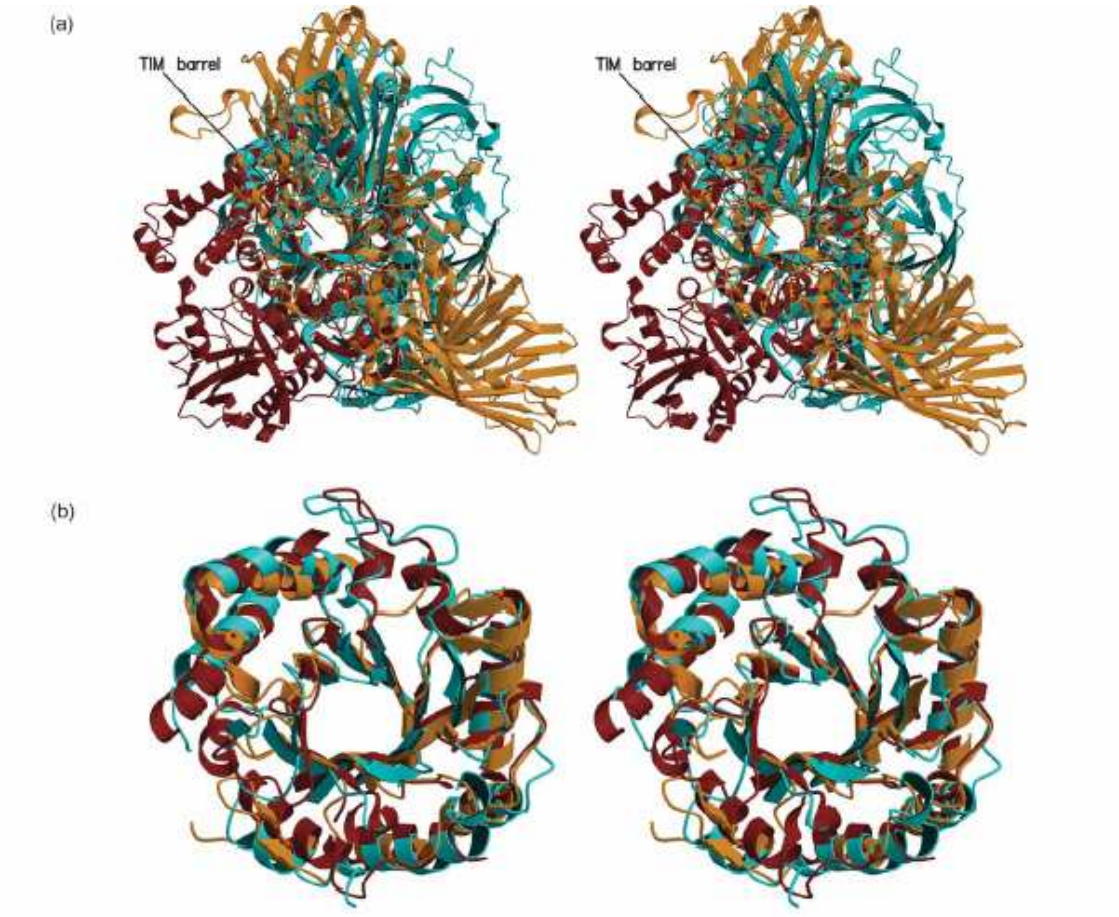


Figure 1.5 (a) Stereo view of the structural superposition of Psp-β-gal (in cyan), EC-β-gal (in orange) and A4-β-gal (in brown). (b) Stereo view of the superposition of their respective TIM barrel domains. (Rojas *et al*, 2004)

1.3. Applications

The use of β-galactosidase, an enzyme capable of hydrolyzing lactose, fills a niche area in food processes in case of utilization of lactose and whey solids. Its main application area is dairy industry low lactose milk production to meet the need of large percentage of population suffering from lactose intolerance,

sensitivity to lactose due to their lack of intestinal lactase. Lactose can also be hydrolyzed using acid, but this causes color formation and fouling of the ion-exchange resins used in processing (www.fst.rdg.ac.uk/courses/fs916/lect12/lect12.htm). Thus, enzyme technology seems to be the most proper way to perform this reaction because enzymes are able to hydrolyze lactose without side reactions leading to losses in organoleptical and alimentary properties of milks, with considerable savings in material and energy in the industrial processes (Gekas and Lopez-Leiva *et al*, 1985). Low-lactose milk is produced in processing plant by adding the liquid enzyme to previously pasteurized milk and holding it for 24 hours. The milk is then repasteurized to halt lactose hydrolysis once appropriate level of reduction has been reached. As well as low-lactose milk with 70% to 100% hydrolysed lactose, lactose reduced cottage cheese, processed cheese, ice-creams are also produced commercially (McBean *et al*, 1998). For low-lactose milk production, enzyme extracted from *Kluyveromyces lactis* or *K. fragilis* which are in GRAS status are used. For oral enzyme tablets against lactose intolerance, enzymes which are active at stomach acidity like lactase from *Aspergillus* species are generally used.

Another use of lactase in dairy industry is to increase sweetness and digestibility of the final product. Since low solubility of lactose leads to its crystallization causing sandy, gritty texture which is a problem for some dairy product such as ice-cream, frozen milk, dry milk, condensed milk, evaporated milk and in confectionary products with a high content of milk. Lactase also improves the utilization of high protein supplements containing milk (Sorensen and Crisan, 1974). Lactose conversion is also used in sweetener production from whey, a biological by-product of cheese processing, since most of the lactose pass to whey during cheese production (http://www.egr.msu.edu/~steffe/handbook/fig151.html).

Environmental pollution caused by by-products of dairy industry is another problem related to lactose. Since the recovery of valuable individual components of whey containing high amount of lactose is not economical, it should be disposed and its disposal becomes a major problem especially after new

regulations forbidding disposal of biologically active compounds (Zarate and Lopez Leiva *et al*, 1990).

Lactose is used in sweets, confectionery, bread and sausages because of its physiological properties such as providing good texture and binding water and color. However, lactose is not fermented by ordinary baker's yeast. Thus, lactase can be used since its hydrolysis products can be readily fermented and improve the toasting of bread and browning of the bread crust due to enhanced Maillard reaction. Feed industries might convert whey lactose to sugars that do not cause diarrhea by incorporating lactase into feed formulas (Pomeranz *et al*, 1964).

In addition, β-galactosidase has found several applications in the laboratories of molecular biologists. Since many organisms completely lack this activity, scientists can splice the gene for enzyme into cells, and detect the presence of the gene by an assay for β-galactosidase. Moreover, lactase can be used as a reagent for determining lactose in blood and other biological fluids. (http://www.ns.purchase.edu/biology/bio1550lab/alt-intro.htm).

Now, by its transferase activity, lactase has another application area, regio-and stereospecific glycoconjugates and oligosaccharide production which can be used as a food ingredient, pharmaceutical or a biologically active compound. Oligosaccharides are largely indigestible in the upper intestine so they can be thought of as low-molecular weight, non-viscous, water-soluble dietary fiber. Thus, they are considered physiologically functional foods. Oligosaccharides increases growth of bifidobacteria present in the colon. This results in decreasing in the activity of putrective bacteria like *Clostridia* and toxic fermentation products (Tomomatsu *et al*, 1994). Oligosaccharides also lead to lower blood cholesterol (Chonan *et al*., 1995; Kikuchi *et al*., 1996), higher Ca absorption, a smaller loss of bone tissue (Chonan and Watanuki *et al*, 1995), lower incidence of colon cancer (Rowland *et al*, 1997), improved liver function (Hawkins *et al*, 1993). Moreover, transgalactosylated oligosaccharides can be used as a suitable replacer of lactose in ice-cream mixtures and other products because their configurations are similar to that of lactose and because they prevent crystallization of this saccharide (Modler *et al*, 1990).

Since β-galactosidases with different properties are necessary for numerous different applications, screening of new sources of this enzyme which is active at different conditions introduces a growing research area.

1.4. Sources of Enzyme

β-galactosidase is produced by various organisms including plants, animals, microorganisms. It exists in many plants like Rosaceae, almonds, tips of wild roses, seeds of soy beans, alfalfa, and coffee (Wallenfels *et al*, 1960). The enzyme has also been found in animals like snails, in the intestine of dogs, rabbits, calves, sheep, goats, rats, rams, bulls, boars (Wallenfels *et al*, 1960; Cajori *et al*, 1935; Conchie 1959a). Moreover, numerous studies has shown the presence of enzyme in human saliva, distribution of lactase in fetuses of primates and farm animals (Heilskov *et al*, 1951), in tissues of rats and mice and in plasma serum and urine of dogs. According to research of Doell and Kretchmer (1962), lactase activity is maximum in the jejunum, moderate in duodenum, least in the cecum and absent in the stomach (Pomeranz, 1964).

Although these various plant and animal sources of the enzyme are present, potential commercial source of the enzyme has been considered as microorganisms due to complexity of other sources. The most thoroughly investigated β-galactosidase with regard to reaction mechanism, three dimensional structure (Jacopson *et al*, 1994), genetic structure is the one obtained from *E. coli*. Other bacterial sources are various strains of *Lactobacillus* (Wallenfels and Malhotra *et al* 1960, Wierzbicki and Kosikowski *et al*, 1971), *Shigella* (Rickenberg *et al*, 1960), *Pneumococci* (Fleming and Neill *et al*, 1927), *Staphylococci* (Creaser *et al*, 1955), *Bacillus megatherium* (Landman *et al*, 1957), *Bacillus stearothermophilus* (Goodman *et al*, 1976), *Thermus aquaticus* (Ulrich *et al*, 1972), *Sclerotina sclerotiorum* (Cristine *et al*, 1992).

1.4.1. Thermophilic Fungi

The first thermophilic true fungus was found as *Mucor pussilus* by Lindt in 1886. Thermophilic fungi grow within a temperature range of 20ºC to 50ºC or higher. Forms such as *Aspergillus fumigatus*, *Absidia ramosa*, and others, which may grow at or near 50ºC but which also grow well at temperatures below 20ºC, are considered thermotolerant and are excluded from thremophilic fungi. (Cooney and Emerson, 1964) While *Thermomyces lanuginosus* can grow at 60ºC which is the highest temperature at which thermophilic fungi can grow, *Humicola stella* is the one which can grow at the lowest possible temperature. This slightly differs from the highest growth temperature of thermotolerant mesophiles such as *Aspergillus fumigatus* (Figure 1.6) (Cooney and Emerson, 1964).

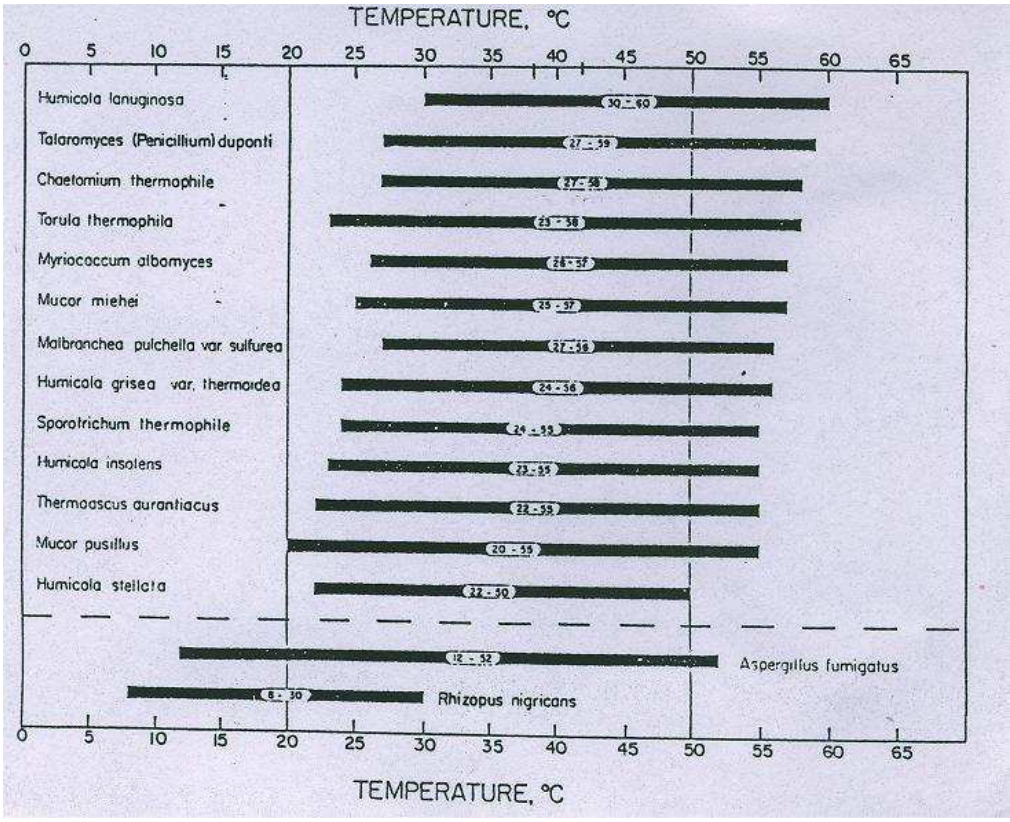


Figure 1.6 Temperature profiles of various thermophilic fungi

Thermophilic fungi are also important for industrial processes such as edible mushroom production, tobacco processing…etc. Preparation of the beds for mushroom usually involves a typical microbial self-heating and composting of the carefully prepared mixtures of horse manure and straw or other ingredients. Atkins (1961) describes this as an aerobic process in which eating occurs to about 60ºC for several days. Fungi take role in transforming the original material into an excellent substratum for the mushroom mycelium and destroying unwanted pests by performing a very effective pasteurization. The decomposition of the readily available nutrients in the raw starting materials also prevents subsequent unwanted heating. The isolations from mushroom compost made by investigators at the Butler County Mushroom Farm in Pennsylvania showed presence of fungi, *Humicola grisea* var. *thermoidea*, *Humicola lanuginosa*, *Torula thermophila*, *Myriococcum albomyces*. It appears that these fungi contribute to the heat that is generated, and are also involved in the critical decomposition processes required for the production of a good mushroom bed (Cooney and Emerson, 1964; p141).

Thermophilic fungi with the highest growth temperature, *Thermomyces lanuginosus* was isolated first in 1899 by Tsiklinskaya, who observed the fungus on patato which had been inoculated with garden soil. This specie is undoubtedly one of the most ubiquitous of the thermophilic fungi. Now the authors uniformly use the original name *Thermomyces lanuginosus*, as proposed by Tsiklinskaya (Table 1.3) (Cooney and Emerson, 1964; p82-83).

Table 1.2 Synonyms of *Thermomyces lanuginosus*

Humicola lanuginosa	Bunce (1961)
Thermomyces lanuginosus	Tsiklinskaya (1899, pp500-505)
Sepedonium lanuginosum	Griffon and Maublane (1911)
Sepedonium thermaphilum cyclosporum and *S. Thermaphilum ovosporum*	Velich (1914)
Acremoniella sp.	Rege (1927)
Acremoniella thermophila	Curzi (1929)
Monotospora lanuginosa	Mason (1933)

Thermomyces lanuginosus can grow easily on YpSs medium (Composition was given in Appendix) at 45ºC. Optimum growth occurs between 45-50ºC. No growth is observed at temperatures either below 30ºC or above 60ºC. It is a real thermophilic fungus, which presumably synthesizes enzymes of higher working temperatures due to its higher optimum temperature and better thermal stability than its mesophilic counterparts. Therefore, it seems to be an ideal source for thermostable enzyme production (Rezessy-Szabó *et al*, 2003; Xiong, 2004).

The colonies appear white and felty at first, less than 1 mm high, but soon turn gray, or greenish gray, beginning at the center of the colony. Gradually the colony turns purplish brown, and at this time the agar substratum stains a deep pink or wine color due to diffusible substances secreted by the colony.

Thermomyces lanuginosus (Figure 1.7 and 1.8) has colorless, septate hyphae of 1.5-4 μ in diameter; short, often septate, unbranched aleuriophores with 10-15μ in long, or branched near the base, single filamentary aleuriospores on each aleuriophore, which are colorless, smooth-walled, spherical when young becoming dark brown, sculptured with age.

Thermomyces lanuginosus is extremely common in all types of self-heating material, guayule rets, hay and grass, garden compost, manure, mushroom compost, potato, oats, straw, and also in birds' nests and sun-heated soils. It colonizes composts after peak-heating and persists throughout the high-temperature phase. However, it cannot degrade cellulose and it seems to live as a commensal with cellulose-decomposing species, sharing some of the sugars released from the plant cell walls by their cellulolytic activities (http://helios.bto.ed.ac.uk/bto/microbes/thermo.htm).

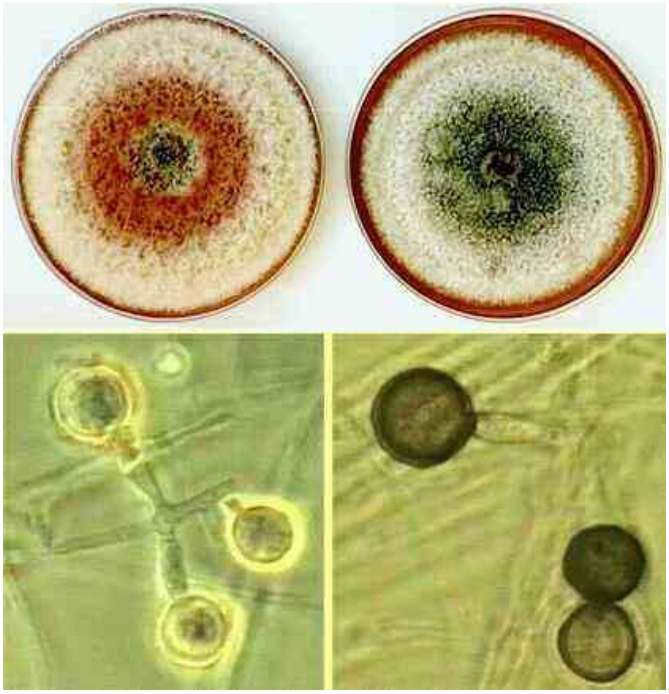


Figure 1.7 *Humicola* (or *Thermomyces*) *lanuginosus*. Colonies growing on potato-dextrose agar (top left) and malt extract agar (top right) at 45°C. This fungus produces single spores by a balloon-like swelling process at the tips of short hyphal branches (bottom, left). At maturity (bottom right) the spores have brown, sculptured walls. (http://helios.bto.ed.ac.uk/bto/microbes/thermo.htm)

Figure 1.8 Spores of *T. lanuginosus* grown on YpSs agar for eight days (Picture was taken in central laboratory in Middle East Technical University by inverse light microscopy.)

1.4.1.1. β-galactosidase from *Thermomyces lanuginosus*

Fischer and co-workers, 1995 have studied β-galactosidase from *Thermomyces lanuginosus* ATCC 16455 strain. Thermophilicity is important not only for application during pasteurization, but also important for less possibility of microbial contamination, decreasing viscosity, so increasing rate of reaction with increasing temperature. Table 1.4 summarizes the results of characterization experiments of intracellular β-galactosidase *from T. Lanuginosus*.

Table 1.3. Properties of intracellular β-galactosidase *from T. lanuginosus* (Fisher *et al*, 1998)*;*

pI	4.4-4.5
pH optima stability	6.7-7.2 6-9
Temperature stability at 50ºC	47% remaining act. after 48 h
Molecular weight (kDa)	200 (tertiary structure) 75-80 (primary structure)
Metal dependency	no
Substrate Specificity	o-NPG>p-NPG>>lactose

o-NPG; o-nitrophenyl-β-D galactopyranoside
p-NPG; p-nitrophenyl-β-D-galactopyranoside

1.4.2. Other Fungal β-galactosidases

Since most of the β-galactosidases from bacterial sources are intracellular and are not in GRAS (generally recognized as safe) status, they are not used especially in food systems. Furthermore, fungi give higher yields and their enzymes show broad stability profiles. Therefore, the commercially available β-galactosidases have been mainly obtained from fungi; yeast and molds. The most important sources *are Kluyveromyces lactis, Kluyveromyces fragilis (Sacchoramyces fragilis)* (Castillo *et al*, 1990; Caputto *et al*, 1948; Wendorff and

Amundson *et al*, 1971) and some *Aspergillus* species which are accepted as GRAS by FDA. (Ladero *et al*, 2002) The enzymes from yeast are generally membrane bound (Pastore and Park *et al*, 1979), active at neutral pH and, thus, employed in milk and sweet whey. The enzymes from fungi are generally extracellular, active at acid pH and are used to hydrolyse lactose in acid whey (Gekas and Lopez-Leiva *et al*, 1985). However, researchers have found that cellulases and proteases produced by *Scytalidium thermophilum*, thermophilic fungus, have generally an optimum pH around neutrality (Arifoğlu, Ögel, 2000; İfrij, Ögel, 2002). This indicates that enzymes of thermophilic fungi may be suitable for dairy processing

Aspergillus oryzae is one of the fungal sources of extracellular β-galactosidase that has been used commercially (Park, De Santi, Pastore *et al*, 1979). Park and co-workers have tried to purify and characterize β-galactosidase from *Aspergillus oryzae*. They have found 50°C as the optimum temperature and 5 as the optimum pH and high pH stability. Galactose was a competitive inhibitor while the other hydrolysis product, glucose was not an inhibitor. Because of the high pH (6.5) of milk, enzyme was found more appropriate for whey application than hydrolysis of lactose in milk.

Studies are also conducted on the purification of fungal β-galactosidases. These studies involve similar purification steps. These steps are ammonium sulfate fractionation, choromatography on DEAE-cellulose, choromatography on CM-cellulose and DEAE-sephadex column choromatography, respectively (Park, De Santi, Pastore *et al*, 1979; Borglum and Sternberg *et al*, 1972). Table 1.2 summarizes general properties of fungal β-galactosidases.

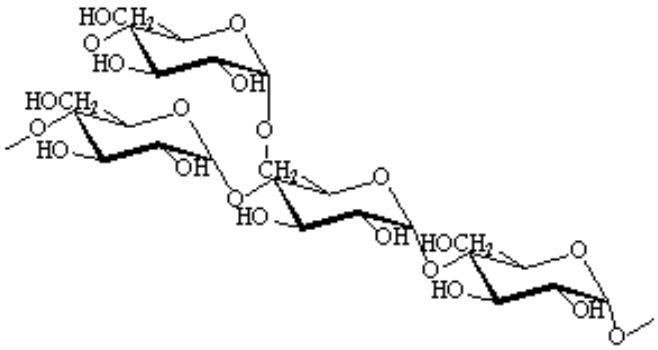
Table 1.4 Some yeast and fungal sources of β-galactosidase and their general properties

Source	opt. pH	opt. T.(ºC)	Location	Reference
Kluyveromyces fragilis	6.3 – 6.5	35-45	Cell bound	Wierzbicki & Kosikowski (1973) Matioli, Moreas & Zanin (2001)
Aspergillus oryzae	4.5 - 5	55-60	E	Park, Santi, Pastore (1979)
Aspergillus foetidus	3.5 - 4	66-67	E	Borglum & Steinberg (1972)
Aspergillus fonsecaeus	2.6 - 4	55-60	E	Gonzalez & Monsen (1990)
Aspergillus niger	3.5 – 4.5	55	not reported	Wallerstein Co. (1974)
Scopulariopsis	3.6 - 5	50-65	E	Wierzbicki & Kosikowski (1973)
Rhizomucor	4.5	60	E	Shaikh, Khire & Khan (1997)
Mucor pusillus	4.4	60	I	Sorensen & Crisan (1974)
Fusarium moniliforme	3.8 - 5	50-60	E	Macris & Markakis (1981)
Neurospora crassa	4		E	Comp & Lester (1971)
Thermomyces lanuginosus	6.7 – 7.2	--	I	Fischer, Scheckermann & Wagner (1995)
Penicillium simplicissimum	4 - 4.6	55-60	E	Cruz & coworkers (1999)

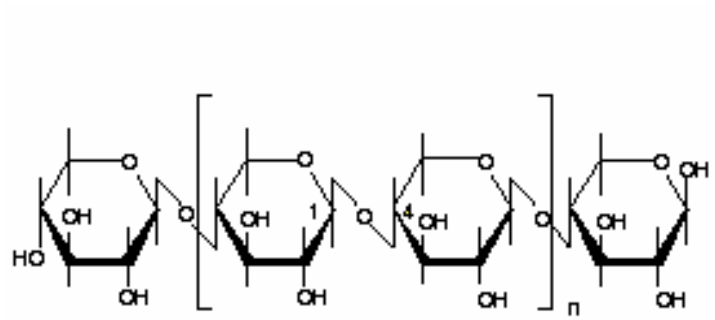
E: extracellular; I;intracellular

Borglum and Sternberg (1972) have found that a mutant strain of *Aspergillus foetidus* (Black Fungi) produces lactase extracellularly and reported its characterization and purification. Results have been very similar to that found in *A. oryzae*. Gonzalez and Monsan also have searched for β-galactosidase activity in *Aspergillus fonsecaeus*. Recent research on β-galactosidase from *Aspergillus niger* revealed that the role of β-galactosidase of *A. niger* in nature is more likely in removing β-linked galactose residues from plant-derived oligo and polysaccharides, than the hydrolysis of lactose. This has been proven by different expression rates on different carbon sources. Highest expression of β-galactosidase encoding lacA gene has been observed on xylose, arabinose, pectin

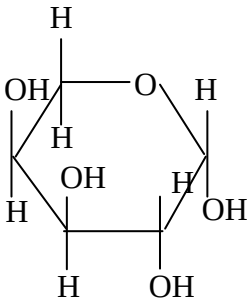
and xylan (Figure 1.9). In contrast to what has been expected, lactose has given low expression, as in the case of glucose, fructose, galactose, mellibiose, raffinose, starchiose and some gums (Vries and co-workers *et al*, 1999).



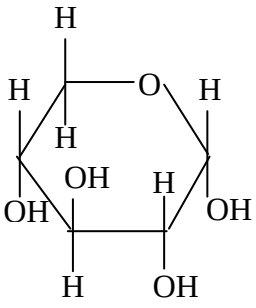
Pectin



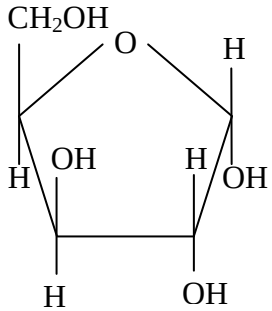
β-1,4-Xylan



β-L arabinose



Xylopyranose



Xylofuranose

Figure 1.9 Chemical structures of pectin, xylan, arabinose, xylopyranose, xylofuranose

Sorensen and Crisan (1974) have also screened themophilic filamentous fungi which exhibit intracellular lactase activity in order to obtain a thermostable enzyme. Selection of themophilic filamentous fungi has been based on exhibition of good growth on lactose containing media. They have found that *Mucor pusillus* was the best. *Thermomyces lanuginosus* and *Torula thermophila* have also shown activity. in this study by Sorensen and Crisan (1974), the main aim of focusing on thermophilic fungi was the possible usage of thermoactive fungal lactases to reduce lactose content in milk during or immediately following pasteurization.

Park and Pastore (1979) have further screened high β-galactosidase producing fungi. They have found that *Scopulariopsis* has shown highest activity between *Spicaria* and *Aspergillus* species. The enzyme of this organism has been found highly thermostable and very active in acid pH, thus suitable for cottage cheese operation and acid whey disposal. In order to find different β-galactosidases for different applications, Macris and Markakis (1981) have focused on β-galactosidase production in *Fusarium moniliforme* grown in whey.

Shaikh, Khire and Khan (1999) has sought *Rhizomucor* sp for both the activity of β-galactosidase and comparison of the solid state and submerged fermentation. Semi-solid fermentation was formerly used to grow *A. oryzae* (Park, De Santi, Pastore *et al*, 1979) and solid state cultivation was employed on *K. fragilis* (Becerra, Gonzalez Sico *et al*, 1996). Becerra and his co-workers also worked on the purification of β-galactosidase from *K. lactis* using FPLC. These authors have compared different methods like gel filtration, ion-exchange chromatography, affinity chromatography. It was concluded that highest purification fold was attained by affinity chromatography; however ion exchange chromatography with FPLC system (Fast performance liquid chromatography) was the fastest procedure even though purification fold was not so high (Becerra *et al*, 1998).

Comp and Lester (1974) studied *Neurospora crassa* and found two β-galactosidases, one being intracellular, the other being extracellular. These two enzymes were active at different pH values. Intracellular one was active mostly at

pH 7, whereas extracellular one was active at pH 4. When β-galactosidase induction was examined using different sugars like D-arabitol, L-arabinose, D-galacticol, D-xylose and D-galactose, it was found that L-arabinose, D-galacticol and L-arabitol were good inducers for both intra and extracellular enzyme. Although lactose on the induced intracellular enzyme at pH 7, it had no effect that much effect on the extracellular enzyme.

1.5. Aim of the Study

Lactose intolerance is common as much as 70-90% of most populations and is the result of low level or low activity of lactase. Some commercial solutions have been found against lactose intolerance such as low lactose milk and oral enzyme tablets. Fungal β-galactosidases are generally used for the production of oral enzyme tablets due to their high stability at stomach acidity. While, yeast β-galactosidases obtained from *K. lactis, K. fragilis* are used for low-lactose milk production. Previous studies indicate that enzymes of thermophilic fungi generally show their activity at or above neutral pH (Arifoğlu and Ögel, 2000; İfrij and Ögel, 2002) in contrast to what is observed in other fungi.

In this respect, objectives of this research were the production and partial purification of β-galactosidase from a thermophilic fungus, determination of conditions for better activity and production, finally the analysis of some of the biochemical properties and lactose hydrolyzing ability of the selected enzyme.

CHAPTER II

MATERIAL AND METHODS

2.1. Materials

Wheat bran, corn and cotton bagasse which were used as carbon source were prepared and turned into a powder by grinding in a miller. L-arabinose (Merck), *o*-nitrophenyl- β-D-galactopyranoside (Sigma-Aldrich), glucose reagent kit (Thermo Electron Corporation), β-galactosidase of *K. lactis* (Sigma) and all other chemicals were of analytical grade.

2.2. Methods

2.2.1. Stock Preparation of Microorganisms

Three thermophilic fungal species, *Humicola insolens* kindly provided by Dr. Mehmet Batum from ORBA A.Ş., *Thermomyces lanuginosus* CBS 288.54 and IMI kindly provided by Prof. Dr. Peter Biely from Slovac Academy of Sciences, *Torula thermophila* which is our own isolate were inoculated into slants containing Potato Dextrose Agar and YpSs medium (Appendix A) and were maintained at 20ºC, 4ºC and -20ºC. Stock cultures were subcultured every 2 months.

2.2.2. Qualitative Detection of Fungal β-galactosidase Activity

X-gal (X-gal 5-bromo 4-chloro 3-indolyl β-D galactoside) (Sigma) was dissolved in N-N-dimethyl-formamide in a 20:1 (mg/ml) ratio in a 1.5 ml eppendorf tube. The tube was covered with aluminum foil and was stored at -20ºC.

Isopropylbetathiogalactoside (IPTG) solution was also prepared by mixing 0.024g IPTG with water to a final volume of 1 ml in 1.5 ml eppendorf tubes. Then the solution was filter sterilized and stored at 4ºC. IPTG is used as an inducer and X-gal as an indicator of β-galactosidase activity in bacterial species.

In order to understand the applicability of X-gal treatment for validity of β-galactosidase existence in fungi, sterile petri plates containing YpSs agar were prepared. 100µl X-gal and 50µl IPTG solutions were spread over the plate by a spreader in aseptic conditions, and then plates were incubated at room temperature for 45 min in order to let X-gal & IPTG diffuse through the agar. A known intracellular β-galactosidase producer, *Thermomyces lanuginosus* was then inoculated to X-gal treated plates and incubated for approximately 5 days until well-growth of fungi and blue colour formation were observed.

For preliminary screening of three thermophilic fungi and carbon source, sterile petri plates were prepared by adding modified YpSs agar containing different carbon sources; pectin, xylan, wheat bran, arabinose, lactose. 100µl X-gal was spread over them and incubated again for 45min. IPTG was not added in order to see solely carbon source effect as inducer. Then, *Humicola insolens*, *Thermomyces lanuginosus* CBS, *Torula thermophila* was inoculated to X-gal treated plates and incubated until well-growth of fungi was observed. Observations were made based on color change on plate. Blue-green color indicated presence of β-galactosidase.

2.2.3. Growth of Microorganisms

Pre-culture and main culture media were based on YpSs broth (Appendix A). 3-5 mg spore from stock slants was inoculated into pre-culture medium volume of which was 2% of the main medium. For the germination of spores, pre-cultures were incubated at 45ºC and 155 rpm for 24 h in rotary shaker incubator. After incubation, pre-cultures were transferred to main-culture, then main culture was incubated at 45ºC, 155 rpm for 12 days. Enzyme activity and biomass were determined by taking samples at each day of growth.

2.2.4. Enzyme Assay

Starting from the first day of incubation, supernatant samples were removed and filtered. Total amount of sample taken throughout the incubation period did not exceed 10% of the initial volume of the main medium not to cause any disturbance in the microbial growth due to volume change. *o*-Nitrophenyl- β-D-galactopyranoside (o-NPG) was used as substrate for enzyme assay. This substrate is converted to galactose and *o*-nitrophenol which is yellow in color and thus intensity of color was determined as absorbance by spectrophotometer at 420 nm wave length.

For enzyme assay, 10 mM substrate was prepared in 0.1 M sodium phosphate buffer at pH 7.0. Supernatants were appropriately diluted. Temperature of water bath and spectrophotometer were adjusted to 60ºC. Diluted sample, substrate and buffer solutions were individually incubated in water bath for 5 min for equilibration of temperature. After 5 min incubation, blank solutions were prepared. For enzyme blank, 0.3 ml sample was added to cuvettes containing 1.2 ml buffer (0.1M sodium phosphate buffer at pH 7.0) and for substrate blank, 0.3 ml buffer was added to cuvettes containing 1.2 ml substrate solution. Then, 0.3 ml sample was added to cuvettes containing 1.2 ml substrate. Absorbance difference was measured at every 25 seconds for approximately 15 min at 420 nm by Shimadzu UV-spectrophotometer and data were recorded by UV-Prope software program connected to the spectrophotometer. For each sample, at least double measurements were performed.

The absorbance values were plotted versus time. Slope (absorbance/min) was determined by linear regression. This value was then converted to enzyme unit per ml with the help of a of standard curve defined (Sec. 2.2.8.)

2.2.5. Measurement of Biomass

In order to determine dry mass of fungi and to observe growth daily, the biomass on the filter paper was incubated at 60ºC for 1 day and weighted after waiting in desiccator for 10 min. The ratio of difference in weight of biomass before and after drying to the volume of supernatant sample gave the value of dry biomass per ml of sample.

2.2.6. Standard Curve Preparation

One unit of enzyme activity is defined as the amount of enzyme liberating 1 μmol of *o*-nitrophenol per min under the assay conditions described above (Park *et al*, 1979; Shaikh *et al*, 1997; Sorensen and Crisan, 1974). For stock solution, *o*-nitrophenol was dissolved in 4 ml ethanol (96%) & 2 ml 0.1 M sodium phosphate buffer at pH 7.0 to final concentration of 62.5mM. Blank solution was prepared by mixing 4ml ethanol and 2ml of the buffer used for stock solution preparation. Then, serial dilutions from the stock solution were performed as shown in Table 2.1.

Blank was prepared by adding 0.3 ml buffer to 1.2 ml blank solution. Sample was prepared by adding 0.3 ml buffer to 1.2 ml *o*-nitrophenol (ONP) in appropriate dilution. Then, absorbance of sample was measured at 420 nm in reference to blank by Shimadzu UV-VIS spectrophotometer at 60ºC until a constant absorbance was observed. This procedure was repeated for all dilution rates tabulated below. Finally, absorbance values were plotted against concentration of the solution. For each dilution, double samples were used and experiment was carried out in triplicate. The slope and range in which linearity was observed was determined. Slope (abs/mM ONP) was used as the conversion factor in determining enzyme activity to enzyme Units/ml.

U/ml = Activity (abs/min) / slope (abs/mM ONP) * (1,5/0,3)

1,5/0,3 is the ratio of total volume of cuvette to crude enzyme volume in cuvette. Slope of the standard curve which is conversion factor was found to be 2,6 abs/mM ONP and the resulting standard curve is presented in Appendix B.

Table 2.1 Serial dilutions of *o*-nitrophenol for standard curve preparation

Dilution Factor	**Preparation**	**Molarity of the *o*-nitrophenol solution (mM)**	**Molarity in the cuvette (mM)**
Stock		62.5	50
10X	1 ml stock + 9 ml B	6.25	5
50X	2 ml 10X + 8 ml B	1.25	1
100X	1 ml 10X + 9 ml B	0.625	0.5
200X	2 ml 50X + 6 ml B	0.3125	0.25
400X	2 ml 100X + 6 ml B	0.15625	0.125
500X	2 ml 100X + 8 ml B	0.125	0.1
1000X	2 ml 200 X + 8 ml B	0.0625	0.05
2000X	2 ml 400 X + 8 ml B	0.03125	0.025
5000X	1 ml 1000X + 4 ml B	0.0125	0.01
10000X	0.5 ml 1000X + 4.5 ml B	0.00625	0.005
20000X	0.5 ml 2000X + 4.5 ml B	0.003125	0.0025
50000X	0.5 ml 5000X + 4.5 ml B	0.00125	0.001

B-0.1 M sodium phosphate buffer at pH 7.0

2.2.7. Determination of Storage Stability

Supernatant (crude enzyme) samples were taken from the 4^{th} and 10^{th} day of growth; the days at which peak activities of enzyme were observed. At least 30 ml sample from both was stored at 4ºC in refrigerator in 1.5 ml epppendorf tubes. Another 30 ml sample was mixed with glycerol in amount of 5% of the sample and final solution was placed in refrigerator at -20ºC in 1.5 ml epppendorf tubes. Every two days of storage, one eppendorf tube was taken from 4ºC and -20ºC, 2X

diluted and the activity was determined as described in Sec. 2.4. In order to determine storage stability of crude enzyme samples, this procedure was applied for approximately 1 month.

2.2.8. Optimization of Temperature

Crude enzyme samples were taken from 4^{th} and 10^{th} day of growth of *Thermomyces lanuginosus* CBS; the days at which peak activities of enzyme were observed. The temperature of the water bath and the UV-VIS spectrophotometer was adjusted to 30, 40, 45, 50, 55, 60, 65, 70, 75ºC sequentially. At each temperature, enzyme activity of the samples from both days was measured as in Sec. 2.2.4 at pH 7.0. Finally, activity versus temperature graph was plotted to determine optimum temperature of the crude enzyme.

2.2.9. Optimization of pH

Crude enzyme samples were taken from 4^{th} and 10^{th} day of growth of *Thermomyces lanuginosus* CBS; the days at which peak activities of enzyme were observed. 0.1 M acetate buffer was prepared at pH values of 4, 5, 5.5 and 0.1 M sodium phosphate buffer was prepared at pH values of 6, 6.5, 6.6, 6.8, 7, 7.2, 7.4, 7.5, 7.6, 8 and finally Tris-HCl buffer was prepared at pH values of 8 & 9. 10 mM substrate solution was prepared for each pH value by mixing substrate with the buffers defined above. Then, enzyme activities of the samples from both days were measured as in Sec. 2.2.4 at 60ºC. Finally, activity versus pH was plotted to determine the optimum pH of the crude enzyme.

2.2.10. Kinetic Parameters of Enzyme

Substrate (*o*-NPG) solutions in buffer at pH 7.0 were prepared at concentrations of 1, 5, 10, 30, 45, 60 mM. Each substrate solution and crude enzyme was incubated in water bath at 60ºC for 5 minutes. Activity of crude

enzyme sample was also measured at 60ºC for each substrate concentration as described in Sec. 2.2.4. Finally, unit activity of enzyme, in other words velocity of reaction was plotted against substrate concentration. Michaelis-Menten constant, K_m and maximum velocity, V_{max} were determined with the help of the method described by Lineweaver and Burk (1934).

2.2.11. Fungal Morphology

Biomass samples of *Thermomyces lanuginosus* CBS 288.54 were taken from each day of growth under aseptic conditions from 3rd day to 12th day. Photographs of these samples were taken by Nikon digital camera. Moreover, these samples were observed under inverse light microscope by 100X magnification in the central laboratory in Middle East Technical University. Macro and micro views of the samples was used to visualize the growth day by day and to compare it with the biomass data measured as described in Sec. 2.2.3.

2.2.12. Determination of Protein

Protein was determined by Bradford method (Bradford M.M., 1976) for samples gathered from Ammonium Sulfate Precipitation. Bovine serum albumin was used as standard protein. However, for samples gathered from gel filtration and anion-exchange chromatography, rough estimation of protein were performed by measuring absorbance of the sample at 280 nm (Walker, 2002) and protein profile was drawn based on these data.

2.2.13. Electrophoretic Analysis

Electrophoretic analysis was performed after ammonium sulfate precipitation and anion exchange chromatography according to the procedure defined by Laemmli (Laemmli U. K., 1970). 21 µl sample was loaded to each well. PageRuler from Fermentas was used as marker and 5 µl was loaded to well.

2.2.14. Optimization of Fractional Ammonium Sulfate Precipitation

In order to find ammonium sulfate concentration which would give better yield and purification for β-galactosidase, optimization was needed to be carried out. First, percentages of ammonium sulfate used were determined as 30%, 30-45%, 45-60%, 60-75%, 75-85% (http://www.biotech.iastate.edu/facilities/protein/manuals/542B_Sec7.pdf). 4 L of 0.05 M sodium phosphate buffer at pH 7.0 was prepared and used for dialysis after precipitation. Supernatant sample (crude enzyme) taken from 11^{th} day of growth were continuously stirred by magnetic stirrer at 4ºC and ammonium sulfate was added to 30% saturation by gradually adding salt within 45 min-1 h, avoiding foaming on the surface of the sample. After adding all the salt enough to make the sample saturated to the desired value, sample was kept for 1 h for precipitation. After 1 h, sample was centrifuged at 10480 rpm (12000 rcp) for 30 min at 4ºC. While centrifuging, dialysis tubes were prepared by dipping them into boiling water containing 0.1 % EDTA and 0.1% sodium bicarbonate for 5 min. After centrifuging, precipitated part was separated and dissolved in 1 ml buffer, before dialysis. Then, samples were put in dialysis bags and placed in 2 L dialysis buffer stirred by magnetic stirrer. Ammonium sulfate was added to the remaining aqueous phase to make the concentration 45% from 30% and the procedure was repeated. Dialysis was carried for 1 day and buffer was refreshed every 12 h. Samples were investigated for their protein amount and enzyme activity in order to find yield and purification fold of each saturation percentage.

2.2.15. Anion-Exchange Chromatography

Anion-exchange chromatography was performed by FPLC (Fast Performance Liquid Chromatography) in Central Laboratory in Middle East Technical University. Commercially available, ready to use strong anion-exchange column, HiPrep 16/10 Q XL was used. 1 ml sample gathered from 60-

85% ammonium sulfate precipitation of crude enzyme from *Thermomyces lanuginosus* CBS 288.54 was loaded to the column. Flow rate was adjusted to 1 ml/min. 0.02 M sodium phosphate buffer at pH 7.0 was used for equilibration and also as start buffer. Column was run for 3.5 hr. Gradient elution was performed by 0.02 M sodium phosphate elution buffer at pH 7.0 containing 0.68M NaCl by linearly increasing salt concentration from 0 to 0.68 M. Eluted fractions containing 2 ml sample were collected every 2 min. While weakly binding proteins to the column were eluted first, stronger binding proteins were eluted last, they required higher salt concentration. UV-detector connected to the FPLC was used for drawing protein profile by plotting absorbance of the samples at 280 nm with respect to time. Fractions in the vicinity of peak protein values were analysed for their enzyme activities and then electrophoretic analysis of the fractions showing higher activity were carried out by SDS-PAGE as in Sec. 2.2.13.

2.2.16. Determination of Molecular Weight

Molecular weight of the sample gathered from 60-85 % ammonium sulfate precipitation was measured by gel filtration with FPLC (Fast Performance Liquid Chromatography) in central laboratory in Middle East Technical University. Commercially available, ready to use, Superdex 200 prep grade column which is selective for proteins with a molecular weight range of 10 000- 600 000 kDa was used for gel filtration. 1 ml sample gathered from 60-85 % ammonium sulfate precipitation of crude enzyme from *Thermomyces lanuginosus* CBS 288.54 was loaded on the column. Flow rate was adjusted to 0.8 ml/min. 0.05M sodium phosphate buffer at pH 7.0 was used for equilibration and also as the start buffer and elution buffer. Proteins with high molecular weight were eluted first. UV-detector connected to the FPLC column was used for drawing protein profile by plotting absorbance of the samples at 280 nm with respect to time. Fractions in the vicinity of peak protein values were analysed for their enzyme activities in order to understand which peak value corresponded to β-galactosidase. Proteins

with known molecular weights were loaded before the sample under the same conditions and standard curve was drawn. Based on this curve, the molecular weights of fractions were estimated.

2.2.17. Standard Curve Preparation for the Determination of Glucose Concentration

Liquid glucose reagent –glucose oxidase-peroxidase enzyme kit- was obtained from Thermo Electron Corporation. This reagent oxidizes glucose to gluconic acid and hydrogen peroxide by glucose oxidase, then hydrogen peroxide is converted to red quinoneimine dye in the presence of peroxidase, HBA (4-hydroxybenzoic acid) and 4-aminoantipyrine. The intensity of color is proportional to the glucose concentration and can be measured photometrically between 460-560 nm. In order to measure glucose concentration of sample, standard curve was needed to be prepared before each experiment. First, 35 mM (maximum concentration that can be measured accurately by the reagent) glucose solution was prepared. Then serial dilutions were carried out as in Table 2.2. Solutions were incubated at 37ºC for at least 5 min. Blank was prepared by adding 10 µl distilled water to 1.5 ml glucose reagent solution. Sample was prepared by adding 10 µl glucose solution in appropriate dilution to 1.5 ml reagent solution. Then, absorbance of sample was measured at 500 nm in reference to blank by Shimadzu UV-VIS spectrophotometer until a constant absorbance was observed. Finally, absorbance values were plotted against concentration of glucose. For each dilution, double samples were used. Slope was used for conversion of absorbance values to glucose concentration. One example of resulting standard curve is presented in Appendix C.

Table 2.2 Serial dilutions of glucose solution for standard curve preparation

Dilution Factor	Preparation	Molarity (mM)
X	Stock	35
1.4X	2 ml X + 0.8 ml dH_2O	25
3.5X	1 ml 1.4X + 1.5 ml dH_2O	10
7X	1 ml 1.4X + 4 ml dH_2O	5
35X	0.5 ml 3.5X + 4.5 ml dH_2O	1
70X	1 ml 35X + 1 ml dH_2O	0.5

dH_2O; distilled water

2.2.18. Determination of Lactose Hydrolysis

Lactose is hydrolyzed into glucose and galactose by β-galactosidase and glucose is known to be much less reactive in transferase reactions than galactose as described in Sec. 1.2. Thus, glucose amount can be used as an estimation of lactose hydrolysis. First, 15 ml samples of 5% lactose solutions in 0.1 M sodium phosphate buffer at pH 7.0 -percentage near to lactose amount in milk- and 15 ml of 10% sodium bicarbonate were prepared in falcon tubes by using distilled water. The activity of enzyme obtained from 60-85 % ammonium sulfate precipitation of crude enzyme from *Thermomyces lanuginosus* CBS 288.54 was determined. 1.35 unit of enzyme was added to 13.5 ml 5% lactose solution (1 U: 10 ml 5% lactose) and solution was started to be incubated at 60ºC in water bath. Time of addition was recorded. After 1 h incubation, 1 ml sample was taken and reaction was stopped by adding 1 ml 10% sodium bicarbonate prepared previously. Next, 10µl sample with sodium bicarbonate was added to 1.5 ml glucose reagent and incubated for 5 min at 37ºC or 15 min at room temperature, then absorbance was measured at 500 nm in reference to blank containing 10 µl distilled water and 1.5 ml reagent. This procedure was repeated for 3, 6, 20, 24, 29, 51, 98, 144, 240 hours of incubation. Finally, %lactose conversion was plotted with respect to time.

2.2.19. Temperature Effect on Lactose Hydrolysis

Enzyme obtained from 60-85 % ammonium sulfate precipitation of culture supernatant from *Thermomyces lanuginosus*, was added into 5% lactose solution in ratio of 1 Unit to 10 ml 5% lactose solution. Three falcon tubes prepared accordingly were incubated at 30ºC, 40ºC and 50ºC separately and the same procedure as in Sec. 2.19 was applied. Data were taken at 2, 4, 20, 44, 68, 98, 144, 240 hours of incubation. In order to observe whether lactose was hydrolysed spontaneously with time at high temperature, conversion to glucose in 5 % lactose solution without addition of enzyme was also investigated with respect to time.

2.2.20. Comparison with Commercial Enzyme

The activity of enzyme obtained from 60-85 % ammonium sulfate precipitation and that of the commercially available β-galactosidase of *Kluyveromyces lactis* (Sigma) were determined. Same unit of enzyme from both organisms was added into 5% lactose solution in ratio of 1 Unit to 10 ml 5% lactose solution. Three samples prepared accordingly were incubated at 30ºC, 40ºC and 50ºC and the same procedure as in Sec. 2.19 was applied. Data were taken at 2, 4, 20, 44, 68, 98, 144, 240 hours of incubation.

CHAPTER III

RESULTS AND DISCUSSION

The aim of this study was the production and partial purification of extracellular β-galactosidase from thermophilic fungi. In this respect, a number of thermophilic fungi were first subjected to β-galactosidase activity screening using the chromogenic substrate X-Gal.

3.1. β-galactosidase Production by Thermophilic Fungi

X-Gal is used in conjunction with IPTG for the detection of β-galactosidase activity in bacterial colonies in a colorimetric assay to distinguish recombinants (white) from non-recombinants (blue). X-Gal is cleaved at the β 1-4 bond between galactose and the 5-Bromo-4-chloro-3-indolyl part of X-Gal by β-Galactosidase via hydrolysis. The cleavage of X-Gal results in the production of a water insoluble blue dichloro-dibromo-indigo precipitate at the site of enzymatic cleavage.
(http://www.apolloscientific.co.uk/otherProducts_Lifesciences_Enzyme.htm).
However, since the function of X-gal and IPTG is based on the working principle of the lac operon in *E. coli*. There was no information available to know if X-gal could be equally, efficiently used to detect fungal β-galactosidase activity or not.

In this research, X-gal was first tried on *Thermomyces lanuginosus*, a known intracellular β-galactosidase producer (Fisher *et al*, 1995), in order to examine the applicability of X-gal treatment. Figure 3.1 shows formation of green color, which indicates that X-gal can be used not only for detection of bacterial β-galactosidase activity but also for the detection of fungal β-galactosidases.

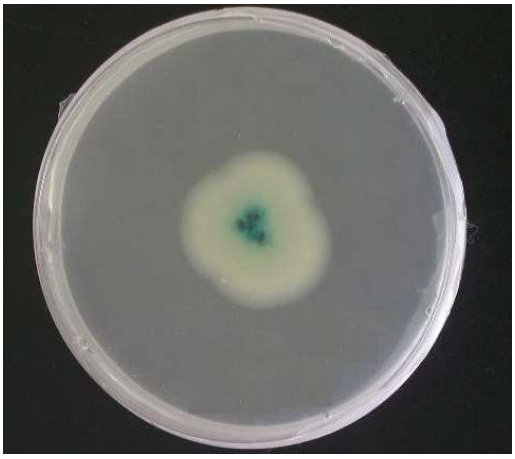


Figure 3.1 *Thermomyces lanuginosus* CBS grown on YpSs agar (Appendix A) containing X-Gal and IPTG.

After validation of the applicability of X-gal for the detection of fungal β-galactosidase producers, this technique was used for screening a number of available thermophilic fungi and for the investigation of carbon source inducing β-galactosidase production. Therefore IPTG was not added into the growth media. The fungus showing best β-galactosidase activity was found to be *Thermomyces lanuginosus* CBS. Furthermore, *Thermomyces lanuginosus* could grow and give a significant green color on all carbon sources. It can grow on arabinose and xylan more easily. All three fungus species grow and show β-galactosidase activity on arabinose and wheat bran. (Figure 3.2 and Table 3.1)

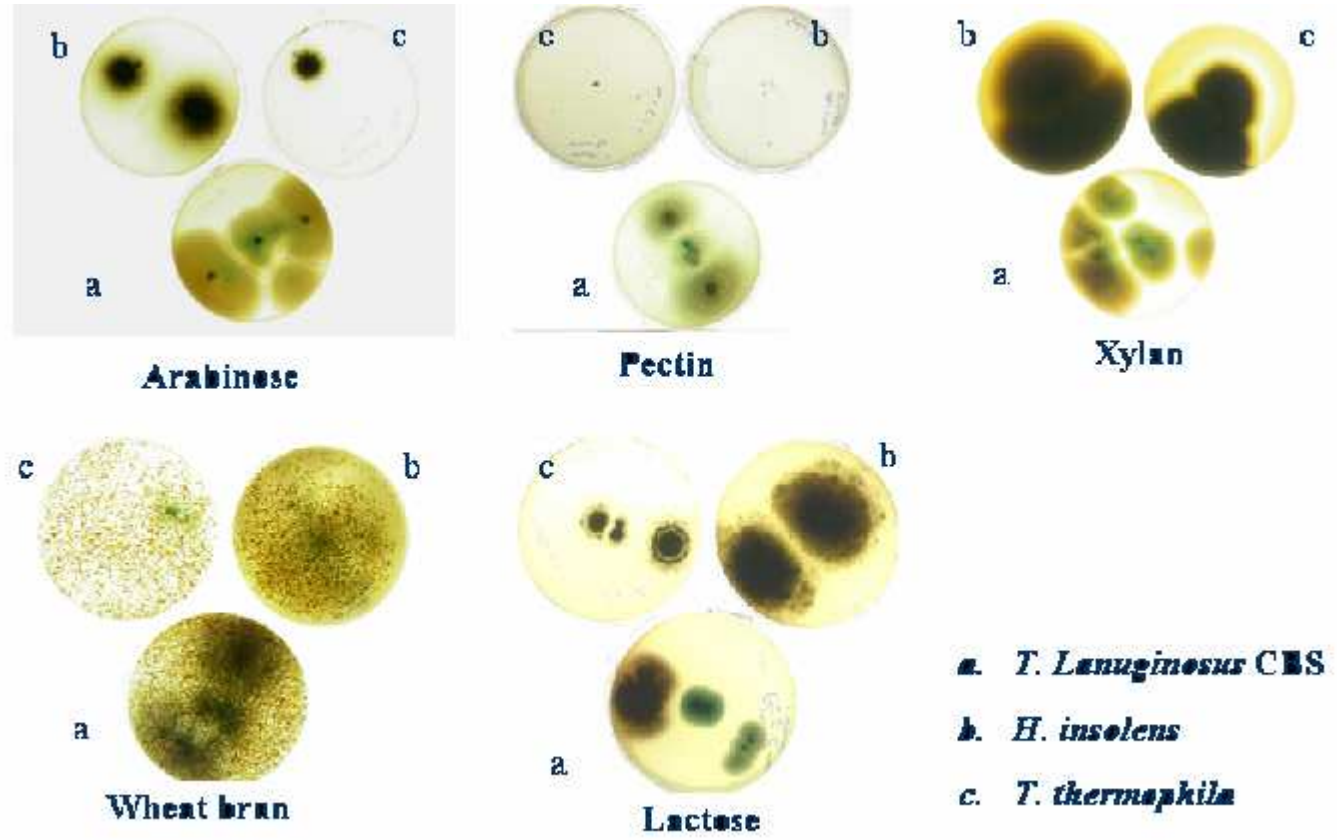


Figure 3.2 Growth of thermophilic fungi on various carbon sources for the detection β-galactosidase activity by X-gal treatment (a) *Thermomyces lanuginosus* CBS, (b) *Humicola insolens*, (c) *Torula thermophila* grown on modified YpSs medium containing arabinose, wheat bran, lactose, xylan and pectin as carbon source instead of starch (Appendix A).

Table 3.1 Tabulated form of Figure 3.2

	Arabinose		**Xylan**		**Wheat Bran**		**Pectin**		**Lactose**	
	g	c	g	c	g	c	g	c	g	c
***T.lanuginosus* CBS**	+	+	+	+	+	+	+	+	+	+
H. insolens	+	+	+	–	+	+	–	–	+	–
T. thermophila	+	+	+	–	+	+	–	–	+	–

g; growth;
c; colour change

3.2. Selection of the Best Carbon Source for β-galactosidase Production by *Thermomyces lanuginosus*

After preliminary screening of carbon source by X-gal treatment, quantitative analysis based on activity measurement was performed in order to determine the best inducing carbon source of all six; pectin, wheat bran, arabinose, lactose, corn bagasse, cotton bagasse. Pre-cultures and modified YpSs broths (main culture) containing these carbon sources were prepared as described in Sec. 2.2.3. Enzyme activity of samples from each flask and at each day of incubation was measured according to the procedure given in sec. 2.4. The carbon source at which maximum activity was observed throughout the incubation period was selected as the best inducing one of all defined above. Results of the experiments showed that *Thermomyces lanuginosus* CBS could not grow well in submerged liquid cultures when hardly miscible carbon sources like cotton bagasse, corn bagasse were used. The carbon source that induced the highest extracellular β-galactosidase production was found as arabinose showing 100 fold higher production of lactase as compared to that of lactose (Table 3.2). This result is in accord with the literature. Vries and coworkers (1999) revealed that higher expression of β-galactosidase encoding *lac*A gene in *Aspergillus niger* occurs on

xylose, pectin, xylan and arabinose (highest) than on lactose, in contrast to what was expected. Wheat bran also induced expression of *lac*A possibly due to the presence of arabinoxylan in its structure (Vries *et al*, 1999). Moreover, research on β-galactosidase secreted by *Neurospora crassa* showed that there were two β-galactosidases, one was intracellular, the other one extracellular. Highest inducing sugar for both enzymes was found to be L-arabinose and lactose was inducing approximately 20 fold less than L-arabinose (Comp and Lester, 1971).

In nature, arabinose is generally found in the structure of polysaccharides like hemicelluloses which can not pass into cells unless they are hydrolyzed by enzymes secreted to the extracellular environment. The presence of arabinose within the medium might act in signalling the organism the presence of polysaccharides in the medium, which would explain the induction of extracellular β-galactosidase.

Table 3.2 Maximum β-galactosidase activities of *Thermomyces lanuginosus* grown on different carbon sources by submerged liquid cultivation

Carbon Source	**Arabinose**	**Wheat Bran**	**Pectin**	**Lactose**	**Corn bagasse**	**Cotton bagasse**
Max Activity (U/ml)	0,25 ±0,02	0,0127 ±0,01	0,02 ±0,005	0,026 ±0,01	No significant growth	No significant growth

When activity and biomass was analysed throughout the growth of *Thermomyces lanuginosus* CBS in YpSs medium containing arabinose as the sole carbon source, two peak points in activity and decrease of biomass after first peak point of activity were observed (Figure 3.3). Activity gave first peak point on either 3rd or 4th day of growth, afterwards a slight decrease was followed by a sharp increase in activity giving a second peak on 10th or 11th days of growth. It

was suggested that there might be two enzymes, one is secreted extracellularly during initial days of growth and the second one which is located perhaps on the cell-wall is released following the initiation of biomass degradation at day 3 as a result of fragmentation of the mycelium.

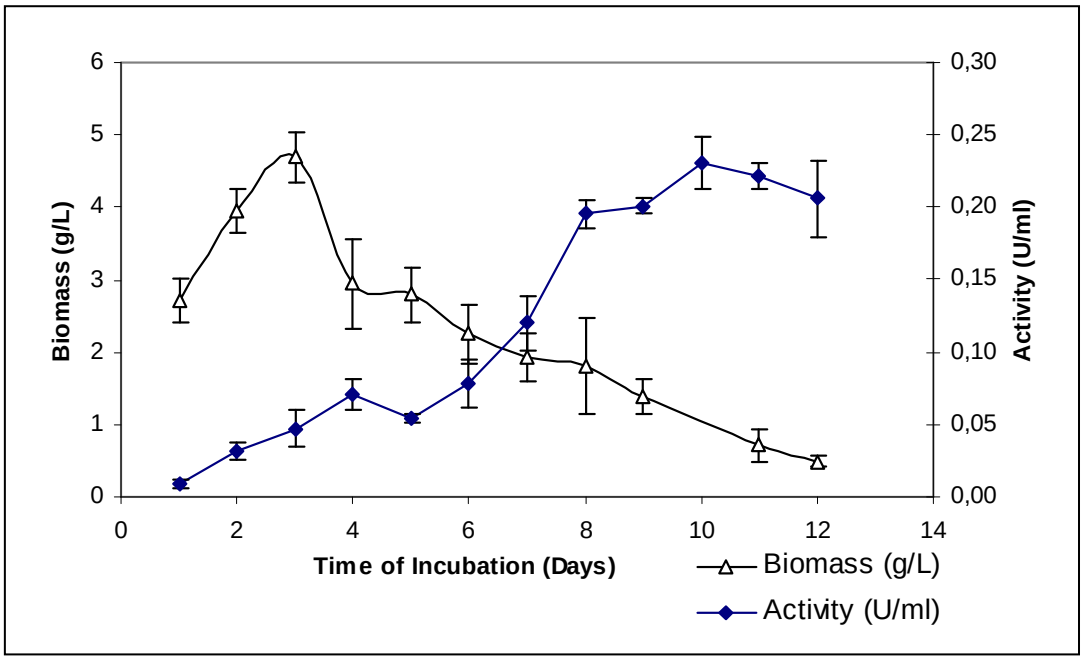


Figure 3.3 Activity and biomass change throughout the growth of *Thermomyces lanuginosus* CBS in modified YpSs medium containing 1.5% arabinose as the sole carbon source.

3.3. Optimization of Assay Conditions

Enzyme assay was conducted based on the continuous measurement of absorbance versus time, rather than the stop assay. Thus, there was no need to optimize the assay time. First of all, it was required to test the substrate to see if it is decomposed spontaneously without enzyme or not. It was observed that even at 75ºC, substrate did not self-decompose.

Activity was also measured every day throughout the growth of the fungus by using both enzyme and substrate blank. It was found that blank type did not have a significant effect on activity. However, since culture supernatants had a natural yellow color and substrate also turned into yellow color in the presence of β-galactosidase, it was thought that color of supernatant could mask the activity, causing misleading results. For this reason, supernatants were diluted and enzyme blank was used instead of substrate blank.

Activity of crude enzyme at different dilution rates was examined in order to assure that there was no effect of buffer (0.1 M sodium phosphate buffer at pH 7.0) on activity of the enzyme. It was observed that there was no positive or negative effect of the buffer on activity (Figure 3.4). According to the results, ½ dilution was selected for further experiments.

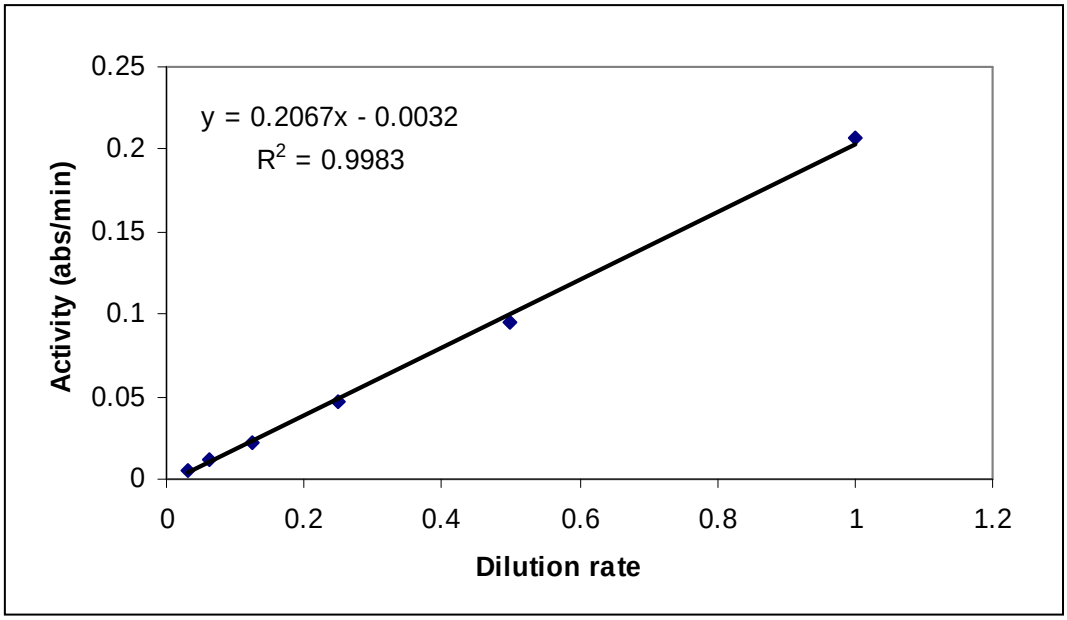


Figure 3.4 Effect of dilution rate on activity of crude β-galactosidase of *Thermomyces lanuginosus* CBS grown on modified YpSs medium containing arabinose as carbon source.

3.3.1 Optimization of pH

Samples from first and second peak point of activity were taken. Activities were determined at 60ºC and at different pH values ranging from 4 to 8 as described in Sec. 2.2.9.

For the sample taken from the first peak point of activity, at day 4, optimum pH was found to be 7.6 (Figure 3.5) whereas the value was 6.8 for the sample taken from the second peak point of activity, at day 10 (Figure 3.6). Previously, the intracellular β-galactosidase of *Thermomyces lanuginosus* was examined and pH optima was found as 6.8 (Fisher *et al*, 1995) which correlates with the results of the second enzyme. The observation of two different pH optima supports the existence of two different β-galactosidases (Sec. 3.2). One is secreted extracellularly during the initial days of growth, while the second one may be located on the cell wall or cell membrane and is excreted as a result of the fragmentation of the mycelium. Such a fragmentation is in fact an advantage since it eliminates the need for expensive and difficult cell disintegration techniques as in Fisher's research; where the authors have ruptured cells by using glass bead mill (Fisher *et al*, 1995).

In the literature, fungal β-galactosidases are known to be acidic. β-galactosidase from *Aspergillus* species was investigated and pH optima was found between 3-5 (Park *et al*, 1979; Borglum and Steinberg, 1972; Gonzalez and Monsen, 1990). pH optima of some other fungal β-galactosidases are listed in Table 1.4. Due to this property of fungal lactases, they are generally used in production of oral enzyme tablets or whey processing. Previous studies have shown that thermophilic fungi generally show their activity at or above neutral pH (Arifoğlu and Ögel, 2000; İfrij and Ögel, 2002). Results found for *Thermomyces lanuginosus* β-galactosidase are in accord with these previous observations. A fungal enzyme with optimum pH at neutral-alkaline pH suggests potential usage of this enzyme of *Thermomyces lanuginosus* in the dairy industry.

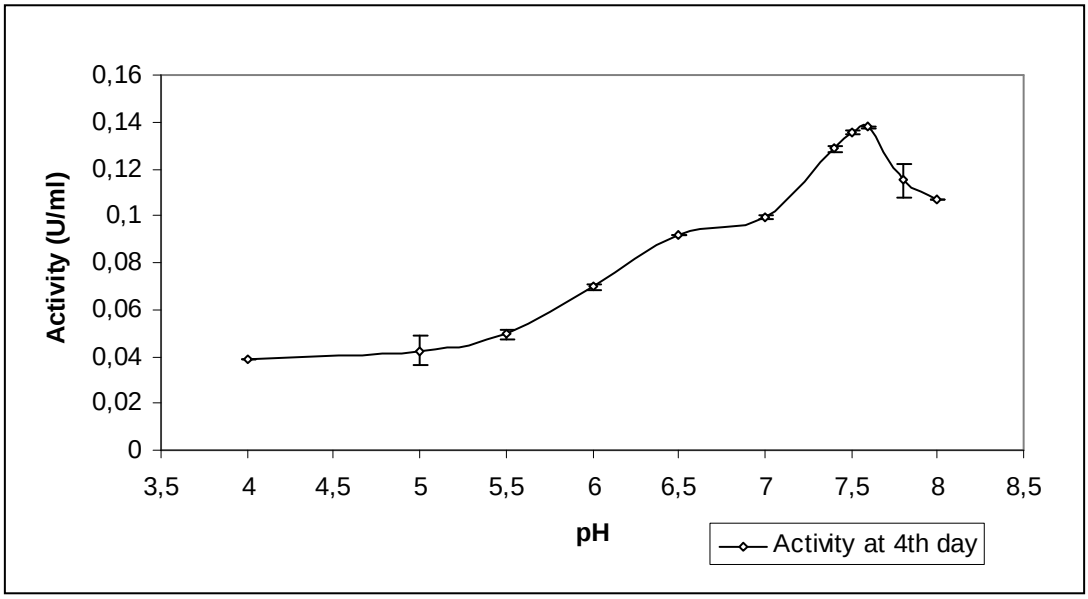


Figure 3.5 Optimization of pH of crude enzyme taken from 4th day of growth on modified YpSs medium containing 1.5% arabinose as carbon source.

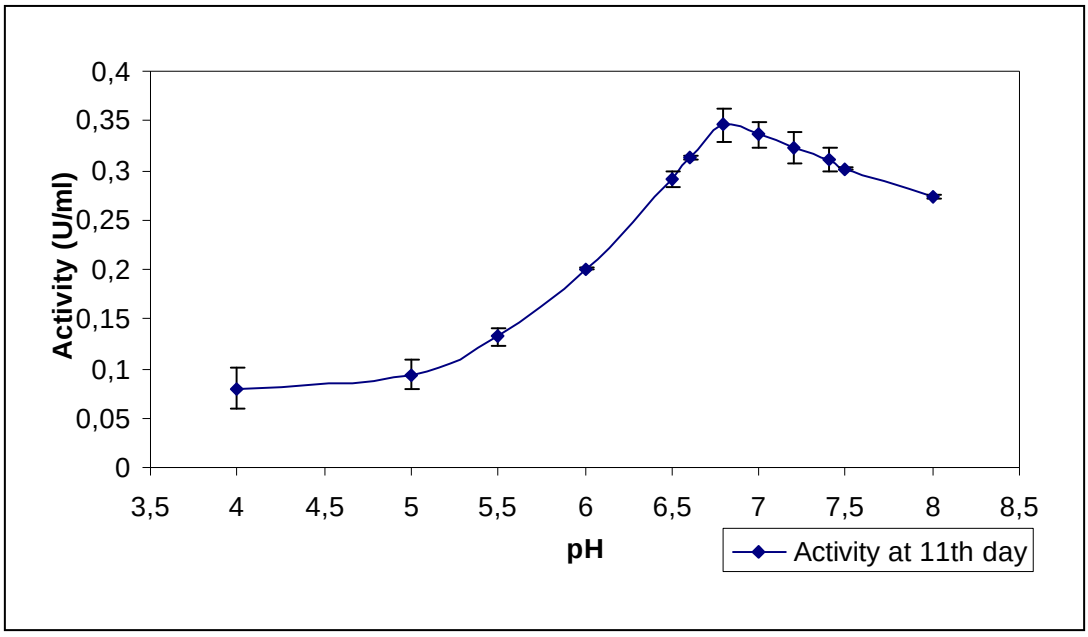


Figure 3.6 Optimization of pH of crude enzyme taken from 11th day of growth modified YpSs medium containing 1.5% arabinose as carbon source.

3.3.2 Optimization of Temperature

In order to find the temperature at which β-galactosidase shows maximum activity under the defined assay conditions, activities were determined at pH 7.0 and at different temperatures ranging from 30ºC to 75ºC as described in Sec. 2.2.8.

For both enzyme components, optimum temperature was found to be 65ºC (Figure 3.7). Thermophilic fungi produce thermostable enzymes and they are less prone to microbial contamination, which is important especially during the long operating time of immobilized enzyme systems. Moreover, higher temperature decreases viscosity so increases rate of reaction. Such enzymes can also improve the economy of the lactose hydrolysis as a result of their greater stability (Maciunska *et al*, 1998). When such an enzyme is added to milk before pasteurization, it can retain most of its activity at the end of pasteurization. Essentially, thermostable β-galactosidases have received considerable attention because of their possible utilization in the industrial processing of lactose-containing fluids. Even lactase of the extreme thermophile *Thermus aquaticus* and *Thermoanaerobium* sp. 2905 have temperature optima at 80ºC (Ulezlo *et al*, 2001; Ulrich *et al*, 1972). Archeabacterium *Sulfolobus solfariticus* shows maximum activity over 90ºC (Pisani *et al*, 1990). However, when fungal and yeast β-galactosidases are compared (Table 1.4), it can be concluded that *Thermomyces lanuginosus* grown on YpSs medium containing arabinose as carbon source produces one of the most thermostable fungal β-galactosidases.

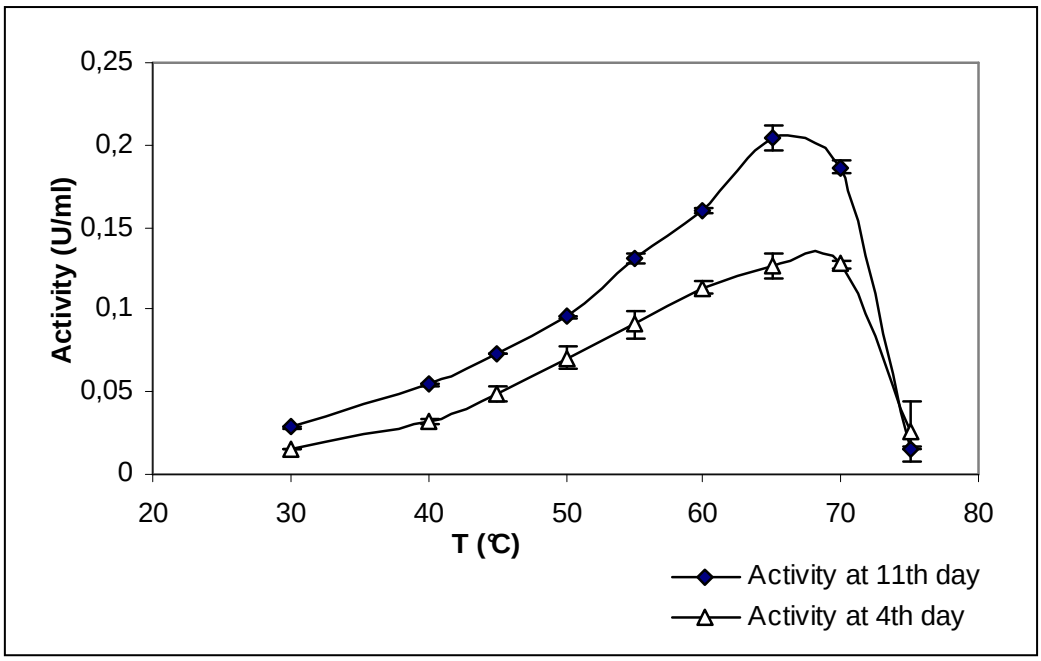


Figure 3.7 Optimization of temperature of crude enzyme taken from 11th and 4th days of growth on modified YpSs medium containing 1.5% arabinose as carbon source.

3.4. Effect of Storage on Enzyme Activity

Crude enzyme samples were stored at 4ºC and -20ºC for about 1 month. When activity was measured, it was concluded that samples at both 4ºC and -20ºC preserved their activity at the end of a month (Figure 3.8 and Figure 3.9).

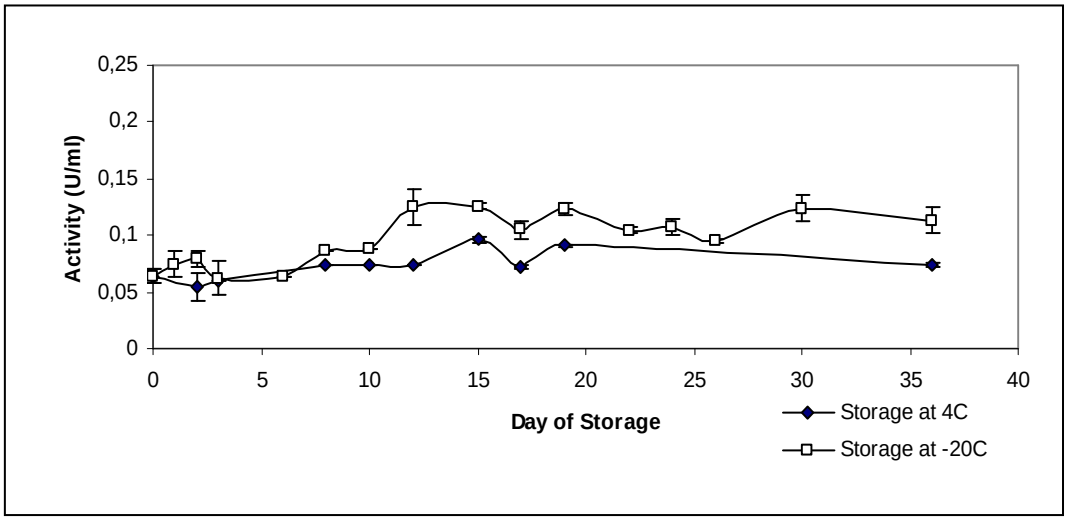


Figure 3.8 Storage effect on crude enzyme taken from 4th day of growth.

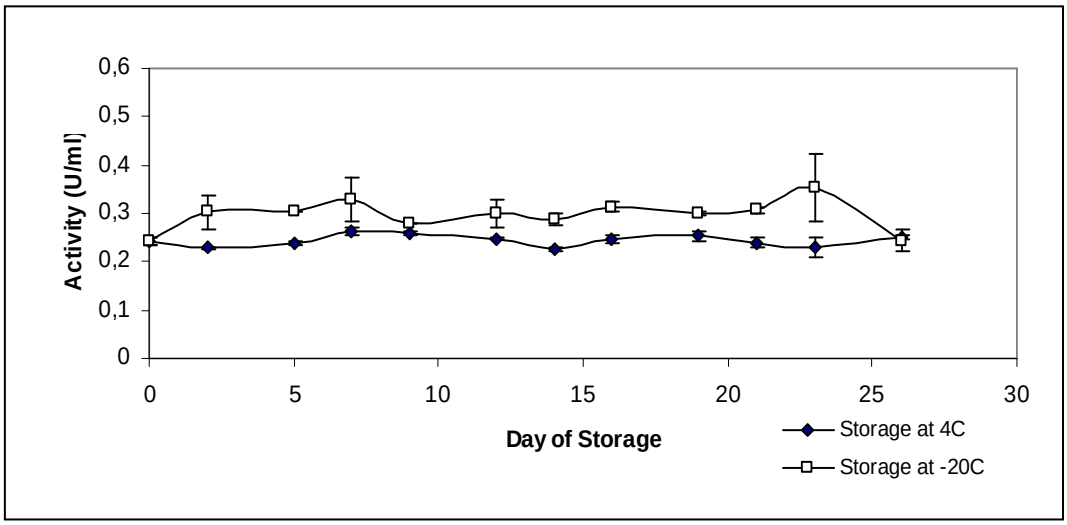


Figure 3.9 Storage effect on crude enzyme taken from 10th day of growth.

3.5 Kinetic Parameters of Enzyme

According to the results, further kinetic studies were conducted on the enzyme excreted at day 11 (named as β-galactosidase-B). In order to understand the affinity of substrate for enzyme, Michaelis-Menten constant, K_m can be determined. Lower the value shows higher affinity of enzyme for its substrate. The Michaelis constant (K_m) can be seen to be the substrate concentration for which the (initial) rate of reaction reaches half (1/2) of the maximum. Figure 3.10 represents the reaction velocity with respect to concentration of substrate, *o*-nitrophenol-β-D- galactopyranoside. The data were also plotted by the method described by Lineawer and Burk (1934) (Figure 3.11). K_m and V_{max} were found as 2.48 mM and 0.44 U/ml, respectively. While K_m value was slightly higher than that of other organisms, it was less than the K_m of the purified intracellular β-galactosidase of *Thermomyces lanuginosus* (Table 3.3). This might be the result of strain differences or reduction of affinity for substrate due to purification as in case of *Rhizomucor* sp. (Shaikh *et al*, 1999)

Table 3.3 Km value of different organisms for *o*-NPG

Organism	**Km value (mM)**	**Reference**
Thermomyces lanuginosus (purified, intracellular)	11,3	Fisher *et al*, 1995
Rhizomucor sp. (purified)	1,32	Shaikh *et al*, 1999
Rhizomucor sp.	0,785	Shaikh *et al*, 1997
Aspergillus fonsecaeus	1,78	Gonzalez & Monsan, 1991
Aspergillus foetidus	1,613	Borglum & Sternberg, 1972
Aspergillus oryzae	0,77	Park *et al*, 1979
Thermomyces lanuginosus (in this research)	2,48	

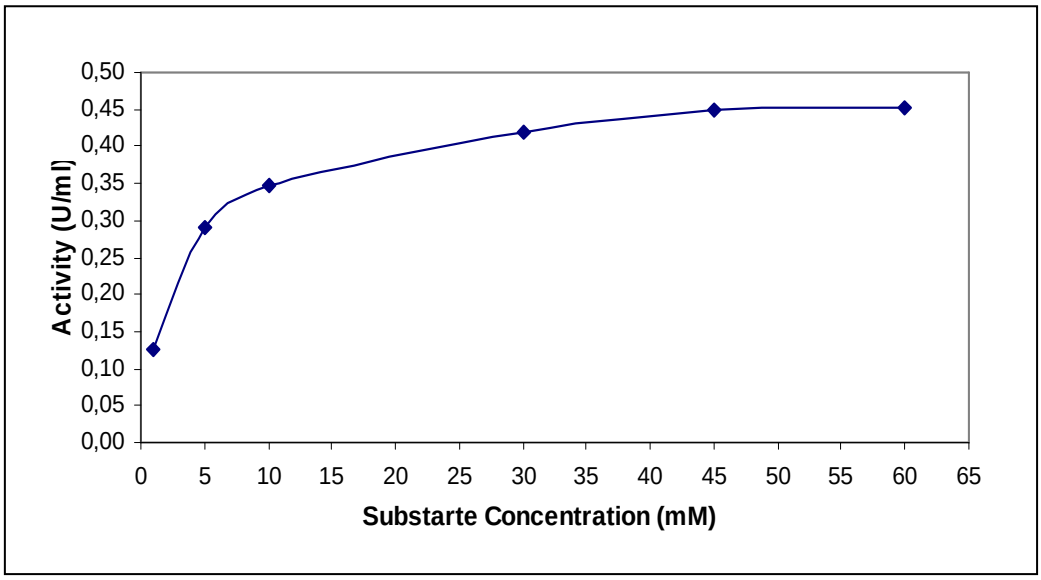


Figure 3.10 Activity of *Thermomyces lanuginosus* β-galactosidase-B versus substrate concentration (substrate: *o*-nitrophenol-β-D- galactopyranoside)

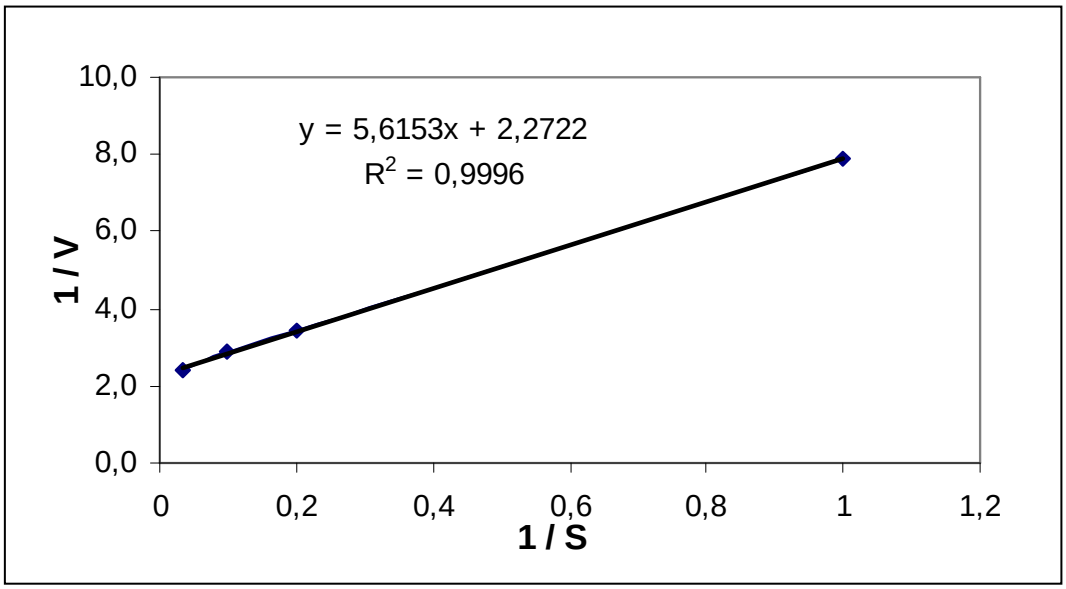
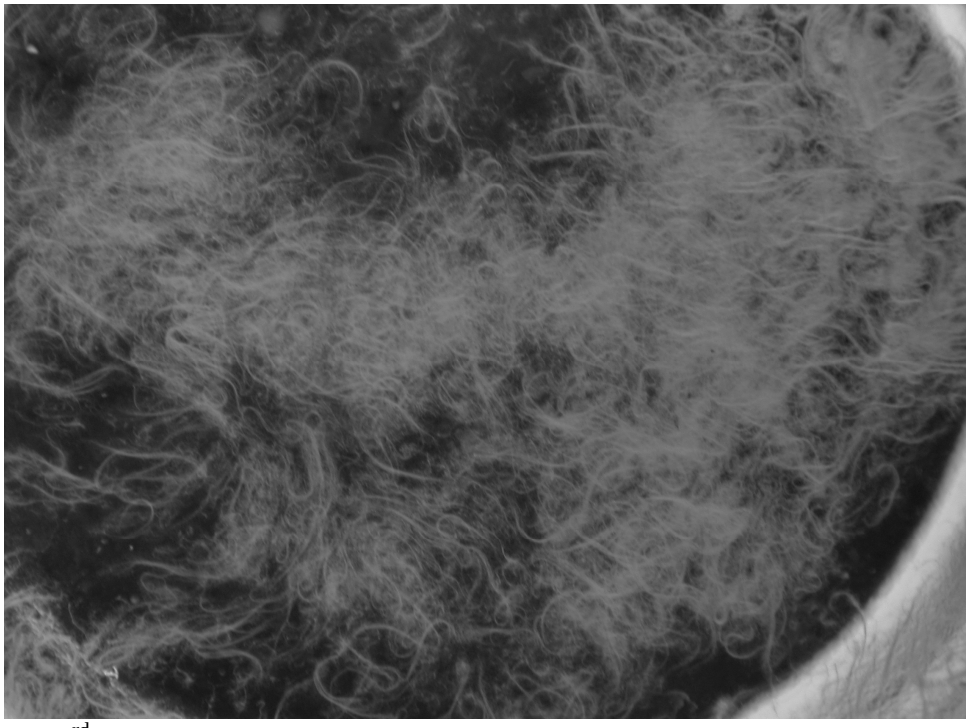


Figure 3.11 Lineweaver-Burk Plot for β-galactosidase-B of *Thermomyces lanuginosus*

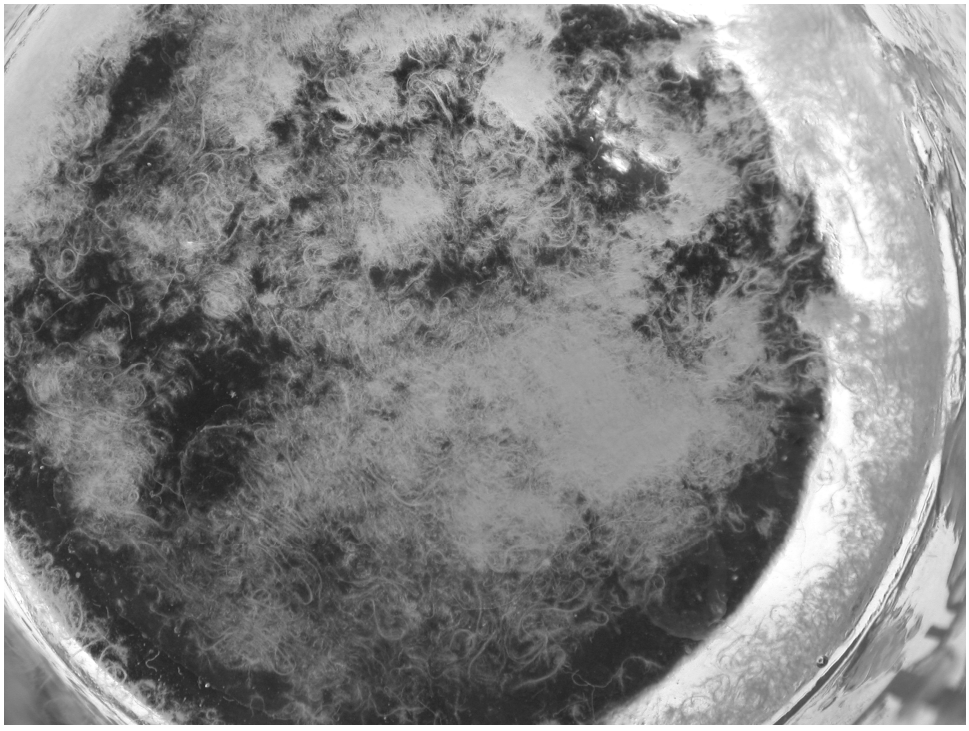
3.6. Fungal Morphology During Growth in Submerged Culture

In order to understand the reason behind the increase in β-galactosidase activity after decrease in biomass on the 3rd day (Figure 3.3), photographs of *Thermomyces lanuginosus* grown on modified YpSs broth containing arabinose as carbon source was taken by both a Nikon digital camera (Figure 3.12) and an image analysis system composed of a computer with a software program connected to an inverse light microscope present in Central Laboratory in METU (Figure 3.13). When looked at those figures, it can easily be detected that filaments are broken into pieces in the days following third day of growth. Moreover, as time continues, fragmentation increases. Increase in fragmentation explains the decrease in biomass after 3rd day of growth and strengthens the suggestions about the existence of cell wall-bound β-galactosidase which is excreted and shows increasing activity after 3rd day.

Figure 3.12- confirms the above suggestions. At the first days of growth, filaments were intact, however, later, number of septums, vacuoles in the cells and dimensions of vacuoles increased. Additionally, less movement in the cytoplasm was observed and it became harder to see the nuclei. Especially, after the 6th day, integrity of cells were lost. Arrows on the Figures 21, 22 and 23 show the break points of filaments. After 8th day, sporulation begins. Finally, after 10th day, cells appear to have entered the death phase. At this stage, cells have very large vacuoles with almost no cytoplasm. It is likely that at this stage proteases are released from the vacuoles causing loss of enzyme activity.

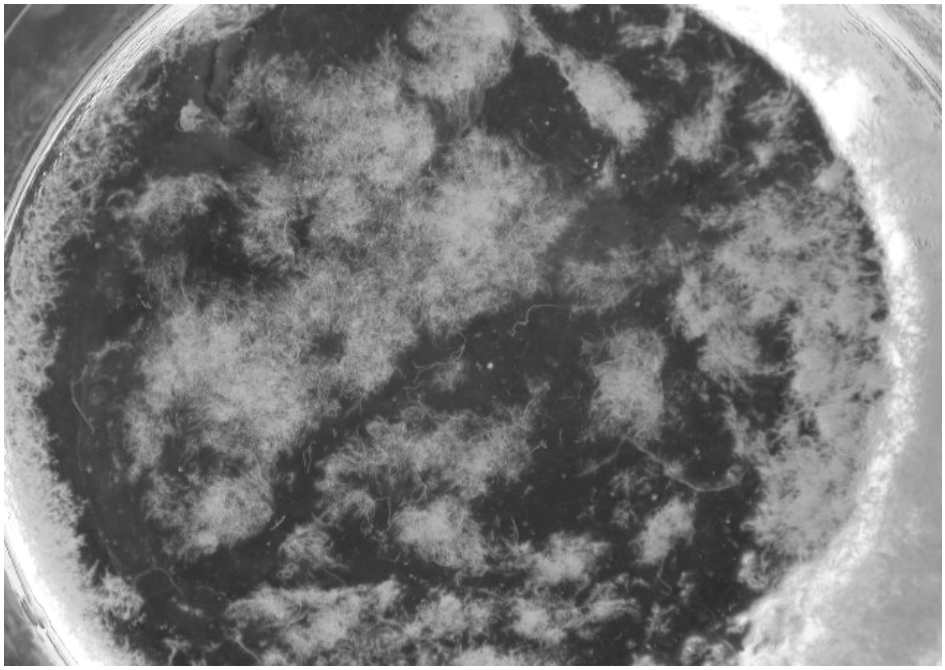


a. 3rd day of growth

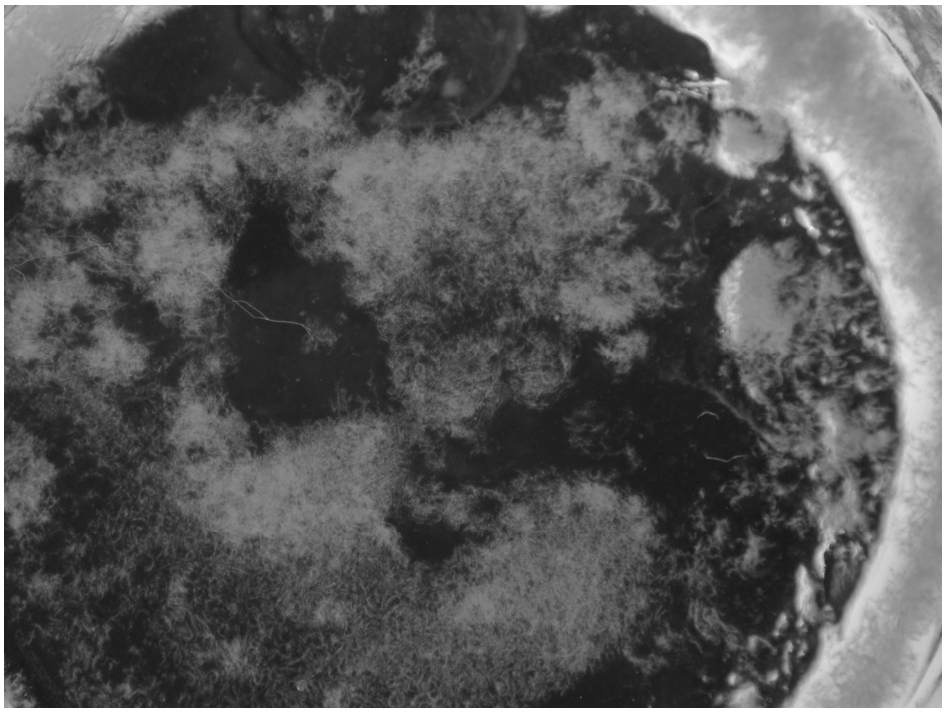


b. 4th day of growth

Figure 3.12 Photographs of *Thermomyces lanuginosus* CBS 288.54 grown on modified YpSs broth containing arabinose as carbon source. (a-b, days 3-4; c-d, days 5-6; e-f, days 7-8; g-h, days 9-10; i-j, days 11-12)

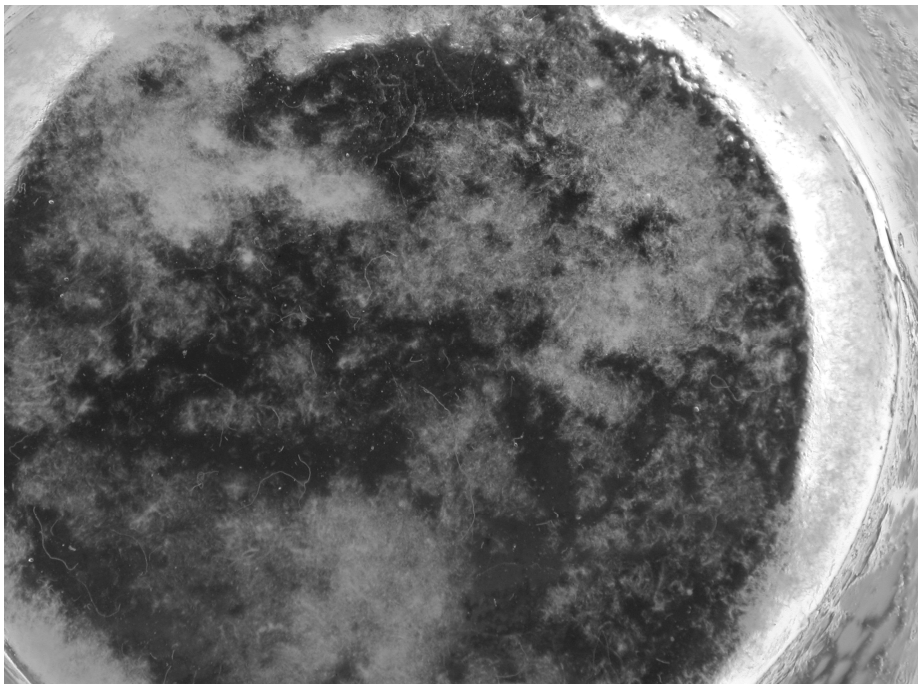


c.5th day of growth

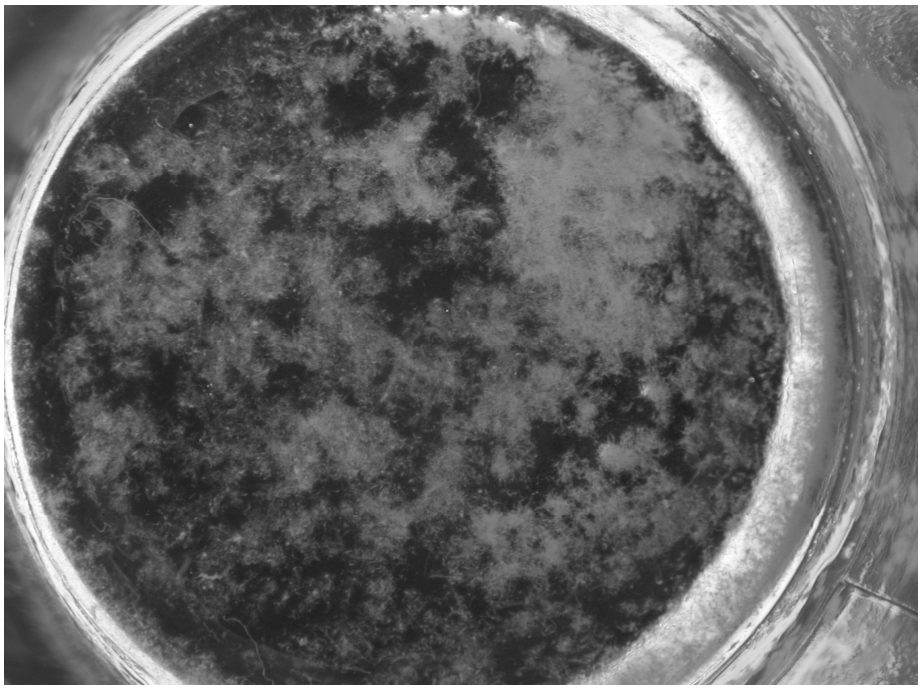


d. 6th day of growth

Figure 3.12 continued

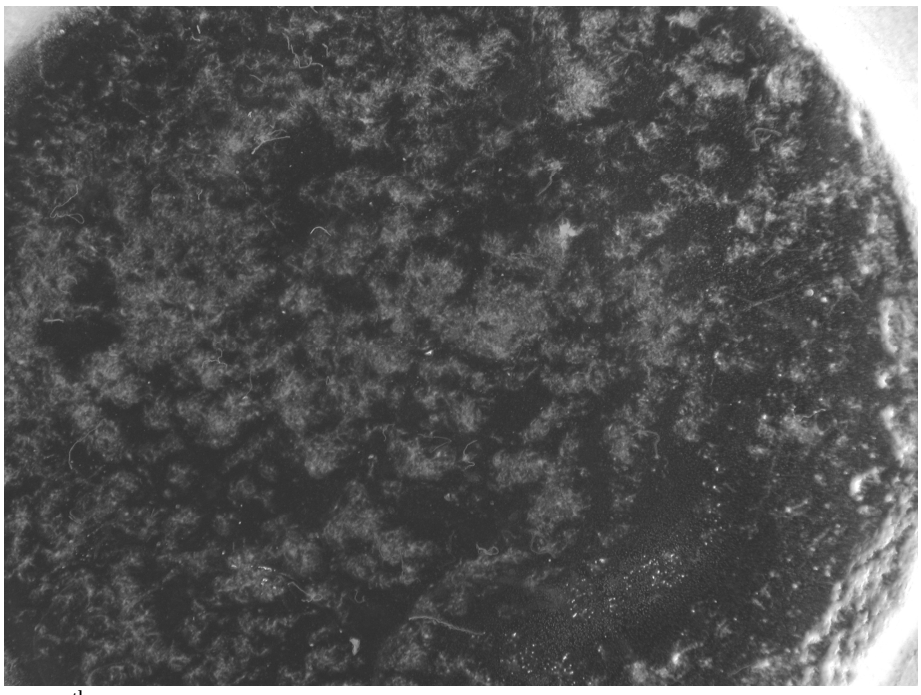


e. 7th day of growth



f. 8th day of growth

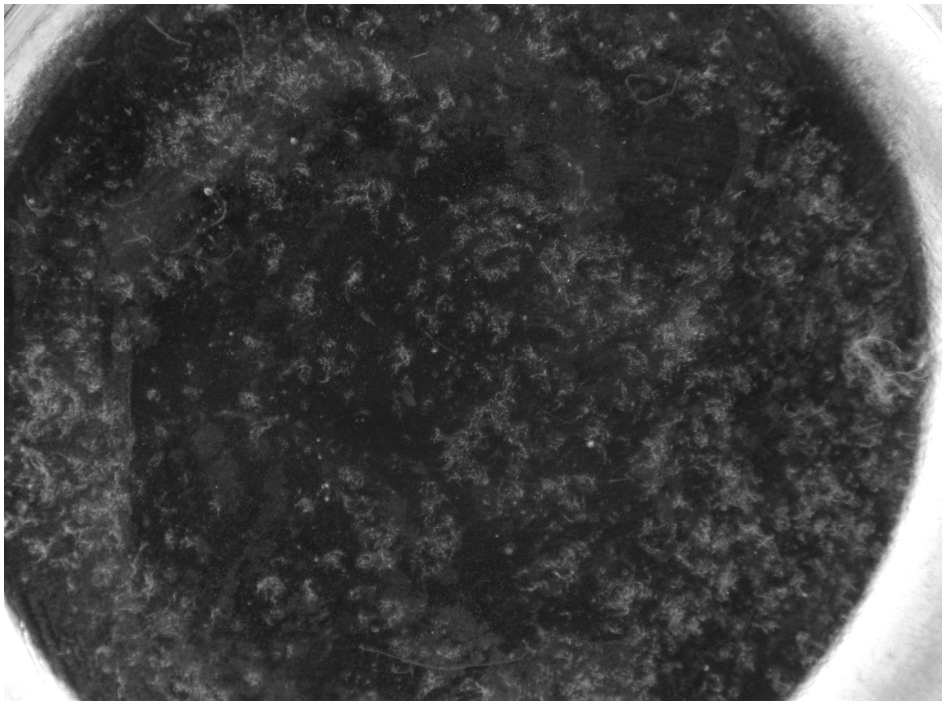
Figure 3.12 continued



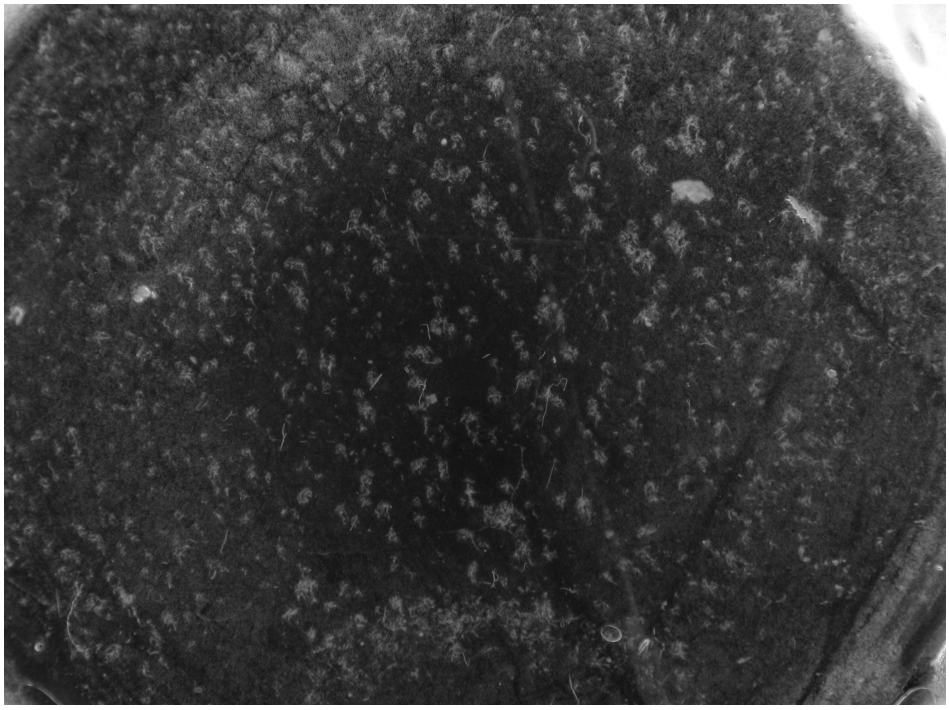
g. 9th day of growth

h. 10th day of growth

Figure 3.12 continued

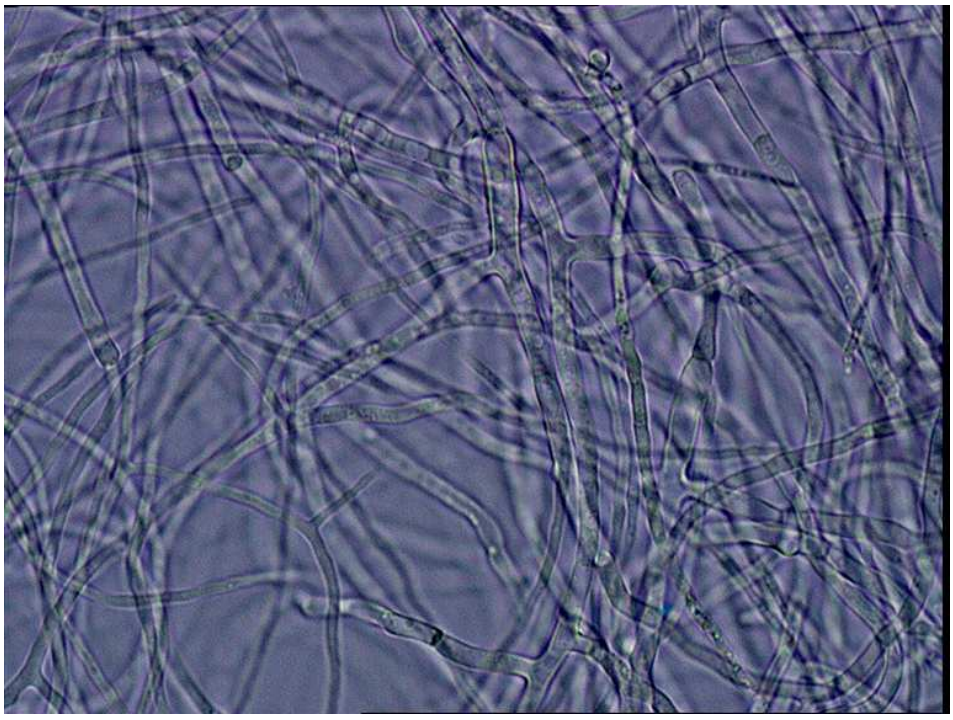


i. 11th day of growth



j. 12th day of growth

Figure 3.12 continued

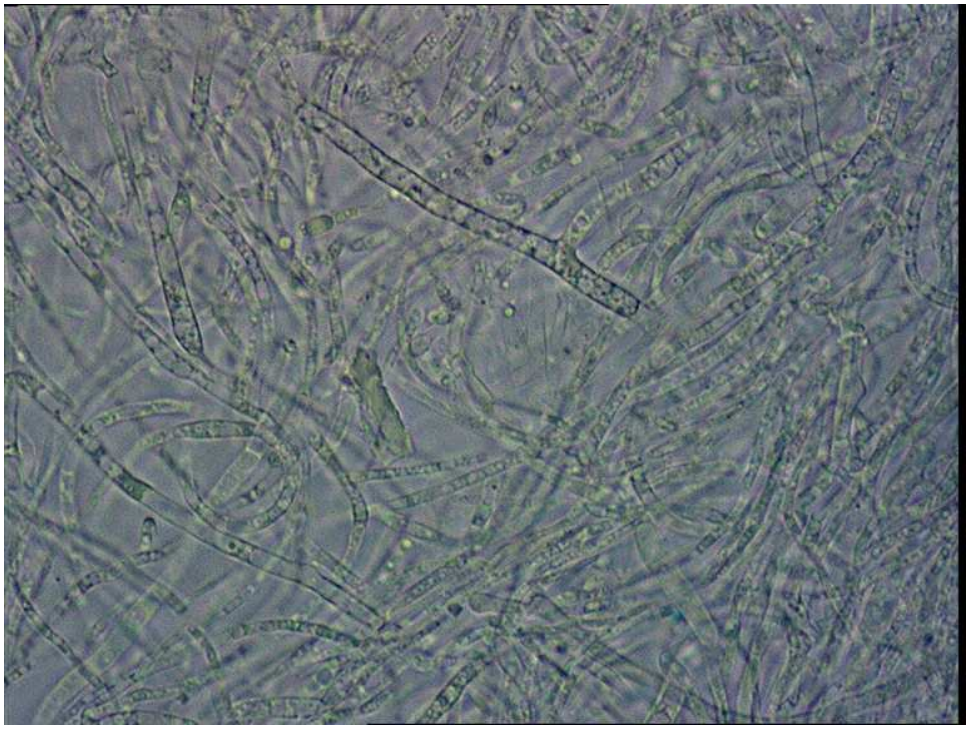


a.3th day of growth

b. 4th day of growth

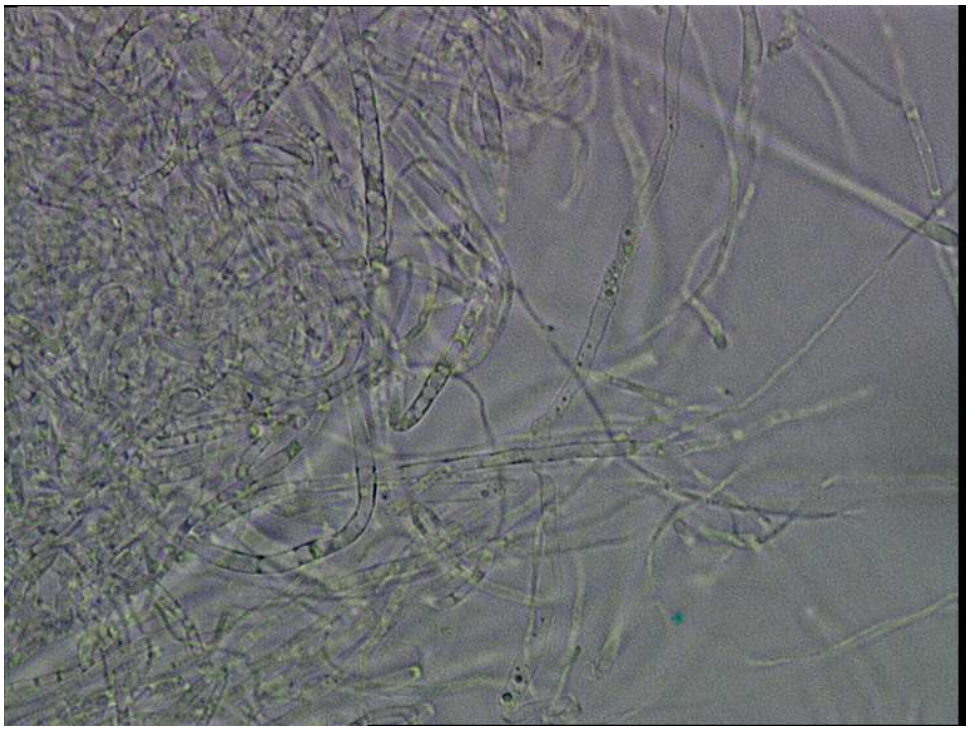
Figure 3.13 1000X magnified images of *Thermomyces lanuginosus* CBS 288.54 taken by inverse light microscope grown on modified YpSs broth containing arabinose as carbon source (a-b, days 3-4; c-d, days 5-6; e-f, days 7; g-h, days 8-9; i-j, days 10-11; k, day 12)

c. 5th day of growth



d. 6th day of growth

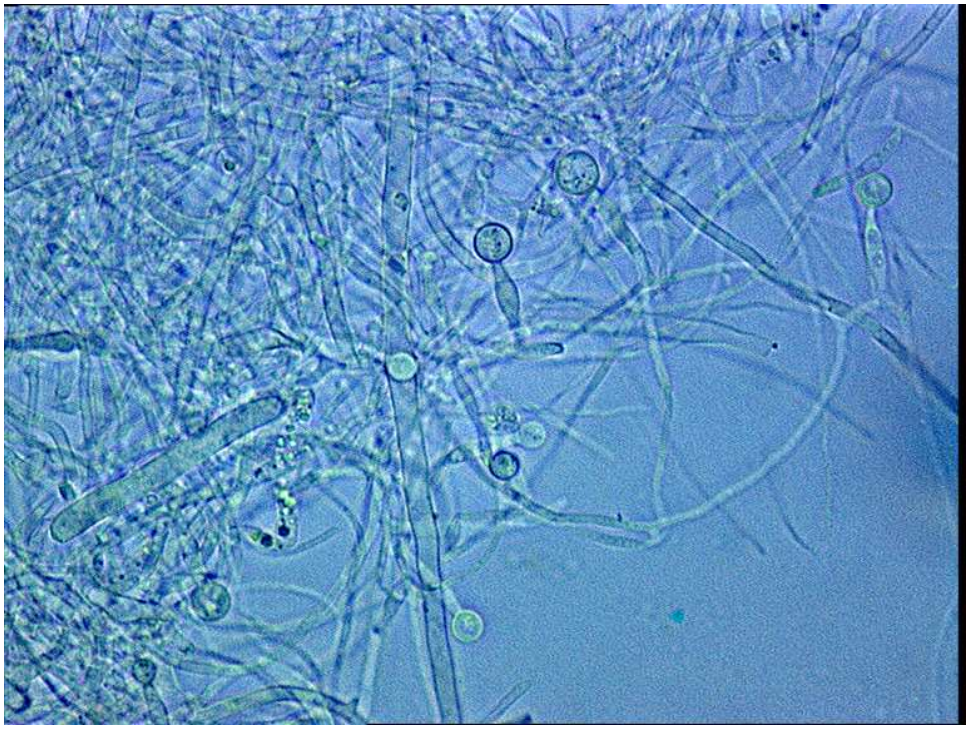
Figure 3.13 continued.



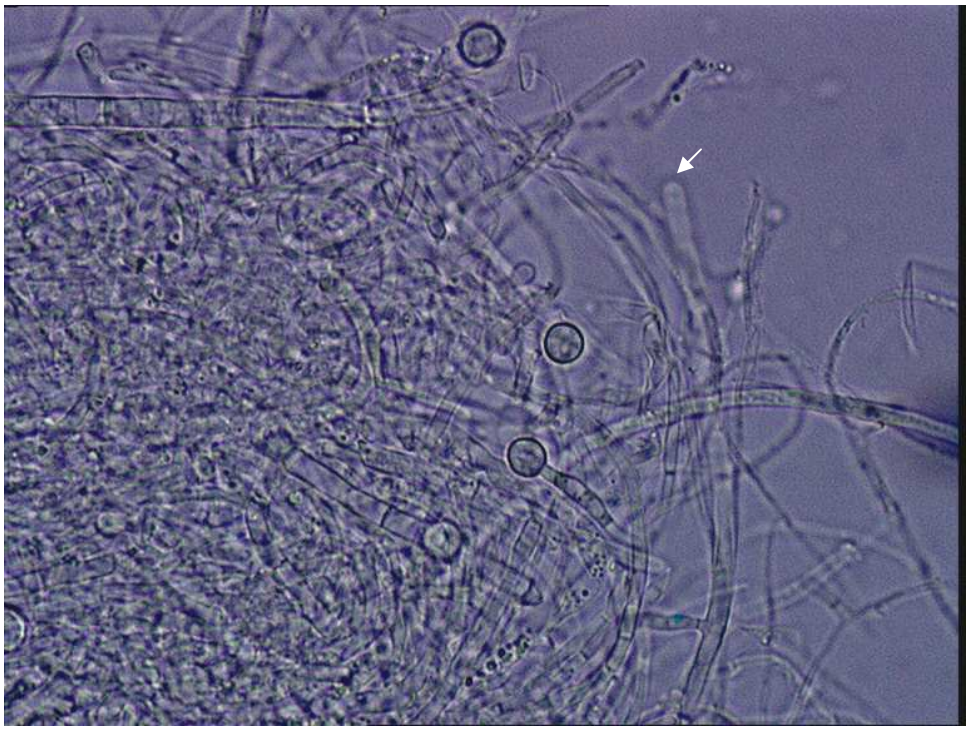
e. 7th day of growth (1000X)

f. 7th day of growth (100X)

Figure 3.13 continued.

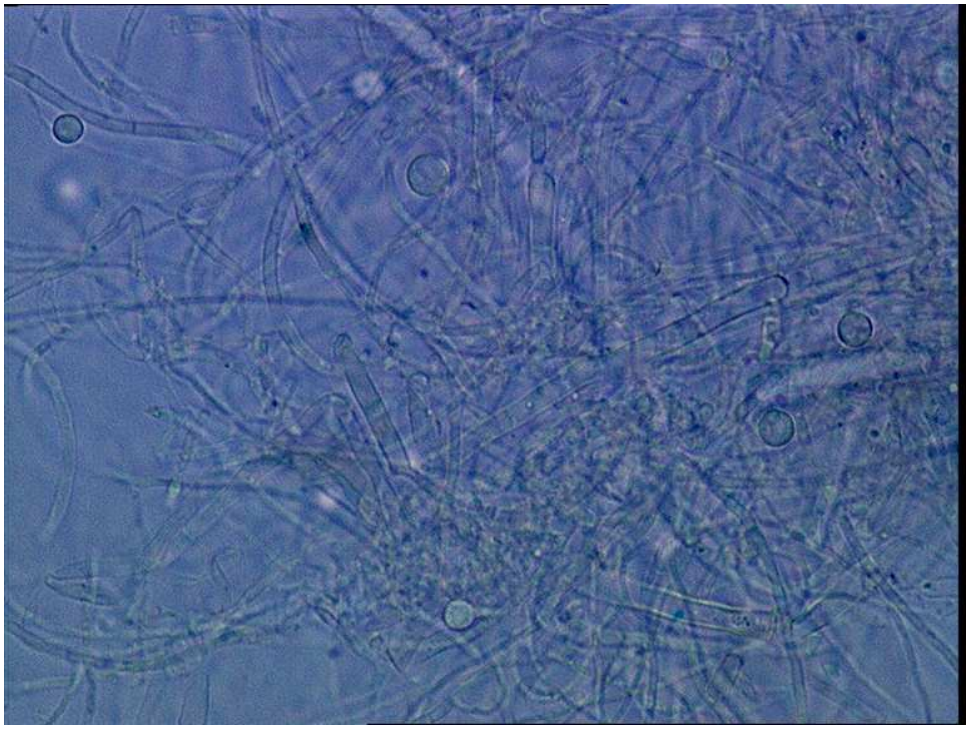


g. 8th day of growth

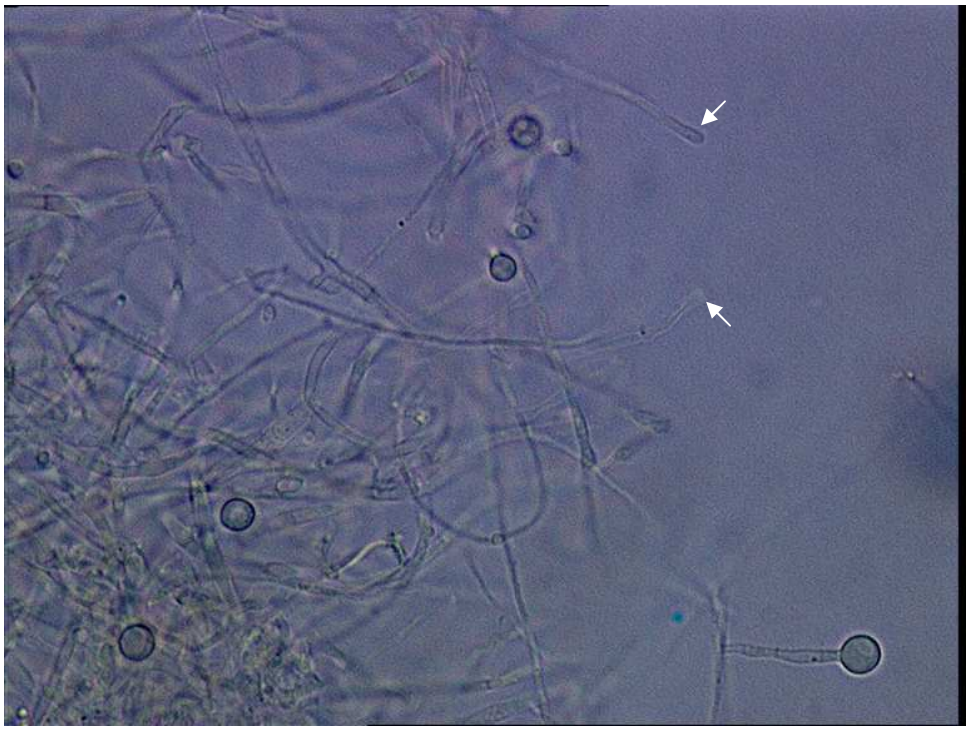


h. 9th day of growth

Figure 3.13 continued.
Arrows indicate break point of filaments.

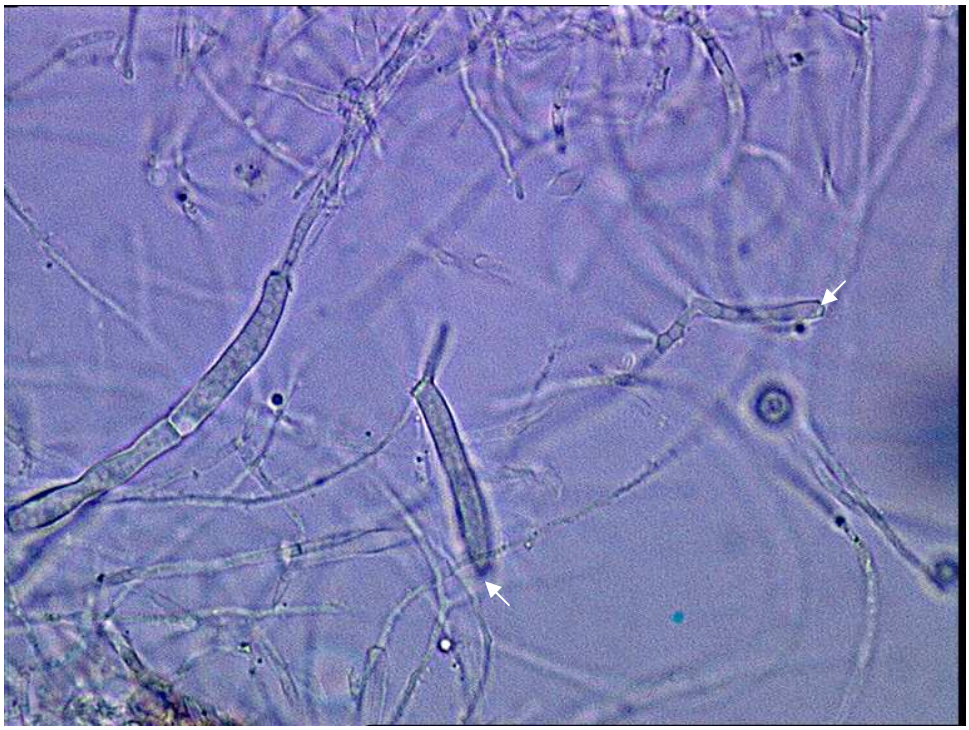


i. 10th day of growth



j. 11th day of growth

Figure 3.13 continued.
Arrows indicate break point of filaments.



k. 12th day of growth

Figure 3.13 continued.
Arrows indicate break point of filaments.

3.7. Partial Purification of *Thermomyces lanuginosus* β-galactosidase

In this research, extracellular enzyme was of interest since intracellular enzymes require expensive and difficult cell disintegration techniques and they are harder to purify due to many cell components appearing after burst of the cell. Since extracellular enzymes are very diluted due to the large volume of the medium, they need to be concentrated before purification. In this study, β-galactosidase-B of *Thermomyces lanuginosus* was partially purified and concentrated by fractional ammonium sulfate precipitation and anion-exchange chromatography.

3.7.1. Optimization of Fractional Ammonium Sulfate Precipitation

Thermomyces lanuginosus was grown as described in Sec. 2.2.3. Supernatant sample was taken by filtering the growth medium using coarse filter paper when the activity of β-galactosidase was maximum, on 10^{th} day of growth. Ammonium sulfate precipitation is a simple and efficient method used for concentration of proteins (Park *et al*, 1979, Fisher *et al*, 1995, Shaikh *et al*, 1999). In order to purify the β-galactosidase to some extend together with concentration, fractional ammonium sulfate precipitation was chosen as a method. In order to find the optimum salt concentration giving the best yield and purification fold, the activity of enzyme and amount of protein was found after precipitation by increasing salt concentration stepwise as 30%, 30-45%, 45-60%, 60-75%, 75-85% (Sec. 2.2.14).

Table 3.4 summarizes the results of optimization experiments. The activity and purification fold was found higher at salt concentrations between 60-85%. Yield results also show that approximately 63.5% of the β-galactosidase is recovered during precipitation between 60-85%. Highest purification fold was at 75-85%

In the literature, purified intracellular β-galactosidase of *Thermomyces lanugisosus* by fractional ammonium sulfate precipitation has shown 1.4 purification fold with 62,8% yield which is similar to our findings (Fischer *et al*, 1995). However, in our study purification fold was found as approximately 5 between 60-85% of salt concentration which is higher than the literature value. Compared to other results in literature, β-galactosidase of *Rhizomucor* sp. has shown 88% yield and 3.18 purification fold at 90% saturation of ammonium sulfate (Shaikh *et al*, 1999).

Table 3.4 Optimization of ammonium sulfate concentration for the partial purification of *Thermomyces lanuginosus* β-galactosidase

	Vol (ml)	Protein (mg/ml)	Activity (U/ml)	Total Pro. (mg)	Total Act. (U)	Spec. Act. (U/mg)	Purfn. Fold	Yield
Crude extract	89	0,144	0,148	12,816	13,179	1,028	1,000	100
0-30% NH4SO4 prec.	5,8	0,2396	0,201	1,390	1,168	0,841	0,817	8,864
30-45% NH4SO4 prec.	4,6	0,0573	0,068	0,264	0,311	1,181	1,149	2,363
45-60% NH4SO4 prec.	2,5	0,09445	0,212	0,236	0,531	2,248	2,186	4,027
60-75% NH4SO4 prec.	5,9	0,2085	0,662	1,230	3,906	3,175	3,088	29,637
75-85% NH4SO4 prec.	5,9	0,107	0,756	0,631	4,461	7,067	6,872	33,852
over 85% NH4SO4 prec.	213,4	0,03	0,022	6,402	4,596	0,718	0,698	34,876

Samples from each step of ammonium sulfate precipitation were also analysed by SDS-PAGE in order to be able to make estimation about the molecular weight of the enzyme (Figure 3.24). Three bands designated by arrows were mainly observed at approximately 118, 80 and 45 kDa molecular weights. Polyacrylamide gradient and homogenous gel analysis of intracellular β-galactosidase of *Thermomyces lanuginosus* was revealed that the molecular weight of the enzyme in the primary structure is between 75-80 kDa (Fisher *et al*, 1995). In our study, the second band in the figure was near 80 kDa suggesting that this might be β-galactosidase-B.

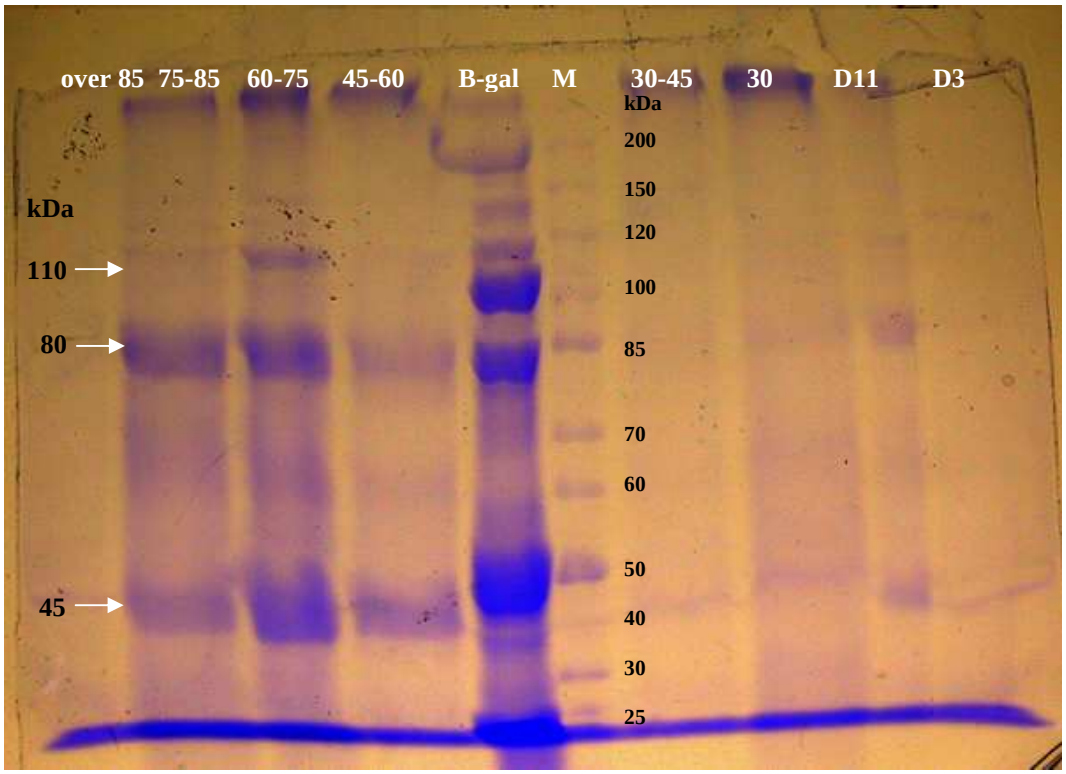


Figure 3.14 SDS-PAGE analysis of samples collected from ammonium sulfate precipitation. Numbers above show the ammonium sulfate percentages used for precipitation at each step. B-gal; β-galactosidase of *Kluyveromyces lactis* (15 unites), M; marker proteins, D11 & D3; crude enzyme samples from 11th and 3rd day of growth.

3.7.2. Anion Exchange Chromatography

In previous studies, after ammonium sulfate precipitation, choromatography on DEAE-cellulose, choromatography on CM-cellulose and DEAE-sephadex column choromatography were used respectively in order to purify β-galactosidase (Park, De Santi, Pastore *et al*, 1979; Borglum and Sternberg *et al*, 1972). Fisher and co-workers (1995) used 4 step of chromatography -combination of hydrophobic interaction and anion exchange chromatography with pH shift- to purify intracellular β-galactosidase of *Thermomyces lanuginosus*. In a recent study, Becerra and co-workers (1998) tried

several methods for the purification of *Kluyveromyces lactis* β-galactosidase. They concluded that affinity chromatography gave the highest purification factor while gel filtration and ion-exchange in the FPLC system has demonstrated to be faster (each run takes about 0,5-1 hour whereas each affinity chromatography run takes about 8 hours) accurate and reproducible.

In this research, as a second step of purification, anion-exchange chromatography in the FPLC system was used as described in Sec. 2.2.15. Amount of protein was determined by measuring absorbance of each fraction at 280 nm. Three peaks at 25^{th}, 54^{th} and 135^{th} min were observed in the chromatograph (Figure 3.15). First peak at 25^{th} minute is likely to be a contaminant. When the resulting chromatograph (Figure 3.15) was compared with β-galactosidase activity in each fraction (Figure 3.16), it was observed that peak values of activity and protein amount overlapped with each other. SDS-PAGE analysis of fractions with higher β-galactosidase activity shows three concentrated bands designated by arrows at 48 kDa, 70-85 kDa and 100-120 kDa, respectively (Figure 3.17). This result is in accord with Figure 3.14. However, in order to obtain complete purification, further studies must be conducted to get mainly fractions between 30-60 min by changing salt concentration used in the elution buffer.

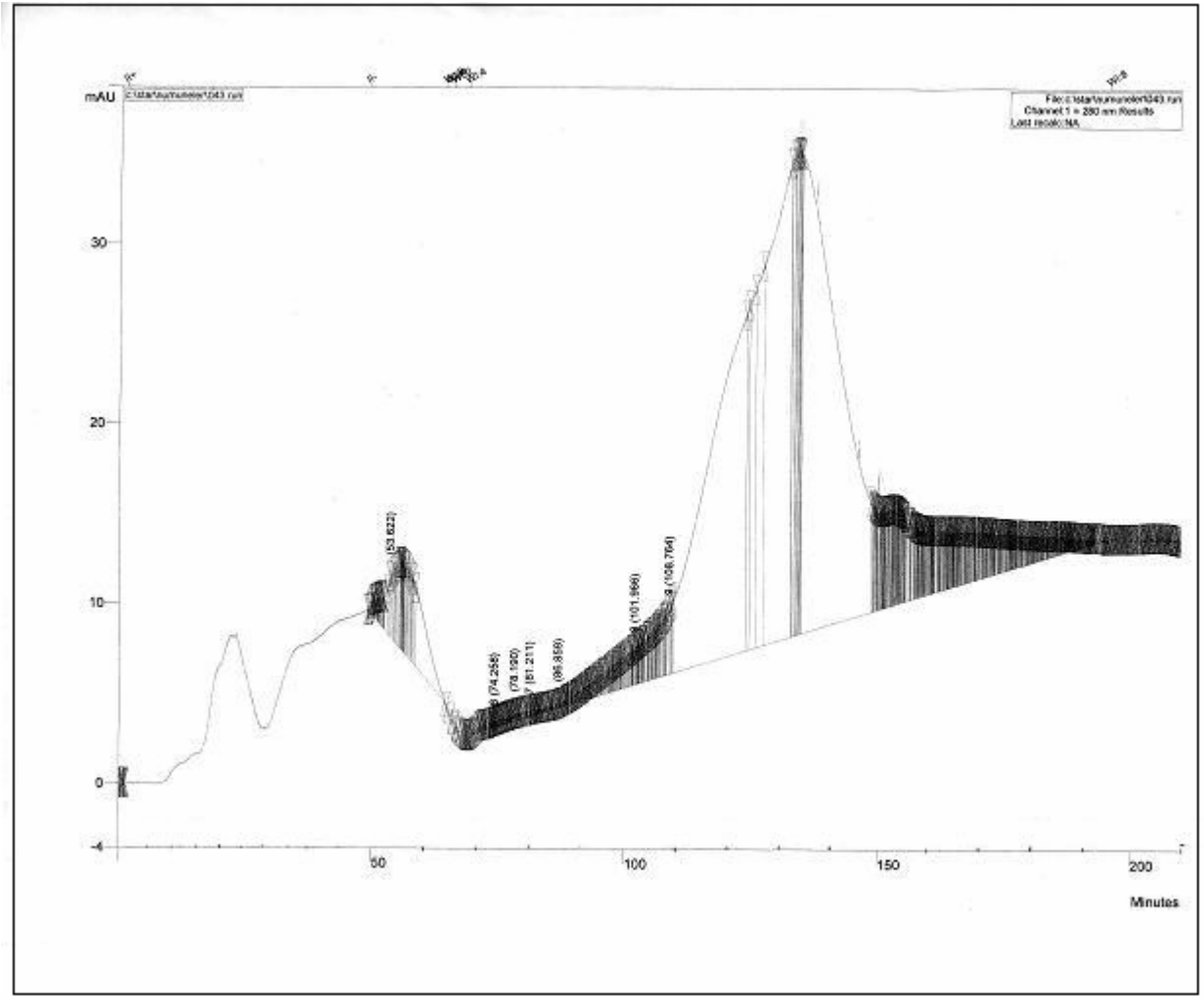


Figure 3.15 Protein profile of fractions collected from Anion-exchange chromatogrphy of β-galactosidase of *Thermomyces lanuginosus*.

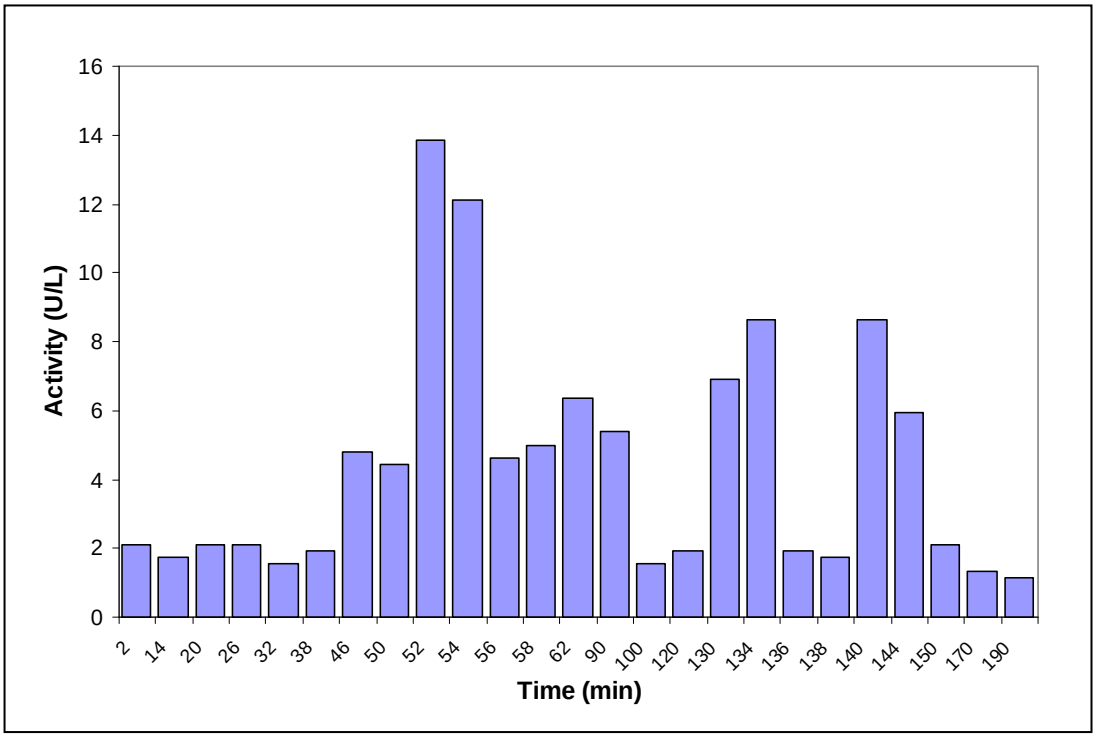


Figure 3.16 β-galactosidase activity of fractions collected from anion exchange column connected to FPLC system.

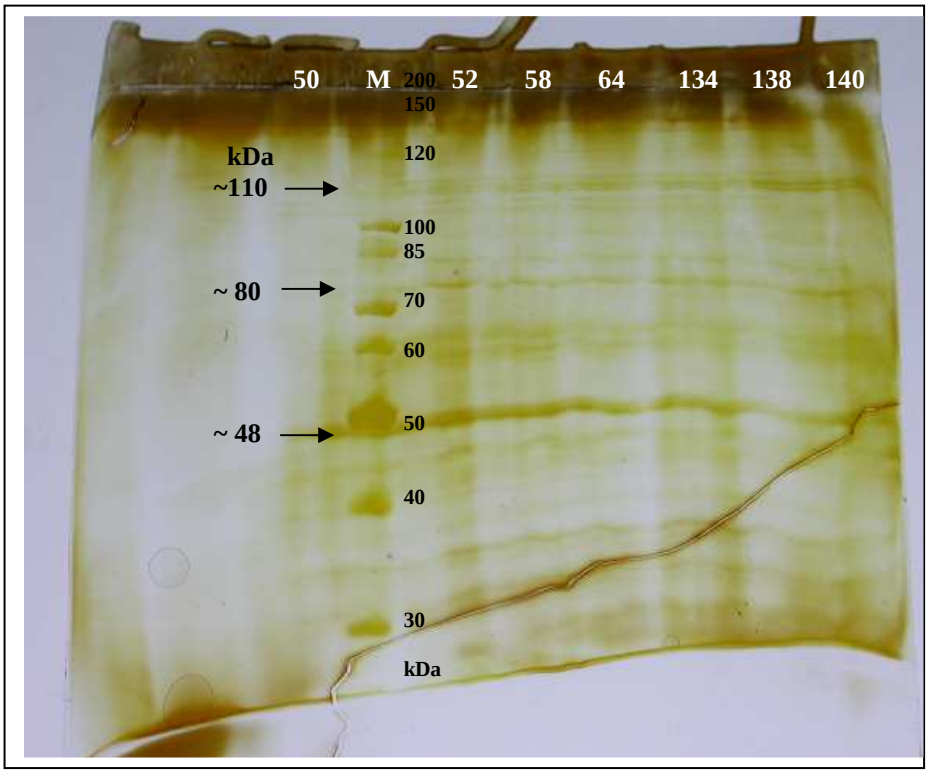


Figure 3.17 SDS-PAGE analysis of collected from anion exchange chromatography. Numbers above shows the elution time (min). M; marker proteins.

3.8. Determination of Molecular Weight by Gel Filtration

Molecular weight of native β-galactosidase from *Thermomyces lanuginosus* grown on modified YpSs medium containing arabinose as carbon source was determined by gel filtration column connected to a FPLC system. Amount of protein was determined by measuring absorbance of each fraction at 280 nm. Proteins were separated from each other according to their molecular weight; protein with highest molecular weight is eluted first. The molecular weight of proteins at peak points showing high amount of protein was determined by comparing with chromatograph of proteins of known molecular weight. Three

peaks were observed in the chromotagraph (Figure 3.18). First peak at 55th minute is likely to be a contaminant. Molecular weights, are corresponding to the remaining peaks are designated on the resulting Figure 3.18.

When the resulting chromatograph (Figure 3.18) was compared with β-galactosidase activity in each fraction (Figure 3.19), it was observed that a peak exists 85th minute, which was near the second peak of chromatograph at 87.313 minute corresponding to 122.154 kDa. The molecular weight of protein eluted at 85th minute was calculated as 140-156 kDa. Fisher and coworkers (1995) determined the molecular weight of intracellular enzyme as 200-220 kDa by size exclusion chromatography. 156 kDa is approximately twice the molecular weight of the possible monomeric form of the enzyme estimated as 80 kDa by SDS-PAGE by Fisher and co-workers (1995). Such a result may strengthen the suggestions made by Fisher and co-workers (1995) such that the β-galactosidase of *Themomyces lanuginosus* has a dimeric structure.

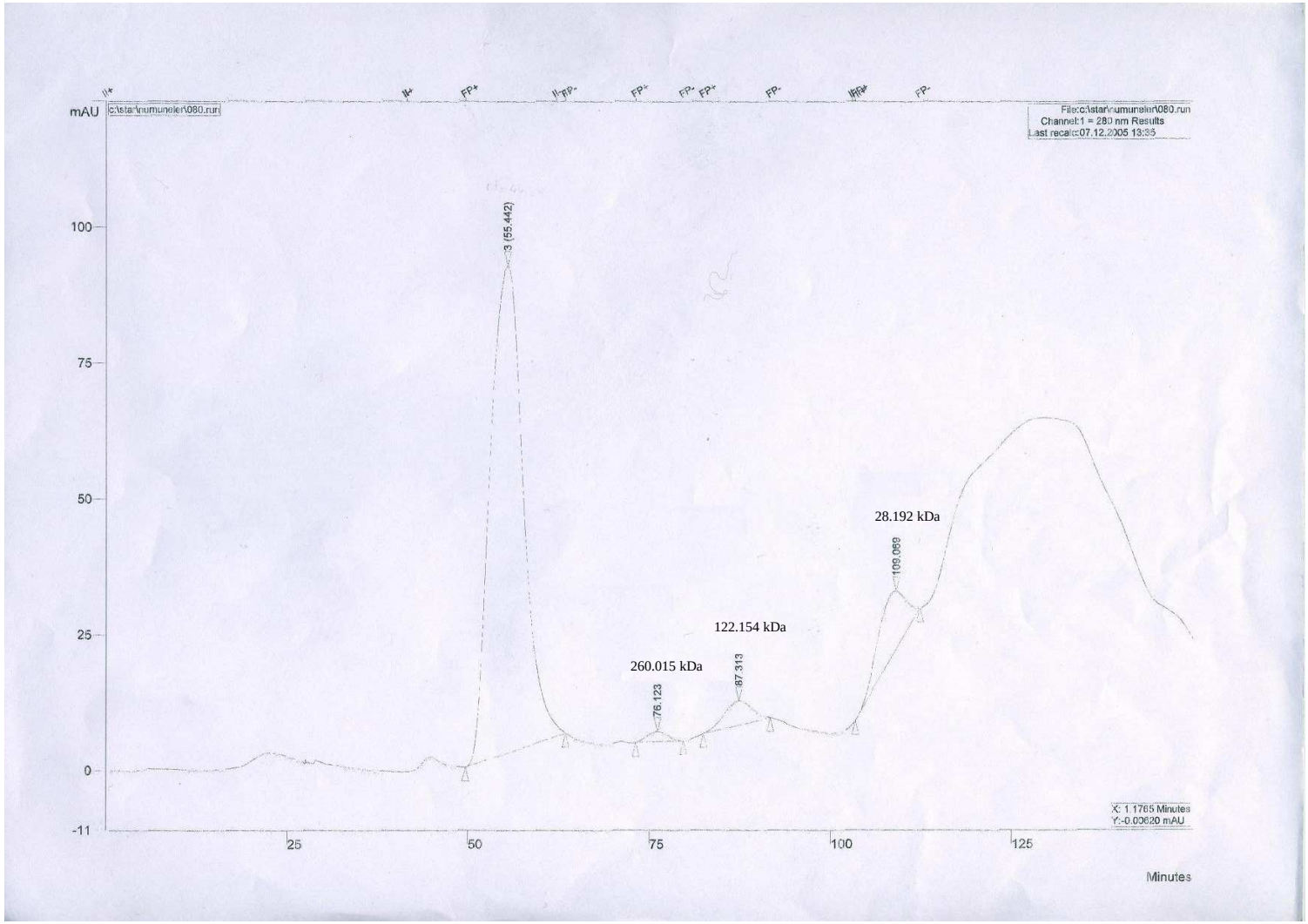


Figure 3.18 Protein profile of fractions collected from gel-filtration chromatography of β-galactosidase-B of *Thermomyces lanugisosus*.

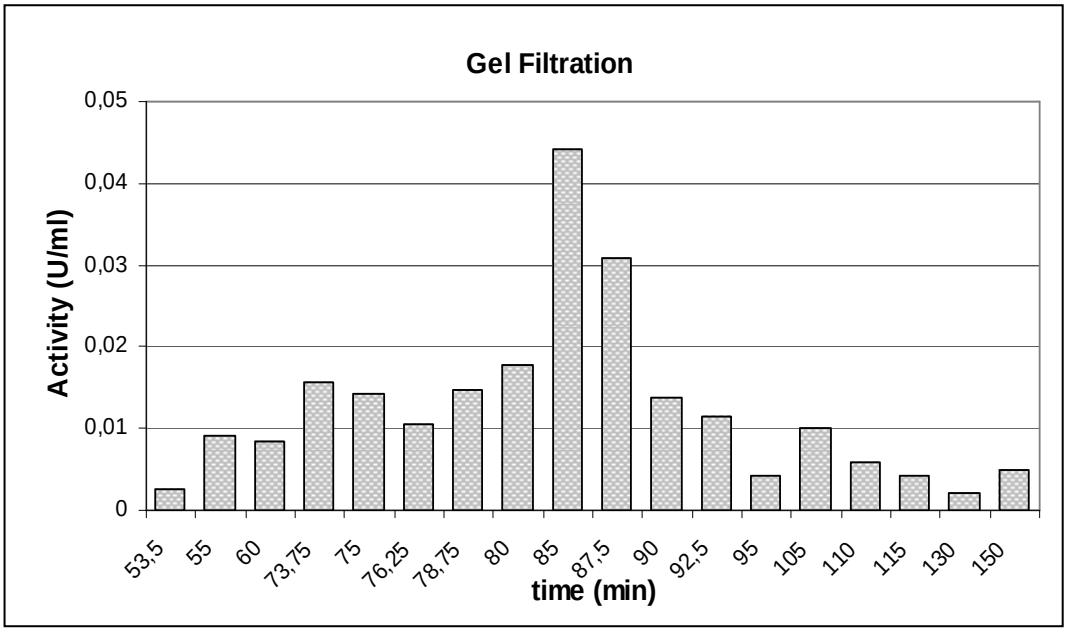


Figure 3.19 β-galactosidase-B activity of fractions collected from FPLC gel filtration column.

3.9. Determination of Lactose Hydrolysis

In order to determine the lactose hydrolysis efficiency of enzyme obtained from ammonium sulfate precipitation at 60-85% saturation, glucose oxidase-peroxidase assay was used (Sec.2.2.18). Before each measurement of lactose hydrolysis, reagent standard curve was plotted as described in Sec. 2.2.17. (Appendix C). Slope of the standard curve was used to determine amount of glucose formed through lactose hydrolysis. Figure 3.20 and Figure 3.21 show lactose conversion by β-galactosidase of *Thermomyces lanuginosus* at different temperatures. Accordingly, enzyme has shown best activity for lactose hydrolysis at 50ºC, which is different than the optimum temperature of activity at 65ºC (Figure 3.7). After 3 days of incubation at 50ºC, almost 50% of lactose was converted to glucose and galactose. This may be due to the higher stability of the enzyme at 50ºC.

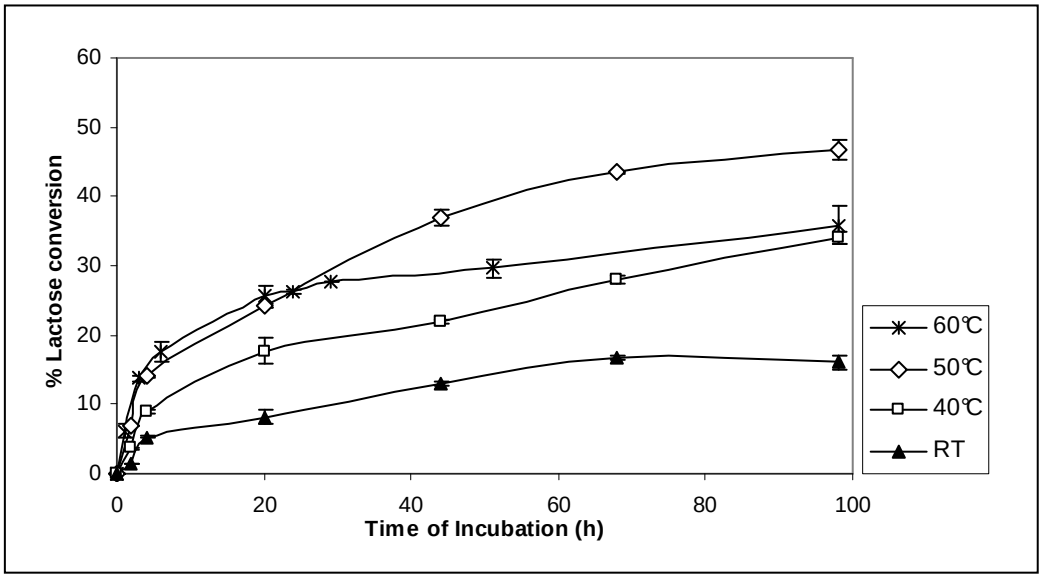


Figure 3.20 Percent lactose conversion by β-galactosidase-B of *Thermomyces lanuginosus* at different temperatures. (RT: Room Temperature)

Prolonged incubation up to 10 days, only slightly increased conversion to 60% (Figure 3.21). Such a slow down in the efficiency of enzyme might be the result of product inhibition due to accumulation of galactose or glucose. Most of the β-galactosidases from different organisms have proved to have such a characteristic. Borglum and Sternberg found that galactose inhibited the β-galactosidase of *Aspergillus foetidus* whereas glucose did not. Same result was also observed for the enzyme *A. oryzae*, while 25 mM galactose showed 15% inhibition, glucose had no effect on enzyme activity even at high concentration of glucose up to 400 mM (Park *et al*, 1979). It can be suggested that galactose inhibits fungal β-galactosidases rather than glucose. However, yeast enzyme was found to be inhibited 60% by glucose, 7% by galactose (Wendorff and Amundson, 1971). *Sulfolobus solfataricus* enzyme also showed inhibition mainly by glucose rather than galacose (Pisani *et al*, 1990).

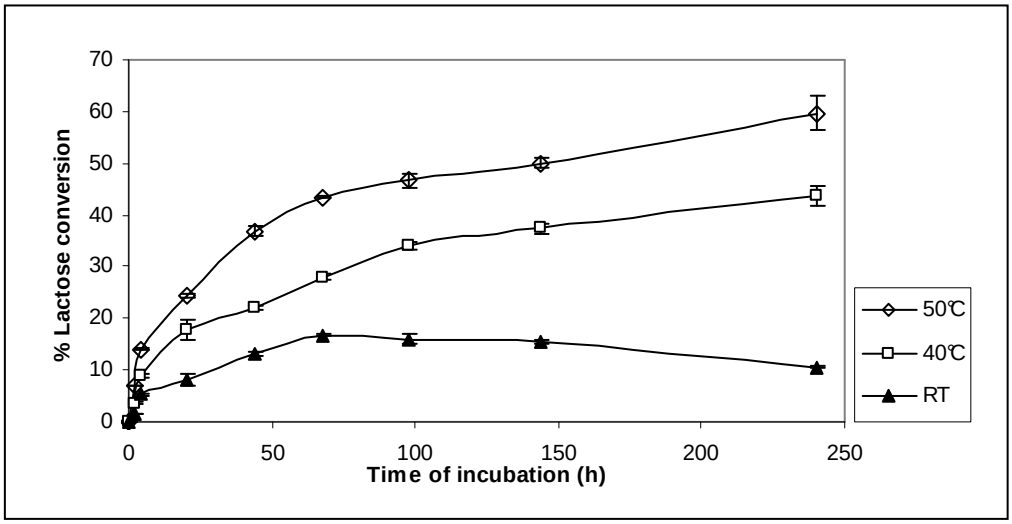


Figure 3.21 Percent lactose conversion by β-galactosidase-B of *Thermomyces lanuginosus* at room temperature (RT), 40ºC and 50ºC for prolonged time.

In order to compare the yield of β-galactosidase of *Thermomyces lanuginosus* with the commercial enzyme, lactose hydrolysis experiment was repeated using commercial enzyme obtained from *Kluyveromyces lactis* together with β-galactosidase from *Thermomyces lanuginosus*. Same unit of enzyme was used in the lactose hydrolysis assays as described in Sec. 2.2.20. Results are presented in Figure 3.22. Accordingly, *K. lactis* enzyme was most efficient at room temperature. However, when looked at the overall results, even at room temperature the enzyme of *T. lanuginosus* was 1.3 times more efficient than the enzyme of *K. lactis*. At 50ºC, lactose hydrolysis by β-galactosidase of *T. lanuginosus* reached to 4.7 fold the β-galactosidase of *K. lactis*.

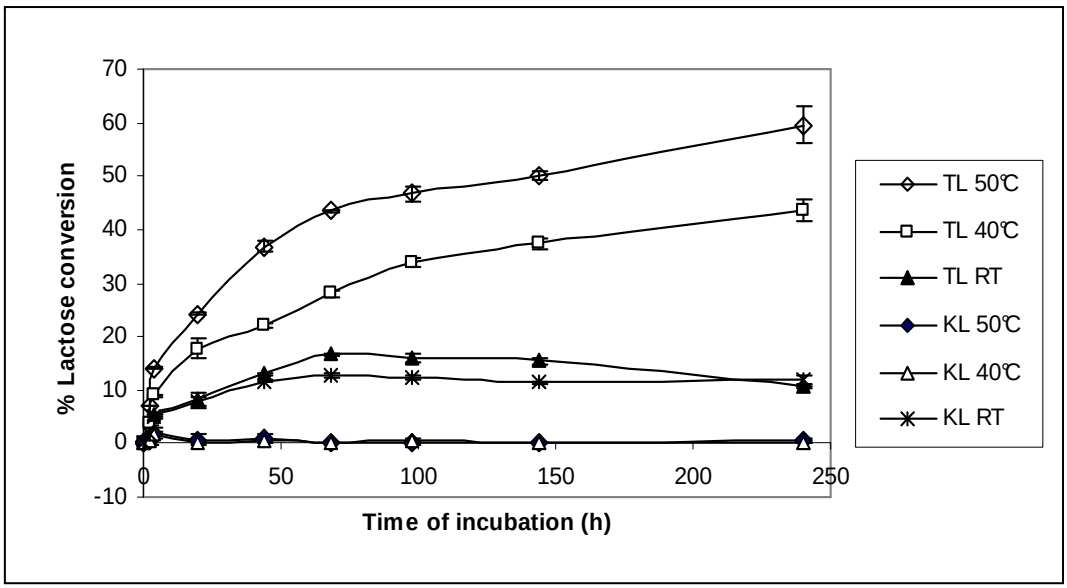


Figure 3.22 Percent lactose conversion by β-galactosidase of *Kluyveromyces lactis* (KL) and *Thermomyces lanuginosus* (TL) at room temperature, 40ºC and 50ºC.

CHAPTER 4

CONCLUSION AND RECOMMENDATIONS

In this study, production of β-galactosidase active at neutral pH from thermophilic fungi was analyzed in order to apply for the purpose of low lactose milk production.

As a result of screening for extracellular β-galactosidase activity, arabinose was found as best inducing carbon source possibly by signaling the organism the presence of polysaccharides in the medium. When biomass and activity was analysed throughout the growth, two peak points in activity and decrease in biomass after first peak point of activity were observed. Moreover, optimum pH of the enzymes collected at the peak points of activity was found as 7,6 and 6,8. Presence of two peak points in activity and different optimum pHs was suggested to be result from the presence of two different enzymes that the one is an extracellular enzyme while the other is a cell-wall bound one. In addition, in order to be able to explain biomass reduction after first peak point of activity, photographs of the growth medium were taken on each day of growth. These photos show that fragmentation begins after first peak point of activity, which confirms our suggestion as the presence of a cell-bound enzyme liberating following fragmentation. Auto-fragmentation of mycelium eliminates the need for usage of expensive cell-disintegration techniques.

Optimum temperature of the enzyme was found as 65ºC. The enzyme was stable over a period of one month when stored at 4ºC and -20ºC. K_m and V_{max} were found as 2.48 mM and 0.44 U/ml, respectively. Molecular weight of the enzyme is estimated as 140-156 kDa by gel-filtration.

Purification fold and yield obtained at the end of ammonium sulfate precipitation was approximately 5 and 65%, respectively. For complete purification, anion exchange chromatography requires further study by changing buffer and its concentration used in elution.

Partially purified β-galactosidase-B of *Thermomyces lanuginosus* hydrolyses 50 % of lactose within 3 days at 50ºC. Time of incubation can be shortened by using pure and concentrated enzyme. Commercial enzyme from *Kluyveromyces lactis* hydrolysed only 13% lactose at room temperature. Yields were much lower at higher temperatures.

Further studies may be carried out by using hemicelluloses containing arabinose as a carbon source of growth medium in fermentors. In addition, transferase ability of the β-galactosidase of *Thermomyces lanuginosus* and galacto-oligosaccharide formation during lactose hydrolysis can be studied.

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APPENDICES

A. Composition of Growth Medium

Table A.1 Composition of YpSs medium

	YpSs agar	**Modified YpSs agar**
Yeast Extract	0.4 g	0.4 g
K_2HPO_4	0.1 g	0.1 g
$MgSO_4.7H_2O$	0.05 g	0.05 g
Carbon source	1.5 g Soluble starch	1.5 g X
Agar	2 g	2 g
Water	100 ml	100 ml

	YpSs broth	**Modified YpSs broth**	**Pre-culture medium**
Yeast Extract	0.4 g	0.4 g	0.4 g
K_2HPO_4	0.1 g	0.1 g	0.1 g
$MgSO_4.7H_2O$	0.05 g	0.05 g	0.05 g
Carbon source	1.5 g Soluble starch	1.5 g X	1.5 g Glucose
Water	100 ml	100 ml	100 ml

X: one of the carbon sources listed below;
arabinose, pectin, xylan, corn bagasse, cotton bagasse, wheat bran, lactose

B. Standard Curve for Enzyme Activity

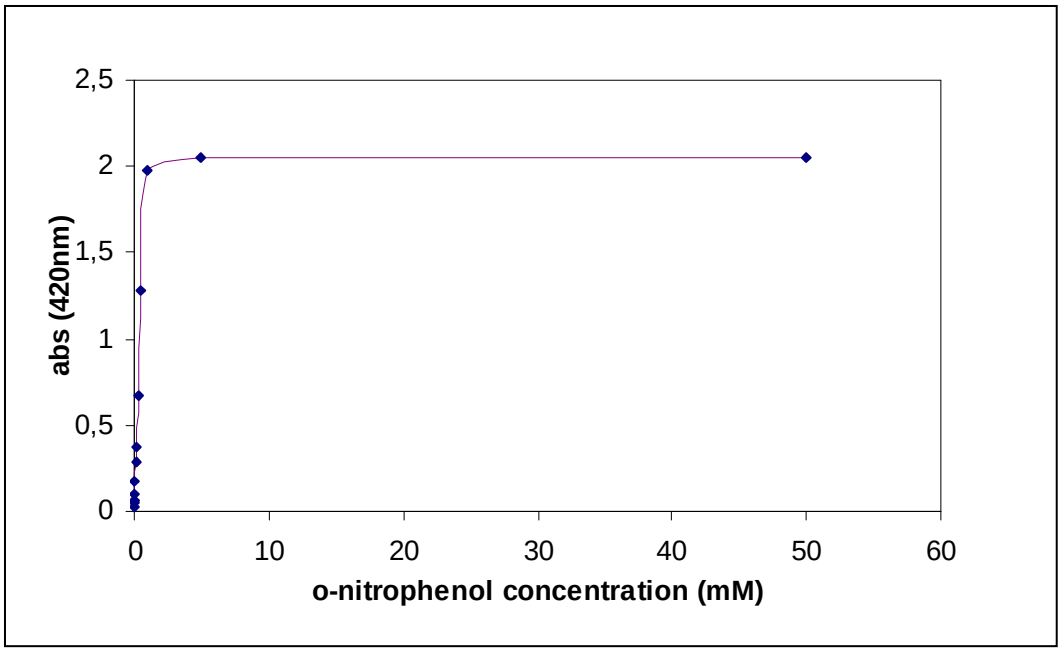

Figure B.1 Absorbance of hydrolysis product (*o*-nitrophenol) at dilution rates described in Table 2.1

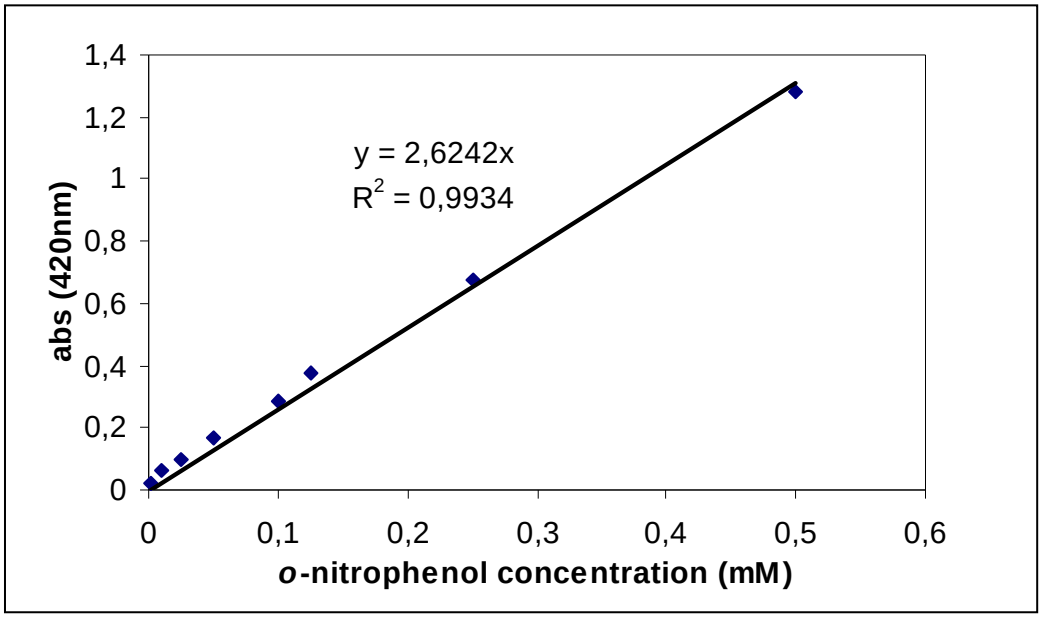

Figure B.2 Absorbance of hydrolysis product (*o*-nitrophenol) at dilution rates corresponding to the linear range.

C. Standard Curve of Glucose oxidase-peroxidase Assay

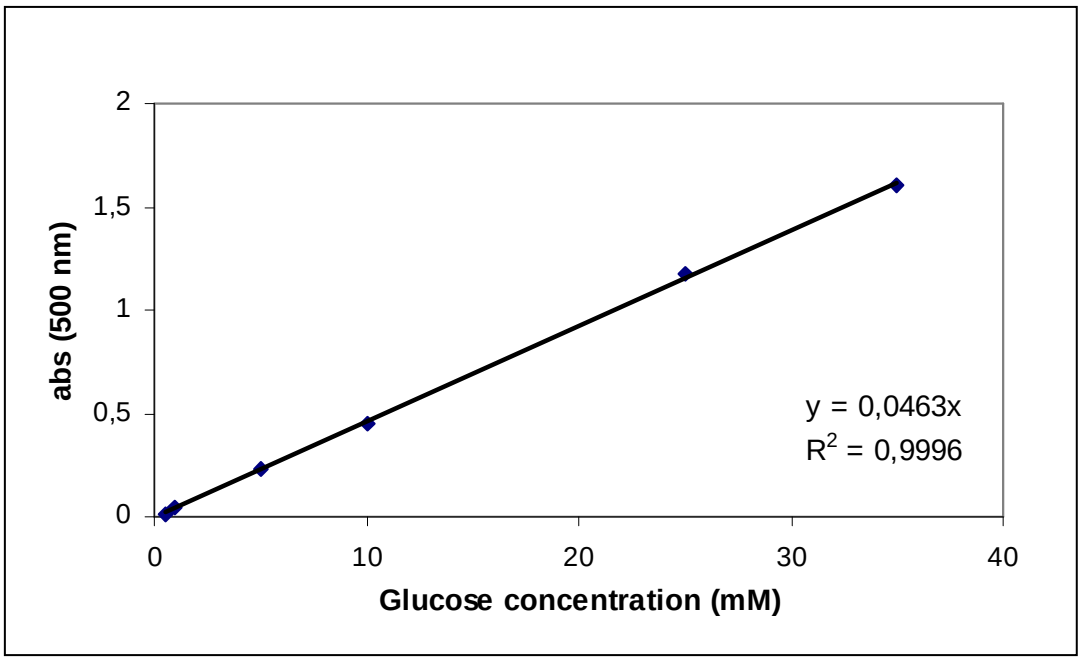

Figure C.1 Standard curve of Glucose oxidase-peroxidase assay for use in the determination of lactose hydrolysis.