# AN INVESTIGATION OF BACTERIAL AND FUNGAL XYLANOLYTIC SYSTEMS

**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 DOCTOR OF PHILOSOPHY**
**IN**
**BIOTECHNOLOGY**

**NOVEMBER, 2006**

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## ABSTRACT

### AN INVESTIGATION OF BACTERIAL AND FUNGAL XYLANOLYTIC SYSTEMS

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November 2006, 194 pages

Endo-β-1,4 xylanases (EC. 3.2.1.8) are typically produced as a mixture of different hydrolytic enzymes such as β-1,4-xylosidase (EC. 3.2.1.37) , α-L-arabinofuranosidases (EC. 3.2.1.55), and feruloyl esterase (EC 3.1.1.73) that hydrolyze xylan molecule, which constitutes 20-30% of the weight of wood and agricultural wastes. Thus, xylan, a renewable biomass, can be utilized as a substrate for the preparation of many products such as fuels, solvents and pharmaceuticals. Besides, xylanolytic enzymes themselves are also used in food, feed, textile industries and pre-bleaching of kraft.

In the first part of the study, xylanolytic systems of a soil isolate *Bacillus pumilus* SB-M13 and a thermophilic fungus *Scytalidium thermophilum* were investigated. Production rate and type of xylanolytic changed depending on the carbon source and the microorganism. However, xylanolytic enzyme production was found to be sequential, in synergy and under the control of carbon catabolite repression for both microorganisms.

In the second part, *B. pumilus* SB-M13 β-1,4 xylanase was purified and

biochemically characterized. The enzyme was stable at alkaline pHs and highest activity was observed at 60°C and pH 7.5. Enzyme $K_m$ and $k_{cat}$ values were determined as 1.87 mg/ml and 43,000 U/mg, respectively.

*B. pumilus* SB-M13 and *S .thermophilum* α-L-arabinofuranosidases were also purified and biochemically characterized. Although produced from a mesophilic microorganism, *B. pumilus* SB-M13 arabinofuranosidase was quite thermostable. Moreover, unlike other fungi, *S. thermophilum* produced alkaline stable arabinofuranosidases. Both enzymes were multimeric, alkaline stable and most active at 70°C and pH 7.0. However, when compared to *S. thermophilum*, catalytic power of *B. pumilus* SB-M13 arabinofuranosidase was higher.

Keywords: Xylanolytic systems, xylanase, arabinofuranosidase, *Bacillus pumilus* and *Scytalidium thermophilum*.

# ÖZ

## BAKTERİYEL VE FUNGAL KSİLANOLİTİK SİSTEMLERİNİN ARAŞTIRILMASI

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Kasım 2006, 194 sayfa

Endo-β-1,4 ksilanazlar (EC. 3.2.1.8) tipik olarak β-1,4-ksilosidaz (EC. 3.2.1.37) , α-L-arabinofuranosidaz (EC. 3.2.1.55) ve feruloyl esteraz (EC 3.1.1.73) gibi farklı hidrolitik enzimler ile birlikte üretilirler ve doğada ağaç ve bitkisel atıkların ağırlığının % 20-30 'lık kısmını oluşturan ksilan molekülünü hidrolize ederler. Böylece geri dönüşümü olan ksilan molekülü, substrat olarak kullanılarak yakıt, çözücü ve kozmetik ürünlerinin hazırlanmasında kullanılabilir. Ayrıca, ksilanolitik enzimler gıda, yem, tekstil ve kağıt beyazlatma işlemlerinde de kullanılırlar.

Çalışmamızın ilk aşamasında, bir toprak izolatı olan *Bacillus pumilus* SB-M13 ve termofilik bir küf olan *Scytalidium thermophilum*'un ksilanolitik enzim sistemleri araştırılmıştır. Ksilanolitik enzimlerin tipi, çeşitliliği ve üretim seviyesi karbon kaynağı ve mikroorganizmaya bağlı olarak değişmiştir. Fakat, her iki mikroorganizma için de, ksilanolitik enzimlerin sentezinin sırasal, sinerjik ve karbon catabolit engellemesi altında olduğu bulunmıştur

Çalışmanın ikinci kısmında, *B. pumilus* SB-M13 β-1,4 ksilanaz enzimi saflaştırılıp biyokimyasal olarak karakterize edilmiştir. Enzim alkalin pH'larda dayanıklı olup,

enzime ait en yüksek aktivite 60°C ve pH 7.5'ta gözlenmiştir. Enzime ait $K_m$ ve $k_{cat}$ değerleri sırasıyla 1.87 mg/ml and 43,000 U/mg olarak belirlenmiştir. *B. pumilus* SB-M13 ve *S .thermophilum* α-L-arabinofuranosidaz enzimleri saflaştırılıp, biyokimyasal karakterizasyonu yapılmıştır. Mezofilik bir mikroorganizma tarafından üretilmesine rağmen, *B. pumilus* SB-M13 arabinofuranosidaz enzimi ısıya dayanıklıdır. Üstelik, diğer küflerin tersine, *S. thermophilum* alkalin dayanıklı arabinofuranosidaz üretmiştir. Her iki enzim de mutimerik, alkalin dayanıklı ve 70°C ve pH 7.0'de en aktiftir. Fakat, *S. thermophilum* arabinofuranosidazı ile karşılaştırıldığında, *B. pumilus* SB-M13 arabinofuranosidazının substrat seçiciliği ve katalitik gücü daha fazladır.

Anahtar Kelimeler: Ksilanolitik sistemler, ksilanaz, arabinofuranosidaz, *Bacillus pumilus* ve *Scytalidium thermophilum.*

To My Mother and **My Husband**

**ACKNOWLEDGMENTS**

I am deeply greatful to Prof. Dr. Ufuk Bakır for her valuable guidance, professional advice, constructive discussions, suggessions, understanding and encouragement throughout this study.

I am also grateful to Prof. Dr. Zümrüt B. Ögel for her help and support in all phases of my study.

I wish to express my deepest gratitude to Prof. Dr. Hüseyin Avni Öktem, and Prof. Dr. Cumhur Çökmüş for their constructive criticisms.

I wish to specially thank Prof. Dr. Pınar Çalık, Prof. Dr. Ufuk Gündüz and Prof. Dr. Güngör Gündüz for their constant encouragement.

I am lucky enough to have had the support of many good friends. My special thanks are devoted to Alev Deniz Öztürk, Gül Sarıbay, Burcu Mirkelamoğlu, Işık Haykır, Vahideh Anghardi, Hande Levent, M.Ali Orman, Nuriye Korkmaz, Mine Görkey, Özlem Ak, Beril Korkmaz, Selin Aytar, Arda Büyüksungur, Eda Çelik, Işıl Işık, Ela Eroğlu and Belma Soydaş.

No words can suffice to acknowledge the immense support rendered by my mother Ayşe Ersayın, *my husband Yüce Oğul Yaşınok*, sisters Semra Ersayın, Belgin Ersayın Akdemir, Selda Yolcu, brothers-in-law Ahmet Yılmaz Akdemir and Yaşar Yolcu and all other close relatives.The knowledge that they will always be there to pick up the pieces is what allowed me to go ahead.

Last but not the least, I love and thank all the memories of my father.

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## ABBREVIATIONS

| | |
|---|---|
| CBH | Cellobiohydrolase |
| CR | Carbon catabolite repression |
| CRE | Catabolire responsive elements |
| DNSA | Dinitrosalicylic acid |
| BSA | Bovine serum albumin |
| FPLC | Fast protein chromatography system |
| AF | α-L-Arabinofuranosidase |
| GAL | β-galactosidase |
| XYL | β-xylosidase |
| GLU | β-glucosidase |
| XYN | Endo-β-1,4-xylanase |
| p-NPAraf | p-Nitrophenyl-α-L-arabinonofuranoside |
| p-NP | *p*-Nitrophenol |
| BAF | *Bacillus pumilus* SM-M13 α-L-Arabinofuranosidase |
| STAF | *Scytalidium thermophilum* α-L-Arabinofuranosidase |

# CHAPTER 1

# GENERAL INTRODUCTION

## 1.1 Plant cell wall

Plant cell walls are composed of several layers of polysaccharides and protein complexes. In Figure 1, cell wall model of maize bran is shown. The outer layer, the primary wall, comprises 20% of cellulose and 50% of hemicellulose. Cellulose fibers are randomly oriented in loose array in the primary cell walls which also contains hemicellulose, pectin, and proteins in its structure. The protein structure crosslinks the amorphous polysaccharides and forms a closely knit network. Moreover, the area between adjacent cell walls is composed predominantly of pectic materials.

The secondary cell wall, formed inside the primary wall, consists of mainly cellulose and hemicellulose in the range of 50-90% and 25%, respectively. The secondary wall also has distinct layers in which cellulose fibers are woven together in various ways.

After ceasing growth, lignification process takes places and lignin, representing 40% of total lignin in older plant tissues, is deposited through the layers of secondary cell wall structure (La Grange, 1999).

Plant cell wall polysaccharides are the most abundant organic compounds found in nature. They can be divided into three groups: cellulose, hemicellulose and lignin.

Cellulose represents the most abundant and renewable biopolymer on the earth and consists of a linear polymer of β-1,4-linked D-glucose residues (Figure 2). Cellulose polymers are present as ordered structures and their main function is to ensure the rigidity of the plant cell wall.

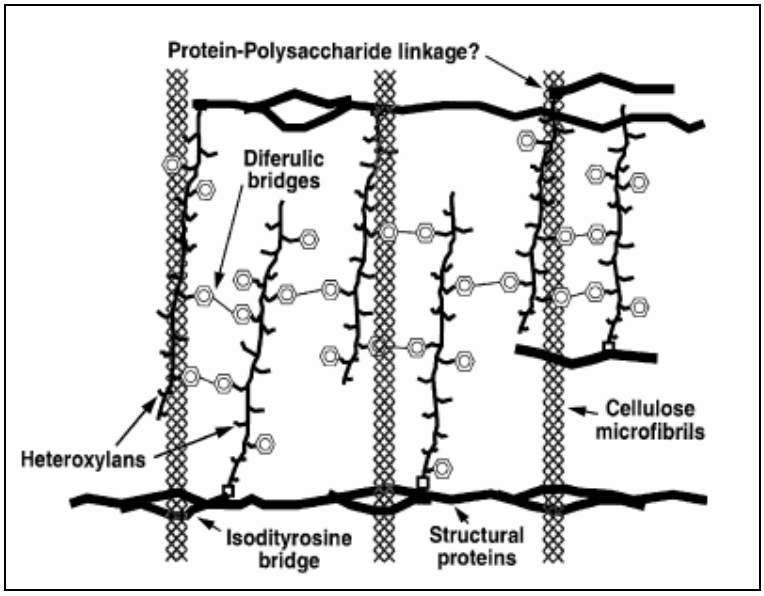


Figure1. Maize bran cell walls model (Saha, 2000).

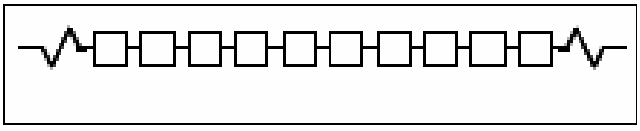

Figure 2. Schematic structure of cellulose. (□: β-D-glucose; Compier, 2005).

Alhough chemical composition of cellulose is simple, its physical structure and morphology is heterogeneous and complex. The polymeric chain of cellulose involve over 10,000 D-glucose residues linked with β-1,4glycosidic bonds which makes resulting chains insoluble. They adhere to each other in parallel fashion and form crystalline microfibrills.

The native cellulose has both highly crystalline and less ordered amorphous regions. Presence of hemicellulose, lignin, and pectin present along with cellulose as plant cell wall components, increase the complexity of the native cellulose. Consequently, its efficient hydrolysis requires presence of different enzymes in a typical cellulolytic enzyme complex (Vyas, 2004).

Hydrolytic enzymes such as endoglucanase [1,4-β-D glucan glucanohydrolase; (EC 3.2.1.4)], cellobiohydrolase (CBH) [1,4-β-D glucan cellobiohydrolase; (EC 3.2.1.91)], 1,4-β-D-glucan glucohydrolase (EC 3.2.1.74) and β-glucosidase [1,4-β-D glucoside glucohydrolase, (EC 3.2.1.21)] are involved in degradation of crystalline cellulose to glucose.

Unlike cellulose, hemicellulose is not a homogenous polymer. It contains different heterogeneous polymer such as xylan, mannan and galactan.

Xylan, the major hemicellulose in most plants, represents one-third of the renewable biomass on the earth. The xylan percentages of the plant dry weight vary depending on plant source. That of hardwoods, softwoods, and annual plants is in the range of 15 to 30, 7 to 10 and up to 30, respectively (Bakir, 2005).

Mannans occur in moderate amounts in certain secondary cell walls. These polysaccharides serve as carbohydrate reserves in a variety of plant species. Mannose containing polysaccharides include (galacto)mannans and (galacto)glucomannans. (Galacto)glucomannan, consisting of a backbone of β-1,4-linked mannose and glucose residues and galactose side groups, are the second

hemicellulosic structure commonly- in soft- and hardwoods. Softwoods contain mainly galactoglucomannan whereas in hardwoods glucomannan is the most common form (Compier, 2005).

Pectins form another group of heteropolysaccharides, which consist of a backbone of α-1,4-linked galacturonic acid residues (Figure 3). In specific 'hairy' regions the galacturonic acid backbone is interrupted by α-1,2-linked rhamnose residues. Long side chains consisting mainly of L-arabinose and D-galactose residues can be attached to these rhamnose residues. In pectins of some origins (e.g. sugar beet and apple) ferulic acid is present as terminal residues attached to O-5 of the arabinose residues or O-2 of the galactose residues (deVries, 1999).

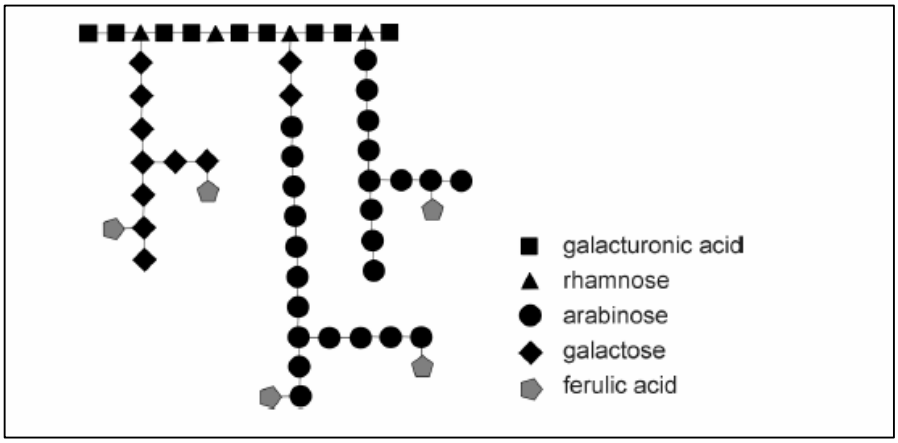


Figure 3. Schematic presentation of the hairy region of the pectin (de Vries, 1999).

Lignin is the most complex cell wall constituents (Figure 4). It composed of polyphenolic polymer formed from three types of phenyl propane units. Coniferyl, sinapyl, and p-coumaryl alcohols, precursor of the lignin, condensed by free

radical polymerization to form huge and heterogenous polymer. The polymerization process of these phenoxy radicals is rondom, consequently lignin has variable structures like hemicelluloses. About 15-35% of lignin is present in the supporting tissues of higher plants and it is effective barrier against microbial hydrolysis (La Grange, 1999).

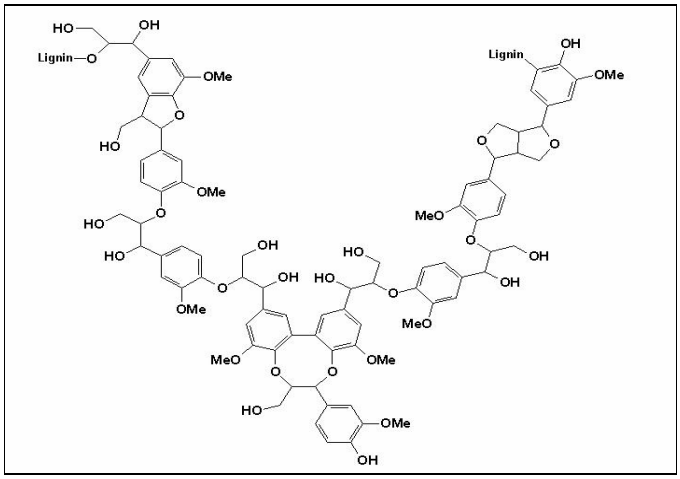


Figure 4. Structure of a small piece of lignin polymer.
(http://en.wikipedia.org/wiki/Lignin)

## 1.2 Xylan

### 1.2.1 The structure of the xylan

As written in the previous part, β-1,4-Xylan, mainly found in the secondary walls of plants, does not have a homogenous chemical composition, except those present in esparto grass and tobacco. Xylan polymer largely consists of a β-D-1,4- linked

D-xylose backbone substituted with acetyl, arabinosyl, and glucuronosyl side chains (Figure 5). As shown in Table 1, the frequency and composition of the branches are dependent on the source of xylan.

Hardwood is acetyl-4-O-methylglucuronoxylan with a degree of polymerization of about 200 (Figure 6). D-xylopyranose units forming the xylan backbone are substituted at C-2 and/or C-3 positions with acetic acid and with acetyl-4-O-methylglucuronic acid at the C-2 position. In addition, most hardwood xylan comprises small amounts of D-galacturonic acid and D-rhamnose as well (Eriksson *et al*., 1990; Komerlink and Voragen, 1993; Coughlan and Hazlewood, 1993).

Softwood xylan is arabino-4-O-methlyglucuronoxylan with a degree of polymerization greater than 120 (Figure 6). The xylan backbone is substituted with 4-O-methly-α-D-glucuronic acid at a position of C-2 and L-arabinose at a position of C-3. Moreover, most softwood xylan also contains D-galacturonic acid and D-rhamnose, and D-xylose reducing end group as well.

Xylans from grasses contain small amount of 4-O-methly-α-D-glucuronic acids. However, some are also arabino-4-O-methlyglucuronoxylan with a degree of polymerization of 70. The graminaceous plant xylans have a large amount of L-arabinosyl side chains attached to backbone at positions of C-2 and/or C-3. This group of xylan also contains O-acetly groups linked to C-2 or C-3 of the D-xylopyranose units. Grass cell walls have 1-2% by weight of phenolic acid substituents which are esterified to position 5 of the arabinose substituents. However, relative proportions of the grass arabinoxylan components vary from species to species, and from tissue to tissue within a single species (Coughlan and Hazlewood, 1993).

### 1.2.2 Arabino xylan structures of agricultural by-products

Composition and relative proportions of the various components of arabinoxylan varies from species to species, and from tissue to tissue within a single species (Caoughlan and Hazlewood, 1993).

Sandra and coworkers (2003) reported that wheat bran comprises very-highly-branched arabinoxylans consisting of linear β-D-(l,4)-linked xylopyranose backbones to which α-L-arabinofuranose units are attached as side residues by means of α-1,3 and α-1,2 linkages. Moreover, β-D-glucuronic acid and other substituents attached to xylan backbone at a position of C(O)-2. Arabinose oligomers, containing two or more arabinofuranosyl residues linked via 1-2, 1-3, and 1-4 linkages.

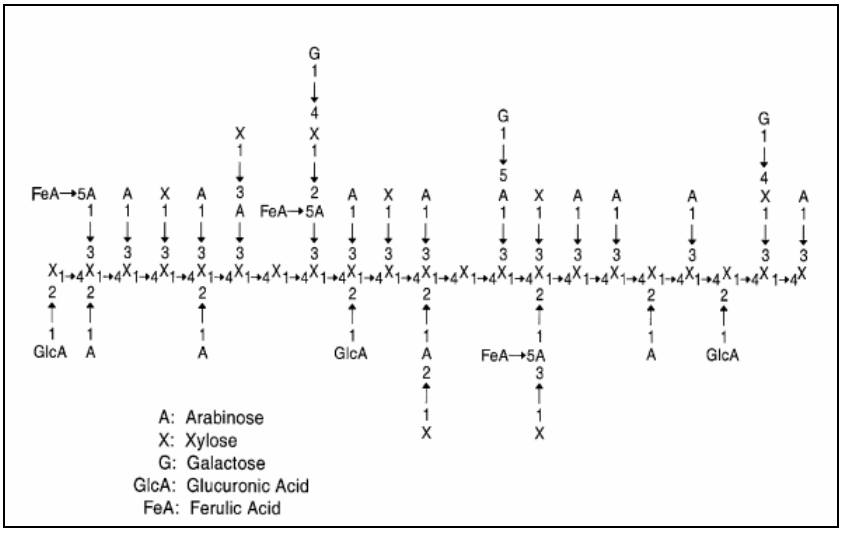


Figure 5. Schematic maize bran heteroxylan structure. (Received from Saha, 2000).

Table 1. Source dependent xylan composition variation (Saha, 2000 and Ak, 2006).

| Xylan source<br>% Xylan composition | Birchwood xylan | Rice bran neutral xylan | Wheat arabino-xylan | Corn fiber hemi-cellulose | Cotton stalk |
|---|---|---|---|---|---|
| Xylose | 89.3 | 46.0 | 65.8 | 48.0-54.0 | 82.9 |
| Arabinose | 1.0 | 44.9 | 33.5 | 33.0-35.0 | 0.0 |
| Glucose | 1.4 | 1.9 | 0.3 | 0.0 | 7.8 |
| Galactose | 0.0 | 6.1 | 0.1-0.2 | 5.0-11.0 | 0.0 |
| Mannose | 0.0 | 0.0 | 0.1-0.2 | 0.0 | 0.0 |
| Anhydrouronic acid | 8.3 | 1.1 | 0.0 | 0.0 | 0.0 |
| Glucuronic acid | 0.0 | 0.0 | 0.0 | 3.0-6.0 | 9.3 |

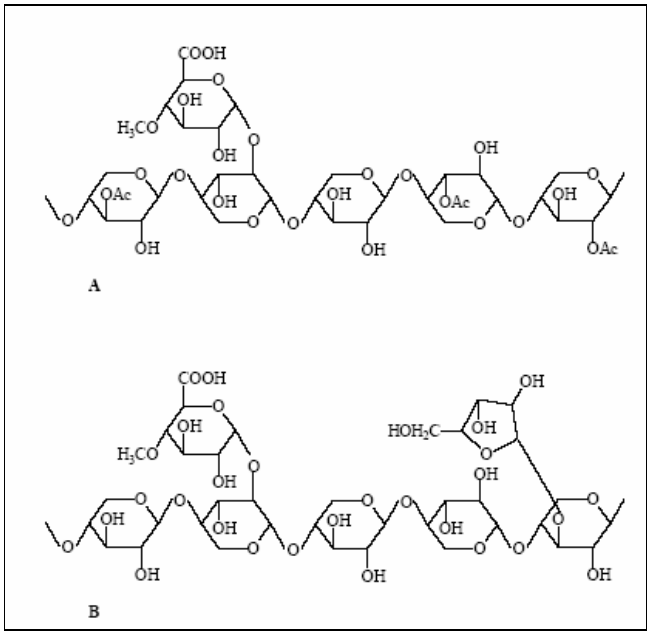


Figure 6. Schematic illustration of xylan. Partial xylan structures; A- from hardwood chain, B- from softwood chain.

Genotype dependent variation in soluble nonstarch polysaccharide (NSP) content within the same plant species was investigated by William and coworkers (2000). Analysis of 22 wheat cultivars which was performed by Saulnier *et al.* (1995) indicated that total arabinoxylan, soluble arabinoxylan and relative viscosity varied depending on the source. Moreover, soluble arabinoxylan content determines the viscosity and the arabinose:xylose ratio and the structure and molecular weight of total arabinoxylan are also determined by plant genotype. Moreover, Choct and Annison (1990) also classified different plants based on their total NSP content from low to high as follows: rice, sorghum, maize, wheat, rye and barley. A majority of polysaccharides, when dissolved in water, give viscous solutions.

Schooneveld-Bergmans and coworkers (1999) investigated the structure of the (glucurono)arabinoxylan extracted from water-unextractable wheat bran cell wall. According to their results, approximately one third of the extracted (glucurono) arabinoxylan was very lowly substituted (Ara/Xyl≈0·2), and arabinose, predominant substitution, was found at the O-3 position of xylose residues. Enzymatic degradation of wheat arabinoxylan showed that substituents are rondomly distributed and they are probably interrupted by 6 or more adjacent unsubstituted xylose residues. Moreover, more than half the extracted (glucurono) arabinoxylan was heavily substituted (Ara/Xyl≥1). Due to the complexity of the structure and the presence of considerable amounts of branched arabinose and terminal xylose, enzymatic degradability of the structure was very poor. In this type of arabinoxylan, substitution of xylose was positioned not only through O-3 mono-, O-2 and O-3 disubstitution by terminal arabinose and O-2 monosubstitution by (4-O-methyl)glucuronic acid, but also through dimeric arabinose, xylose and possibly galactose containing branches as well as through 2,3-linked arabinose. The remaining (glucurono)arabinoxylan (15%) was either intermediately substituted (Ara/Xyl≈0·5) or very highly substituted (Ara-Xyl≈1·2) (Schooneveld-Bergmans *et al*., 1999a).

Rice bran contains a substantial quantity of arabinoxylan similar to that found in wheat. Although structures of the both arabinoxylan are similar, arabinoxylan from wheat are much more viscous in solution (Table 2) than that of rice bran which is probably a indication of their more branched nature (Choct and Annison, 1990).

Schematic structure of the arabinoxylan from maize was given in Figure 5 (Saulnier *et al*., 1995). As can be seen from the figure, maize bran xylan is very complex structure and it comprises various side chain residue type and composition. In general, maize bran contains phenolic acids (4% dry matter; mainly ferulic acid and also diferulic acid, heteroxylans (50%), and cellulose (20%). When maize pericarp was treated with 0.05 M trifluoroacetic acid at 100°C for 2 h, 90% of the heteroxylans and 90% of the ferulic acid and its esters were released. After fractionation of the products, main feruloylated oligosaccharides ($F_3$–$F_7$) were isolated and characterized as 30% of the ferulic acid, and 2% of the neutral sugars. $F_7$ has been previously isolated from other monocots especially from wheat bran and soluble arabinoxylans from wheat flour; whereas presence of $F_6$ and $F_3$ oligosaccharides was first reported by Saulnier *et al.* (1995). They suggested that these oligomers are side-chain constituents of xylan backbone in maize bran and ferulic acid is probably partly responsible for the insolubility of heteroxylans by coupling polysaccharide chains through ferulic acid dimers.

Table 2. Chemical and physical properties of isolated soluble non-strach polysaccharide from wheat and rice bran: molar proportions of the different sugars (Choct and Annison, 1990)

| Source | Arabinose | Xylose | Mannose | Galactose | Glucose | A/X | *Viscosity cp |
|---|---|---|---|---|---|---|---|
| Wheat | 0.35 | 0.60 | - | - | 0.05 | 0.58 | 64 |
| Rice bran | 0.40 | 0.32 | 0.03 | 0.17 | 0.08 | 1.23 | 1.6 |

*Viscosity of the 1% (w/v) solution in 0.1M NaCl at 25°C.

### 1.2.3 Enzymatic xylan degradation

The complexity of the xylan molecules requires the action of the different hydrolytic enzymes (Figure 7). The most effective one is endo- β-1,4-xylanase (β-xylanase or xylanase) that cleaves the β -1,4 bonds of xylan backbone and produces xylo-oligosaccharides. These oligosaccharides are further hydrolyzed to D-xylose by the action of β -1, 4-xylosidase.

A series of accessory debranching enzymes also take part in effective full degradation of xylan. α-L-arabinofuranosidases (EC 3.2.1.55, AF) and α-glucuronidase (EC 3.2.1.131) remove the arabinose and 4-O-methly glucuronic acid substituents, respectively, from the xylan backbone. Esterases hydrolyze the ester linkages between xylose units of the xylan and acetic acid (acetylxylan esterase, EC 3.1.1.72**.**6) or between arabinose side chain residues and phenolic acids, such as ferulic acid (ferulic acid esterase, EC 3.2.2.73) and p-coumaric acid (p-coumaric acid esterases, EC 3.1.1-).

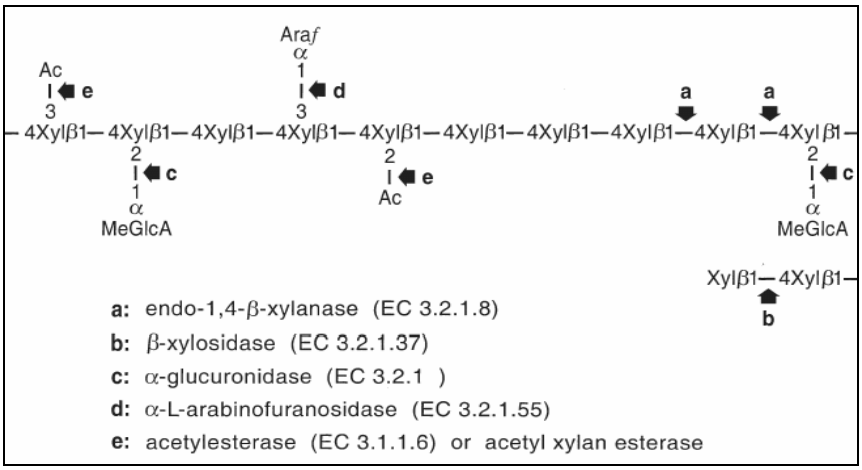


Figure 7. Representative plant xylan and attack sites of xylan hydrolyzing enzymes for xylan degradation (Saha, 2000).

The enzymes liberating xylan substituents act synergistically with the depolymerizing xylanases. Debranching enzymes create new sites on the main chain for formation of productive complex with xylanases. Indeed, complete degradation of arabinoxylans to monosachharides can only be achieved by synergistic act of both side chain cleaving and depolymerizing enzyme activities. Enzymatic cleavage of the side chains requires action of several accessory activities, such as α-L-arabinofuranosidases (EC. 3.2.1.55), feruloyl esterase (EC. 3.1.1.73), α-glucuronidases (EC.3.2.1.139) (Sørensen *et al*., 2005).

In addition to hemicelluloytic enzymes, synergistic action between cellulose- and hemicellulose-degrading enzymes was also investigated (Tenkanen *et al*., 1999). Tenkanen and coworkers (1999) reported that residual lignin in birch kraft pulp is linked at least to xylan and a minor portion may also be linked to cellulose. The linkages between lignin and cellulose and hemicelluloses may be either native or

formed during pulp processing, and cellulose- and hemicellulose-degrading enzymes act synergistically on pulp fibers, consequently degradation of xylan clearly enhanced the action of enzymes on cellulose.

There are many different types of β-xylanases, produced by numerous different fungal species and bacterial species (Reilly, 1981). Very few species are likely to produce all the different types of β-xylanases. Many xylanases are isozymes of each other. They have similar specifities, but because they are encoded by multiple genes differ in amino acid or carbohydrate content (Coughlan *et. al.,* 1993; Millward-Sadler *et. al.,* 1994). This leads to differences in the isoelectric points, relative stability and in the optimum pH for activity and stability for different isozymes (Lehninger, 1982; Mahler and Cordes, 1966). Different authors have classified β-xylanases in different ways. Reilly identified six types of β-xylanases, based on product they from (Reilly, 1981). Henrissat and Bairoch (1996) have classified glycosidases into several families originally on the basis of homologies in the structural elements, hydrophobic clusters, which are derived from two dimensional representations of the amino acid sequences (Törrenen and Rouvinen, 1994).

Törrenen classified xylanases into two families, families F/10 and G/11. The low molecular weight xylanases (Familiy 11) are more abundant than the high molecular weight ones (Family 10). The family 11 is composed of highly specific low molecular weight endoxylanases from eukaryotic and bacterial species, in which the sequence identity varies from 40% to 90% (Törrenen *et. al*., 1992). Multiple sequence alignment among low molecular weight xylanases shows that xylanase of *T. viride* is highly homologous to bacterial and other fungal xylanases, 48% identity to *Clostridium acetobullicum* and 52% *Bacillus pumilis*.

Biochemical characterization of endoxylanases regardless of its source has a strong relationship between their molecular weight and isoelectric point (pI) values. Molecular weight and pI values are in the range of 11,000 to 85,000 Da

and 3.6 to 10.3, respectively (Wong *et. al*., 1988). Many endoxylanases also fall into a pattern of high molecular weight/acidic pI value (F/10) and low molecular weight/basic pI (G/11).

### 1.2.4 Relationships between activities of xylanases and xylan structures

In general, different xylanases have different activities against various xylan structures. The key factors that affect the rate of xylan hydrolysis are chain length and degree of substitution. For example, Liab and coworkers (2000) reported that family 11 xylanase produced by *Aureobasidium pullulans* is most effective on long chain xylans (greater than 19 xylose residues), and also effective against substituent groups, as well. Moreover, *Trichoderma longibrachiatum* xylanase, can rapidly hydrolyze xylans that have a chain length greater than 8 xylose residues, and substituents on the xylan backbone have no impact on their hydrolytic rates. *Thermatoga maritima* xylanase is also more active on a long xylan chain (greater than 19 xylose residues); whereas it's hydrolytic rate is significantly reduced by substituents on xylan backbones.

Some xylanases may involve a binding region that may encompass four or five xylose residues (Bieley *et al*., 1992). Xylanase binding to the xylan backbone may be sterically hindered by arabinose side groups. Indeed, cleavage of the xylose backbone within several residues around an arabinose side group by *Polyporus tulipiferae* xylanase (Brillouet *et al*., 1987) was blocked by presence of side groups. In contrast to *P. tulipiferae*, *Butyrovibrio fibrisolvens* H17c xylanases is not hindered by arabinose side chains and can cleave at or near xylose residues that contain arabinose side chains (Hespell and Cotta, 1995).

### 1.2.5 Diversity of the microbial xylanolytic system

Microbial xylanolytic systems are composed of xylanases and other glycosidases which act synergistically for full hydrolysis of complex xylan structures. Variety

of different genera and species of bacteria, filamentous fungi, and yeasts show xylanolytic activity. Characteristics of some fungal and bacterial xylanases are tabulated in Tables 3 and 4, respectively.

### 1.2.6 Xylanase and xylanase producers

Many xylanolytic fungal (*Trichoderma* spp, *Aspergillus* spp.), and bacterial species (*Bacillus* spp.) have been recognized. Extensive biochemical analysis of xylan degrading enzymes of both fungal and bacterial origin has been conducted, and a large number of enzymes have been purified and characterized. Gene cloning, sequencing and expression studies of some xylanases have also been performed.

Certain strains of *Bacillus polymyxa*, *Bacillus pumilus, Bacillus subtilis, Cellulomonas fimi*, *Clostridium acetobutylicum, Streptomyces lividans, Streptomyces flavogriseus, Aspergillus fumigatus, Neurospora crassa, Trichoderma viride, Pichia stipitis,* and *Candida shehatae* secrete xylanases under mesophilic growth conditions (Gosalbes *et al*., 1991; Wong *et al*., 1988).

Moreover, xylanases from thermophilic organisms, *Thermomyces lanugiosus* (DSM 5826) (Schlacher *et al*., 1996), *Clostridium thermocellum* (DSM 1237) (Royer *et al*., 1989), and actinomycetes *Thermomonaspora alba* ULJB1 (Blanco *et al*., 1997) have been investigated.

Hyperthermophilic eubacteria that grow at temperatures above 80°C have also been isolated. These microbes include *Thermotoga maritima* (Winterhalter and Liebl, 1995), *Caldocellum saccharolyticum* (Lüthi *et al*., 1990), and *Rhodothermus marinus* (Dahlberg *et al.*, 1993).

Table 3. Comparative physicochemical properties of fungal xylanases.

| Microorganisms | $M_W$ (kDa) (SDS-PAGE) | pI | Optimum temperature (°C) | Optimum pH |
|---|---|---|---|---|
| *Acrophialophora nainiana (Cardoso and Filho, 2003)* | 27.5 | n.d | 55 | 6.5 |
| *Aspergillus oryzae (Kitanono et al., 1999)* | 35 | n.d. | 60 | 5.0 |
| *Fusarium oxysporum f.sp. lycopersici* (*Ruiz et al*., 1997) | 40 | 3.7 | 40 | 3.7 |
| *Humicola grisea var. thermoidea (Neto and Filho, 2004)* | 29 | n.d | 55-60 | 4.5-6.5 |
| *Rhizopus oryzae (Bakir et al., 2001)* | 22 | n.d | 55 | 4.5 |
| *Sporotrichum thermophile (Tokapas et al., 2003)* | 90-120 | 4.0 | 70 | 5.0 |
| *Trichoderma reesei (Törrenen et al., 1992)* | 19-20 | 5.2-9.0 | - | - |
| *Trichoderma harzianum* E58 (Tan *et al*., 1987; Tan *et al*., 1985; Wong *et al*., 1986) | 20, 22, and 29 | 9.4, 8.5, 9.5 | 50, 45-50, 60 | 5.0, 4.5-5.0, 5.0 |
| *Trichoderma koningii* IMI 73022 (Wood and McCrae, 1986) | 17.7, 29.0 | 7.3, 7.2 | 50, 60 | 4.5-5.5, 4.5-6.0 |

Table 4. Comparative physicochemical properties of bacterial xylanases.

| Microorganisms | $M_W$ (kDa) (SDS-PAGE) | pI | Optimum temperature (°C) | Optimum pH |
|---|---|---|---|---|
| *Bacillus circulans* WL-12 (Esteban et al.,1982) | 15.0*, 85.0* | 9.1, 4.5 | n.d | 5.5-7 |
| *Bacillus pumilus* SB-M13 (Our study) (Biran et al., 2006) | 24.8 | 9.2 | 60 | 7.5 |
| *Bacillus* sp. strain 41M-1 (Nakamura et al., 1993) | 36.0 | 5.3 | 50 | 9.0 |
| *Bacillus stearothernophilus* T-6 (Khasin et al., 1993) | 43.0 | 9.0 | n.d | 6.5 |
| *Clostridium acetobutylicum* ATCC 824 (Lee and Fosrberg, 1987) | 29 | 8.5 | 60 | 5.5-6.0 |
| *Streptomyces* sp. strain KT-23 (Nakajima, et al., 1984) | 44.0 | 6.9 | 55 | 5.5 |

* *Mw was determined using gel filtration chromatography, n.d: Not defined.*

Use of microbial xylanases at temperatures above 50°C and in alkaline conditions is especially desirable for kraft pulp treatment in the paper industry. Although, fungal xylanases are active in neutral or acidic pH, bacterial xylanases generally have higher optimal pH and thus, are more alkaline stable and more suitable for applications in the paper and pulp industry.

Wide applications of xylanases in industry direct researches to investigate new, high level xylanase producers.

### 1.2.7 Xylanase production

In general regulation of polymeric substrate degrading enzymes is that low constitutive levels of hydrolytic enzymes produce small soluble 'signal' fragments which are able to enter cell and induce synthesis of the corresponding enzyme taking role in polymer hydrolysis. Constitutive xylanases degrade xylan to xylooligosaccharides and xylobiose which are taken up by the cell, consequently induce the other xylanase genes. The β-xylosidases which may be produced constitutively and/or inducibly, convert xylobiose to xylose and may subsequently transxylanate or tranglycosylate it to XylB1-2 Xyl and GlcB1-2Xyl. Therefore, this reaction relieves repression effect of the xylose. In the subsequent stage, modified compounds are taken up by cell and serve as an additional inducer instead of repressor which allows further expression of genes encoding xylonolytic enzymes (Thomson, 1993).

Xylan has been shown to be the best inducer of xylanase production (Nakamura *et al*., 1992) but, few organisms show constitutive production of the enzyme (Debeire *et al*., 1990). Hemicellulosic substrates like corn cob, wheat bran, rice bran, rice straw, corn stalk and bagasse have also been found to be most suitable for the production of xylanase in certain microbes. Maximum xylanase production (285-350 U/ml) was obtained when *Aspergillus tamarii* was grown on in media supplemented with 5-8% (w/v) of corn cobs (Kadowaki *et al*., 1996). Corn cob was also the most suitable substrate for the production of xylanase by an alkalothermophilic *Thermomonospora* sp. (George *et al*., 2001). Moreover, the highest xylanase activity, 260 IU/ml, was obtained when *Rhizopus oryzae* utilizing 3% of corn cobs (Bakir, 2001). Wheat bran was found to be the best substrate for xylanase production by alkalophilic *Streptomyces* VP5 (Vyas *et al*., 1990), and *Streptomyces* T-7 (Keskar *et al*., 1992).

When compared to bacteria, fungi produce higher levels of xylanases (Table 5). However, these xylanases are generally associated with cellulases (Steiner *et al*.,

1987). Cellulase-free xylanases are advantageous in the paper and pulp industry, because cellulase adversely affect the quality of the pulp. Investigations of naturally occurring microorganisms capable of selectively secreting high levels of xylanase have yielded promising results. Cellulase-free xylanase have been isolated from *Thermomyces lanuginosus* (Gomes *et al*., 1993), *Streptomyces roseiscleroticus* (Grabski and Jeffries, 1991) and *Streptomyces* T-7 (Keskar *et al*., 1992). Moreover, *Bacillus pumilus* SB-M13 has very low level of 0.003 FPU cellulase activity; Hence, the culture filtrate can most possibly be used for treating pulp without further purification (Biran *et al*., 2006).

Table 5. Comparisons of activities of enzymes in the xylanolytic systems in different culture filtrates (Eriksson *et al*., 1990; Poutanen *et al*., 1987).

| **Organisms** | **xylanase nkat/ml** | **xylanase U/ml** |
|---|---|---|
| *Trichoderma reesei* | 2170 | 130 |
| *Aspergillus awamori* | 200 | 12 |
| *Bacillus subtilis* | 311 | 19 |
| *Streptomyces olivochromogenes* | 90 | 5 |

*1 nkat: 0,06 U*

nkat activity: production of the reducing equivalent of 1 nmol xylose per second

U: production of the reducing sugar equivalent of 1μmol xylose per minute.

Activity measurement method for all was DNSA.

### 1.2.8 Carbon catabolite repression

Most of the xylanolytic enzymes producing organisms are under the control of the carbon catabolite repression. Carbon catabolite repression (CR) in bacteria is a regulatory mechanism to guarantee sequential utilization of carbohydrates. In this mechanism presence and the absence of carbon sources that can be well metabolized determines the expression of genes involved in catabolism of many other substrates. Specific control proteins regulate the all carbohydrate catabolic genes or operons and high level of expression necessitate inducers. By these mechanisms, bacteria are able to create a hierarchy of sugar utilization.

Gene expressions in *Bacillus* are under the control of three components involved in catabolite repression; the cis-acting catabolite responsive element (CRE), and the trans-acting factors CcpA and HPr. Similarities between CcpA, Lac and Gal repressors propose binding of CcpA to CRE. Moreover, HPr mutant, comprising exchange of serine to alanine in HPr, is deficient in phosphorylated HPr, consequently lacks CR of several catabolic activities. Indeed, HPr, a component of the phosphoenolpyruvate:sugar phosphotransferase system, undergoes regulatory phosphorylation at a serine residue by a fructose-1,6-diphosphate-activated kinase. In brief, direct protein-protein interaction between CcpA and HPr(Ser-P) was demonstrated and constitutes a link between metabolic activity and CR repression (Hueck and Hillen, 1995). Moreover, the components mediating carbon catabolite repression in *B. subtilis* are also investigated in many other gram-positive bacteria containing low GC content (Stülke and Hillen, 2000).

In the fungal system, the major system responsible for carbon repression in *Aspergillus* is well documented and it is mediated via the carbon catabolite repressor protein CreA. In the presence of well metabolized substrates, such as glucose or fructose, CreA binds to specific sites in the promoters of a wide variety of target genes and inhibits or decreases the expression of these genes.

Repression impact of CreA in *Aspergillus* was detected for genes encoding arabinases, L-arabinose catabolic enzymes, and several other xylanolytic enzymes, such as β-xylosidase, arabinoxylan arabinofuranohydrolase. Besides glucose and fructose, other monomeric carbon sources also result in CreA mediated repression of gene expression. For example, in contrast to high concentrations, lower xylose concentrations cause higher expression levels of xylanolytic enzymes. Moreover, high concentrations of glucose, xylose and cellobiose decrease the cellulase production as well.

### 1.3 Arabinose containing polymers

#### 1.3.1 The structure of the arabinose containing polymers

Arabinan, arabinoxylan, arabinogalactan are the arabinose containing polysaccharides which are present in different tissues of the plant; such as roots, seeds, leaves and flowers (Luonteri *et al*., 1998).

Arabinan molecules are composed of α-1,5-linked arabinofuranose backbone some of which are a densely branched with arabinofuranose molecules at C-2 and/or C-3 positions. The arabinan structure of wood of maritime pine is given in Figure 8. Backbone of it consists of α-1,5-linked arabinose units with side chains of arabinose units bound by α-1,3 linkages. Moreover, sugar beet arabinan consists of a 1,5-α-linked backbone to which 1,3-α-linked (and possibly some 1,2-α-linked) L-arabinofuranosyl residues are attached (Figure 9). Approximately 60% of the main-chain arabinofuranosyl residues are substituted by single 1,3-linked arabinofuranosyl groups. The reducing terminal arabinosyl residue is attached through rhamnose to fragments of the rhamnogalacturonan backbone of the native pectin molecule.

In β-1,3-arabinogalactans, backbone is also substituted by α-L-arabinofuranose side chains at varying degree at the C-3 and C-6 positions. Indeed, larchwood

arabinogalactan, consisting of a β-1,3-linked D-galactopyranose units, is highly branched at C6. The side chains are composed of β-1,6-linked D-galactose, D-galactose and L-arabinose units or single L-arabinose units and single D-glucuronic acid units. Arabinose exists in furanose and pyranose forms in the ratio of 1:2 (Figure 10).

The xylan molecules contain a homopolymeric β-1,4-linked xylose backbone, but structure of it differs strongly depending on the plant cell wall origin. Xylans contain large quantities of L-arabinose are referred to as arabinoxylans and arabinose is bound to the xylan backbone by means of α-1,2- or/and α-1,3-linkage as single residues or as short side chains. These side chains can also contain galactose which can be either β-1,5-linked to arabinose or β-1,4-linked to xylose, and xylose, β-1,2-linked to arabinose. Figure 11 shows the softwood arabinoglucorunoxylan molecule which has a backbone of β-1,4-linked xylopyranose units to which single-unit side chains of 4-O-methyl-D-glucuronic acid units attached by α-1,2 linkage, on average one unit per 5-6 xylose units. Moreover L-arabinose units are also attached by α-1,3-linkage, on the average one unit per 5-12 xylose units (Figure 11).

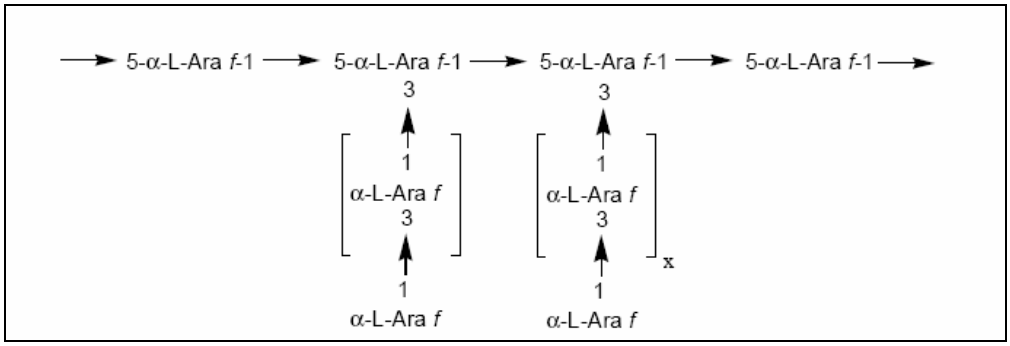


Figure 8. Structure of the maritime pine wood arabinan. (Received (in modified form) from Laine, 2005).

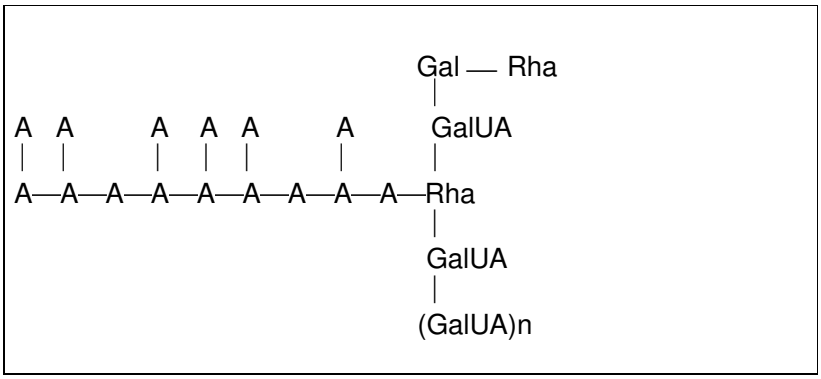


Figure 9. Structure of sugar beet arabinan (Megazyme). (A: arabinose, Gal: galactose, GalUA: galacturonic acid, Rha: rhamnose; A:Gal:Rha:GalUA= 88:3:2:7).

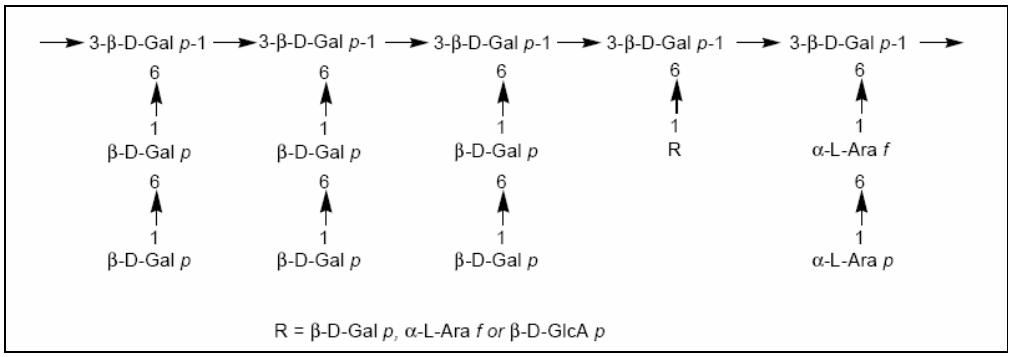


Figure 10. Structure of the larchwood arabinogalactan. R; Gal: galactose, Ara f: arabinofuranose, Ara p: arabinopyranose, Glc A: glucuronic acid. (Received (in modified form) from Laine, 2005).

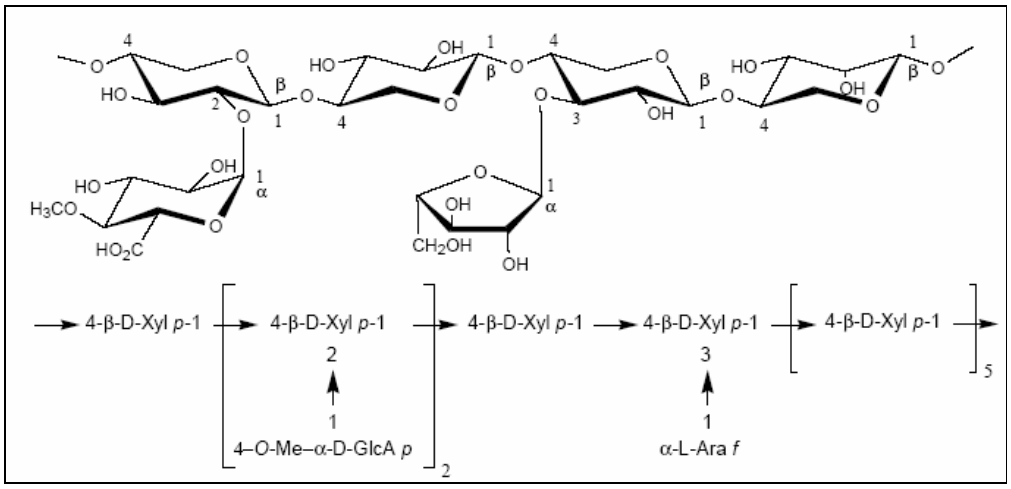


Figure 11. Structure of softwood xylan. Xylp: xylopyranose, GlcA: glucuronic acid. (Received (in modified form) from Laine, 2005).

### 1.3.2 Arabinose containing polymers degradation

L-Arabinosyl residues are extensively distributed in some plant polysaccharides, such as arabinoxylan, arabinogalactan, arabinan, and gum arabic. Total hydrolysis of polymers is frequently limited by presence of arabinose residues attached to the main backbones as side chains (Sakamoto and Kawasaki, 2003).

Extensive hydrolysis of heteroxylan requires endo-β-1,4-xylanase, β-xylosidase and several accessory enzymes, such as AF, α-glucuronosidase, acetyl xylan esterase, ferulic acid esterase which are involved in removal of side chains. Some xylanases do not cleave glycosidic bonds between xylose units substituted. The presence of large amounts of subtituents may avoid enzyme-substrate complex formation and block enzyme hydrolysis (Kormelink and Voragen, 1993). The AFs, part of the xylanolytic enzyme system, represent potential rate limiting enzyme for full hydrolysis of arabinoxylan, particularly substrates from agricultural by-

products like corn fiber, corn stover, and rice straw (Saha and Bothast, 1999b). Therefore, α-L-arabinofuranosidase (AF, EC 3.2.1.55) takes significant role in arabinoxylan hydrolysis. In general, arabinose residues on oligosaccharides and/or polysaccharides can be removed by arabinofuranosidases (exo-1,5-α-L-arabinofuranosidase, AF, EC. 3.2.1.55) which enables endo-arabinase (endo-1,5-α-L-arabinofuranosidase, EC. 3.2.1.99) to hydrolyse the α-1,5-linkages of arabinan polysaccharides. In summary, respected enzymes act synergistically to enhance the efficiency of arabinan degradation.

### 1.3.3 Types of α-L-arabinofuranosidases (AFs)

Arabinose releasing enzymes have been classified into four families of gylcanases (families 43, 51, 54, and 62). The two families (51 and 54) have also been classified further depending on their mode of action and substrate specifity (Beldman *et al*., 1997). Type A AFs, inactive towards arabinosyl linkages present in polysaccharides, preferentially degrade α-1,5-L- arabinofurano-oligosaccharides to monomeric arabinose. Type B AFs debranches L-arabinose residues from side chains of arabinan and arabinoxylan. Both types of AFs attack on synthetic p-nitrophenyl-α-L-arabinofuranoside. The third type of AFs, called α-L-arabinofuranohyrdolases, act on arabinosidic linkages in oat spelt, wheat and barley arabinoxylan, but do not show any activity towards p-nitrophenyl-α-L-arabinofuranoside, arabinans, and arabinogalactans (*Aspergillus awamori* α-L-arabinofuranohyrdolases, Kormelink *et al.*, 1991a). Substrate specifity of the some microbial AFs are tabulated in Table 6.

Table 6. Substrate specifity variation in microbial AFs.

| Microorganism | AF Active on | AF Inactive against |
|---|---|---|
| *S. lividans* (Manin et al., 1994) | Gramineae xylan, small arabinoxylooligosides | Oat spelt xylan and arabinoxylan |
| *S. purpurascens* (Komae *et al*., 1982a) | | Arabinan and arabinogalactan |
| *A.pullulans* (Saha and Bohast, 1998b), *Streptomyces* sp. Strain 17-1 (Kaji and Tagawa, 1970), *B. subtilus* 3-6 (Komae *et al*., 1982b) | α-1,3 and α-1,5-linked nonreducing terminal arabinosyl residues of substrates | Internal α-arabinosyl linkages of the substrates |
| *A. awamori* AF: AXH (Kormenlink *et al*., 1993a) | Arabinoxylan | pNP-α-L-arabinofuranoside, arabinan, arabinogalactan |
| *A. awamori* AF: AF I (Kaneko *et al*., 1998a) | α-1,5 linkage of branched arabinotrisaccahrides, nonreducing terminus of arabinan (arabinose release), PNP-α-L-arabinofuranoside | *O*-β-D-xylonopyranosyl-1,2-*O*-α-L-arabinofuranosyl-1,3-*O*-β-D-xylanopyranosyl-1,4-*O*-β-D-xylanopyranosyl-1,4-D-xylopyranose |
| *A. awamori* AF: AF II (Kaneko *et al*., 1998a) | α-1,3- linkage of branched arabinotrisaccahrides, arabinosyl side chain linkage of arabinan, PNP-α-L-rabinofuranoside | *O*-β-D-xylonopyranosyl-1,2-*O*-α-L-arabinofuranosyl |

Table 6 (Continued). Substrate specifity variation in microbial AFs.

| Microorganism | AF Active on | AF Inactive against |
|---|---|---|
| *B. adolescentis* (Van Laere *et al.*, 1999) | Arabinoxylan ( Remove arabinosyl residues from double substituted xylose units in arabinoxylan) | Sugar beet arabinan, soy arabinogalactan, arabinoolgosaccharides, and, arabinogalacto-oligosaccharides |
| *Cytophaga xylanolytica* (Renner and Breznak, 1998) | Rye, wheat, corn cob and oat spelt arabinoxylan, and sugar beet arabinan (arabinose release) | Arabinogalactan |
| *B. polymyxa* (Morales *et al.*, 1995a) | 1,5-α-L-arabinooligosaccharides *Arabinoxylan when together with xylanase | Linear 1,5-α-L-arabinan, arabinogalactan, arabinogalactan and *Arabinoxylan |

### 1.3.4 AF producers and the physicochemical characteristics of AFs

Several reports on AFs from various sources are available in literature such as culture broth of *Bacillus* (Gilead and Shoham, 1995; Kaneko *et al*., 1994), Clostridium (Lee and Forsberg, 1897; Kaneko *et al*., 1993), *Aspergillus* (Kaneko *et al*., 1998; *Luonteri et al., 1995;* Kaneko *et al*., 1993) many microorganisms, leaf (Hirano *et al*., 1994), cell culture (Konno *et al*.**,** 1994) and seeds of some plant (Ferré, 2000). Extensive biochemical analysis of AFs has been conducted, and a large number of enzymes have been purified and characterized. Gene cloning, sequencing and expression studies have also been performed (Crous *et al*., 1996, Margolles-Clark *et al*., 1999).

Most AFs exist as monomers, but dimeric, tetrameric and octameric forms have

also been found. In terms of microbial AFs, *Penicillium purpurogenum* AF is monomer with $M_W$ of 58 kDa and pI of 6.5 (De Ionnes, 2000). AF purified from soil isolate *Bacillus pumilus* SB-M13 has native $M_W$ of 210 kDa and subunit $M_W$ of 53.3 kDa (our study). *Scytalidium thermophilum* produced AF with native and subunit $M_W$s of 160 and 38 kDa, respectively (our study). Two AFs, purified from *Aspergillus awamori* IFO 4033 culture broth, have $M_W$s of 81 kDa and 62 kDa, and pI of 3.3 and 3.6, respectively (Kaneko *et al*., 1998a). Being monomer enzymes, AFs produced from *Clostridium acetobutylicum* ATCC 824 *and A. niger* have $M_{Ws}$ of 94 and 67 kDa, respectively (Lee and Forsberg, 1987; Kaneko *et al*., 1993). *Streptomyces diastaticus* produced two different AFs with respective molecular weight of 38 and 60 kDa, and pI of 8.8 and 8.3 (Tajana *et al*., 1992). Having a native $M_W$ of 210 kDa and a subunit $M_W$ of 105 kDa, the AF from *Aureobasidium pullulans* is a homodimer (Saha and Bothast, 1998b). *Bacillus stearothermophilus* T-6 AF consists of four identical subunits of $M_W$ 64 kDa with pI of 6.5 (Gilead and Shoham, 1995). Moreover, $M_W$ of AF from *Streptomyces purpurascens* IFO 3389 (Komae *et al*.,1982a) and *Butyrivibrio fibrosolvens* GS 113 (Hespell and O'Bryan, 1992) are about 495 and 240 kDa, containing eight equal subunits and eight subunits of $M_W$ 31 kDa, respectively.

The pH and temperatures at which AFs from both fungal and bacteria origins are most active was found in the range of 3.5-7.0 and 50-75$^0$C, respectively. Uesaka *et al*. (1978) showed that *Rhodotorula flava* AF is highly acid stable. Enzyme having optimum pH 2.0 retains 82 % of its activity after 24 h incubation at pH 1.5 and 30$^0$C.

### 1.4 Use of xylanases and α-L-arabinofuranosidases in industry

In recent years, interest in xylan degrading enzymes have been increased due to their applications in various agro-industrial processes, such as hemicellulosic biomass conversion to fuels and chemicals, paper pulp delignification, animal feedstock digestibility enhancement, juices clarification, beer consistency

improvement, fruit juices and wines aroma enhancement, pentose containing disaccharides synthesis (Rahman *et al*., 2001 and; Makkonen *et al*., 2005; Wong *et al*., 1988; Gunata *et al*., 1990 and Spagna *et al*.,1998; Rémond *et al*., 2004). Wastes from agriculture or forestry are available in large amounts and their excess accumulation causes environmental problems. Abundance of xylan clearly indicates that the xylanolytic enzymes can play an important role in bioconversion. Biodegradation of this biomass by xylanolytic enzymes not only eliminates accumulation of waste but also generates numerous products such as fuels, single cell protein, xylooligosaccharides, xylose, and xylitol. The enzymes liberating xylan substituents act synergistically with the depolymerizing xylanases. Debranching enzymes create new sites on the main chain for productive complex formation with xylanases. Indeed, complete degradation of arabinoxylans to monosachharides can only be achieved by synergistic act of both side chain cleaving and depolymerizing enzyme activities. In feed industry, presence of fiber content in animal feed negatively effect the digestion and uptake of nutritive part of the feed. Partial xylan hydrolysis of animal feed was solved uptake problem and improved nutritional value of the food (Senior et al, 1992). Moreover, in grasses arabinoxylan hydrolysis, enzymes cleaving α-L-arabinofuranosidic linkages can act synergistically with xylanases, consequently enhanced the animal feed digestibility further (Graham and Inborr, 1992).

Additionally, hydrolysis of other plant polysaccharides also requires enzyme complexes. For instance, sugarbeet pulp can be hydrolysed into pectin, cellulose, and arabinose using AF and/or endoarabinase in combination with other polysaccharide degrading enzymes and ultrafiltration process. Besides, ferulic acid is found esterified to the arabinose and galactose residues in the pectin side chains. The complete degradation of this complex molecules comprising esterified ferulic acid requires mixture of both main chain and side chain hydrolyzing enzymes which act synergistically. The ester linked substituents on the backbone may impede the action of glycanases as observed for a *Butyrovibrio fibrosolvens* (Hespell and O'Bryan, 1992). When incubated with endo-arabinase and α-L-

arabinofuranosidases, ferulic acid release from sugarbeet pulp by ferulic acid esterase was increased (Kroon and Williamson, 1996)

Furthermore, the use of xylanases in pulp bleaching has been considered as one of the most important biotechnological application of these enzymes. Xylanase based pre-bleaching resulted in a 20 to 30% reduction of toxic organic chlorine treatments (Senior *et al*., 1992). Treatment of pulp with AF alone led 2.3% lignin release. Xylanase treatment together with AF contributed enzyme synergy in delignification process, resulted in 19.2 % release of lignin (Saha, 2000).

In baking industry, xylanases alone improve the volume, appearance, shelf life, and texture of the bread (Hoseney, 1994). Xylanases and AF's are also used in other biotechnological applications like clarification and thinning of fruit juices. Due to hydrolysis of grape monoterpenly α-L-arabinofuranoslyglucosides, AFs are also used in vine aroma enhancement process.

**1.5 Molecular biology of xylanases and AFs**

Use of xylanases in paper manufacturing (Wong *et al*., 1988) makes xylanases valuable. The feasibility of using xylanases and AFs for cellulose purification will be highly depent on the absence of cellulolytic activities. The best strategy for obtaining cellulase free xylanases and higher level of gene expression is the cloning of xylanase and AF genes into noncelluloytic organisms (Wong *et al.,* 1988).

*Saccharomyces cerevisiae* can neither degrade nor utilize complex polysaccharides, including xylan. Through recombinant DNA technology*, S. cerevisiae* can be complemented by heterologous polysaccharase-encoding genes, thereby broadening its substrate range and facilitating a direct bioconversion of polysaccharides to valuable products, such as ethanol.

There are many studies reported the successful cloning and expression of fungal and bacterial xylanase and arabinofuranosidase genes in *S. cerevisiae* (La Grange *et al*., 1990, Crous *et al*., 1996; La Grange *et al.*,1996 for xylanase, and; Crous *et al*., 1996, Margolles-Clark *et al*., 1996 for AFs).

Besides *S. cerevisiae*, prokaryotic non-xylonolytic host *E. coli* was also utilized for cloning of xylan hydrolyzing enzyme gene. First study for cloning of xylanase from *Bacillus* to *E. coli* was reported by Bernier (1983). Later many other xylan degrading enzyme cloning studies have been done (Pechan *et al*., 1989, La Grange *et al*., 1996). (Pechan *et al*., 1989, Kubata *et al*., 1997 for xylanase, and; Schwartz *et al*., 1995 for AFs )

## REFERENCES


Annison, 1990, cited by William, P.E.V., Geraert, P.A., Uzu, G., and Annison, G., 2000, "Factors affecting non-starch polysaccharide digestibility in poultry", *CIHEAM-Options Mediterraneennes*.

Bakir, U., Yavascaoglu, S., Guvenc, F., and Ersayin, A., 2001, "An endo-1,4-β-xylanase from *Rhizopus oryzae*: production, partial purification and biochemical characterization", *Enzyme and Microbial Technology*, Vol. 29, pp. 328-334.

Bakır, U., 2005, "In: Microbial Enzymes: Production and Applications*"*, *The Haworth Press, NY*, pp. 592-600.

Bernier, R., Driguez, H., and Desrochers, M., 1983, "Cloning of xylanase from *Bacillus* to *E. coli"*, *Gene*, Vol.26, pp. 59-85.

Biely, P., Mislovicova, D., and Toman, R., 1985, "Soluble chromogenic substrates for the assay of endo-1,4-β-xylanases and endo-1,4-β-glucanases", *Analytical Biochemistry,* Vol. 144, pp. 142-146.

Bieley et al., 1992, cited by Hespell, R.B., and O'Bryan, P.J., 1992, "Purification and characterization of α-L-arabinofuranosidases from *Butyrivibrio fibrosolvens* GS13", *Applied and Environmental Microbiology* Vol. 58, pp. 1082-1088.

Biran, S., Ersayin, A., Kocabas, A., and Bakir, U., 2006, "A novel xylanase from a soil isolate, *Bacillus pumilus*: Production, purification, characterization and one-step separation by aqueous-two-phase system" *Journal of Chromatography*, in rewiev.

Blanco, J., Coque, J.R.R., Velasco, J., and Martin, J.F., 1997, "Cloning, expression in *Streptomyces lividans* and biochemical characterization of a thermostable endo-β- 1,4-xylanase of *Thermomonospora alba* ULJB1 with cellulose-binding ability", *Applied microbiology and Biotechnology,* Vol. 48, pp. 208-217.

Brillouet et al., 1987, cited by Hespell, R.B., and O'Bryan, P.J., 1992, "Purification and characterization of α-L-arabinofuranosidases from *Butyrivibrio fibrosolvens* GS13", *Applied and Environmental Microbiology* Vol. 58, pp. 1082-1088.

Cardoso, O.A., and Filho, X.F., 2003, "Purification and characterization of a celluloase- free xylanase from *Acrophialophora nainiana", FEMS Microbiology Letters,* Vol. 223, pp. 309-314.

Choct and Annison, 1990, cited by William, P.E.V., Geraert, P.A., Uzu, G., and Annison, G., 2000, "Factors affecting non-starch polysaccharide digestibility in poultry", *CIHEAM-Options Mediterraneennes.*

Compier, M., 2005. "Hemicellulose biosynthesis and degradation in tobacco cell walls, PhD Thesis, Wageningen University, Netherlands.

Coughlan, M.P., and Hazlewood, G.P., 1993, "β-1,4-D-xylan-degrading enzyme systems: biochemistry, molecular biology and applications", *Biotechnology and Applied Biochemistry*, Vol. 17, pp. 259-289.

Crous, J.M., Pretorius, I.S., and Van, Z.W.H., 1996, "Cloning and expression of the α-L-arabinofuranosidases gene (ABF2) of *Aspergillus niger* into *Saccharomyces cerevisiae", Applied Microbiology and Biotechnology*, Vol. 46, pp. 256-160.

Dahlberg, L., Holst, O., and Kristjansson, J.K., 1993, "Thermostable xylanolytic enzymes from *Rhodothermus marinus* grown on xylan", *Applied Environmental Microbiology,* Vol. 40, pp. 63-68.

Debeire, P., Priem, B., Strecker, G., and Vigonan, 1990, "Purification and properties of an endo-1,4-xylanase excreted by a hydrolytic thermophilic anaerobe, *Clostridium thermolacticum*.A proposal for its action mechanism on larchwood 4-O-methylglucuronoxylan", *European Journal of Biochemistry*, Vol. 187, pp. 573-575.

De Ioannes, P., Peirano, Steiner, J., and Eyzaguirre, J., 2000, "An α-L-arabinofuranosidase from Penicillum purpuragenum: production, purification and properties", *Journal of Biotechnology*, Vol. 76, pp. 253-258.

De Vries, R.P., 1999, "Accessory enzymes from Aspergillus involved in xylan and pectin degredation", PhD Thesis, Wageningen University, Netherlands.

Erikkson, K.E.L., Blanchette, R.A., and Ander., P., 1990, "In: Microbial and enzymatic degredation of wood and wood components", *Springer,* Verlag, NY.

George, S. P., 2000, Nath, D.S., 2005, "Studies on xylan degrading enzymes from an alkalophilic thermophilic *Bacillus* sp", PhD Thesis, Biochemical Sciences Division, National Chemical Laboratory, Pune.

.

Gilead, S., and Shoham, Y., 1995, "Purification and characterization of α-L-arabinofuranosidase from *Bacillus stearothermophilus* T-6", *Applied and Environmental Microbiology*, Vol. 61, pp. 170-174.

Gomes, J., Gomes, I., Terler, K., Gubala, N., Ditzelmüller, G., Steiner, W., 2000, "Optimization of culture medium and conditions for α-L-arabinofuranosidases production by the extreme thermophilic eubacterium *Rhodotermus marinus", Enzyme and Microbial Technology*, Vol. 27, pp. 414-422.

Gosalbes, M.J., Pérez-González, J.A., González, R., and Navarro, A.,1991, "Two β-glycanase genes are clustered in *Bacillus polymyxa*: molecular cloning, expression, and sequence analysis of genes encoding a xylanase and an endo-β-(1,3)-(1,4)-glucanase", *Journal of Bacteriogy*, Vol. 173, pp. 7705-7710.

Grabski, A.C., and Jeffries, T.W., 1991, "Production, purification, and characterization of β-(1-4)-endoxylanase of *Streptomyces roseiscleroticus"*, *Applied Environmental Microbiology*, Vol. 57, pp. 987-992.

Graham, H., and Inborr, J., 1992, "Application of xylanase-based enzymes in commercial pig and poultry production", p. 535-538. In Visser, J., Beldman, G., Someren, M. A. K., and Voragen, A. G. J., Xylan and xylanases. Elsevier Science, Amsterdam.

Gunata, Z.Y., Brillouet, J.M., Voirin, S., Baumes, R., and Cordonnier, R., 1990, "Purification and some properties of an α-L-arabinofuranosidase from Aspergillus niger. Action on grape monoterpenyl arabinofuranosyl-glucosides", *Journal of Agric. Food. Chem.*,Vol. 38, pp. 772-776.

La Grange, D.C., 1999, "Genetic engineering of the yeast *Saccharomyces cerevisiae* to degrade xylan", Ph.D Thesis, University of Stellenbosch.

Henristad, B., and Bairoch, A., 1996, "Updating the sequence based classification of glycosyl hydrolayses", Biochemical Journal, Vol. 316, pp. 695-596.

Hespell, R.B., and O'Bryan, P.J., 1992, "Purification and characterization of α-L-arabinofuranosidases from *Butyrivibrio fibrosolvens* GS13", *Applied and Environmental Microbiology* Vol. 58, pp. 1082-1088.

Hespell, R.B., and Cotta, M.A, 1995, "Degradation and utilization by *Butyrivibrio fibrisolvens* H17c of xylans with different chemical and physical properties", *Applied and Environmental Microbiology*, Vol. 61, pp. 3042–3050.

Hirano, Y.; Tsumuraya, Y.; Hashimoto, Y., 1994, "Characterization of spinach leaf α-L-arabinofuranosidases and β-galactosidases and their synergistic action on an endogenous arabinogalactan-protein", *Physiol Plant*, Vol. 92, pp. 286-296.

Hoseney, R.C., 1994a, "In yeast-leaven the products, *In Principles of cereal science and technology"*, American Association of Cereal Chemist, Inc, Minnesota, pp. 229-173.

Hueck, C.J., and Hillen, W., 1995, "Catabolite repression in *Bacillus subtilis*: a global regulatory mechanism for the gram positive bacteria", *Moleculer Microbiology*, Vol. 15, pp. 395-401.

Kadowaki, M.K., Souza, C.G.M., Simão, R.C.G., and Peralta, R.M., 1996, "Xylanase production by *Aspergillu tamari"*, *Applied Biochemistry and Biotechnology*, Vol. 66, pp. 97-106.

Kaji, A., and Tagawa, K., 1970, "Purificaion, crystallization and amino acid composition of α-L-arabinofuranosidase from *Aspergillus niger", Biochimica et Biophysica Acta*, Vol. 297, pp. 456-464.

Kaneko, S.T., Shimasaki, T., Kusakabe, I., 1993, "Purification and some properties of intracellular α-L-arabinofuranosidases from *Aspergillus niger* 5-16", *Bioscience Biotechnology and Biochemistry*, Vol. 57, pp. 1161-1165.

Kaneko, S., Arimoto, M., Ohba, M., Kobayashi, H., Ishii, T., and Kusabeke, I., 1998, "Purification and substrate specifities of two α-L-arabinofuranosidases from *Aspergillus awamori* IFO 4033", *Applied and Environmental Microbiology*, Vol. 64, pp. 4021-4027.

Keskar, S. S., 1992, cited by Nath, D.S., 2005, "Studies on xylan degrading enzymes from an alkalophilic thermophilic *Bacillus* sp", PhD Thesis, Biochemical Sciences Division, National Chemical Laboratory, Pune.

Khasin, A., Alchanati, I., and Shoham, Y., 1993, "Purification and characterization of a thermostable xylanase from Bacillus stearothermophilus T-6", *Applied and Environmental Microbiology*, Vol. 59, pp. 1725-1730.

Komae, K., Kaji, A., and Sato, M., 1982a, "An α-L-arabinofuranosidases from *Streptomyces purpurascens* IFO 3389", *Agricultural and. Biological Chemistry*, Vol. 46, pp. 1899-1905.

Komae, 1982b, cited by Saha, 2000, "Monoterpenyl arabinofuranosyl-glucosides". *Journal of Agric. Food Chem.* 1990, Vol. 38, pp. 772-776.

Kormelink, F.J.M., Leeuwen M.J.F.S.V., Wood, T.M., and Voragen, A.G.J., 1991a, "Purification and characterization of a 1,4-β-D-arabinoxylan arabinofuranohydrrolase from *Aspergillus awamori*", *Applied Microbiology and Biotechnology*, Vol. 35., pp. 753-758.

Kormelink, F.J.M., Gruppen, R.J., and Voragen, A.G.J., 1993a, "Mode of action of the xylan-degrading enzymes from *Aspergillu awamori* on alkali-extracable cereal arabinoxylans", *Carbohydrate Research*, Vol. 249, pp. 355-367.

Kormelink, F.J.M., and Voragen, A.G.J., 1993, "Degradation of different [(glucurono)arabino]xylans by a combination of purified xylan-degrading enzymes", *Applied Microbiology and Biotechnology*, Vol. 38, pp. 688-695.

Kroon and Williamson, 1996, cited by Luonteri, E., Kroon, P.A., Tenkanen, M., Teleman, A., and Williamson, G., 1999, "Activity of an A*spergillus terreus* α-L-arabinofuranosidases on phenolic-substituted oligosaccharides, *Journal of Biotechnology*, Vol. 67, pp. 41-48.

Konno, H.; Tanaka, R., Katoh, K., 1994, "An extracellular α-L-arabinofuranosidase secreted from cell suspension cultures of carrot", *Physiol Plant*, pp. 454-460.

Kubata, B.L., Suzuki, T., Ito, Y., and Naito, H., 1997, "Cloning and expression of xylanase I gene (xyn A) of *Aeromonas caviae* ME-1 in *Escherichia coli"*, *Journal of Fermentation and Bioengineering,* Vol. 3, pp. 292-295.

La Grange, D.C., Pretorius, I.S., and Van Zyl, W.H., 1996, "Expression of a *Trichoderma reesei* beta-xylanase gene (XYN2) in *Saccharomyces cerevisiae"*, *Applied and Environmental Microbiology*, Vol.62, pp. 1036-1044.

Laine, C., 2005, "Structures of hemicellulose and pectins in wood and pulp", PhD Thesis, Helsinki University of Technology, Espoo, Finland.

Lee, F., and Forsberg, C.W., 1987, "Purification and characterization of an α-L-arabinofuranosidase from *Clostridium acetobutylicum* ATCC 824", *Canadian Journal of Microbiology*, Vol. 33, pp. 1011-1016.

Lehninger, A. L., 1982, "Principles of biochemistry", *Worth Publishers*, Inc., New York.

Liab, K., Azadi, P., Collins, R., Tolan, J., Kim, J.S., and Erikkson, J.L., 2000, "Relationships between activities of xylanases and xylan structures", *Enzyme and Microbial Technology*, Vol. 27, pp. 89-94.

Luonteri, E., Beldman, G., Tenkanen, M., 1998, "Substrate specifities of *Aspergillus terreus* α-L-arabinofuranosidase", *Carbohydrate Polymers*, Vol. 31, pp. 131-141.

Luonteri, E., Kroon, P.A., Tenkanen, M., Teleman, A., and Williamson, G., 1999, "Activity of an A*spergillus terreus* α-L-arabinofuranosidases on phenolic-substituted oligosaccharides, *Journal of Biotechnology*, Vol. 67, pp. 41-48.

Lüthi, E., Jasmat, N.B., and Bergquist, P.L., 1990, "Xylanase from the extremely thermophilic bacterium "*Caldocellum saccharolyticum*": over expression of the gene in Escherichia coli and characterization of the gene product", *Applied Environmental Microbiology,* Vol. 56, pp. 2677-2683.

Mahler, H.R., and Cordes, E.H., 1966, "*Biological Chemistry",* Harper & Row Publisher, New York.

Makkonen, H.P.; Nakas, J.P., 2005, "Use of xylanase and arabinofuranosidase for arabinose removal from unbleached kraft pulp", *Biotechnology Letters*, Vol. 27, pp. 1675-1679.

Manin, C., Shareek, F., Morosoli, R., and Kluepfel, D., 1994, "Purification and characterization of an α-L-arabinofuranosidase from *Streptomyces lividans* 66 and DNA sequence of the gene (abfA)", *Canadian Biochemical Journal*, Vol. 302, pp. 443-449.

Margolles-Clark, E., Tenkanen, M., Teleman, A., and Williamson, G., 1999, "Cloninng of genes encoding α-L-arabinofuranosidases and xylosidase from *Trichoderma reesei* by expression in *Saccharomyces cerevisiae*", *Applied and Environmental Microbiology,* Vol. 62, pp. 3840-3846.

Millward-Saddler, S.J., Poole, D.M., Henrissat, B., Hazlewood. G.P., Clarke, J.H., and Gilbert, F.J., 1994, "Evidence for a general role for high affinity non-catalytic cellulose binding domains in microbial plant cell wall hydrolases", *Molecular Microbiology*, Vol. 11, pp. 375-382.

Morales, P., Madarro, A., Flors, A., Sendra, J.M., and Gonzalez, P.J.A., 1995, "Purification and characterization of a xylanase and an arabinofuranosidase from *Bacillus polymyxa", Enzyme and Microbial Technology,* Vol 44, pp. 112-117.

Nakajima, K., Tsukamoto, K.I., Watanabe, T., Kainuma, K., and Matsuda, K., 1984, "Purification and some properties of an endo-1,4-β-xylanase from *Streptomyces* sp.", *Journal of Fermentation Technology,* Vol. 62, pp. 269-276.

Nakamura, S., Aono, R., Wakabayashi, K., and Horikoshi, K.,1992, In: *Xylan and Xylanases* Prog. Biotechnol. vol 7 (Visser, J., Beldman, G., Kusters-van Someren, M. A. and Voragen, A. G.J., eds.) pp 443-446, Elsevier, Amsterdam.

Nakamura, S., Wakabayashi, K., Nakai, R., Aono, R., and Horikoshi, K., 1993, "Purification and some properties of an alkaline xylanase from alkaliphilic *Bacillus* sp. Strain 41M-1", *Applied and Environmental Microbiology,* Vol. 59, pp. 2311-2316.

Nath, D.S., 2005, "Studies on xylan degrading enzymes from an alkalophilic thermophilic *Bacillus* sp", PhD Thesis, Biochemical Sciences Division, National Chemical Laboratory, Pune.

Neto, L.S.S., and Filho, F.E.X., 2004, "Purification and characterization of a new xylanase from *Humicola grisea var thermoidea*", *Brazilian Journal of Microbiology*, Vol. 35, pp. 86-90.

Pechan, P., Barak. I., Jucovic, Jucoviv, M., Bieley, P., and Timko, J., 1989, *Biologica*, Vol. 44, pp. 1137-1145.

Poutanen, K., Rattö. J., Puls, J., and Viikari, L., 1987, "Evaluation of different microbial xylanolytic systems", *Journal of Biotechnology*, Vol. 6, pp. 49-60.

Rahman, A.K.M.S, Kato, K., Kawaki, S., Takamizawa, K., 2003, " Substrate specifity of the α–L-arabinofuranosidase from *Rhizomucor pusillus* HHT-1", *Carbohydrate Research*, Vol.338, pp.1469-1476

Reilly, P.J., 1981, "Xylanases: Structure and function", *Basic Life Sciences (New York, NY)*, Vol. 18, pp. 11-129.

Remond, C., Plantier-Royon, R., Aurby, N., Maes, E., Bliard, C., and O'Donohue, M.J., 2004, "Synthesis of pentose containing disaccharides using a thermostable α–L-arabinofuranısidase" *Carbohydrate Research*, Vol. 339, pp. 2019-2025.

Renner, M.J., and Brznak, J.A., 1998, "Purification and properties of Arf1, α-L-arabinofuranosidase from *Cytophaga xylanolytica*, *Applied Environmental Microbiology,* Vol. 64, pp. 43-52.

Royer, J.C., and Nakas, J.P., 1989, "Xylanase production by *Trichoderma longibrachiatum"*, *Enzyme and Microbiology Technology*, Vol. 11, pp. 405-410.

Ruiz, M.C., Pietro, A.D., and Roncero, M.I.G., 1997, "Purification and characterization of an acidic endo-1,4-β-xylanase from tomato pathogen *Fusarium oxysporum f. sp. Lycopersici*", *FEMS Microbiology Letters,* Vol. 148, pp. 75-82.

Saha, B.C., and Bothast R.J., 1998b, "Purification and characterization of a novel thermostable α-L-arabinofuranosidase from a color-variant strain of *Aureobasidium pullulans"*, *Applied and Environmental Microbiology*, 1998b, Vol. 64, pp. 216-220.

Saha, B.C., and Bothast R.J., 1999b, "Pretreatment and enzymatic saccharification of corn fiber", *Applied Biochemistry and Biotechnology,* Vol. 76, pp. 65-77.

Saha, B.C., 2000, "α-L-arabinofuranosidases: biochemistry, molecular biology and application in biotechnology", *Biotechnology Advances*, Vol. 18, pp. 403-423.

Sakamoto, T., and Kawasaki, H., 2003, "Purification and properties of two type-B α-L-arabinofuranosidases produced by *Penicillium crysogenum", Biochimica et Biophysica Acta*, Vol. 1621, pp. 204-210.

Sandra, E., Chaplin, M.F., Blackwood, A.D., and Dettmar, P.W., 2003, "Primary structure of arabinoxylans of ispaghula husk and wheat bran", *Proceedings of the Nutrition Society,* Royal College of Physicians, Edinburgh, UK, Vol. 62, pp. 217-222.

Saulnier, L., Vigouroux, J., and Thibault, J.Ç., 1995, "Isolation and partial characterization of feruloylated oligosaccharides from maize bran", *Applied and Environmental Microbiology*, Vol. 61, pp. 3042-3050.

Schlacher, A., Holzman, K., Hayn, M, Steiner, W., and Schwab, H., 1996, "Cloning and characterization of the gene for the thermostable xylanase XynA from *Thermomyces lanuginosus"*, *Journal of Biotechnology*, Vol. 49, pp. 211-218.

Schooneveld-Bergmans, M.E.F., Beldman, G., and Voragen, A.G.J, 1999, "Structural features of (Glucurono) arabinoxylans extracted from wheat bran by barium hydroxide", *Journal of Cereal Science*, Vol. 29, pp. 63-75.

Schwartz, W.H., Bronnenmeier, K., Krause, B., Lottspeich, F., and Staudenbauer, W.L., 1995, "Debranching of arabinoxylan: properties of the thermoactive recombinant α-L-arabinofuranosidases from *Clostridium stercorarium* (ArfB)", *Applied Microbiology and Biotechnology,* Vol. 43, pp. 856-860.

Senior, D.J., Hamilton, J., and Bernier, R.L., 1992, "Use of *Streptomyces lividans* for Biobleaching of Kraft Pulps"*, In: Xylan and Xylanases* (Visser, J., Beldmen, G., Voragen, A.G.J., Someren, M.A.K.), pp. 371-379.

Spagna, G., Romagnoli, D., Angela, M., Bianchi, G., and Pifferi, P.G., 1998, "Simple method for purifying glycosidases: α-L-arabinofuranosidase and β-D-glucopyranosidase from *Aspergillus niger* to increase the aroma of wine. PartI", *Enzyme and Microbial Technology*, Vol 22, pp. 298-304.

Sørensen, H.R., Pedersen, S., Nielsen, A.V., and Meyer, A.S., 2005, "Efficiencies of designed enzyme combinations in releasing arabinose and xylose from wheat arabinoxylan in industrial ethanol fermentation residue", *Enzyme and Microbial Technology*, Vol. 36, pp. 773-784.

Steiner *et al*., 1987, Nath, D.S., 2005, "Studies on xylan degrading enzymes from an alkalophilic thermophilic *Bacillus* sp", PhD Thesis, Biochemical Sciences Division, National Chemical Laboratory, Pune.

Stülke, J, and Hillen W., 2000, "Regulation of carbon catabolism in *Bacillus* species", *Annual Review of Microbiology*, Vol. 54, pp. 849-880.

Tan et al., 1987; Tan et al., 1985; Wong et al., 1986, Esteban et al., 1982, cited by Wong, K.K.Y., Tan, L.U.L., and Saddler, J.N., 1988, "Multiplicity of β-1,4-Xylanase in microorganisms: Function and applications", *Microbiological Reviews*, Vol. 52, pp. 305-317.

Tajana, E.; Fiechter, A.; Zimmermann, W. Purification and characterization of two α-L-arabinofuranosidases from *Streptomyces diastaticus.* Appl Environ Microbiol.1992, 58, 1447-1450.

Tenkanen, M., Tamminen, T., and Hortling, B., 1999, "Investigation of lignin-carbohydrate complexes in kraft pulps by selective enzymatic treatments", *Applied Microbiology and Biotechnology*, Vol. 51, pp. 241-248.

Thomson, J.A., 1993, "Molecular Biology of xylan degradation", *FEMS Microbiology Reviews*, Vol. 104, pp. 65-82.

Topakas, E., Katapodis, P., Kekos, D., Macri, B.J., Cristakopoulos., 2003, "Production and partial characterization of xyalanse by *Sporotrichum thermophile* under solid-state fermentation", *World Journal of Microbiology and Biotechnology,* Vol. 19, pp. 195-198.

Törrönnen, A., Affenzeller, K., Hofer, F., Myohanen, T.A., Blaas, D., Harkki, A., and Kübicek, C.P., 1992, "The major xylanase of *Trichoderma reesei*: purification, and production of monoclonal antibodies", *In: Xylan and Xylanases* (Visser, J., Beldman, G., Voragen, G.J.), pp. 501-504, Elsevier Sciemce, Amsterdam.

Törrönnen, A., Harkki, A., and Rouvinen, J., 1994, "Three dimensional structure of endo-1,4-β-xylanase II from *Trichoderma reesei*: two conformational states in the active site", *European Molecular Biology Organization Journal*., Vol. 13, pp. 2493-2501.

Uesaka, E., Sato, M., Raiju, M., Kaji, A., 1978, "α-L-arabinofuranosidases from *Rhodotorula flava"*, *Journal of Bacteriology*., Vol. 133, pp. 1073-1077.

Van Laere *et al*., 1999, cited by Saha, B.C., 2000, "α-L-arabinofuranosidases: biochemistry, molecular biology and application in biotechnology", *Biotechnology Advances*, Vol. 18, pp. 403-423.

Vyas, P., Chauthaiwale, V., Phadatare, S., Deshpande, V., and Srinivasan, M. C., 1990, "Studies on the alkalophilic *Streptomyces* with extracellular xylanolytic activity", *Biotechnology Letters*, Vol. 12, pp. 225-228.

Vyas, S.R., 2004, "Characterization of alkali stable fungal cellulases and their potential industrial applications", PhD thesis, University of Pune.

William, P.E.V., Geraert, P.A., Uzu, G., and Annison, G., 2000, "Factors affecting non-starch polysaccharide digestibility in poultry", *CIHEAM-Options Mediterraneennes*.

Winterhalter, C., and Liebl, W., 1995, "Two extremely thermostable xylanases of the hyperthermophilic bacterium *Thermotoga maritima* MSB8", *Applied Enviromental Microbiology*, Vol. 61, pp. 1810-1815.

Wood, T.M., and McCrae, S.I., 1986, "Studies of two low molecular weight endo-(1,4)-β-D-xylanases constituvely synthesized by the cellulolytic fungus *Trichoderma koningii*", *Carbohydrate Research*, Vol. 148, pp. 321-330.

# PART I

## INVESTIGATION OF THE MICROBIAL XYLANOLYTIC ENZYMES from A SOIL ISOLATE *Bacillus pumilus* SB-M13 and THERMOPHILIC FUNGUS *Scytalidium thermophilum*

# CHAPTER 1

## INTRODUCTION

Complete degradation of arabinoxylans to monosachharides can only be achieved by synergistic act of both side chain cleaving and depolymerizing enzyme activities. Endo-β-1,4-xylanase (β-xylanase or xylanase) cleaves the β-1,4 bonds of xylan backbone and produces xylo-oligosaccharides which are further hydrolyzed to D-xylose by the action of β-1,4-xylosidase. Enzymatic cleavage of the side chains requires action of several accessory activities, such as α-L-arabinofuranosidases (EC. 3.2.1.55), feruloyl esterase (3.1.1.73), α-glucuronidases (EC.3.2.1.139). Besides, synergistic action between cellulose- and hemicellulose-degrading enzymes is important as reported by Tenkanen and coworkers (1999).

In general, different endo-β-1,4- xylanases have different activities against various xylan structures. The key factors that affect the rate of xylan hydrolysis are substrate chain length, degree and type of substitutions. Some xylanases, most effective on long chain xylans might also be effective against substituent groups, as well (*Aureobasidium pullulans, Trichoderma longibrachiatum*). However, *Thermatoga maritima* xylanase's hydrolytic rate is significantly reduced by substituents on xylan backbones (Liab, 2000). Moreover, *Polyporus tulipiferae* xylanase hydrolysis was also blocked by presence of side groups (Brillouet *et al*., 1987, cited by Hespell and Cotta, 1995).

Xylan has been shown to be the best inducer of xylanase production for many microorganisms (Nakamura *et al*., 1992); however, few organisms show constitutive production of the enzyme (Debeire *et al*., 1990). Hemicellulosic

substrates like corn cob, wheat bran, rice bran, rice straw, corn stalk and bagasse have also been found to be most suitable for the production of xylanase in certain microorganisms. Corn cobs are the best inducer for *Aspergillus tamarii* (Kadowaki *et al*., 1996), *Thermomonospora* sp. (George *et al*., 2000), *Rhizopus oryzae* (Bakir, 2001), *Scytalidium thermophilum* (This study), whereas wheat bran was the top one for the *Streptomyces* VP5 (Vyas *et al*., 1990), *Bacillus pumilus* SB-M13 (This study), and *Streptomyces* T-7 (Keskar *et al*., 1992).

In general, regulation of polymeric substrate degrading enzymes is that low constitutive levels of hydrolytic enzymes produce small soluble 'signal' fragments which are able to enter cell and induce synthesis of the corresponding enzyme taking role in polymer hydrolysis.

Most of the xylanolytic enzyme producing organisms are under the control of the carbon catabolite repression. Carbon catabolite repression (CR) is a regulatory mechanism to guarantee sequential utilization of carbohydrates (Stülke and Hillen, 2000; Hueck and Hillen, 1995).

Extensive biochemical analysis of xylan degrading enzymes of both fungal and bacterial origin has been conducted, and a large number of enzymes have been purified and characterized such as *Rhizopus* spp. (Bakir *et al*., 2001), *Aspergillus* spp. (Kadowaki *et al*., 1996), *Scytalidium* spp. (Ögel *et al*., 2001; Düsterhöft *et al*., 1997; Zanoelo *et al*., 2004, and this study), *Thermomonaspora* spp. (Tuncer, 2000), *Bacillus* spp. (Biran *et al*., 2006; Avcioglu *et al*., 2005; Rahim and Lee, 1992; Kocabas and Dizbay, 1999, Degrassie *et al*., 1995; Degrassie *et al*., 2003).

### 1.1 Aim of the study

The main purpose of the study was to investigate xylanolytic systems of *Bacillus pumilus* SB-M13 and *Scytalidium thermophilum* to characterize and determine their potential application areas in industry. Accordingly, microorganisms were

grown on agricultural by-products, namely corn cobs, wheat bran, and rice bran and their xylanolytic enzyme production profiles were assessed up to 7 days and then, results were comparatively evaluated.

# CHAPTER 2

# MATERILAS AND METHODS

## 2.1 Xylanase producing microorganisms and culture maintenance

### 2.1.1 Isolation and maintenance of soil isolate *Bacillus* species

The *Bacillus pumilus* used in this study was the best xylanase-producing microorganism isolated from soil samples collected from different regions in Turkey among hundreds of others. Microbial isolation was performed by using the method given elsewhere (Avcioglu *et al*., 2005) and the isolate was identified as *Bacillus pumilus* according to both API 50 CH-API 50 CHB/E medium kit (BioMérieux), fatty acid analysis, and FAME profiling (Sasset *et al*., 1990, and Khoodoo *et al*, 2005). The isolate was maintained at 37°C on agar plate containing (w/v): 1.0%; glucose, 0.5%; peptone, 0.5%; yeast extract, 0.1%; $KH_2PO_4$, 0.02%; $MgSO_4$ and 2.0%; agar to which sterile 0.01%; $Na_2CO_3$ added separately.

### 2.1.2 Identification of the soil isolate *Bacillus* species

Combination of the following microbiological and biochemical techniques were used for identification of soil isolate after microscopic evaluation, gram staining, catalase and motility test (Biran, 2001)

#### 2.1.2.1 Endospor staining

Schaeffer-Fulton method, the most common endospore staining technique, was

employed to investigate capability of the soil isolate to produce endospore (http://howie.myweb.uga.edu/staining.html). Accordingly, bacterial smear was prepared aseptically on a clean slide, air dried, and gently fixed by heat. Slide, covered with a piece of paper towel, was placed on a staining rack over the water bath. Then, the paper towel on the slide was saturated with Malachite green (primary stain), and slide was steamed for 5 min. After removing the paper towel, the slide was taken out from water bath to cool the slide. Then, the slide was rinsed with distilled water to remove excess dye. Excess water was also poured off, and safranin, a counterstain, was applied to the slide for 2 min. Excess dye was rinsed with distilled water and the slide was blotted with the piece of paper towel. In the subsequent step, the slide was examined with light microscope under oil immersion.

#### 2.1.2.2 API 50 CH-API 50 CHB/E medium kit analysis

API 50 CH-API 50 CHB/E medium kit (BioMérieux), proposed for genus *Bacillus*, was utilized according to manifacturer's instructions. Identification system based on fermentation of 49 carbohydrates on the kit strip (Appendix A and B ). Decrease in pH due to fermentation of carbohydrates was detected by color change of the indicator and test results were deduced as -/+ color change. After reading, the biochemical profile obtained for the strain was identified using identification software with data base (V3.0) provided by Health Center (METU).

#### 2.1.2.3 Fatty acid analysis

Soil isolate was also identified at Yeditepe University by means of fatty acid extraction and fatty acid methyl esters (FAME) profiling using Gas chromatography (GC) which was followed by comparison of FAME profile with standard profiles already available in the library (Sasser *et al*., 1990, and Khoodoo *et a*l, 2005).

### 2.1.3 *Scytalidium thermophilum* and culture maintenance

*Scytalidium thermophilum* type culture *Humicola insolens* was provided from ORBA Inc. Microorganism was grown at 40°C on YpSs agar plates containing (w/v): 0.4; yeast extract, 0.1; $K_2HPO_4$, 0.05; $MgSO_4.7H_2O$, 1.5; soluble starch and 2.0 ; agar. Culture was maintained at room temperatures, subcultured monthly. Cultures were maintained at -20°C on agar slants for long term storage.

## 2.2 Enzyme production from *B. pumilus* SB-M13 and *S.thermophilum*

Xylanolytic enzyme production by *Bacillus pumilus* SB M-13 was performed in 250-ml shake-flask bioreactors containing 100-ml medium at 30°C, 175 rpm for 7 days. *Bacillus pumilus* SB M-13 fermentation medium contained 0.5% NaCl, 0.25% yeast extract, 0.1% $KH_2PO_4$, 0.02% $MgSO_4$, 0.1% $Na_2CO_3$ and 3% steam hydrolyzed and ground agricultural by-products such as; corn cobs, wheat bran and rice bran as sole carbon source and inducer.

*S. thermophilum* ($10^4$spores/ml) was also cultured in 250-ml shake-flask bioreactors containing 100 ml medium at 45°C, 155 rpm for 7 days. The medium contained 1.0% yeast extract, 1.0% tryptone, 0.2% $(NH_4)_2SO_4$, 0.03% $MgSO_4.7H_2O$, 0.03%$FeSO_4$, 0.03% $CaCl_2$, and 3.0% ground and steam hydrolyzed agricultural by-products such as; corn cobs, wheat bran and rice bran as sole carbon source and inducer.

Then, time course of xylanolytic enzymes (α-L-arabinofuranosidase, β-galactosidase, endo-β-xylanase, and β-xylosidase) production was followed in fermentation cultures. In addition to xylanolytic enzymes, production of β-glucosidase, involved in cellulose hydrolysis by releasing terminal, non-reducing β-D-glucose was also examined as a function of time. Additionally, to observe the effect of arabinose on the expression of xylanoltic enzymes, arabinose at a final concentration of 1.0% and 0.5% (w/v) was added to fermentation medium

containing 3% of corn cobs. Agricultural by-products, ground corn cobs, wheat bran, and rice bran were subjected to steam hydrolysis to facilitate utilization by the microorganism (Bakir *et al*., 2000). The fermentation medium was centrifuged for 40 min at 11,000 x *g* and the supernatant was used as crude enzyme extract.

**2.3 Xylanolytic enzyme assays**

AF (α-L-arabinofuranosidase), GAL (β-galactosidase), XYL (β-xylosidase), and GLU (β-glucosidase) activities were measured using synthetic *p*-nitrophenol glycosides- p-nitrophenyl-α-L-arabinonofuranoside, p-nitrophenyl-β-D-galactopyranoside, p-nitrophenyl-β-D-xylopyranoside, and p-nitrophenyl-β-D-glucopyranoside. Substrate concentrations used in the assays were 2 mM for p-nitrophenyl-α-L-arabinonofuranoside and 10 mM for the other substrates.

The reaction mixture containing 0.5 ml of a 2 mM p-NPAraf solution (Kaneko *et al*., 1998)-10mM of other substrates, 0.4 ml of 50 mM phosphate buffer at pH 7.0 and 0.1 ml of the enzyme solution, was incubated at 40°C and 60°C for *B. pumilus* SB-M13 and *S. thermophilum* enzymes, respectively. Amount of *p*-nitrophenol (p-NP) release was followed spectrophotometrically at every 15 sec within a period of 5 min at 410 nm and the initial reaction rates were measured. One unit of enzyme activity was defined as the amount of enzyme required for the secretion of 1 μmol of p-NP per min from p-NP glycoside under the assay conditions.

XYN (endo-β-xylanase) assay was performed using 1% of birchwood xylan in 50 mM phosphate buffer at pH 7.0. The reaction mixtures contained 0.1 ml of properly diluted enzyme and 1.0 ml of substrate solution. The reaction mixtures were incubated at 40°C for *B. pumilus* SB-M13 and at 60°C for *S. thermophilum* xylanase and the samples were taken at 15 second intervals up to 1 min to measure the initial reaction rates. Dinitrosalicylic acid (DNSA) method was used to

determine reducing sugar concentration by using xylose as a standard. One unit of enzyme was defined as the amount of enzyme releasing 1μmol of xylose equivalent per min under assay conditions.

# CHAPTER 3

# RESULTS AND DISCUSSION

## 3.1 Identification of the soil isolate

The *Bacillus species* used in this study was isolated from soil samples collected from different regions in Turkey, and identified using endospor staining method in combination with API 50 CH-API 50 CHB/E medium kit (BioMérieux), and fatty acid analyses (Sasser at al., 1990, and Khoodoo et al, 2005).

This strain was previously partially characterized by Biran (1999) as catalase positive, molitile, gram positive bacilli. In the present study, endospor staining result (Figure 12) showed that isolate is an endosporulating bacterium. After API 50 CH-API 50 CHB/E medium kit (Appendix A and B), and fatty acid analyses, isolate was definitely identified as *Bacillus pumilus*, and it was referred as *Bacillus pumilus* SB M-13.

## 3.2 *B. pumilus* SB-M13 xylanolytic system

*B. pumilus* SB-M13 was grown on 3% of ground agricultural by-products, corn cobs, wheat bran and rice bran, on shake-flask bioreactors and production of xylanolytic enzymes were assessed in the culture supernatant up to 7 days using the standard assay conditions. Moreover, the effect of arabinose at a final concentration of 1.0% and 0.5% on induction of the xylanolytic enzyme production was also followed in the fermentation medium containing 3% of corn cobs as a sole carbon source.

Cellulose degrading enzymes-free xylanase preparations is very important for applications in the paper and pulp industry. *B. pumilus* SB-M13 has very low level of 0.003 FPU cellulase activity (Biran *et al.*, 2006). In addition to xylanolytic enzymes, production of β-glucosidase, another enzyme involved in cellulose hydrolysis, was also assessed in this study.

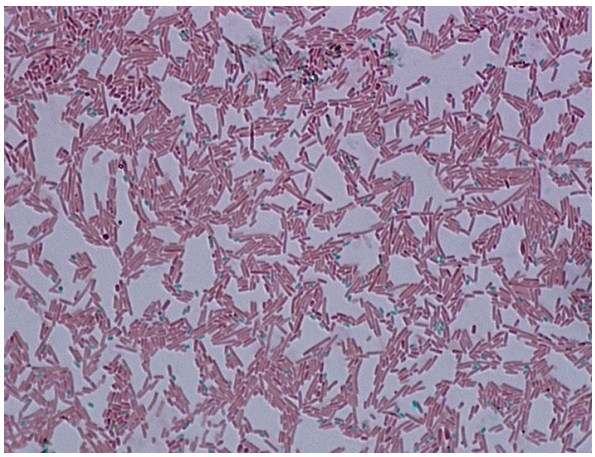

Figure 12. *Bacillus pumilus* SB-M13 endospore staining picture. (The green structures are endospores, and the pink structures are vegetative cells under light microscope).

### 3.2.1 Time course of extracellular xylanolytic enzyme production by *B. pumilus* SB-M13

When grown on 3% of corn cobs, *B. pumilus* SB-M13 produced AF, XYN and GAL (Figure 13). XYN and AF syntheses, beginning with the bacterial cultivation, increased rapidly up to 4$^{th}$ day of fermentation at which maximum activities of 1.3 U/ml and 108 U/ml were obtained for AF and XYN, respectively. Then, on the

subsequent days AF activity was almost stable at 1.3 U/ml, however probably because of protease accumulation and enzyme deactivation, XYN activity gradually decreased and it was 67 U/ml on 7$^{th}$ day of cultivation. Unlike AF and XYN, GAL synthesis delayed for one day and enzyme production started on 2$^{nd}$ day of fermentation. Moreover, when compared to AF and XYN, GAL titer in *B. pumilus* SB-M13 crude enzyme extract was noticeably low and the maximum GAL activity of 0.2 U/ml was attained on 4$^{th}$ day of cultivation. GAL production profile resembled that of AF as well. Unlike AF, XYN, and GAL, there were no XYL and GLU activities detected in crude enzyme extract.

In summary, when the time course of xylanolytic enzyme production considered, it was found that, syntheses of both AF and XYN were started immediately during cultivation; whereas GAL activity was detected later. AF and XYN activities in the beginning of fermentation may facilitate GAL synthesis in the subsequent days. All enzyme activities reached at maximum values on the 4$^{th}$ day of fermentation. For the first 4 days, it was remarkable that as production of XYN increased, AF production increased significantly which may be a sign of possible synergistic action between these two enzymes, as well. We may conclude that as AF removes arabinose residues from the xylan backbone, resultant structure becomes better substrate (inducer) for XYN and consequently, XYN production increased. Increase in titer of the XYN, acting on xylan backbone, produced more xylo-oligosaccharides which served as a better inducer for AF expression.

Xylanolytic enzyme production profile obtained with 3% of wheat bran (Figure 14) was slightly different than that obtained with corn cobs. AF and XYN production initiated at the beginning of cultivation, but GAL production started on the 2$^{nd}$ day of cultivation. The maximum level of GAL activity (0.3 U/ml) was achieved on 4$^{th}$ day of fermentation, and that of AF (5.0 U/ml) and XYN (105 U/ml) production were attained on the 6$^{th}$ and 7$^{th}$ days of fermentation, respectively. AF production was at the same level on the first 3 days of fermentation, but within this period slight increase in XYN titer induced the AF

production in the subsequent days. Indeed, starting from the 3rd day of fermentation, AF activity sharply increased and the maximum AF of 5 U/ml was reached on the 6th day of fermentation. On the other hand, XYN activity was stable between the 3rd and 6th days of fermentation. Furthermore, in the subsequent stage, after the 6th day of cultivation, accumulation of AF titer in the fermentation culture induced the XYN production. Besides, GAL production was rather low when compared to AF and XYN, and unlike AF, XYN, and GAL, there was no XYL and GLU activities detected in crude enzyme extract.

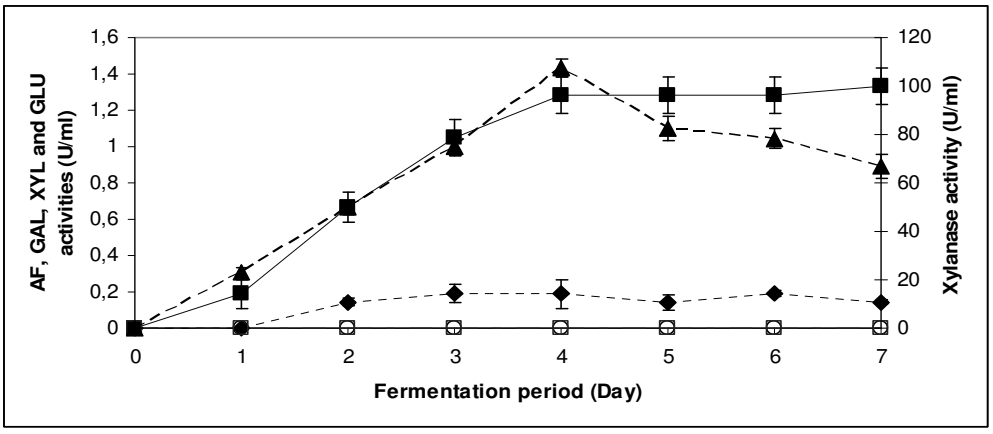


Figure 13. Xylanolytic activities in crude enzyme of *B. pumilus* SB-M13 grown on 3% corn cobs as a sole carbon source and inducer. Bacterium was shake-cultured at 37°C, 175 rpm for 7 days. Enzyme activities were measured using standard assay at 40°C and pH 7.0. (Enzyme activities; ■: AF, ◆:GAL, ▲:XYN, ○: XYL □: GLU).

In brief, when time course of productions were considered, it was found that xylanolytic enzyme production was sequential and AF and XYN were preliminary

enzymes in wheat bran containing culture filtrate, followed by GAL. Although AF, XYN, and GAL productions were not parallel, enzyme production profiles showed the possible synergy among these enzymes. Initial AF and XYN production might induce GAL production which might induce production of AF and/or XYN in the subsequent period. Level of GAL and AF enzymes reached their maximum values on the $4^{th}$ and $6^{th}$ day of fermentations, and then it was almost steady until the end of fermentation (7 days), but XYN production increased as fermentation period increased and reached maximum value on the $7^{th}$ day. Therefore, accumulated GAL and AF activities may increase amount of XYN production by utilizing and modifying xylan molecules which serve as a better inducer for XYN production. Additionally, as can be seen from Figure 14, XYN and AF productions have a significant relation and due to accumulation the XYN during a specific fermentation period, amount of arabinose containing xylo-oligosaccharides increased, which later possibly induced the AF expression. Then, higher level of AF present in fermentation culture possibly removes arabinose residues from xylan backbone/xylo-oligosaccharides and produces better inducer for XYN and consequently, XYN expression level was enhanced.

When grown on rice bran, B. *pumilus* SB-M13 produced AF, XYN, and rather low level of GAL with the beginning of cultivation (Figure 15). However, enzymes reached their maximum values one after the other. The maximum AF activity of 1.5 U/ml was reached on $2^{nd}$ day of fermentation and it was kept at same level until the end of the fermentation period. Then the maximum GAL activity of 0.1 U/ml was attained on $3^{rd}$ day of fermentation, and the enzyme production ceased significantly on the succeeding days and terminated on $5^{th}$ day. The XYN activity was stable for the fist $4^{th}$ day of fermentation. Then, XYN production increased fairly and reached to the maximum level (16 U/ml) on $7^{th}$ day of cultivation. However, rice bran containing crude enzyme extract did exhibit neither XYL nor GLU activities.

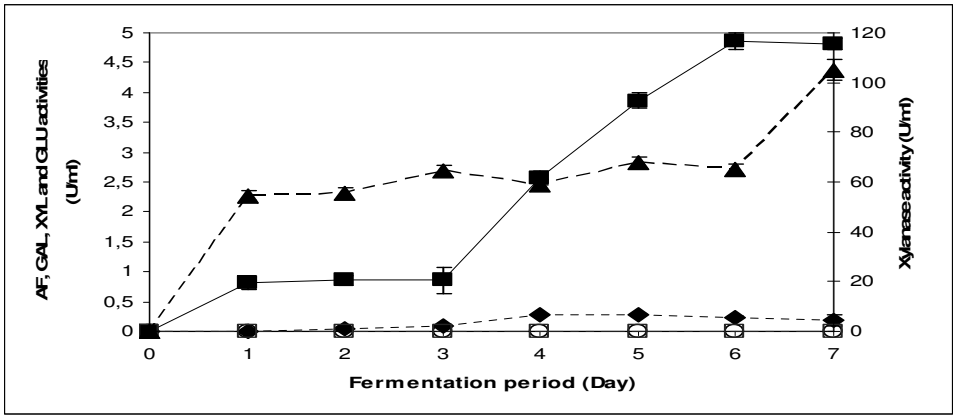


Figure 14. Xylanolytic activities in crude enzyme of *B. pumilus* SB-M13 grown on 3% wheat bran as a sole carbon source and inducer. Bacterium was shake-cultured at 37°C, 175 rpm for 7 days. Enzyme activities were measured using standard assay at 40°C and pH 7.0. (Enzyme activities; ■: AF, ◆:GAL, ▲:XYN, ○: XYL □: GLU).

XYL and GLU activities were absent in all fermentations. Actually, *B. pumilus* XYL is an intracellular enzyme (La Grange, 1999) and it was not supposed to be present in *B. pumilus* SB-M13 crude enzyme extract. Moreover, an intracellular β-glucosidase have been investigated from *Bacillus circulans* subsp. alkalophilus (Paavilainen *et al*., 1976). Therefore, although there have been no extracellular GLU activity investigated, *B. pumilus* SB-M13 may have intracellular GLU activity.

In brief, attaining the maximum level of enzyme activities in sequence (AF, GAL, and later XYN) reflects possible synergy among AF, GAL, and XYN. Synthesis of one may enhance that of others or/and combined effect between two may enhance the production of the third one. We assumed that during the fermentation, AF debranches the arabinoxylan (AX) molecule by releasing arabinose side chains

from the backbone, and makes AX molecule better inducer for XYN. That is why, after certain period of fermentation, XYN production increased noticeably.

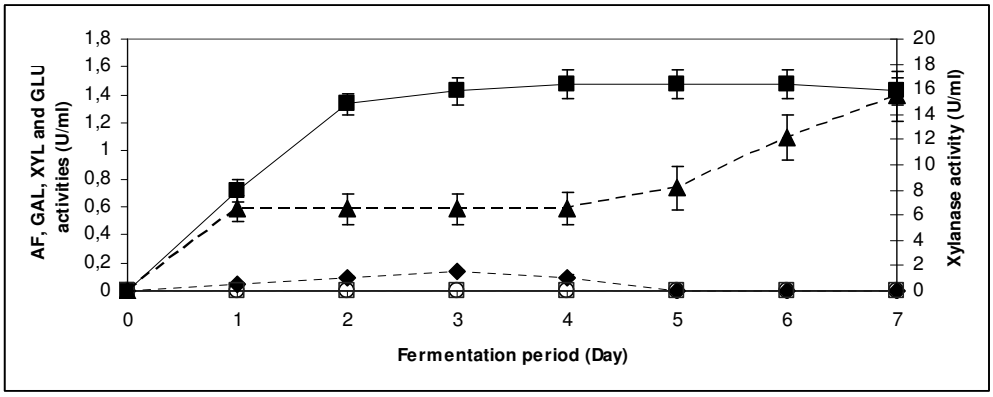


Figure 15. Xylanolytic activities in crude enzyme of *B. pumilus* SB-M13 grown on 3% rice bran as a sole carbon source and inducer. Bacterium was shake-cultured at 37°C, 175 rpm for 7 days. Enzyme activities were measured using standard assay at 40°C and pH 7.0. (Enzyme activities; ■: AF, ◆:GAL, ▲:XYN, ○: XYL □: GLU).

### 3.2.2 Effect of carbon source on xylanolytic enzyme production induction

#### 3.2.2.1 Effect of agricultural by-products

The *B. pumilus* SB-M13 was grown on 3% of various carbon sources, corn cobs, wheat bran, rice bran, and xylanolytic enzyme induction power of each was determined (Table 7).

It was investigated that all carbon sources induced AF production and the highest

AF activity of 5.0 U /ml was obtained on 6$^{th}$ day of fermentation containing wheat bran (Figure 16 and Table 7). When the carbon source was replaced with corn cobs and rice bran, both of the AF titer and fermentation period decreased. In fact, the maximum AF activities of 1.3 U/ml and 1.5 U/ml were obtained on 4$^{th}$ and 5$^{th}$ day of fermentation on corn cobs and rice bran, respectively.

XYN, cleaving the xylan backbone, was the main extracellular enzyme produced in all fermentation cultures (Figure 17). Induction power of corn cobs and wheat bran are similar and 7 times higher than that of rice bran (Table 7). The highest XYN activities of 108 U/ml (on 4$^{th}$ day) and 105 U/ml (on 7$^{th}$ day) were obtained with corn cobs and wheat bran, respectively, whereas it was only 16 U/ml (on 7$^{th}$ day) for rice bran.

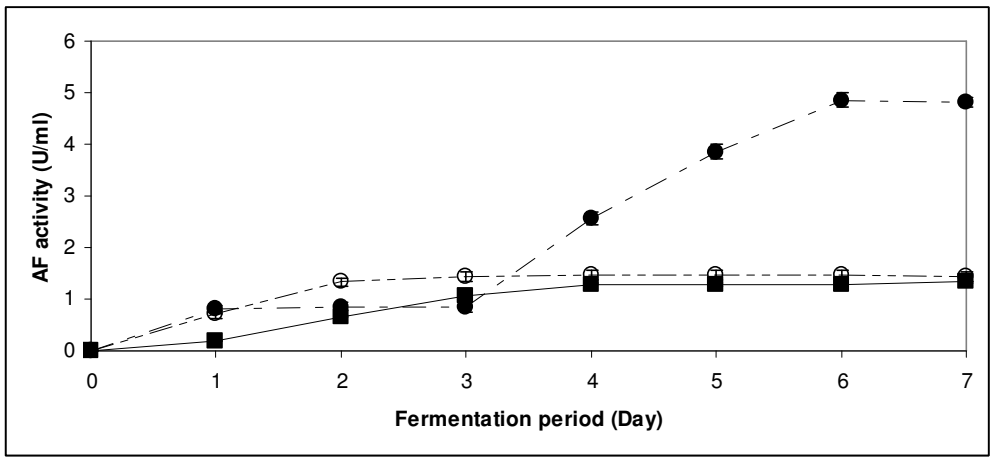


Figure 16. Effect of carbon sources on the production of AF by *Bacillus pumilus* SBM-13. (■: 3% corn cobs, ●: 3% wheat bran, ○: 3% rice bran). AF activities were measured at 40°C at pH 7.0 using standard AF activity assay.

Table 7. Xylanolytic activities in culture filtrate of *B. pumilus* SB-M14.

| Enzymes | Enzyme production initiation on (Day) | Maximum enzyme production on (Day) | Maximum enzyme activity (U/ml) |
|---|---|---|---|
| **α-L-arabinofuranosidase** | | | |
| *Corn cobs* | 1 | 4 | 1.3 ± 0.0 |
| *Wheat bran* | 1 | 5 | 5.0 ± 0.1 |
| *Rice bran* | 1 | 5 | 1.5 ± 0.1 |
| **β-galactosidase** | | | |
| *Corn cobs* | 2 | 2 | 0.2 ± 0.0 |
| *Wheat bran* | 2 | 4 | 0.3 ± 0.0 |
| *Rice bran* | 1 | 3 | 0.1 ± 0.0 |
| **Endo-β-xylanase** | | | |
| *Corn cobs* | 1 | 4 | 108.0 ± 4.0 |
| *Wheat bran* | 1 | 7 | 105.0 ± 2.0 |
| *Rice bran* | 1 | 7 | 16.0 ± 1.5 |
| **β-xylosidase** | | | |
| *Corn cobs* | n.d | n.d | 0.0 |
| *Wheat bran* | n.d | n.d | 0.0 |
| *Rice bran* | n.d | n.d | 0.0 |
| **β-glucosidase*** | | | |
| *Corn cobs* | n.d | n.d | 0.0 |
| *Wheat bran* | n.d | n.d | 0.0 |
| *Rice bran* | n.d | n.d | 0.0 |

* Enzyme was involved in cellulose degradation, n.d; activity was not detected in crude enzyme extract under standard assay conditions.

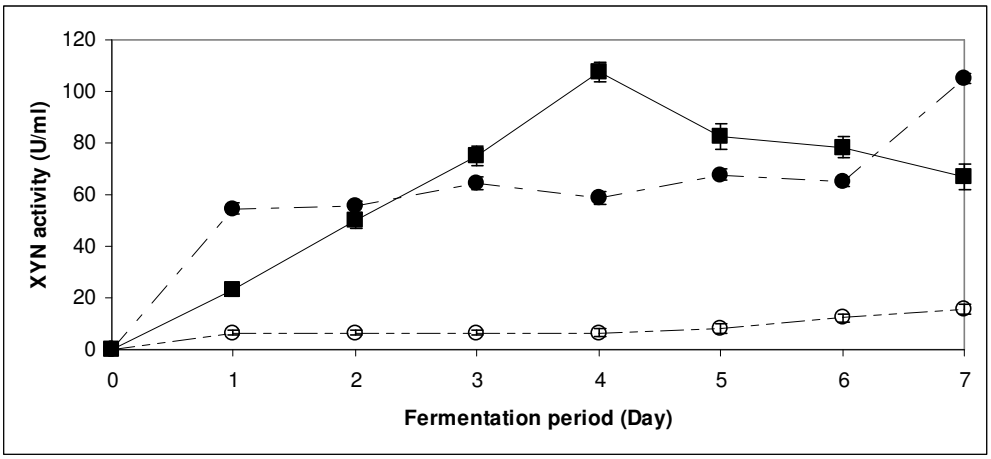


Figure 17. Effect of carbon sources on the production of XYN by *Bacillus pumilus* SBM-13. (■: 3% corn cobs, ●: 3% wheat bran, ○: 3% rice bran). AF activities were measured at 40°C at pH 7.0 using standard XYN activity assay.

Wheat bran with 0.3 U/ml activity seemed to be the most effective inducer for GAL secretion, followed by corn cobs-0.2 U/ml- and rice bran-0.1 U/ml (Figure 18 and Table 7 ). GALs of *Bacillus* species are intracellular (Rahim and Lee, 1992; Kocabas and Dizbay, 1999; Mabrouk et al., 2002). Therefore, as predicted, in our experiment very low level of extracellular GAL activities were measured in all fermentations.

Apart from AF, XYN, and GAL, there were no XYL and GLU activities detected in any of the culture supernatant. Actually, *B. pumilus* XYL is an intracellular enzyme (La Grange, 1999) and it was not supposed to be present in *B. pumilus* SB-M13 crude enzyme extract. In addition, there being no GLU activity in any of the culture supernatant, crude enzyme extracts of all fermentation culture can directly be used in bleaching pulp and xylooligosachharide production process. Moreover, *B. pumilus* SB-M13 produced very low level of extracellular cellulase (0.003 FPU) (Biran *et al*., 2006).

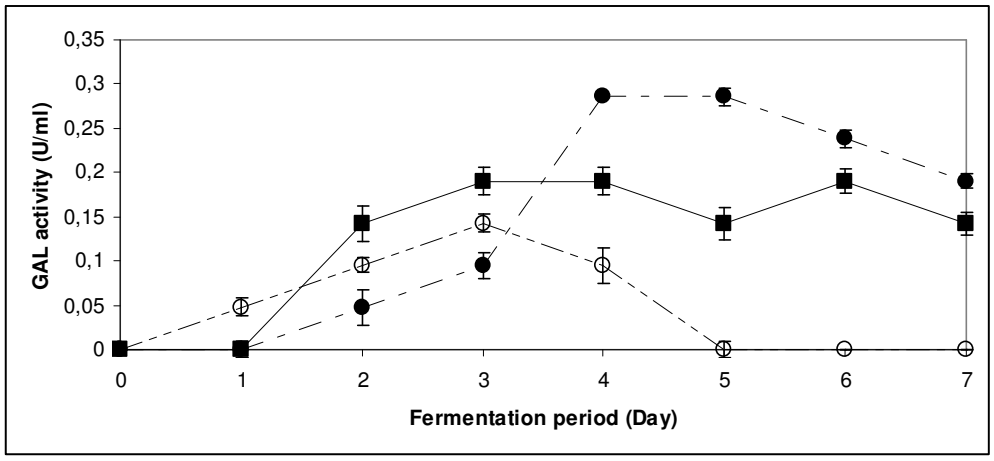


Figure 18. Effect of carbon sources on the production of GAL by *Bacillus pumilus* SBM-13. (■: 3% corn cobs, ●: 3% wheat bran, ○: 3% rice bran). AF activities were measured at 40°C at pH 7.0 using standard GAL activity assay.

GLU is the part of the cellulose degrading enzyme system. Endo-1,4-β-glucanase (EC 3.2.1.4) and exo-1,4-β-glucanase (EC 3.2.1.91) act directly cellulose fiber and relase oligosaccharides of different size and cellobiose. β-glucosidase (GLU, EC 3.2.1.21) hydrolyses cellobiose and release glucose (Zanoelo *et al.*, 2004a) and an intracellular β-glucosidase have also been investigated from *Bacillus circulans* subsp. *alkalophilus* (Paavilainen *et al.*, 1976). Therefore, although there have been no extracellular GLU activity investigated, *B. pumilus* SB-M13 may have intracellular GLU activity.

Duarte and coworkers (2003) assessed extracellular xylanolytic enzymes of *B. pumilus* CBMAI 0008 in fermentation medium containing three different xylan sources; birchwood, *Eucalyptus grandis*, and oat at alkaline and acidic conditions. *B. pumilus* CBMAI 0008 produced β-xylanase, mannanase, α-glucuronosidase, β-xylosidase, β-glucosidase, arabinosidase, α-galactosidase and filter paper cellulose

at varying level on various carbon sources. The maximum XYN activity of 328 U/ml was obtained with birchwood xylan at pH 9.0. The maximum mannanase activity of 6.0 U/ml and α-glucuronosidase activity of 1.5 U/ml was attained in *Eucalyptus grandis* containing cultures at pH 4.0. Moreover, β-glucosidase has maximum activity of 0.02 U/ml on both birchwood xylan and *Eucalyptus grandis* at pH 9.*0.* Maximum arabinosidase, α-galatosidase and filter paper cellulose activities were 0.07 U/ml, 5.0 U/ml, 0.01 U/ml, and 0.02 U/ml on oat xylan. In our study fermentation was performed at pH 7.0, and enzyme activites were measured under the standard assay conditions and maximum XYN, AF, and GAL activities of 105 ± 2.0 U/ml, 5 ± 0.1 U/ml, and 0.3 ± 0.0 U/ml were obtained, respectively on wheat bran. When compared to of *B. pumilus* CBMAI 0008, *B. pumilus* SB-M13 produced XYN in lower titer, AF at same level, but rather higher GAL.

In brief, enzyme activity results showed that high level of XYN and AF, and constitutive level of GAL formed when *B. pumilus* SB-M-13 grown on different carbon sources. Arabinoxylan, abundant hemicellulosic polymer in all utilized carbon sources, was assumed to be inducer. Nature, frequency and the position of the branches vary through xylan sources and are dependent on the source of xylan.

When *B. pumilus* SB-M13 was grown on fermentation culture containing 3% of corn cobs, wheat bran, and rice bran as a sole carbon sources, extracellular xylanolytic enzyme production profile showed variation. Hemicellulose and xylan content, xylan accessibility including particle size, lignin and xylan structure and composition of the ground agricultural by-products, solubility of the arabinoxylan molecules, and viscosity of the fermentation medium were the key parameters concluding xylanolytic enzyme production profile.

Unlike wheat bran, rice bran and corn cobs were ground. Actually, natural size of wheat bran was tiny and did not require any grinding process. When form was considered, wheat bran and rice bran were sheet like, whereas ground corn cobs were granulated which may put limitation in enzyme diffusion through the

substrate. Moreover, depending on their total soluble polysaccharide contents, viscosity of each fermentation medium varied (visual detection). The fermentation cultures containing wheat bran and rice bran was significantly more viscous than corn cobs containing culture medium, which can affect the enzyme production profile. Having small size was a disadvantage for granulated corn cobs. On the other hand, using granulated corn cobs and production of less viscous medium was an advantage.

Arabinoxylan contents of the maize bran (Saulnier *et al*., 1994), wheat (Schooneveld-Bergmans *et al*., 1999) and rice bran (Choct and Annison, 1990) have been well documented (See details in part 1.1). Accordingly, it was indicated that xylan component of all carbon sources is very complex and it comprises various side chain residue type and composition.

Schooneveld-Bergmans *et al.* (1999) investigated the structure of the (glucurono)arabinoxylan extracted from water-unextractable wheat bran cell wall. According to results, arabinose, predominant substitution, was found at the O-3 position of xylose residues. Moreover, more than half the arabinoxylan, substitution of xylose was positioned not only through O-3 mono-, O-2 and O-3 disubstitution by terminal arabinose and O-2 monosubstitution by (4-O-methyl)glucuronic acid, but also through dimeric arabinose, xylose and possibly galactose containing branches as well as through 2,3-linked arabinose. Enzymatic degradation of wheat arabinoxylan showed that substituents are rondomly distributed and they are probably interrupted by 6 or more adjacent unsubstituted xylose residues.

Side chains have a positive effect of increasing solubility of hemicelluloses in aqueous solution (La Grange, 1999). Rice bran also contains a substantial quantity of arabinoxylan similar to that found in wheat. However as of having more branched structure wheat bran arabinoxylan is more soluble than that of rice bran (Wlliam *et al.*, 2000).

Corn cobs arabinoxylan molecule is a complex structure in which main side chain substituents, arabinose monomers, attach to xylan backbone at a position of O-2 and/or O-3. However, in wheat bran arabinoxylan molecule arabinose oligomers, consisting of two or more arabinofuranosyl residues, also linked to xylan backbone via 1-2, 1-3, and 1-4 linkages (Sandra *et al*., 2003). Moreover, solubility degree of corn cobs is between that of wheat bran end rice bran. Besides, being ground structures may negatively affect the release of some molecules which directly effect production and release of the enzyme.

In general wheat bran and rice bran have similar arabinoxylan structure and when they are supplemented in fermentation culture, media become quite viscous. However, in our experiments, when compared to wheat bran, xylanolytic enzyme production level on rice bran is significantly low. This may be explained by the solubility difference between these two arabinoxylans. As of being more soluble, wheat bran arabinoxylan is more accessible in fermentation culture and then, enzymes involved in arabinoxylan degradation can easily reach to substrate and enhance enzyme production. Although viscosity is a problem for wheat and rice bran containing cultures, unlike rice bran, the more soluble wheat bran arabinoxylan was able to overcome this problem and induced xylanolytic enzyme production. Not only solubility but also structure (presence of arabinooligo side chains) of the wheat bran arabinoxylan contributed enzyme production. Indeed, the highest level of AF was obtained with wheat bran. Moreover, induction power of corn cobs and wheat bran are similar in XYN production, and the highest XYN activities of 108 U/ml (on $4^{th}$ day) and 105 U/ml (on $7^{th}$ day) were obtained with corn cobs and wheat bran, respectively. Delay in fermentation period for production of maximum level of XYN in wheat bran, may due to viscous fermentation culture and resistance in enzyme diffusion.

Corn cob containing culture medium viscosity is low so high viscosity dependent diffusion problem was not present. Therefore, xylanolytic enzymes, produced in corn cob containing cultures, might attain their maximum values more quickly

than that of wheat and rice bran. However, corn cobs have granulated structure which may limit the diffusion of enzymes thorough substrates, and consequently negatively affect xylan hydrolysis and xylanolytic enzyme production.

In general, constitutive levels of hydrolytic enzymes produce small soluble 'signal' fragment which are able to enter cell and induce synthesis of corresponding polymeric substrate degrading enzymes. Accordingly, presence of constitutive xylanases degrades xylan to xylooligosaccharides and xylobiose which are taken up by the cell, consequently induce the other xylanase genes. Overall, different xylanases and other xylanolytic enzymes have different activities against various xylan structures (substrate specifity variation).

Therefore, substrate specifities and physicochemical properties of the xylanolytic enzymes are also other crucial factors determining arabinoxylan hydrolyses and enzyme production level. Accordingly, substrate specifity of the xylanolytic enzymes determine hydrolysis degree of various xylan molecules. For example, *Aureobasidium pullulans* and *Trichoderma longibrachiatum* xylanases are most effective on long chain xylans (19 and 8 xylose residues, respectively). Moreover, substituents on the xylan backbone have no impact on their hydrolytic rates. However, although *Thermatoga maritima* xylanase is also more active on a long xylan chain (greater than 19 xylose residues); whereas it's hydrolytic rate is significantly reduced by substituents on xylan (Liab *et al*., 2000). Furthermore, physicochemical properties of enzymes may also affect their access to substrate. Indeed, enzymes which easily diffuse in viscous medium can reach to substrate more quickly and initiate enzyme induction-production progress.

Overall, lignin composition of agricultural products has also impact on their hydrolysis and enzyme production intensity. Tenkanen and coworkers (1999) investigated the covalent bonds between residual lignin and polysaccharides in birch and pine kraft pulps by specific enzymatic treatments. According to results, residual lignin in birch kraft pulp is linked at least to xylan and a minor portion

may also be linked to cellulose. Moreover, in pine kraft pulp some of the residual lignin seemed to be linked to cellulose, glucomannan and xylan. The linkages between lignin and cellulose and hemicelluloses may be either native or formed during processing. The results also presented new information on the synergistic action of cellulose- and hemicellulose-degrading enzymes on pulp fibres.

#### 3.2.2.2 Effect of L-arabinose

The effect of L-arabinose on xylanolytic enzyme production level was also investigated in fermentation media containing 3% corn cobs. Accordingly, 0.5% and 1% arabinose were supplemented to fermentation culture and xylanolytic enzyme (AF, XYN, and GAL) syntheses were investigated. Then, the results were compared with those obtained in the control experiment without arabinose.

As shown in Figure 19, arabinose was utilized by the bacterium very efficiently. Cells consumed all the arabinose within 3 and 5 days depending on the arabinose concentration. The highest dry weights were obtained using 1% arabinose.

Arabinose is the readily utilized carbon sources for *B. pumilus* SB-M13. Therefore, when supplemented in fermentation culture it was consumed preferentially over complex carbon sources. As a result, xylanolytic enzyme productions were blocked as shown in Figures 20-22. As time passed, due to bacterial consumption, arabinose concentration decreased in fermentation culture which forced microorganism to synthesize xylanolytic enzymes and utilize xylan sources.

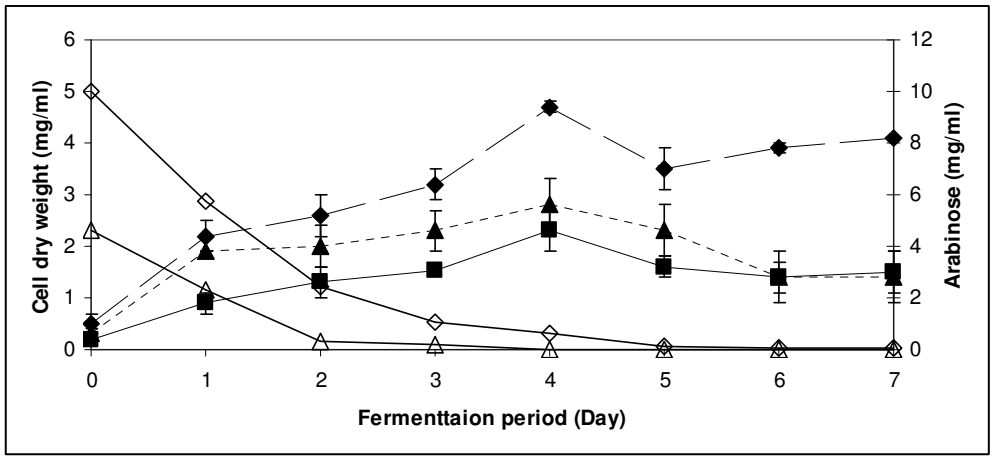


Figure 19. Effect of arabinose addition on *B. pumilus* SB-M1cultivation in 100-ml shake flask culture at 37°C, 175 rpm for 7 days. (■: 3% corn cobs, ◆: 3% corn cobs + 1% arabinose, ▲: 3% of corn cobs + 0.5% arabinose, arabinose in fermentation cultures; ◊: 1%, and Δ: 0.5% at the beginning).

Effect of arabinose on *B. pumilus* SB-M13 AF production was illustrated in Figure 20. According to the figure, very low level of AF synthesized during first 2-3 days of fermentation period. When 0.5% arabinose considered, bacterium consumed almost all of arabinose in fermentation culture within two-three days and AF synthesis increased sharply. On 3$^{rd}$ day of fermentation AF production level was same in both 0.5% arabinose-containing and arabinose-free cultures. After 3$^{rd}$ day of fermentation, AF production in 0.5% arabinose-containing culture was remarkably higher than that in arabinose-free cultures. However, during fermentation, AF production level in 0.1% arabinose-containing cultures was lower than that in both arabinose free and 0.5% arabinose containing cultures.

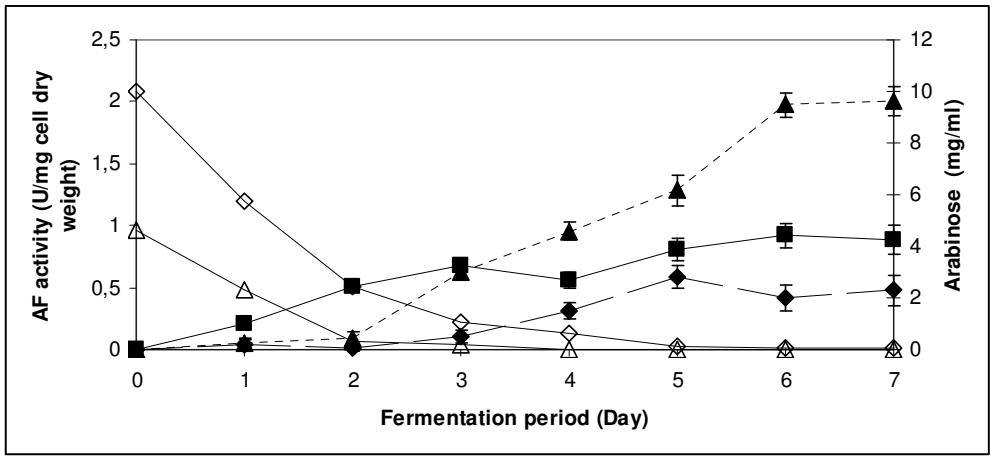


Figure 20. Effect of arabinose on the production of AF by *Bacillus pumilus* SB-M13. (■: 3% corn cobs, ◆: 3% corn cobs + 1% arabinose, ▲: 3% of corn cobs + 0.5% arabinose, arabinose in fermentation cultures; ◊: 1%, and Δ: 0.5% at the beginning). Activities were measured at 40°C at pH 7.0 using standard AF activity assay.

Figure 21 indicated the effect of arabinose on *B. pumilus* SB-M13 XYN production level. When compared to arabinose-free culture, at the beginning of fermentation, little amount of XYN was synthesized in both 0.5% and 1% arabinose containing cultures. Due to microbial consumption as arabinose concentration decreased, XYN synthesis increased. During fermentation period, XYN production level in 0.1% arabinose containing culture was lower that in arabinose-free one. However, due to arabinose consumption, XYN synthesis in 0.5% arabinose containing culture increased and on 6$^{th}$ day of fermentation it was rather higher than that in arabinose-free culture.

Suppression profile of arabinose on *B. pumilus* SB-M13 GAL production was similar to AF and XYN (Figure 22). As arabinose concentration decreased, suppression power of arabinose on GAL production was ceased in arabinose

containing cultures. Overall, on 4th , 5th, 6th and 7th day of fermentation, GAL production was the highest in 0.5% arabinose containing culture.

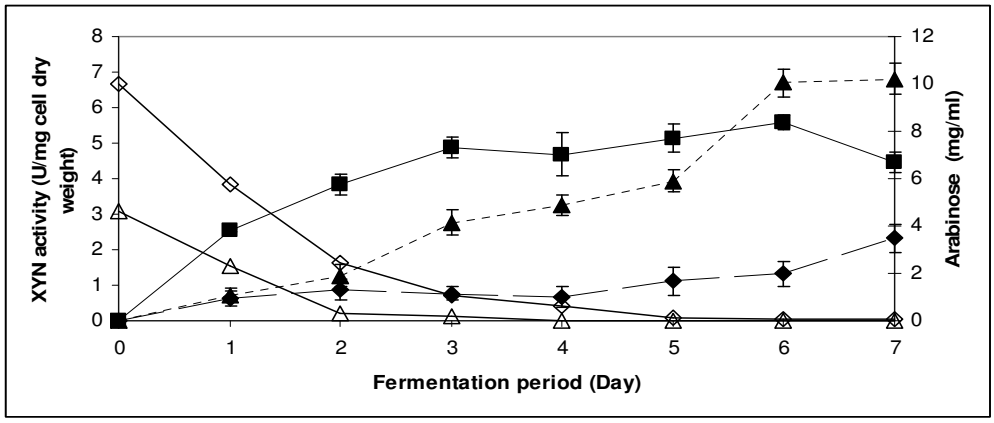


Figure 21. Effect of arabinose addition on the production of XYN by *Bacillus pumilus* SBM-13. (■: 3% corn cobs, ◆: 3% corn cobs + 1% arabinose, ▲: 3% of corn cobs + 0.5% arabinose, arabinose in fermentation cultures; ◊: 1%, and Δ: 0.5% at the beginning). Activities were measured at 40°C at pH 7.0 using standard XYN activity assay.

The results clearly indicated carbon catabolite repression of all *B. pumilus* SB-M13 xylanolytic enzymes. Hence, with addition of arabinose in fermentation culture, xylanolytic enzyme syntheses were suppressed at the beginning of the fermentation, but later as microorganisms preferentially consumed arabinose, suppression effect significantly decreased and enzyme productions took place

Duration and degree of suppression was dependent on arabinose concentration, and suppression impact of 1.0% arabinose was more effective than that of 0.5%. When 7th day of fermentations considered, all arabinose was consumed in 0.5% arabinose containing cultures, and when compared to arabinose-free cultures, AF,

XYN, and GAL activities were increased 2.2, 1.5, and 1.4 folds, respectively. However, 54%, 52%, and 37% activity decrease of AF, XYN, and GAL was obtained in 1% arabinose containing culture. Depending on enzyme production profiles, if the fermentation had been continued beyond the 7$^{th}$ day, xylanolytic enzyme titers might have been increased in 1% arabinose containing cultures.

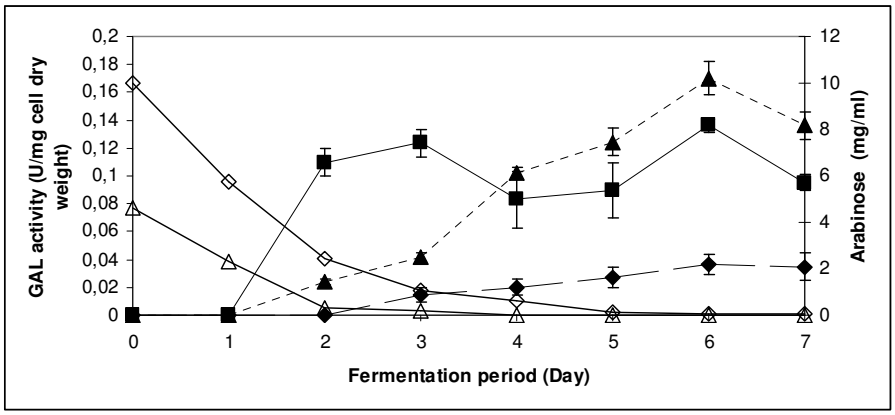


Figure 22. Effect of arabinose addition on the production of GAL by *Bacillus pumilus* SBM-13. (■: 3% corn cobs, ◆: 3% corn cobs + 1% arabinose, ▲: 3% of corn cobs + 0.5% arabinose, arabinose in fermentation cultures; ◊: 1%, and Δ: 0.5% at the beginning). Activities were measured at 40°C at pH 7.0 using standard GAL activity assay.

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Degrassie and coworkers (2003) investigated the effect of various carbon sources on *B. pumilus* PS213 AF production. They indicated that AF induction mechanism was different from those reported for other AFs and enzyme production was not induced by 1% of L-arabitol, L-sorbose, L-arabinose, or oat spelt xylan. However, in our study we clearly observed the significant induction power of the L-arabinose on *B.pumilus* SB-M13 AF (Figure 20). Indeed, L-arabinose (0.5%) was

able to increase AF activity by 2.2 fold. Furthermore, L-arabinose was also found to be the best inducer for other AFs in *Aureobasidium pullulans* (Saha and Bothast, 1998) and *B. stearothermophilus* (Gilead and Shoham, 1995), *Rhodothermus marinus* (Gomes *et al*., 2000).

### 3.3 Xylanolytic system of *Scytalidium thermophilum*

Xylanolytic system of *S. thermophilum*was was studied using the same conditions used for *B. pumilus* SB-M13. *S. thermophilum* was cultivated on 3% of ground corn cobs, wheat bran and rice bran, and production of xylanolytic enzymes were investigated in the culture supernatant up to 7 days using the standard assay conditions. Moreover, the effect of arabinose at a final concentration of 1.0% and 0.5% on induction of the xylanolytic enzyme production was also followed in the fermentation medium containing 3% of corn cobs as a sole carbon source.

#### 3.3.1 Time course of xylanolanolytic enzyme production by *S. thermophilum*

In this part of the experiments, *S. thermophilum* was cultivated in a fermentation medium containing 3% of ground corn cobs, wheat, and rice bran, and then xylanolytic enzyme production profile was investigated in culture supernatant up to 7 days using standard assay conditions.

When utilizing corn cobs, *S. thermophilum* produced all four extracellular xylanolytic (AF, XYN, GAL, and XYL) and one cellulolytic (GLU) enzyme (Figure 23, Table 8). Syntheses of all enzymes began with bacterial cultivation, and productions increased as fermentation period increased. Then, all xylanolytic enzymes and GLU reached their maximum values on $6^{th}$ and $7^{th}$ day of fermentation, respectively. However, unlike GLU, xylanolytic enzymes production level started to decrease in the following days. The parallelism in xylanolytic enzyme production indicates their possible synergistic action. Synergistic act of one or more xylanolytic enzymes may modify xylan molecules

which serve as better substrate (inducer) for others in the subsequent step in hydrolysis progress.

The XYN with maximum activity of 266 U/ml was the main enzyme in the fermentation culture, followed by XYL (40 U/ml), GAL (10 U/ml), and AF (3.3 U/ml). Moreover, a high level of GLU, involved in cellulose hydrolysis progress, was also observed in culture filtrate. It has maximum activity of 80 U/ml in 7$^{th}$ day of fermentation; its production profile gave the impression that enzyme production will be continued for the subsequent days.

Additionally, xylanolytic enzyme associated GLU synthesis may indicate the possible synergy between xylanolytic enzymes and GLU. Indeed, Tenkanen and coworkers (1999) reported that cellulose- and hemicellulose-degrading enzymes act synergistically on pulp fibers; consequently degradation of xylan clearly enhanced the action of enzymes on cellulose.

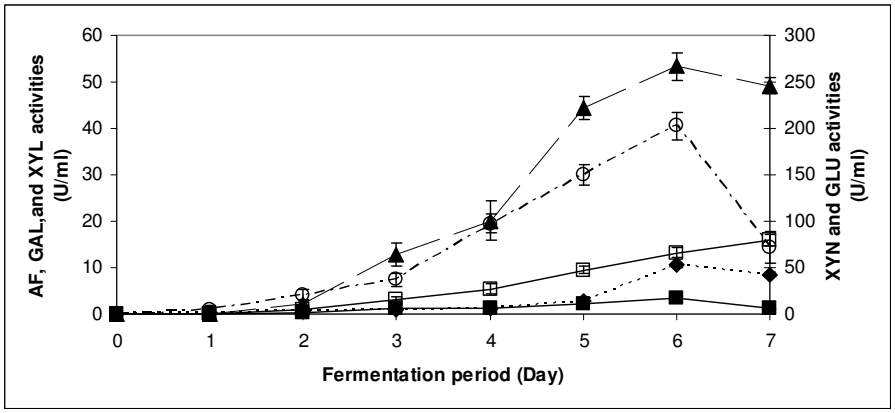


Figure 23. Xylanolytic enzyme production profiles of *S. thermophilum* grown on 3% corn cobs as a sole carbon source and inducer. The fungus was shake-cultured at 45°C, 155 rpm for 7 days. Enzyme activities were measured using standard assay at 60°C and pH 7.0. (Enzyme activities; ■: AF, ◆:GAL, ▲:XYN, ○: XYL □: GLU).

Table 8. Xylanolytic activities in culture filtrate of *Scytalidium thermophilum*.

| Enzymes | Enzyme production initiation on (Day) | Maximum enzyme production on (Day) | Maximum enzyme activity (U/ml) |
|---|---|---|---|
| **α-L-arabinofuranosidase** | | | |
| *Corn cobs* | 1 | 6 | 3.3 ± 0.3 |
| *Wheat bran* | 4 | 6 | 0.2 ± 0.0 |
| *Rice bran* | 4 | 6 | 0.3 ± 0.0 |
| **β-galactosidase** | | | |
| *Corn cobs* | 1 | 6 | 10.0 ± 00 |
| *Wheat bran* | 1 | 7 | 1.0 ± 0.0 |
| *Rice bran* | 1 | 7 | 5.0 ± 0.5 |
| **Endo-β-xylanase** | | | |
| *Corn cobs* | 1 | 6 | 266 ± 15.0 |
| *Wheat bran* | 1 | 6 | 61 ± 6.5 |
| *Rice bran* | 1 | 6 | 77 ± 11.0 |
| **β-xylosidase** | | | |
| *Corn cobs* | 1 | 6 | 40.0 ± 3.0 |
| *Wheat bran* | 1-4 | 5 | 3.5 ± 0.0 |
| *Rice bran* | 1-4 | 6 | 2.4 ± 1.0 |
| **β-glucosidase*** | | | |
| *Corn cobs* | 1 | 7 | 80.0 ± 6.0 |
| *Wheat bran* | 1 | 6 | 17.0 ± 3.0 |
| *Rice bran* | 1 | 6 | 16.0 ± 3.0 |

* Enzyme was involved in cellulose degradation, n.d; activity was not detected in crude enzyme extract under standard assay conditions.

When wheat bran was used as the sole carbon source in the fermentation medium, it was found that *S. thermophilum* xylanolytic enzymes productions were sequential (Figure 24, Table 8). XYN (1st- 4th day), GAL (1st day), and GLU (1st

day), were the preliminary enzymes determined in wheat bran containing culture filtrate, followed by AF (4th day) and XYL (4th day). As predicted, XYN was the main enzyme in culture filtrate, followed by GLU, XYL≈AF, and GAL.

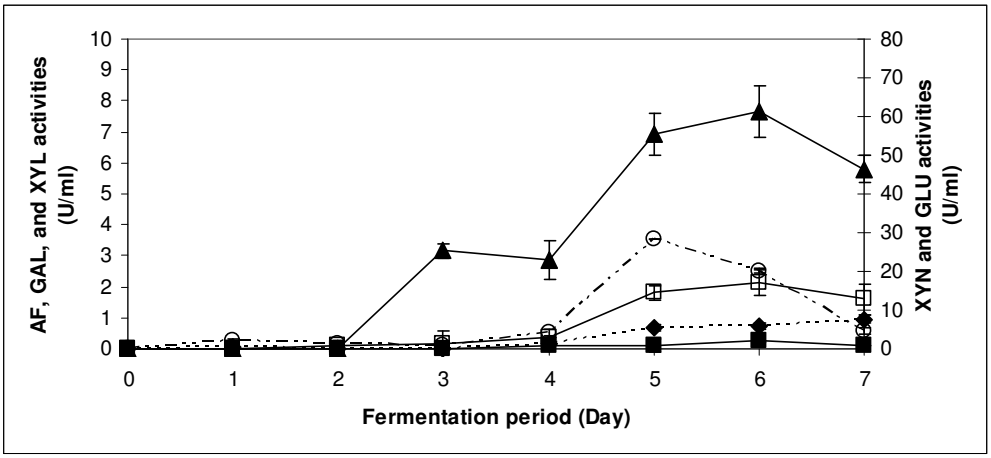


Figure 24. Xylanolytic activities in crude enzyme of *S. thermophilum* grown on 3% wheat bran as a sole carbon source and inducer. The fungus was shake-cultured at 45°C, 155 rpm for 7 days. Enzyme activities were measured using standard assay at 60°C and pH 7.0. (Enzyme activities; ■: AF, ◆:GAL, ▲:XYN, ○: XYL □: GLU).

XYL synthesis reached it maximum level of 3.5 U/ml on 5th day of cultivation. Then, the maximum level of AF (0.2 U/ml), XYN (61 U/ml) activities were obtained in 6th day of fermentation culture. Finally, GAL was produced and its maximum activity was measured as 1 U/ml on the 7th day. Although the enzyme productions were not parallel, enzyme production profiles showed the possible synergy among these enzymes. Attaining maximum XYL activity prior to XYN may indicate that, increase in xylooligosaccharides (inducer of XYN) titer

enhanced the production of XYN. Moreover, primary XYN, GAL and GLU syntheses might induce the AF and XYL in the next stage, and then they might induce production of others in the subsequent periods (i.e., GAL production). Indeed, except GAL, enzyme production diminished after 6$^{th}$ day.

GLU production, beginning with cultivation, reached maximum level of 17 U/ml on 6$^{th}$ day of fermentation, whereas next day it gradually decreased to 13 U/ml. Moreover, as explained for corn cobs, xylanolytic enzyme associated GLU synthesis may indicate the possible synergy between xylanolytic enzymes and GLU in wheat bran hydrolysis.

When grown on rice bran, *S. thermophilum* produces five of the enzymes at the beginning of fermentation, except AF which was synthesized on 4$^{th}$ day cultivation (Figure 25). Moreover, very minute amount of XYL was synthesized within the first 4 days of fermentation but enzyme titer became detectable after the 4$^{th}$ day. Unlike XYN, syntheses of rest of the enzymes were gradual. Moreover, all the enzymes reached their maximum values on 6$^{th}$ day, except GAL. The maximum GAL activity of 5 U/ml was obtained on the 7$^{th}$ day and according to production profile, GAL seemed to be produced more in the succeeding days. This situation can be explained by synergistic act of the xylanolytic enzymes to generate better substrate for GAL synthesis.

The maximum XYN activity of 77 U/ml was attained in the rice bran containing culture filtrate, and maximum activities of GLU, GAL, XYL, and AF were calculated as 16 U/ml, 5 U/ml, 2.4 U/ml, and 0.3 U/ml, respectively. When compared to wheat bran, induction power of rice bran was similar to that of wheat bran, whereas GAL which is significantly higher in rice bran containing culture. Moreover, corn cobs were the best inducer among the other agricultural by-products for synthesis of respective enzymes.

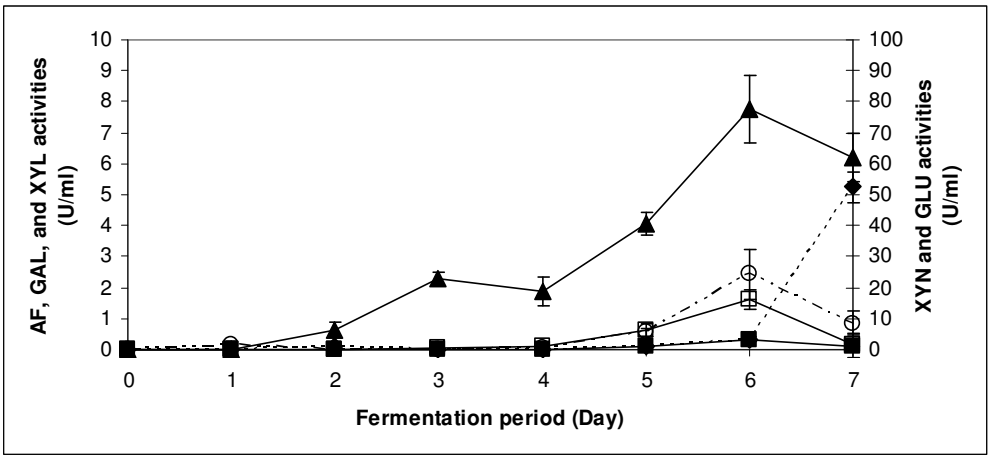


Figure 25. Xylanolytic activities in crude enzyme of *S. thermophilum* grown on 3% rice bran as a sole carbon source and inducer. The fungus was shake-cultured at 45°C, 155 rpm for 7 days. Enzyme activities were measured using standard assay at 60°C and pH 7.0. (Enzyme activities; ■: AF, ◆:GAL, ▲:XYN, ○: XYL □: GLU).

### 3.3.2 Effect of carbon source on xylanolytic enzyme production-induction

#### 3.3.2.1 Effect of agricultural by-products

In this study, *S. thermophilum* xylanolytic enzyme induction power of 3% of corn cobs, wheat bran, and rice bran was determined (Table 8).

All carbon sources induced syntheses of xylanolytic enzymes (AF, XYN, XYL, and GAL) at varying degree. As expected, XYN was the main extracellular enzyme produced in all fermentation cultures (Figure 26). XYN induction power of wheat bran (61 U/ml) and rice bran (77 U/ml) were similar but it was considerably lower than that of corn cobs (266 U/ml).

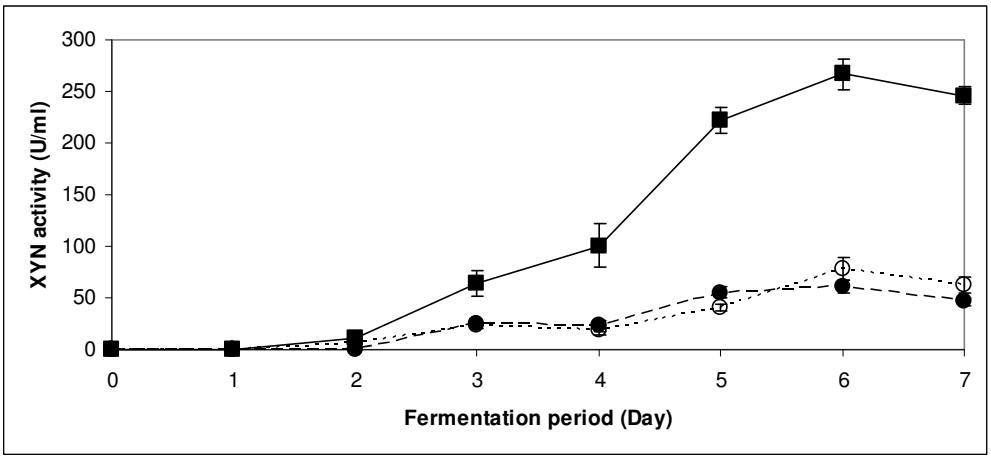


Figure 26. Effect of carbon sources on the production of XYN by *S. thermophilum*. (■: 3% corn cobs, ●: 3% wheat bran, ○: 3% rice bran). XYN activities were measured at 60°C at pH 7.0 using standard XYN activity assay.

When AF production was considered, the highest AF activity of 3.3 U /ml was obtained on 6$^{th}$ day of fermentation containing corn cobs (Figure 27 and Table 8). When the carbon source was replaced with wheat bran and rice bran, the AF activities decreased to 0.2 U/ml and 0.3 U/ml, respectively. Accordingly, corn cobs were the best inducer for AF production, and low level AF productions were obtained with wheat and rice bran.

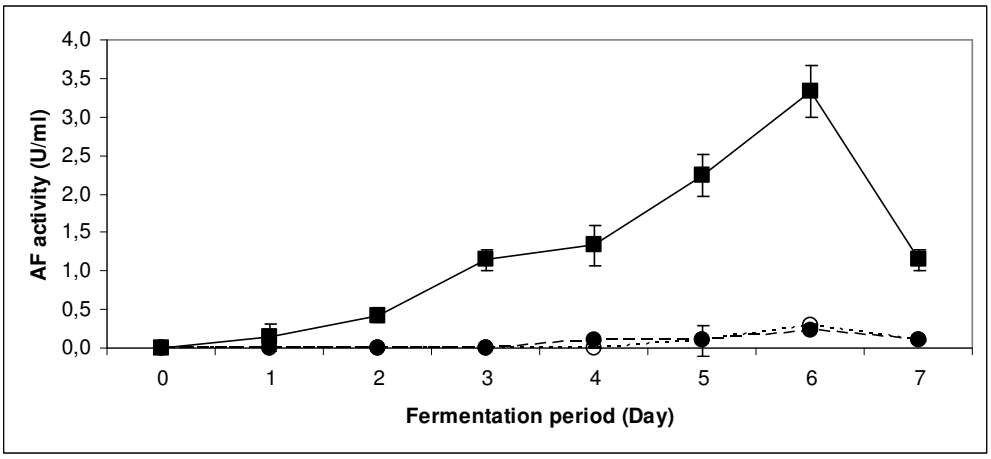


Figure 27. Effect of carbon sources on the production of AF by *S. thermophilum*. (■: 3% corn cobs, ●: 3% wheat bran, ○: 3% rice bran). AF activities were measured at 60°C at pH 7.0 using standard AF activity assay.

Although XYL production was the highest with corn cobs (40 U/ml), it was significantly poor with wheat bran (3.5 U/ml) and rice bran (2.4 U/ml) (Figure 28). Besides, GAL induction efficiency of the carbon sources from high to low as follows; corn cobs (10 U/ml), rice bran (5 U/ml), and wheat bran (1U/ml) (Figure 29, Table 8).

GLU, enzyme involved in cellulose hydrolysis progress, were also measured in all fermentations (Figure 30). GLU production efficiency of the corn cobs (80 U/ml) was the best, whereas that of wheat bran (17 U/ml) and rice bran (16 U/ml) were similar but notably lower.

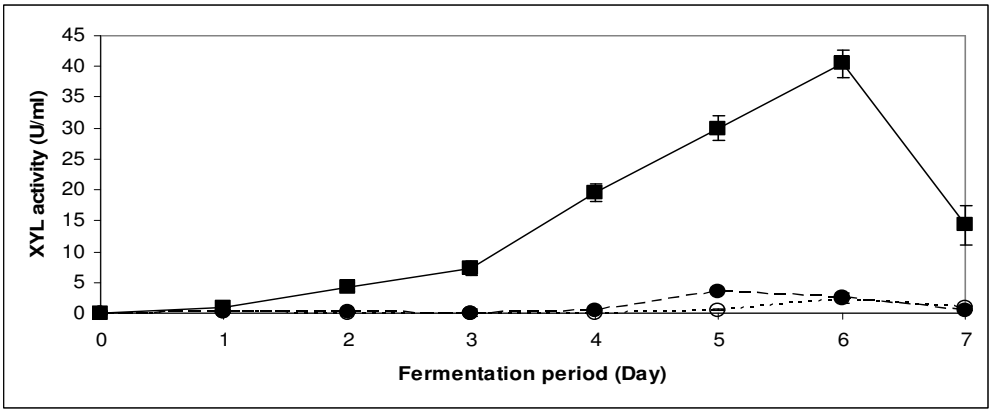


Figure 28. Effect of carbon sources on the production of XYL by *S. thermophilum*. (■: 3% corn cobs, ●: 3% wheat bran, ○: 3% rice bran). XYL activities were measured at 60°C at pH 7.0 using standard XYL activity assay.

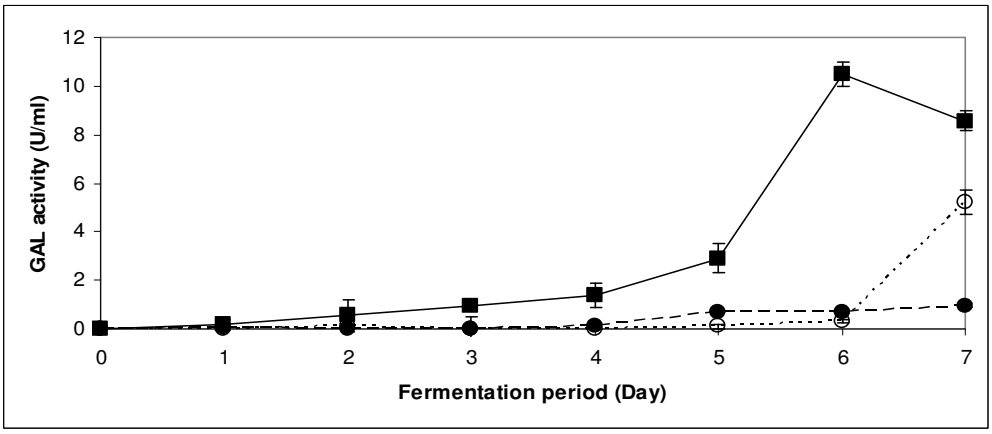


Figure 29. Effect of carbon sources on the production of GAL by *S. thermophilum*. (■: 3% corn cobs, ●: 3% wheat bran, ○: 3% rice bran). GAL activities were measured at 60°C at pH 7.0 using standard GAL activity assay.

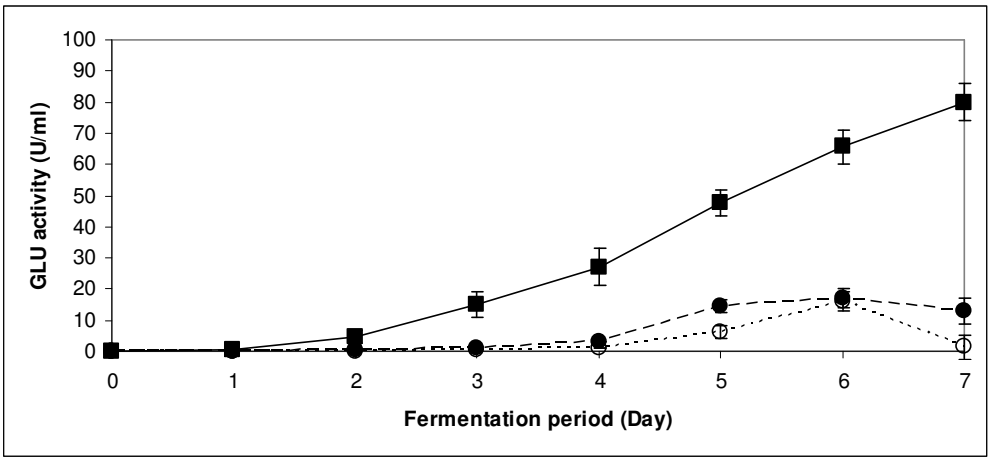


Figure 30. Effect of carbon sources on the production of GLU by *S. thermophilum*. (■: 3% corn cobs, ●: 3% wheat bran, ○: 3% rice bran). GLU activities were measured at 60°C at pH 7.0 using standard GLU activity assay.

In literature, there are many xylanolytic enzymes from *Scytalidium thermophilum* investigated. For example, several endoxylanases have been isolated from *Scytalidium thermophilum* type culture *Humicola insolens* by Düsterhöft and coworkers (1997).

Moreover, *S. thermophilum* β-xylosidase was also studied. Zanoelo (2004) reported the cellular distribution of *S. thermophilum* β-xylosidase in fermentation cultures containing 1% xylan. Accordingly, after 24 h growth, 69% of the enzyme was surface bound, and could be partially removed by s succeeding washings with water or buffer. Twelve percent of the enzyme stayed adsorbed to cell wall, whereas 19% was detected only after cell wall disruption. Therefore, Zanoelo (2004) suggested a physiological role for this enzyme in the final steps is xylan degradation which support the studies of Erikkson and coworkers (1990). They suggested that, unlike bacteria, fungi produce β-xylosidases which remains cell-associated during early stages of growth but may be secreted during later stages.

Moreover, Zanoelo (2004) also assessed effect of several sugars on β-xylosidase synthesis, and the highest levels of enzyme activity were obtained with xylan as the main carbon sources, followed by starch, avicel (crystalline cellulose), CM-cellulose, and xylose; whereas very low level of both *S. thermophilum* growth and β-xylosidase production induction were obtained with sucrose. Indeed, in our experiments, *S.thermoplium* produced minute amount β-xylosidase in wheat bran and rice bran containing cultures (extracellular β-xylosidase) during first 4 day of fermentation, but later, enzyme production level, significantly increased. Although induction efficiency of corn cobs was notably higher than that of wheat and rice bran, similar enzyme production profiles were obtained with corn cobs.

Zaneolo and coworkers (2004a) also showed that after 48 h growth, about 87% of the β-glucosidase activity was surface bound and 13% of it was detected only after cell wall disruption (intracellular), and with aging the enzyme was partially released in the culture medium. Moreover, in our experiments, time dependent GLU titer increment was definitely remarkable in corn cobs containing cultures.

Studies covering cellulase (Kaur *et al*., 2005) and endogulucanase (Ögel *et al*., 2001) production from *S. thermophium* have also been reported. However, no data have been reported in the topic of *S.thermophilum* AF production, purification and characterization studies, yet.

When *S. thermophilum* was cultivated in a fermentation medium containing 3% of corn cobs, wheat bran, and rice bran as a sole carbon sources, extracellular xylanolytic enzyme production profile showed variation. As discussed in the Bacillus part hemicellulose and xylan content, xylan accessibility including particle size, lignin and xylan structure and composition of the ground agricultural by-products, solubility of the arabinoxylan molecules, viscosity of the fermentation medium were the key parameters concluding xylanolytic enzyme production profile (general introduction, 1.2.2).

Xylanolytic enzyme production system of S. *thermophilum* was also assessed as followings. In general, 3% of all carbon sources (corn cobs, wheat bran, and rice bran) induced production of four xylanolytic (XYN, AF, XYL, and GAL) and one cellulolytic (GLU) enzymes at various degrees. Corn cobs were the best carbon source among the others and consequently, the highest enzyme activities were attained with corn cobs.

Theoretically, wheat bran, having the oligoarabinose side chains, was supposed to induce AF production better than the other two substrates. Although, there is a structure similarity between wheat and rice bran, when compared to rice bran, due to the rather higher solubility, the higher induction power was expected for wheat bran.

Although wheat bran arabinoxylan was more soluble and accessible in the culture, when compared to corn cobs, very low level of enzyme productions was observed. Viscosity is high in both wheat and rice bran containing *S. thermophilum* cultures. When wheat bran was replaced with rice bran in fermentation culture, roughly similar low level of enzymes production was obtained. The situation can be explained by physicochemical properties of the enzymes themselves. Indeed, enzyme molecules which can easily diffuse in viscous medium can reach to substrate more quickly and initiate enzyme induction-production . Accordingly we may conclude that when compared to *B. pumilus* SB-M13, S. *thermophilum* xylanolytic enzymes have diffusion restriction against viscous medium, can not overcome viscosity problem and reach substrate. Then, low level of enzyme production was obtained for both wheat and rice bran containing fermentation cultures.

Although, corn cobs have granulated structure which may limit the diffusion of enzymes through the substrates, corn cobs-containing cultures were of low viscosity and lack of viscosity dependent diffusion problem. Accordingly, the highest level of enzyme production was attained in corn cobs containing cultures.

Moreover, substrate specifities of the xylanolytic enzymes are also other crucial factors determining arabinoxylan hydrolyses and enzyme production level (general introduction, 1.2.4). Accordingly, substrate specifity of the xylanolytic enzymes determine hydrolysis degree of various xylan molecules. Accordingly, different xylanases and other xylanolytic enzymes have different activities against various xylan structures (substrate specifity variation).

Overall, lignin composition and its interaction with other cell wall components of agricultural products have also impact on xylan hydrolysis and enzyme production intensity.

In summary, followings can be interpreted for xylanolytic enzyme production systems of *B. pumilus SB-M13* and *S. thermophilum*.

When grown on 3% of corn cobs, wheat bran, and rice bran containing fermentation cultures, *B. pumilus* produced extracellular, AF, XYN, and GAL at varying levels. However, there were no XYL and GLU activities measured in culture filtrate. Actually, bacterial β-xylosidases are intracellular and not secreted (La Grange *et al*., 1999). Absence of extracellular XYL activities in culture filtrate is an advantage in xylooligosaccharide production, because, culture filtrate can directly be used without any enzyme purification.

Cellulose degrading enzymes-free xylanase preparations is very important for applications in the paper and pulp industry. *B. pumilus* SB-M13 has very low level of 0,003 FPU cellulase activity (Biran *et al*, 2006). Moreover, in our study it was also found that there was no β-glucosidase in any of the fermentation culture. Therefore, crude enzyme extracts of all fermentation culture can directly be used in bleaching pulp.

When *B. pumilus* SB-M13 xylanolytic enzyme induction effciency of corn cobs, wheat bran, and rice bran were compared, wheat bran was the best carboun source

by which maximum levels of AF (5 U/ml), GAL (0.3 U/ml), and XYN (105 U/ml) were produced. It should also be noted that corn cobs were also able to induce same level of XYN (108 U/ml) as wheat bran did.

When utilizing 3% of corn cobs, wheat bran, and rice bran, *S. thermophilum* produced four xylanolytic (XYN, AF, XYL, and GAL) and one cellulolytic (GLU) extracellular enzymes at various degrees. Corn cobs were the best carbon source among the others and consequently, the highest enzyme activities were attained with corn cobs. Accordingly, The XYN with maximum activity of 266 U/ml was the main enzyme in the fermentation culture, followed by GLU (80 U/ml), XYL (40 U/ml), GAL (10 U/ml), and AF (3.3 U/ml).

Unlike bacteria, fungi produce β-xylosidases which remains cell-associated during early stages of growth but may be secreted during later stages (Eriksson *et al*., 1990). Indeed, *B. pumilus* SB-M13 does not produce XYL, whereas, *S. thermophilum*, thermophilic fungus, produce extracellular XYL. Presence of extracellular XYL is a limitation in xylooligosaccharide production, because it hydrolyzes xylooligosaccharides into xylose monomer. Therefore, *S. thermophilum* crude enzyme extract can not be directly used in xylooligosaccharide production. However, it can be used for xylose production more efficiently than *B. pumilus* SB-M13.

*S. thermophilum* XYN production level (266 U/ml) is almost 2.5 times higher than that of *B. pumlis* SB-M13 (108 U/ml). On the other hand, production of high level of GLU (80 U/ml) restricts direct use of crude enzyme extracts in bleaching pulp.

Besides, *B.pumilus* SB-M13 AF (5 U/ml) production degree was higher than *S. thermophilum* (3.3 U/ml). Moreover, both enzymes can be used in in food industry for L-arabinose production. Recently, interest in L-arabinose has been increasing, because of its low uptake by body, sweet taste, and food additive potential (Rahman *et al*., 2003).

### 3.2.2.2 Effect of arabinose

Unlike *B. pumilus*, SB-M13, *S. thermophilum* was cultivated poorly on arabinose (Figure 31). Presence of arabinose in fermentation culture also suppressed the AF, XYN, GAL, XYL, and GLU activities (Figures 32, 33, 34, 35 and 36), as well. In conclusion, arabinose decreased both growth and xylanolytic enzyme syntheses which are under the control of carbon repression.

Some reports also indicated that growth profile of *S. thermophilum* was determined by carbon sources. For example, Zanoelo (2004) reported that unlike glucose, xylose and fructose, *S. thermophilum* grow very ineffectively on sucrose. Moreover, it was also indicated that presence of 1% glucose in 1% xylan containing culture supernatant, drastically reduced the levels of mycelial β-xylosidase activity, suggesting that *S. thermophilum* β-xylosidase (xylanolytic enzyme) production was dependent on carbon source repression.

Although syntheses of all enzymes were suppressed by arabinose (1% and 0.5%), suppression of GAL syntheses was the most dramatic, followed by GLU. Moreover, duration and degree of suppression was dependent on concentration of arabinose in fermentation culture, and due to consumption associated profile suppression impact of 1.0% arabinose was more effective than that of 0.5%.

In many microorganisms, utilization of polymeric substrates is regulated by presence of the more easly metabolizable carbon sources, and therefore dependent on various mechanisms of carbon control. The carbon catabolite control of xylanases (Pinaga *et al*., 1994; De Graaff *et al*., 1994), and arabinase formation by *Aspergilli* and *Trichoderma reesei* (Mach *et al*., 1996) have been documented.

When effect of arabinose on *B. pumilus* SB-M13 and *S. thermoplium* growth and xylanolytic enzymes was considered, it was found that unlike *S. thermophilum*, arabinose was efficiently consumed by *B. pumilus* SB-M13 which caused increase in *B. pumlus* SB-M13 biomass in fermentation culture. Both *S. thermophilum* and

*B. pumilus* SB-M113 xylanolytic enzymes were under the control of carbon catabolite repression. However, when compared to *S. thermophilum*, due to efficient and rapid consumption, suppression effect of arabinose on *B. pumilus* SB-M13 xylanaolytic enzyme synthesis was relieved quickly.

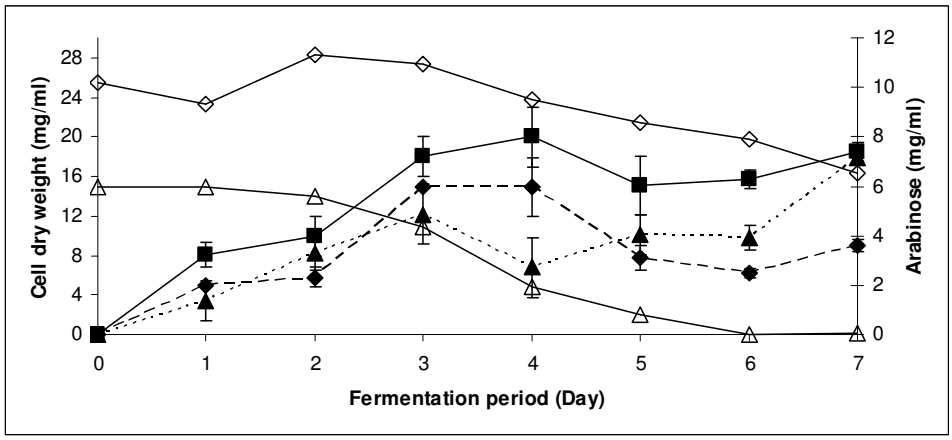


Figure 31. Effect of arabinose addition on *Scytalidium thermophilum* cultivation in 100-ml shake flask culture at 45°C, 155 rpm for 7 days. (■: 3% corn cobs, ◆: 3% corn cobs + 1% arabinose, ▲: 3% of corn cobs + 0.5% arabinose, arabinose in fermentation cultures; ◊: 1%, and Δ: 0.5% at the beginning).

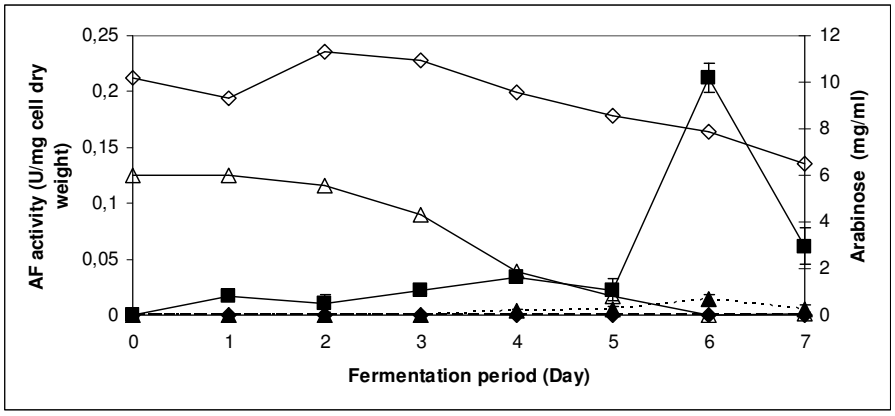


Figure 32. Effect of arabinose addition on the production of AF by *Scytalidium thermophilum*. (■: 3% corn cobs, ◆: 3% corn cobs + 1% arabinose, ▲: 3% of corn cobs + 0.5% arabinose, arabinose in fermentation cultures; ◊: 1%, and Δ: 0.5% at the beginning). Activities were measured at 60°C at pH 7.0 using standard AF activity assay.

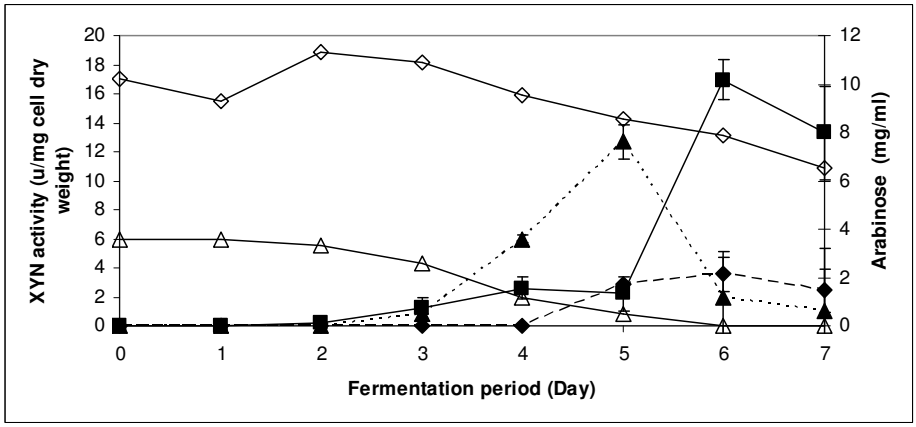


Figure 33. Effect of arabinose addition on the production of XYN by *Scytalidium thermophilum*. (■: 3% corn cobs, ◆: 3% corn cobs + 1% arabinose, ▲: 3% of corn cobs + 0.5% arabinose, arabinose in fermentation cultures; ◊: 1%, and Δ: 0.5% at the beginning). Activities were measured at 60°C at pH 7.0 using standard XYN activity assay.

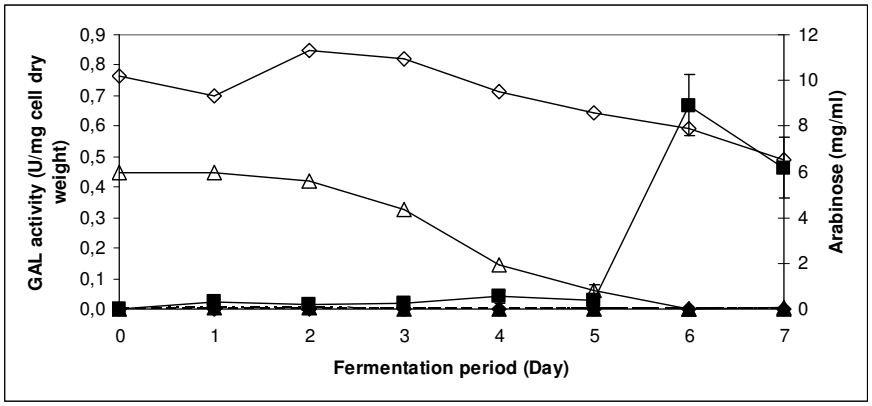


Figure 34. Effect of arabinose addition on the production of GAL by *Scytalidium thermophilum*. (■: 3% corn cobs, ◆: 3% corn cobs + 1% arabinose, ▲: 3% of corn cobs + 0.5% arabinose, arabinose in fermentation cultures; ◊: 1%, and Δ: 0.5% at the beginning). Activities were measured at 60°C at pH 7.0 using standard GAL activity assay.

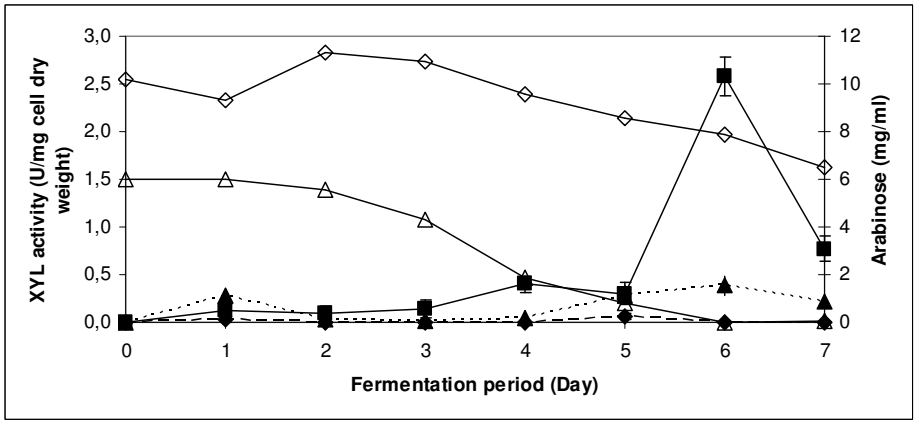


Figure 35. Effect of arabinose addition on the production of XYL by *Scytalidium thermophilum*. (■: 3% corn cobs, ◆: 3% corn cobs + 1% arabinose, ▲: 3% of corn cobs + 0.5% arabinose, arabinose in fermentation cultures; ◊: 1%, and Δ: 0.5% at the beginning). Activities were measured at 60°C at pH 7.0 using standard XYL activity assay.

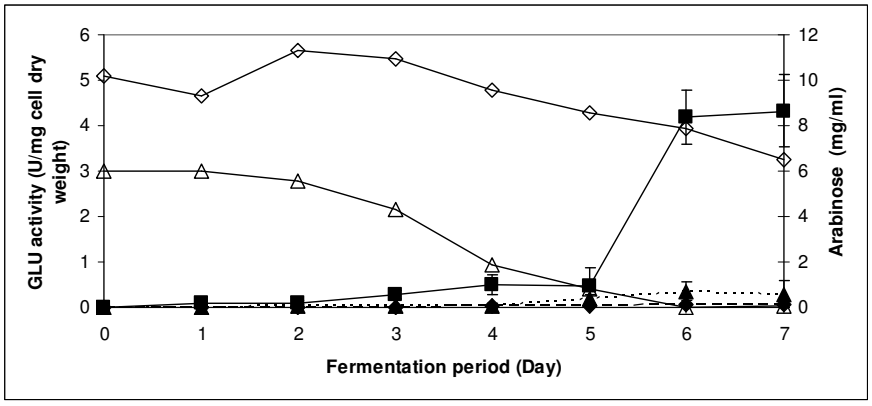


Figure 36. Effect of arabinose addition on the production of GLU by *Scytalidium thermophilum*. (■: 3% corn cobs, ◆: 3% corn cobs + 1% arabinose, ▲: 3% of corn cobs + 0.5% arabinose, arabinose in fermentation cultures; ◊: 1%, and Δ: 0.5% at the beginning). Activities were measured at 60°C at pH 7.0 using standard GLU activity assay.

## REFERENCES


Avcioglu, B., Eyupoglu, B., and Bakir, U., 2005, "Production and characterization of xylanases of *Bacillus* strain isolated from soil", *World Journal of Microbiology and Biotechnology*, Vol. 21, pp. 65-68.

Bakir, U., Yavascaoglu, S., Guvenc, F., and Ersayin, A., 2001, "An endo-β-1,4-xylanase from *Rhizopus oryzae*: production, partial purification and biochemical characterization", *Enzyme Microbial Technology*, Vol. 29(6-7), pp. 328-334.

Bieley et al., 1992, cited by Hespell, R.B., and Cotta, M.A, 1995, "Degradation and utilization by *Butyrivibrio fibrisolvens* H17c of xylans with different chemical and physical properties", *Applied and Environmental Microbiology*, Vol. 61, pp. 3042–3050.

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Biran, S., 2001, "Aqueous two-phase separation of xylanases produced by a *Bacillus* sp.", MS thesis, Middle East Technical University, Ankara, Turkey.

Biran, S., Ersayin, A., Kocabas, A., and Bakir, U., 2006, "A novel xylanase from a soil isolate, *Bacillus pumilus*: Production, purification, characterization and one-step separation by aqueous-two-phase system" *Journal of Chromatography*, in rewiev.

Brillouet., 1987, cited by Hespell, R.B., and Cotta, M.A, 1995, "Degradation and utilization by *Butyrivibrio fibrisolvens* H17c of xylans with different chemical and physical properties", *Applied and Environmental Microbiology*, Vol. 61, pp. 3042–3050.

Choct and Annison, 1990, cited by William, P.E.V., Geraert, P.A., Uzu, G., and Annison, G., 2000, "Factors affecting non-starch polysaccharide digestibility in poultry", *CIHEAM-Options Mediterraneennes*.

Debeire, P., Priem, B., Strecker, G., and Vigonan, 1990, "Purification and properties of an endo-1,4-xylanase excreted by a hydrolytic thermophilic anaerobe, *Clostridium thermolacticum*. A proposal for its action mechanism on larchwood 4-O-methylglucuronoxylan", *European Jounal of Biochemistry,* Vol. 187, pp. 573-575.

De Graaf, L., Van Den Broek, Van Ooijen, A.J.J., and Visser, J., 1994, "Regulation of the xylanase-encoding xlnA gene of *Aspergillus tubigensis*", *Molecular Microbiology*, Vol. 12, pp. 479-490.

Degrassi, G., De Laureto, P.P., and Bruschi, C.V., 1995, "Purification and characterization of ferulate and p-coumarate decarboxylase from *Bacillus pumilus*", *Applied and Environmental Microbiology*, Vol. 61, pp. 326-332.

Degrassi, G., Vindigni, A., and Venturi, V., 2003, "A thermostable α-arabinofuranosidase from xylanolytic *Bacillus pumilus*: purification and characterization", *Journal of Biotechnology*, Vol. 101, pp. 69-79.

Duarte, M.C.T., Da Silva, E.C., Gomes, I.S.B., Ponezi, A.N., Portugal, E.P., Vicente, J.R., and Davanzo, E., 2003, "Xylan-hydrolysing enzyme system from *Bacillus pumilus* CBMAI 0008 and its effects on *Eucalyptus grandis* kraft pulp for pulp bleaching improvement", *Biosource Technology*, Vol. 88, pp. 9-15.

Düsterhöft, E.M., Linssen V.A.J.M., Voragen, A.G.J., and Beldman, G., 1997, "Purification, characterization, and properties of two xylanases from *Humicola inolens", Enzyme and Microbial Technology*, Vol. 20, pp. 437-445.

Erikkson, K.E.L., Blanchette, R.A., and Ander., P., 1990, *In Microbial and enzymatic degredation of wood and wood components*, Springer-Verlag,NY.

George, S. P., 2000, cited by Nath, D.S., 2005, "Studies on xylan degrading enzymes from an alkalophilic thermophilic *Bacillus* sp.", PhD thesis, Biochemical Sciences Division, National Chemical Laboratory, Pune

Gilead, S., and Shoham, Y., 1995, "Purification and characterization of α-L-arabinofuranosidase from *Bacillus stearothermophilus* T-6", *Applied and Environmental Microbiology*, Vol. 61, pp. 170-174.

Gomes, J., Gomes, I., Terler, K., Gubala, N., Ditzelmüller, G., and Steiner, W., 2000, "Optimization of culture medium and conditions for α-L-arabinofuranosidases production by the extreme thermophilic eubacterium *Rhodotermus marinus", Enzyme and Microbial Technology*, Vol. 27, pp. 414-422.

Hueck, C.J., and Hillen, W., 1995, "Catabolite repression in *Bacillus subtilis*: a global regulatory mechanism for the gram positive bacteria", *Moleculer Microbiology*, Vol. 15, pp. 395-401.

Kadowaki, M.K., Souza, C.G.M., Simão, R.C.G., and Peralta, R.M., 1996, "Xylanase production by *Aspergillu tamari", Applied Bichemistry and Biotechnology*, Vol. 66, pp. 97-106.

Keskar, S.S., 1992, cited by Nath, D.S., 2005, "Studies on xylan degrading enzymes from an alkalophilic thermophilic *Bacillus* sp.", PhD thesis, Biochemical Sciences Division, National Chemical Laboratory, Pune.

Khoodoo, M.H.R., Sahin, F., Donmez, M.F., and Fakim, Y.J., 2005, "Molecular characterization of *Xanthomonas* strains isolated from aroids in Mauritus", *Systematic and Applied Microbiology*, Vol. 28, pp. 366-380.

Kocabas**,** E.E., and Dizbay, M., 1999, " *Bacillus stearothermophilus* var. calidolactis C953'ten a-galaktosidaz üretimi", *Turkish Journal of Biology*, Tubitak, Vol. 23, pp. 47-54.

La Grange, D.C., 1999, "Genetic engineering of the yeast *Saccharomyces cerevisiae* to degrade xylan", Ph.D Thesis, University of Stellenbosch.

Liab, K., Azadi, P., Collins, R., Tolan, J., Kim, J.S., and Erikkson, J.L., 2000, "Relationships between activities of xylanases and xylan structures", *Enzyme and Microbial Technology*, Vol. 27, pp. 89-94.

Mach, R.L., Strauss, J., Zeilinger, S., Schindler, M., and Kubicek, C.P., 1996, "Carbon catabolite repression of xylanase I (xyn1) gebe expression in *Trichoderma reesei*", *Molecular Microbiology*, Vol. 21, pp. 1273-1281.

Ögel, Z.B., Yaramgümeli, K., Dündar, H., and Ifrij, I., 2001, "Submerged cultivation of *Scytalidium thermophilum* on complex lignosellulosic biomass for endoglucanase production", *Enzyme and Microbial Technology*,Vol. 28, pp. 689-695.

Paavilainen, S., Hellman, J., and Korpela, T., 1976, "Purification, characterization, gene cloning, and sequencing of a new beta-glucosidase from *Bacillus circulans* subsp. alkalophilus", *Applied and Environmental Microbiology.,* Vol.6, pp. 807–812.

Pinega, F., Fernendez-Espinar, M.T., Valles, S., and Ramon, D., 1994, "Xylanse production in *Aspergillus nidulans*: induction and carbon catabolite repression", *FEMS Microbiology Letters*, Vol. 115, pp. 319-324.

Rahim**,** A.A.K., and Lee, B.H., 1991, "Specificity, inhibitory studies, and oligosaccharide formation by *p*-galactosidase from psychrotrophic *Bacillus subtilis* KL88", *Journal of Dairy Science*, Vol. 74, pp. 6.

Rahman, A.K.M.S, Kato, K., Kawaki, S., Takamizawa, K., 2003, " Substrate specifity of the α–L-arabinofuranosidase from *Rhizomucor pusillus* HHT-1", *Carbohydrate Research*, Vol.338, pp.1469-1476

Saha, B.C., and Bothast, R.J., 1998, "Purification and characterization of a novel thermostable α-L-arabinofuranosidase from a color-variant strain of *Aureobasidium pullulans", Applied and Environmental Microbiology,* Vol. 64, pp. 216-220.

Sandra, E., Chaplin, M.F., Blackwood, A.D., and Dettmar, P.W., 2003, "Primary structure of arabinoxylans of ispaghula husk and wheat bran", *Proceedings of the Nutrition Society,* Royal College of Physicians, Edinburgh, UK, Vol. 62, pp. 217-222.

Sasser M.J., 1990, "Identification of Bacteria by Gas Chromatography of Cellular Fatty Acids", Technical note 101, Microbial ID, Inc., Newark, De.

Saulnier *et al*., 1994, cited by Saha, B.C., 2000, "α-L-arabinofuranosidases: biochemistry, molecular biology and application in biotechnology, *Biotechnology Advances*, Vol. 18, pp. 403-423.

Schooneveld-Bergmans, M.E.F., Beldman, G., and Voragen, A.G.J, 1999, "Structural features of (Glucurono) arabinoxylans extracted from wheat bran by barium hydroxide", *Journal of Cereal Science*, Vol. 29, pp. 63-75.

Stülke, J, and Hillen W., 2000, "Regulation of carbon catabolism in *Bacillus* species", *Annual Review of Microbiology*, Vol. 54, pp. 849-880.

Tenkanen, M., Tamminen, T., and Hortling, B., 1999, "Investigation of lignin-carbohydrate complexes in kraft pulps by selective enzymatic treatments", *Applied Microbiology and Biotechnology*, Vol. 51, pp. 241-248.

Tuncer. M., 2000, “Characterization of β-xylosidase and α-L-arabinofuranosidase activities from *Thermomonaspora fusca* BD25”, *Turkish Journal of Biology*, Vol. 24, pp. 753-767.

Vyas, P., Chauthaiwale, V., Phadatare, S., Deshpande, V., and Srinivasan, M.C., 1990, “Studies on the alkalophilic *Streptomyces* with extracellular xylanolytic activity”, *Biotechnology Letters*, Vol. 12, pp. 225-228.

Zaneolo, F.F., 2004, “Purification and biochemical properties of a thermostable xylose-tolarant β-D-xylosidase from *Scytalidium thermophilum*”, *Journal of Industrial Microbiology and Biotechnology*, Vol. 31, pp. 170-176.

Zaneolo, F.F., Polizeli, M.L.T.M., Terenzi, H.F., and Jorge, J.A., 2004a, “β-glucosidase activity from the thermophilic fungus *Scytalidium thermophilum* is stimulated by glucose and xylose”, *FEMS Microbiology Letters*, Vol. 240, pp. 137-143.

# PART 2

## PRODUCTION, PURIFICATION and CHARACTERIZATION of XYLANASE FROM A SOIL ISOLATE *Bacillus pumilus* SB-M13

# CHAPTER 1

# INTRODUCTION

Numerous microorganisms were investigated for xylanolytic activity (Tables 3 and 4 in general introduction part). Xylanases from fungi are well documented and some of the xylanase producers are *Aspergillus* (Gulati *et al*., 2000), *Trichoderma* (Gomes *et al*., 1992), *Rhizopus* (Bakir *et al*., 2001), and *Penicillium* (Belancic *et al*., 1995). Fungal xylanases are active in neutral or acidic pH. On the other hand, bacterial xylanases generally have higher optimal pH and consequently, are stable at alkaline pHs which makes bacterial xylanases being more suitable for applications in the paper and pulp industry (Subramaniyan and Prema, 2000). The most studied xylanase producers among bacterial sources are *Bacillus* species due to their high yield and stability at alkaline pHs (Pham *et al*., 1998, Beg *et al*., 2001; Avcioglu *et al.,* 2005; Wong and Sandler, 1993; Bakir, 2004). The extensive use of enzymes in industrial applications necessitates the investigation of new xylanase producers.

## 1.1 Aim of the study

The aim of this study was to produce, purify and characterize xylanase suitable for paper-pulp utilization using a *Bacillus pumilus* isolate. Xylanase production was performed on 3% of corn cobs. Moreover, xylanase purification achievements of various liquid chromatographic systmes were assassed. Finally, the enzyme purified by a single step hydrophobic interaction chromatography was biochemically characterized and its kinetic parameters were determined.

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1 Materials

Birchwood xylan (Sigma, USA) was used as a substrate to measure xylanase activity. Agricultural byproducts were obtained from the local market or feed plants. Liquid chromatography columns purchased from Amersham Biosciences (Uppsala, Sweeden). Chemical reagents, markers and apparatus used in gel electrophoresis were supplied from BioRad and Sigma. Vivaspin centrifugal concentrators were used to concentrate protein samples (Sartorius, Germany). All other chemical reagents were analytical grade, obtained either from Merck or Sigma.

## 2.2 Xylanase production

Single colony of *Bacillus pumilus* SB-M13 on agar plate was precultured in 10-ml of liquid media containing (g/L): 0.3; steam hydrolyzed ground corn cobs as sole carbon source, 5.0; NaCl, 2.5; yeast extract, 1.0; $KH_2PO_4$, 2.0; $MgSO_4$ to which sterile 0.1; $Na_2CO_3$ added separately in 100-ml Erlenmeyer flask and incubated at 35°C for 24 hours at 275 rpm. Then, 100-ml of same fermentation media in 250-ml of shake flask was inoculated with preculture and fermentation was performed under same conditions for 4 days. Then, fermentation medium was centrifuged for 40 min at 11,000 x g and the supernatant was used as xylanase extract in all the experiments.

Corn cobs, used as sole carbon source in fermentation medium, were subjected to steam hydrolysis to facilitate microbial consumption (Bakir *et al*., 2000).

### 2.3 Xylanase assay

Xylanase (endo-β-xylanase) assay was performed according to Bailey *et al.* (1992) using 1% of birchwood xylan in 50 mM phosphate buffer at pH 7.0 Xylanase activities were determined using 1% Birchwood xylan in 50mM phosphate buffet at pH 7. The reaction mixtures contained 0.1 ml of properly diluted enzyme and 1.0 ml of substrate solution. The reaction mixtures were incubated at 40°C for *B. pumilus* SB-M13 and the samples were taken at 15 second intervals up to 1 min to measure the initial reaction rates. Dinitrosalicylic acid (DNSA) method (Miller, 1959) was used to determine reducing sugar concentration by using xylose as a standard. One unit of enzyme was defined as the amount of enzyme releasing 1μmol of xylose equivalent per min under assay conditions. All the experiments were performed at least two times and the averages were given.

#### 2.3.1 Substrate solution (Xylan solution)

1 g of birch wood xylan was dissolved in 50 mM pH 7.0 phosphate buffer by heating solution to boiling, and cooling to room temperature with overnight stirring. Xylan solution was centrifuged subsequently to get ride of undissolved xylan molecule at 5,000 g for 30 min at room temperature and maintained at 4°C.

### 2.4 Protein assay

Protein content of the samples was determined according to Bradford (Bradford, 1976) using bovine serum albumin (BSA) as a standard (Appendix G).

## 2.5 Xylanase purification and biochemical characterization

### 2.5.1 Xylanase purification

Xylanase purification studies were performed at room temperature in Fast Protein Liquid Chromatography system (FPLC, ÄKTAprime; GE Healthcare, Amersham Biosciences, Uppsala, Sweden). All columns were purchased from Amersham Biosciences. Xylanase purification attainment of gel filtration and ion exchange chromatography were assessed in the prilimary studies. However, utilization of hydrophobic interaction chromatography yielded the best result. Therefore, hydrophobic interaction chromatograpy was used in subsequent xylanase purification studies.

#### 2.5.1.1 Hydrophobic interaction chromatography

##### 2.5.1.1.1 Assessment of hydrophobic interaction media test kit

In the preliminary experiments, xylanase purification achievement of hydrophobic interaction chromatography media test kit was assessed. The kit contained 1 ml columns prepacked with five different media.The name of the column types provided in this kit were; i- butyl sepharose fast flow, ii- octyl sepharose fast flow, iii- phenyl sepharose fast flow high-binding, iv- phenyl sepharose fast flow low-binding, and v- phenyl sepharose high performance mini columns.

Purification studies were performed at various pHs and NaCl concentrations. Consequently, salt was directly added to crude extract enzyme solution, and then pH of the solution was adjusted to final pH. After filtration with 0.45-micron-pore-size syringe filter (Sartorius), pretreated crude enzyme extract was applied to the column which was previously equilibrated with start buffer. Subsequently, elution of adsorbed proteins was achieved by applying a decreasing NaCl gradient.

Purification studies were carried out using NaCl in 50 mM of three different buffers: citric acid-$Na_2HPO_4$ (pH 4.0-6.0), sodium phosphate (pH 7.0-8.0) and glycine-NaOH (pH 9.0-10.0).

**2.5.1.1.2 Xylanase purification using 20 ml phenyl sepharose high performance column**

As xylanase was successfully purified using phenyl sepharose high performance mini column, bigger volume of the same column type (20 ml) was used in subsequent-large scale- xylanase purification studies.

Furthermore, xylanase purification was performed at room temperature in Fast Protein Liquid Chromatography system (FPLC, ÄKTAprime; GE Healthcare, Amersham Biosciences, Uppsala, Sweden) within the pH range of 6.0-7.5 at constant 3.5 M NaCl concentration. Consequently, crude enzyme solution was fractionated at 3 ml/min flow rate by single-step hydrophobic interaction chromatography.

Accordingly, NaCl was directly added into crude extract enzyme solution to bring the final NaCl concentration to 3.5 M, and then pH of the solution was adjusted to final pH at which purification was performed. After filtration with 0.45µm -pore-size syringe filter (Sartorius), 15 ml of crude extract was applied to the HiLoad 16/10 phenyl sepharose high performance column (Amersham Biosciences, 20 ml, 1.6 cm x 10 cm) previously equilibrated with 50 mM phosphate buffer at determined pH containing 3.5 M NaCl. Later, elution of adsorbed protein was achieved by applying a decreasing (3.5 M -0 M) NaCl gradient. Then, SDS-PAGE electrophoresis was performed on xylanase positive fractions to check its purity, and pure xylanase was subjected to enzyme characterization studies.

### 2.5.2 Biochemical characterization

#### 2.5.2.1 Analytical gel electrophoresis and isoelectric focusing

Xylanase samples concentrated in vivaspin centrifugal concentrators (5-10 kDacut-off, Sartorius) were used in gel electrophoresis. The SDS-PAGE was conducted in 4% stacking and 10-12% separating gels according to Laemmli et al. (1970) using Mini-Protean II Dual Slab cell system (BioRad). The molecular weight standards used were Fermentas (14.4, 18.4, 25, 35, 45, 66.2, and 116 kDa). Gel run was performed at constant voltage settings at 20 V for 15 min, then at 50 V for 1-3 h. The gels were stained by silver staining method according to Blum *et al.* (1987).

Isoelectric point of native enzyme was determined on IEF gel with ampholytes in the pH range of 3.5-10.0 (Sigma) in Biorad 111 Mini IEF Cell. IEF markers were in the pI range of 4.45-9.6 (BioRad). Gel with 3% C monomer concentrate was prepared and casted according to the IEF manual (BioRad). Gel was run at 100 V for 15 min, at 200 V for 15 min, and at 300 V for 30 min to 1 h. Visible markers on the gel left unstained and gels containing xylanase sample were subjected to zymogram analysis to see active xylanase band(s).

#### 2.5.2.2 Zymogram analysis

Xylanase bands were visualized by a zymogram technique described by Wang *et. al.* (1993) after SDS-PAGE and IEF were performed. The gel was soaked in 20mM-Tris/HCl buffer at pH 7.5 containing 0.7% Triton X-100 with gentle shaking for 1h. After repeating this treatment twice, the gel was incubated in the same buffer but without Triton X-100 for 20 min and washed with distilled water. Then, the gel was incubated in 0.1% xylan solution at 37ºC for 2h, washed with distilled water and stained with Congo red (0.5 mg/ml) for 15 min. Destaining was performed by placing the gel in 1 M NaCl solution for at least 15 min. For longer

storage, the gel was treated with 1 M HCl and xylanase bands appeared as white against the blue background.

#### 2.5.2.3 Effects of pH and temperature on xylanase activity

To determine the pH-dependance of the xylanase activity, the following buffers (50 mM) were used over the pH range 6.0-11.0 at 40°C by standard activity assay; citric acid-$Na_2HPO_4$ (pH 6.0), sodium phosphate (pH 7.0-8.0), glycine-NaOH (pH 9.0-10.0) and carbonate-bicarbonate (pH 11.0). The effect of temperature on the xylanase activity was determined at 40-70°C in 50 mM phosphate buffer at pH 7.0 using the standard xylanase assay.

#### 2.5.2.4 Effect of pH and temperature on xylanase stability

After preincubation of xylanase samples at pH range of 6.0 to 10.0 for 100 min and 19 h, and temperature range of 40-70°C for 15 and 45 min, remaining xylanase activities were measured by the standard assay at pH 7.0 and 40°C.

#### 2.5.2.5 Kinetic studies

Initial reaction rates of birchwood xylan hydrolysis were determined at different substrate concentrations ranging from 2-40 mg xylan/ml. Reaction rate vs. substrate concentration curve was plotted to determine whether the enzyme obeys Michaelis-Menten kinetics and the constants were determined by using non-linear regression analysis (Sigma-Plot).

# CHAPTER 3

# RESULTS AND DISCUSSION

## 3.1 Xylanase purification and biochemical characterization

### 3.1.1 Xylanase purification

In general, in this study, xylanase purification success of various liquid chromatographic systems were investigated, and then more detailed assessment was carried out with the one providing best purification profile.

#### 3.1.1.1 Hydrophobic interaction chromatography

##### 3.1.1.1.1 Assessment of hydrophobic interaction media test kit

In the preliminary experiments, to assess xylanase purification achievement of various ligand types, hydrophobic interaction chromatography media test kit (Amersham Biosciences) was examined. This kit contained five different media prepacked in 1 ml columns which are called as; i- butyl spharose fast flow, ii- octyl sepharose fast flow, iii- phenyl sepharose fast flow high-binding, iv- phenyl sepharose fast flow low-binding, and v- phenyl sepharose high performance mini columns.

Purification studies were performed at various pHs using NaCl at various concentrations. Xylanase purification conditions and the results obtained for each of the column types were tabulated in Table 9. According to this table, phenyl

sepharose high performance mini column was the only column to which xylanase bound satisfactorily. Furthermore, when utilizing this column type, 80% of the xylanase activity was recovered in xylanase positive fractions at 3.5 M NaCl and pH 6.0. As a result, bigger volume of the phenyl sepharose high performance column was used in large scale xylanase purification studies.

Moreover, mini column xylanase purification chromatograms were given in Appendix I.

#### 3.1.1.1.2 Xylanase purification using 20 ml phenyl sepharose high performance column

Xylanase was successfully purified when utilizing phenyl sepharose high performance mini column. Consequently, bigger volume of the same column type (20 ml) was used in subsequent xylanase purification studies.

In large scale xylanase purification studies effect of pH on xylanase purification was investigated in the pH range of 6.0 to 7.5 at constant 3.5M NaCl concentration (Table 10). The maximum xylanase purification fold of 372 was obtained at pH 6.3. Any pH value shift beyond pH 6.3, lowered the xylanase purification fold. Indeed, at pH of 6.0, 6.5, 7.0, and 7.5, xylanase purification fold decreased to 300, 220, 224, and 84 fold. Additionally, maximum 81% of original xylanase activity was recovered at pH 6.5. It declined to 60, 68, 38, and 40 % at pHs 6.0, 6.3, 7.0, and 7.5.

In summary, xylanase purification studies were performed at room temperature at various pH, salt type and concentrations by using single step hydrophobic interaction chromatography. Utilization of pH 6.3 and 3.5 M NaCl resulted in best purification providing selective binding of xylanase to the column while the majority of the contaminating proteins passed through the column without binding. To elute adsorbed proteins, decreasing (3.5 M-0 M) NaCl gradient was

applied. Xylanase, strongly bound to the column with a very high selectivity, was eluted at nearly 0 M NaCl (Figure 37). The maximum xylanase purification fold of 372 was obtained with 68% activity recovery. Purity of the concentrated xylanase positive fractions were checked by SDS-PAGE which showed a single xylanase band on the gel after silver staining (Figure 38). Thus, a single step hydrophobic interaction chromatography successfully yielded a very pure xylanase.

Table 9. Comparison of the hydrophobic interaction chromatography mini columns at small scale xylanase purification.

| Column type | NaCl concentration (M) | pH | Xylanase binding to matrix | % Activity recovery |
|---|---|---|---|---|
| Butyl sepharose fast flow | 1.0 | 7.0 | ( - ) | 0.0 |
| | 1.5 | 7.0 | ( - ) | 0.0 |
| | 1.5 | 7.0 | ( - ) | 0.0 |
| Octyl sepharose fast flow | 1.0 | 7.0 | ( - ) | 70.0 |
| | 4.0 | 7.0 | ( - ) | 67.0 |
| Phenyl sepharose fast flow high-binding | 3.0 | 6.0 | ( - ) | 0.0 |
| | 3.0 | 8.5 | ( - ) | 0.0 |
| | 4.0 | 7.0 | ( - ) | 0.0 |
| Phenyl sepharose fast flow low-binding | 3.0 | 8.5 | ( - ) | 24.0 |
| | 3.0 | 6.0 | ( - ) | 67.0 |
| | 3.0 | 7.0 | ( -/+ ) | 60.0 |
| Phenyl sepharose high performance | 3.0 | 7.0 | ( - ) | 50.0 |
| | 3.5 | 6.0 | ( + ) | 80.0 |

Table 10. Xylanase purification using 20 ml of phenyl sepharose high performance column.

| NaCl Concentration (M) | pH | % Activity recovery | *Purification fold |
|---|---|---|---|
| 3.5 | 6.0 | 60 | 300 |
| 3.5 | 6.3 | 68 | 372 |
| 3.5 | 6.5 | 81 | 220 |
| 3.5 | 7.0 | 38 | 224 |
| 3.5 | 7.5 | 40 | 84 |

*Due to very low protein concentrations, purification fold was calculated on computer by analysing chromatogram peak area. Formula used to calculate purification fold as fallows: (total area of the chromatogram/ xylanase peak area) x (applied xylanase activity/ recovered xylanase activity).

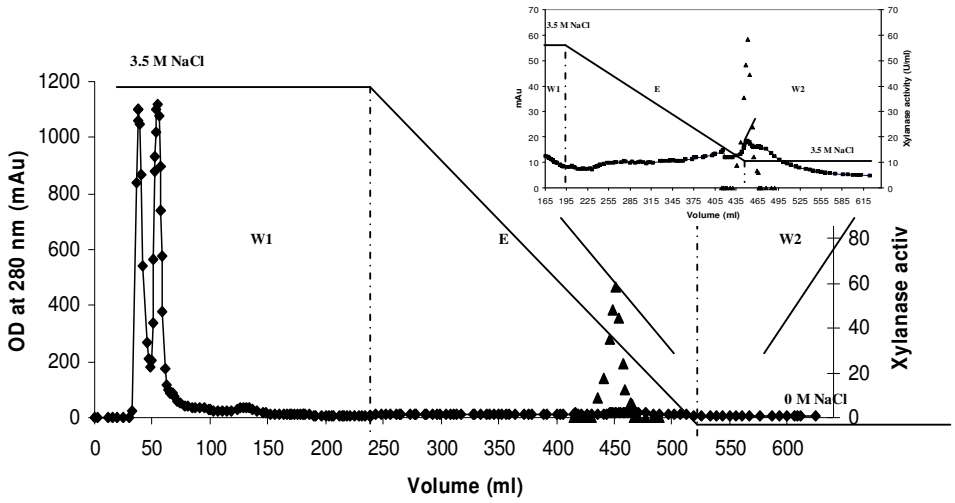



Figure 37. *Bacillus pumilus* SB-M13 xylanase purification by using phenyl sepharose high performance column at pH 6.3 and 3.5 M NaCl. W1: sample injection and wash out in 3.5 M NaCl, E: elution in 3.5 M to 0 M NaCl gradient, W2: wash out in 0 M NaCl. (■: protein concentration, ▲: xylanase activity).

### 3.1.2 Biochemical characterization

#### 3.1.2.1 Molecular weight and isoelectric point determinations

Molecular weight and pI of *Bacillus pumilus* SB M-13 xylanase were determined as 24.8 kDa and pI of 9.2, respectively (Figure 38). Results were confirmed on activity gel by zymogram analysis. A single protein band having xylanase activity on both SDS and IEF gels indicated the purity of the xylanase sample obtained after single step hydrophobic interaction chromatography.

#### 3.1.2.2 Effects of pH and temperature on xylanase activity and stability

Effects of pH and temperature on pure xylanase activity and stability were tested by determining initial reaction rates.

#### 3.1.2.3 The effect of pH on xylanase activity and stability

The effect of the pH on the xylanase activity was determined over the pH range of 6.0-11.0 at 40°C (Figure 39). The enzyme was most active at pH 7.5, but it retained 62% of its activity at pH 8.0. Complete xylanase activity loss was observed at pH 11.0.

After the treatment of xylanase samples at several pHs and temperatures during different periods of time, remaining xylanase activities were assayed at 40°C and pH 7.0. Accodingly, enzyme was active over the pH range of 6.0 to 10.0 and most stable at pH 7.5. It retained 86 and 71% of its original activity in pH 7.5 buffer after 100 min and 19 h incubations, respectively. Enzyme was rather less stable at acidic pH than alkaline pH after 19 h incubation. The original xylanase activity loss was 45% at pH 6.0, but it was 29-30% at pH 9.0 and 10.0, respectively (Figure 39).

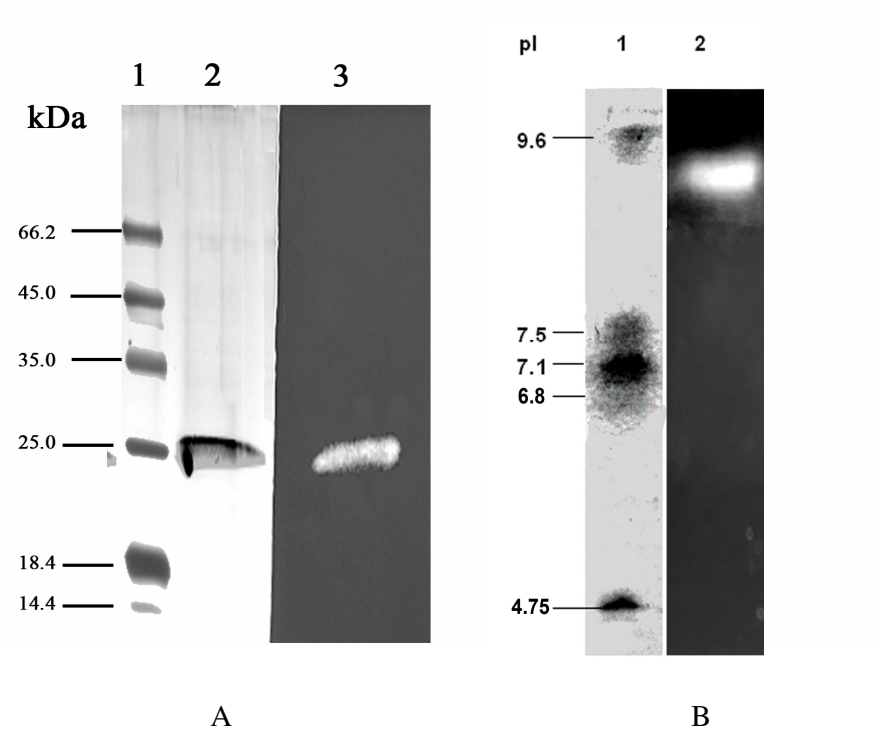



Figure 38. A-SDS-PAGE (12%) and activity zymogram of the 20 fold concentrated *Bacillus pumilus* SB-M13 pure xylanase from hydrophobic interaction chromatography. Lanes: 1- molecular weight markers, 2- pure xylanase. Protein bands were stained with silver stain, 3- activity zymogram of the pure xylanase. B- IEF and activity zymogram of the 20 fold concentrated *Bacillus pumilus* SB-M13 pure xylanase from hydrophobic interaction chromatography; Lanes: 1- visible IEF markers, 2- activity zymogram of the pure xylanase.

### 3.1.2.4 The effect of temperature on xylanase activity and stability

The effect of temperature on xylanase activity was measured over the temperature range of 40-70°C. The highest xylanase activity was obtained at 60°C. Relative activities at 65 and 70°C were determined as 64 and 20%, respectively (Figure 40).

Thermostability of the xylanase was also tested over the temperature range of 40-70°C after 15 and 45 min incubations (Figure 40). When compared to the incubation for 15 min, 45 min incubations caused more dramatic loss in xylanase activity. Xylanase samples incubated at 40 °C for 15 min retained 90% of its original activity, but it was 76% for 45 min. Incubations for 45 min at higher temperatures sharpened the decrease in xylanase activities and respective 56, 40, and 2% xylanase activities retained at 50, 55, and 60°C. Finally, complete activity loss was observed at 70 and 65°C after 15 and 45 min incubations, respectively.

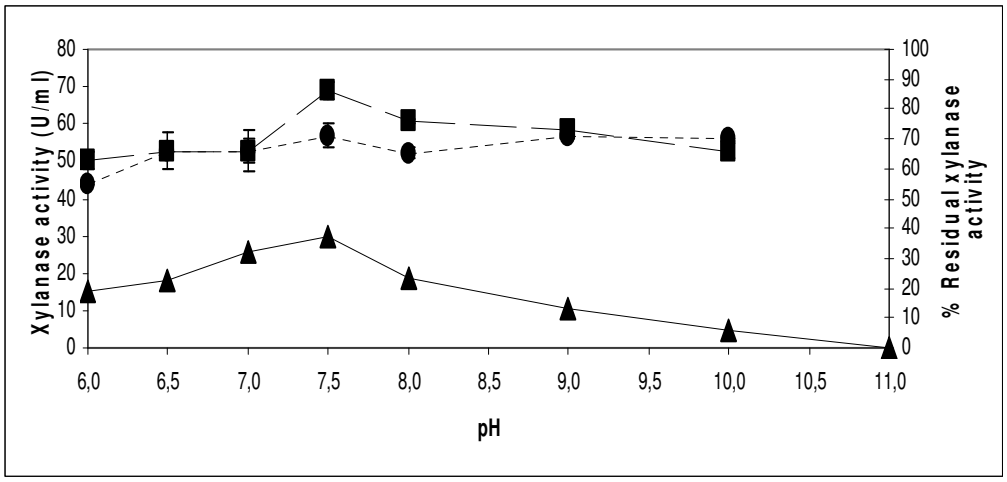



Figure 39. pH-dependance of activity and stability of *Bacillus pumilus* SB-M13 xylanase. (▲: xylanase activity, ■: residual xylanase activity after 100 min incubation, and, ●: residual xylanase activity after 19 h incubation). Xylanase activities were measured at 40°C.

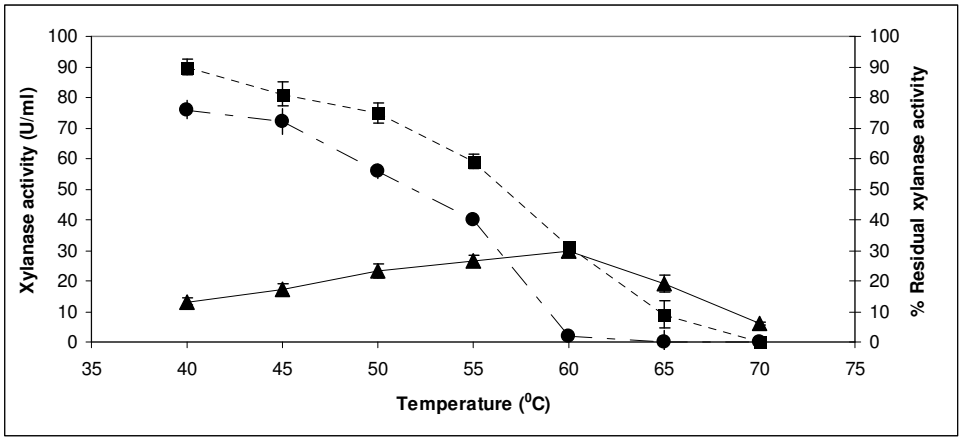



Figure 40. Temperature-dependance of activity and thermal stability of *Bacillus pumilus* SB-M13 xylanase. (▲: xylanase activity, ■: residual xylanase activity after 15 min incubation, and, ●: residual xylanase activity after 45 min incubation). Xylanase activities were measured at pH 7.0.

### 3.1.2.5 Kinetic studies

Initial reaction rates were measured at 40°C in 50 mM phosphate buffer at pH 7.0 in various substrate concentrations ranging from 2-40 mg/ml. Enzyme followed Michaelis-Menten kinetics and constants were determined by using non-linear regression analysis (Sigma-Plot) (Figure 41). The $K_m$, $V_{max}$ and $k_{cat}$ values obtained by using birchwood xylan as a substrate were 1.87 mg/ml, 17.9 U/ml and 42,619 U/mg protein, respectively. In literature, the crude enzymes produced from different *B. pumilus* strains showed $K_m$ value of 1.1-71.4 mg/ml and $V_{max}$ value of 11.0-1,667 U/ml using birchwood xylan (Duarte *et al*., 2000). $K_m$ values reported for some other *Bacillus* strains fall in the range of 0.6-17.7 mg/ml. Furthermore, enzyme $k_{cat}$ values of 0.017-700 U/mg were also calculated with various xylans. Although comparision of $k_{cat}$ cannot give sound results because of the different conditions used in enzyme assays and substrate type, rather higher $k_{cat}$ value

obtained from pure xylanase of *B. pumilus* SB M-13 determined its higher catalytic power. In addition, low $K_m$ value of pure xylanase of *B. pumilus* SB M-13 also revealed its high substrate affinity.

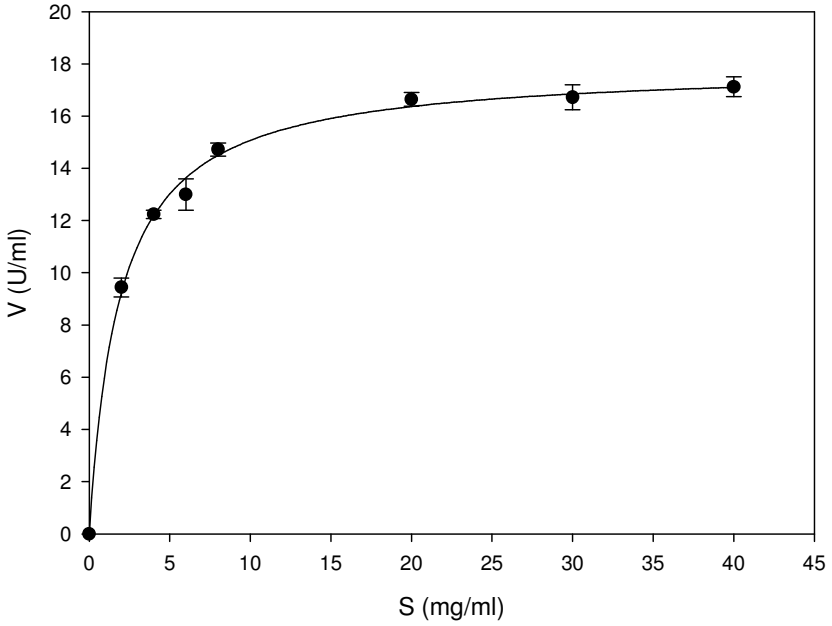



Figure 41. Michaelis-Menten plot for the pure *Bacillus pumilus* SB-M13 xylanase.

### 3.1.2.6 An assessment of pyhsicochemical properties of *B. pumilus* SB-M13 xylanase

The physicochemical properties of the xylanases produced by different *B. pumilus* strains were given in Table 11. *Bacillus pumilus* strains produce multiple xylanases with optimal enzyme activity between pH 5.0-9.0 and 40-60°C. Molecular weight and pI values of the enzymes varied within the range of 22-78.9 kDa and 8.8-9.9, respectively. Xylanase of *B. pumilus* SB M-13 shows similar

pyhsicochemical properties with other *B. pumilus* strains xylanases. Indeed, enzyme, having molecular weight of 24.8 kDa, and pI of 9.2, is most active at pH 7.5 and 60°C.

In terms of thermostability, after treatment for 15 min at 60°C, *B. pumilus* IPO (Panbangred *et al*., 1983) pure xylanase completely lost its activity in contrast with *B. pumilus* SB M-13 xylanase retaining 31% of its original activity. Respective 40 and 35% original xylanase activities remained in pure xylanase solution from *B. pumilus* PRL B12 (de Buyl *et al*., 1998) and crude extract enzyme solution from *B. pumilus* $5_{14}$ (Duarte *et al*., 2000) after treatment of samples at 60°C for 120 min. Therefore, xylanase from *B. pumilus* SB M-13 was more thermostable than *B. pumilus* IPO xylanase, but it is less stable in comparison to *B. pumilus* PRL B12 and $5_{14}$ xylanases. However, because of the possible protein concentration differences in each xylanase sample, and its contribution to enzyme stability, thermostability of the xylanases by different source cannot be compared efficiently.

Moreover, xylanase of *Bacillus pumilus* SB-M13 strain is more stable at alkaline pHs, than alkaline xylanases reported for other *B. pumilus* strains. Alkaline xylanases of four *B. pumilus* strains (Duarte *et al*., 2000), $5_2$, $5_{14}$, 13a, and 4a, retained 30-65% of its original enzyme activity, after being incubated at pH range of 8.0-10.0 for 2 h, but xylanase of *B. pumilus* SB M-13 retained 65-70% of its original activity at given pHs after 19 h incubation.

Table 11. Physicochemical properties of the *Bacillus pumilus* xylanases

| *B. pumilus* strains | MW by SDS-PAGE (kDa) | pI | Optimum pH | Optimum Temperature ($^0$C) | Substrate (w/v) |
|---|---|---|---|---|---|
| *B. pumilus* IPO Panbangred et al. (1983) | 20*-24 | N.D. | 6.5 | 40 | Larchwood xylan (1%) |
| *B. pumilus* DSM 6124 Anker et al. (1993) | 22 | 8.8 | 6.0 | 40-60 | Birchwood xylan (0.5%) |
| *B. pumilus* PRL B12 De Buyl et al. (1998) | 26 | 9.8-9.9 | 5.0-7.0 | 55 | Birchwood xylan (1%) |
| *B. pumilus* Soil isolates Duarte et al. (1999-2000) | 78.9, 63.8, 24.5, 15.5 | N.D. | 8.0-9.0 | 55-60 | Birchwood xylan (1%) |
| *B. pumilus* SB-M13 Our study | 24.8 | 9.2 | 7.5 | 60 | Birchwood xylan (1%) |

*$M_W$ determination by equilibrium centrifugation

## REFERENCES


Anker, L., Bisgard-Frantzen, H., and Halkier, T., 1993, United States Patent, Patent no: 5,457,045.

Avcioglu, B., Eyupoglu, B., and Bakir, U., 2005, "Production and characterization of xylanases of *Bacillus* strain isolated from soil", *World Journal of Microbiology and Biotechnology*, Vol. 21, pp. 65-68.

Bailey, M.J., Biely, P., and Pountanen, K., 1992, "Interlaboratory testing methods for xylanase activity", *Biochimica Biophysica Acta*, Vol. 1117, pp. 252-270.

Bakir, U., Yavascaoglu, S., Guvenc, F., and Ersayin, A., 2001, "An endo-β-1,4-xylanase from *Rhizopus oryzae*: production, partial purification and biochemical characterization", *Enzyme and Microbial Technology*, Vol. 29(6-7), pp. 328-334.

Bakır, U., 2004, "Microbial Xylanase Production in Encyclopedia of Bioresource Technology", *Haworth Press, Ashok Pandey*, p. 592.

Beg, Q. K., Kapoor, M., Mahajan, L., and Hoondal, G.S., 2001, "Microbial xylanases and their industrial applications: a review", *Applied Microbiology and Biotechnology*, Vol. 56, pp. 326-338.

Belancic, A., Scarpa, J., Peirano, A., Diaz, R., Steiner, J., and Eyzayuirre, J., 1995, "*Penicillium purpurogenum* produces several xylanases: purification and properties of two of the enzymes", *Journal of Biotechnology*, Vol. 41, pp. 71-79.

Blum, H., Beier, H., and Grass, H.J., 1987, "Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels", *Electrophoresis*, Vol. 8, pp. 93-99.

Bradford, M.M., 1976, "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding", *Analitical Biochemistry*, Vol. 72, pp. 248-254.

De Buyl, E., Lahaye, A., Ledoux, P., Amory, A., Detroz, R., Andre, C., and Vetter, R., 1998, United States Patent, Patent no: 6,426,211.

Duarte, M.C.T., Portugal, E.P., Ponezi, A.N., Bim, M.A., Tagilari, C.V., and Franco, T.T., 1999, "Production and purification of alkaline xylanases", *Bioresource Technology*, Vol. 68, pp. 49-53.

Duarte, M.C.T., and Pelegrino, A.C.A., Portugal, E. P., 2000, "Characterization of alkaline xylanases from *Bacillus pumilus"*, *Brazilian Journal of Microbiology*, Vol. 31(2), pp. 90-94.

Gomes, I., Gomes, J., Steiner, W., and Esterbauer, H., 1992, "Production of cellulase and xylanase by a wild strain of *Trichoderma viride", Applied Microbiology and Biotechnology*, Vol. 36, pp. 701-707.

Gulati, R., Saxena, R.K., and Gupta, R., 2000, "Fermentation and downstream processing of lipase from *Aspergillus terreus"*, *Process Biochemistry*, Vol. 36, pp. 149-155.

Laemmli, U.K., 1970, "Cleavage of structural proteins during the assembly of the head of bacteriophage T4", *Nature (London)*, Vol. 227, pp. 680-685.

Miller, G.L., 1959, "Use of dinitrosalicyclic acid reagent for determination of reducing sugars", *Analitical Chemistry*, Vol. 21, pp. 426-428

Panbangred, W., Shinmyo, A., Kinoshita, S., and Okada, H., 1983, "Purification and properties of endoxylanase produced by *Bacillus pumilus", Agricultural and Biological Chemistry*, Vol. 47 (83), pp. 957-963.

Pham, P.L., Taillandier, P., Delmas, M., and Strehaiano, P., 1998, "Production of xylanases by *Bacillus polymyxa* using lignocellulosic wastes", *Industrial Crops and Products*, Vol. 7, pp. 195-203.

Subramaniyan, S., and Prema, P., 2000, "Cellulase-free xylanases from *Bacillus* and other microorganisms", *FEMS Microbiology Letters*, Vol. 183, pp. 1-7.

Wang, P., Mason, J.C., and Broda, P., 1993, "Xylanases from *Streptomyces cyaneus*: Their production, purification and characterization", *Journal of General Microbiology*, Vol. 139, 1987-1993.

Wong, K.K.Y., and Saddler, J.N., 1993, "Applications if hemicellulases in the food, feed, and pulp and paper industries", *In: Hemicellulose and Hemicellulases*, Portland Press, London, pp. 127-143.

# PART 3

## PURIFICATION and CHARACTERIZATION OF α-L-ARABINOFURANOSIDASES FROM A SOIL ISOLATE *Bacillus pumilus* SB-M13 and A THERMOPHILIC FUNGUS *Scytalidium thermophilum*

# CHAPTER 1

## INTRODUCTION

Synergistic action of xylanases and accessory side chain cleaving enzymes such as α-L-arabinofuranosidases (AFs), α-glucuronosidases, and esterases are required for complete degradation of hemicelluloses (Gomes *et al*., 2000). AFs are the potential rate limiting enzyme in xylan break down, consequently playing a critical role in complete breakdown of arabinoxylan present in agricultural by-producs such as corn fiber, corn stover and rice straw (Bachman and McCarty, 1991; Gereve et al., 1994; Lee and Forsberg, 1987; Saha and Bothast, 1999).

Being a part of xylanolytic systems, AFs are utilized in successful conversion of plant biomass to fuels and chemicals, and efficient exploitation of plant biomass for feed and food. Besides, AFs act together with endoxylanases in the biobleaching of pulp to reduce amounts of chlorine use and pollution. AFs are also consumed to increase aroma of wines and fruit juices as well as to produce arabinose which has been stated to show antigylsemic effect (Rahman *et al*., 2001 and; Makkonen *et al*.; Wong *et al*., 1988; Gunata *et al*., 1990 and Spagna *et al*.,1998; Rémond *et al*., 2004). Moreover, synergy between AFs and endoxylanases/β-xylosidases has also been reported (de Vries *et al*., 2000).

Beldman (1997) have classified AFs into tree types depending on their substrate specifities: 1- AFs, not active toward arabinose containing polymers; 2- AFs, active towards arabinose containing polymer including arabinoxylan, and 3- arabinofuranohydrolases, specific for arabinoxylans. Substrate specifity of the AFs determines their potential application areas, consequently their biotechnological

importance.

AFs are crucial part of the xylanolytic systems in many bacteria and fungi (Table 13 and 14). Although, wide ranges of microorganisms produce xylanolytic enzymes, there has been limited information presented on the production and characterization of these enzymes, except for endoxylanases.

### 1.1 Aim of the study

In recent years, interest in xylan degrading enzymes have been increased due to their applications in various agro-industrial processes.Therefore, many researches have been directed to find novel and efficient AF producers and understand physicochemical properties of AFs produced.

In this part of the study it was aimed to produce, purify, and characterize AFs from soil isolate *Bacillus pumilus* SB-M13 (BAF) and thermophilic fungus *Scytalidium thermophilum* (STAF). AF production capability of the *S. thermophilum* was first determined and reported in this study. AF of *B. pumilus* PS213 was purified and partially characterized by Degrassie and coworkers (2003). Accordingly, the enzymes BAF and STAF produced on 3% corn cobs were purified using a single step hydrophobic interaction chromatography and biochemically characterized. Kinetic parameters of the enzymes were also determined. After purification and characterization, enzyme characteristics of *B.pumilus* SB-M13 AF were compared with that of *B. pumilus* PS213 AF. Besides, substrate specifity of the BAF was investigated to understand its potential industrial application areas.

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1 Materials

Fast Protein Liquid chromatography (FPLC) system (ÄKTAprime) and a phenyl sepharose column were purchased from Amersham Biosciences (Uppsala, Sweeden). Agricultural byproducts were supplied from the local market or local feed plants. Chemicals and apparatus used in gel electrophoresis were from BioRad and Sigma. Syringe filters and vivaspin centrifugal concentrators were purchased from Sartorius (Germany).

The synthetic *p*-nitrophenol glycosides (*p*-nitrophenyl-α-L-arabinonopyranoside, *p*-nitrophenyl-β-D-glucopyranoside, *p*-nitrophenyl-β-D-xylopyranoside, *p*-nitrophenyl-β-D-galactopyranoside, *p*-nitrophenyl-β-D-mannopyranoside, *p*-nitrophenyl-β-D-galacuronide, *p*-nitrophenyl-β-D-fucopyranoside) were bought from Sigma (USA).

## 2.2 The microorganism and culture maintenance

### 2.2.1 *Bacillus pumilus* SB-M13

The *Bacillus pumilus* SB-M13 used in this study was isolated as xylanase positive *Bacillus* strain from soil samples collected from different regions in Turkey by using the method given elsewhere (Biran *et al*., 2006). The isolate was cultivated at 37°C on agar plate containing (w/v): 1.0%; glucose, 0.5%; peptone, 0.5%; yeast

extract, 0.1%; $KH_2PO_4$, 0.02%; $MgSO_4$ and 2.0%; agar to which sterile 0.01%; $Na_2CO_3$ added separately, maintained at 4°C, and subcultured monthly. Cultures were maintained in liquid media with 30% of glycerol at -20°C long term storage as well.

### 2.2.2 *Scytalidium thermophilum*

*Scytalidium thermophilum* type culture *Humicola insolens* was provided from ORBA Inc. The microorganism was grown at 40°C on YpSs agar plates containing (w/v): 0.4; yeast extract, 0.1; $K_2HPO_4$, 0.05; $MgSO_4.7H_2O$, 1.5; soluble starch and 2.0; agar. It was maintained at room temperature, subcultured monthly, and long term storage was at -20°C on agar slants.

## 2.3 α-L-Arabinofuranosidase (AF) productions

### 2.3.1 *Bacillus pumilus* SB M-13 AF (BAF) production

Arabinofuranosiase production by *Bacillus pumilus* SB M-13 (BAF) was performed in 250-ml shake-flask bioreactors containing 100-ml medium at 30°C, 175 rpm for 4 days. The medium contained 0.5% NaCl, 0.25% yeast extract, 0.1% $KH_2PO_4$, 0.02% $MgSO_4$, 0.1% $Na_2CO_3$ and 3% ground and steam hydrolyzed corn cobs as a sole carbon source and inducer. The fermentation medium was centrifuged for 40 min at 11,000 x ***g*** and the supernatant was used as crude enzyme preparation.

### 2.3.2 *Scytalidium thermophilum* AF (STAF) production

*S. thermophilum* was cultured in 250 ml shake-flask bioreactors containing 100 ml medium at 45°C, 175 rpm for 6 days. The medium contained (w/v): 1.0; yeast extract, 1.0; tryptone, 0.2; $(NH_4)_2SO_4$, 0.03; $MgSO_4.7H_2O$, 0.03; $FeSO_4$, 0.03; $CaCl_2$, and 3.0%; steam hydrolyzed ground corn cobs as sole carbon source and

inducer. The fermentation medium was centrifuged for 40 min at 11,000 x ***g*** and the supernatant was used as the crude enzyme preparation. Crude enzyme could also be prepared using filtration.

**2.4 Enzyme assay**

The α-L-Arabinofuranosidase (AF) activity was assayed with *p*-nitrophenyl-α-L-arabinofuranoside (p-NPAraf) as the substrate. The reaction mixture containing 0.5 ml of a 2 mM p-NPAraf solution, 0.4 ml of 50 mM phosphate buffer at pH 7.0 and 0.1 ml of the enzyme solution, was incubated at 40°C for BAF and at 60°C for STAF. Amount of *p*-nitrophenol (p-NP) release was followed spectrophotometrically at every 15 sec within a period of 5 min at 410 nm and the enzyme activity was calculated from the initial reaction rates determined. This AF activity assay is referred hereafter as the standard assay method. One unit of AF activity was defined as the amount of enzyme required for the release of 1μmol of p-NP per min from p-NPAraf under the assay conditions.

**2.5 Protein assay**

Protein content of the samples was determined by the Bradford method (1976) using bovine serum albumin (BSA) as a standard.

**2.6 AF purifications**

BAF and STAF were purified from pretreated crude enzyme extract at room temperature in the FPLC system (ÄKTAprime; GE Healthcare, Amersham Biosciences, Uppsala, Sweden) using a single-step hydrophobic interaction chromatography. Pretreatment of crude enzyme extract included the followings: NaCl addition into the crude enzyme to a final concentration of 3.5 M, pH adjustment to 6.3, and filtration with 0.45 μm-pore-size syringe filter to remove insolubles.

BAF was fractionated at 3 ml/min flow rate. Accordingly, 7.5-ml of pretreated crude enzyme extract applied to the HiLoad 16/10 phenyl sepharose high performance column (Amersham Biosciences, 20 ml, 1.6 cm x 10 cm) which was previously equilibrated with the start buffer- 50 mM phosphate buffer at pH 6.3 containing 3.5 M NaCl.

On the other hand, STAF in 0.5-ml of crude extract was fractionated at 1 ml/min flow rate on HiLoad phenyl sepharose high performance mini column (1ml, 0.5 cm x 2.6 cm) previously equilibrated with start buffer- 50 mM phosphate buffer at pH 6.3 containing 3.5 M NaCl .

Then, adsorbed proteins were eluted in decreasing NaCl gradient from 3.5 M to 0 M. Subsequently, SDS-PAGE electrophoresis was performed on AF positive fractions to check the enzyme purity. The pure AF was used in the enzyme characterization studies.

**2.7 Analytical gel electrophoresis and isoelectric focusing**

Molecular weight of the enzymes were estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and by gel filtration chromatography on a Hi Load 16/60 Superdex 200 pg (Amersham Biosciences) fast protein liquid chromatography column (FPLC) in which the mobile phase was 50 mM phosphate buffer at pH 7.0, and flow rate was 1 ml/min.

In the first method, pure AF samples concentrated in vivaspin centrifugal concentrators (5-10 kDa cut-off, Sartorius) were applied on SDS gel conducted in 4% stacking and 12% separating gels according to Laemmli *et al*. (1970). The molecular weight standards used for BAF and STAF were; 10, 15, 20, 25, 30, 40, 50, 60, 70, 85, 100, 120, 150, and 116 kDa (Amersham Biosciences) and 14.4, 18.4, 25, 35, 45, 66.2, and 116 kDa (Fermentas), respectively

BAF gel run was carried out at constant voltage settings at 150 V for 30 min, later at 300 V at 3 h in Amersham SDS vertical electrophoreseis system (Amersham Biosciences). Moreover, STAF gel run was performed at constant voltage settings at 20 V for 15 min, then at 50 V for 1-3 h in Mini-Protean II Dual Slab cell system (BioRad). The gels were stained by silver staining method according to Blum *et al.* (1987).

In the second method, concentrated crude enzymes extract was applied on a gel filtration column and fractionated at 1 ml/min flow rate in 50 mM phosphate buffer at pH 7.0. Molecular weight standards used were; 13.7, 25.0, 43.0, 67.0, 158.0, 232.0, 440.0 kDa (Amersham Biosciences).

Isoelectric point of the native BAF and STAF were determined on IEF gel with ampholytes in the pH range of 3.5-10.0 (Sigma) in a Biorad 111 Mini IEF Cell. Each gel with 3% C monomer concentrate was prepared and casted according to the IEF manual (BioRad). The IEF markers were in the pI range of 4.45-9.6 (BioRad). Each gel run was performed at 100 V for 15 min, at 200 V for 15 min, and at 300 V for 30 min to 1 h. The visible markers on the gel left unstained and the part of the gels containing AFs were subjected to zymogram analysis to observe an active AF band if any.

**2.8 Zymogram analysis**

After IEF was performed, the gels were soaked in 2 mM p-NPAraf substrate solution at pH 7.0 and have been incubated at 45ºC until the development of a yellow color on the gel indicating an active AF band.

**2.9 The effects of pH and temperature on BAF and STAF activity**

The enzymatic reactions were carried out in three different buffers: citric acid-$Na_2HPO_4$ (pH 4.0-6.0), sodium phosphate (pH 7.0-8.0) and glycine-NaOH (pH 9.0-10.0).

The activity of the BAF was investigated over the pH range of 5.0-10.0 at 40°C and over the temperature range of 30-80 °C in 50 mM phosphate buffer at pH 7.0, using the standard AF assay method.

Moreover, the activity of the STAF was examined between the pH ranges of 4.0 to 11.0 at 60°C and between the temperature ranges of 40-80 °C in 50 mM phosphate buffer at pH 7.0, using the standard AF assay method.

**2.10 The effects of pH and temperature on BAF and STAF stability**

The pH stabilities of the both BAF and STAF were evaluated at the pH range of 4.0 to 10.0. BAF samples were incubated at defined pHs for 2h, and 24 h at 4°C and 2 h at room temperature. On the other hand, preincubation periods for STAF samples were 24 h at both 4°C, and room temperature and 4 h at room temperature. After incubations, residual AF activities were measured using the standard AF assay method, and pH stability profiles of AFs were determined.

Moreover, thermostability of the both BAF and STAF was investigated over the temperature range of 30-90°C in 50 mM phosphate buffer at pH 7.0. Accordingly, BAF was incubated at determined temperatures for 2 h. On the other hand, STAF samples were incubated at defined temperatures for 15 min or 2h. Then, residual AF activities were measured using the standard AF assay method, and thermostability profiles of AFs were investigated.

**2.11 Kinetic studies**

The initial reaction rates of p-NPAraf hydrolysis by BAF and STAF were determined at different substrate concentrations ranging from 0.1-2.0 mM and 0.5-2.5 mM p-NPAraf, respectively. Reaction rate vs. substrate concentration curve was plotted to determine whether the enzyme obeys Michaelis-Menten

kinetics and the constants were determined by using non-linear regression analysis (Sigma-Plot).

### 2.12 BAF substrate specifity

#### 2.12.1 Subtrates

The synthetic *p*-nitrophenol glycosides (*p*-nitrophenyl-α-L-arabinonopyranoside, (*p-NP*); *p*-nitrophenyl-β-D-glucopyranoside, *p*-nitrophenyl-β-D-xylopyranoside, *p*-nitrophenyl-β-D-galactopyranoside, *p*-nitrophenyl-β-D-mannopyranoside, *p*-nitrophenyl-β-D-galacuronide, *p*-nitrophenyl-β-D-fucopyranoside (Appendix J). Larchwood arabinogalactan was from Sigma. Wheat arabinoxylan (low viscosity), rye flour arabinoxylan, branched and linear sugar beet arabinans were from Megazyme (Ireland).

##### 2.12.1.1 Synthetic p-nitrophenol (p-NP) glycosides

The substrate specifity of *Bacillus pumilus* SB-M14 pure AF (BAF) towards synthetic *p*-nitrophenol (p-NP) glycosides was tested using the standard assay method utilized for *p*-nitrophenyl-α-L-arabinofuranoside (p-NPAraf) except the substrate concentration which was increased to10 mM. Most of the studies in literature utilized 10 mM of p-NP glycosides in this type of analysis, consequently same concentration was exploited in our study to standardize procedure and compare our results with others.

##### 2.12.1.2 Arabinose containing polysaccharides

The effeciency of BAF (0.4 U/ml) on the hydrolysis of various arabinose containing polysaccharides (arabinoxylan, arabinogalactan, arabinan and debranched arabinan; 1% (w/v)) was assessed at 40°C for 24 h. Each reaction mixture contained 0.5-ml BAF (0.4 U/ml), 0.4- ml 50 mM phosphate buffer at pH

7.0, and 0.1-ml of 1% (w/v) polymeric substrate. The hydrolyzed samples were withdrawn after 1, 3, 6, 12 and 24 h incubations and the enzyme was inactivated by boiling for 10 min. The reaction products were determined using HPLC analysis.

Wheat arabinoxylan (low viscosity) sugar composition:
Arabinose, 37%; xylose, 61% ; other sugars, 2%.

Rye flour arabinoxylan sugar composition:
Arabinose, 38%; xylose, 59%; other sugars 3%.

Branched sugar beet arabinan sugar composition:
Arabinose, 88%; galactose, 3%; rhamnose, 2%; galacturonic acid; 7%.

# CHAPTER 3

# RESULTS AND DISCUSSION

## 3.1 *Bacillus pumilus* SB-M13 α-L-arabinofuranosidase (BAF)

### 3.1.1 AF purification

AF was purified with a single-step hydrophobic interaction chromatography using HiLoad phenyl sepharose high performance column (Figure 42). AF bound to the matrix selectively at 3.5 M NaCl and pH 6.3, was eluted nearly at 0.8 M NaCl and 700 fold purified with 66% activity recovery. AF positive fractions were concentrated and its purity was checked by SDS-PAGE. The results showed a single protein band on the gel after silver staining (Figure 43). As a result, a very pure AF was obtained after a single-step hydrophobic interaction chromatography.

### 3.1.2 Analytical gel electrophoresis and isoelectric focusing

Molecular weight of the *Bacillus pumilus* SB-M13 AF (BAF) was determined as 53 kDa and 210 kDa by SDS-PAGE and gel filtration chromatography, respectively. Accordingly, the enzyme may contain four equal subunits of $M_W$ 53 kDa (Figure 43). Moreover, presence of as a single sharp protein band at an estimated $M_W$ of 53 kDa on SDS-PAGE gel, after silver staining also proved that AF was purified successfully by single-step hydrophobic interaction chromatography. Additionally, the pI value of the *Bacillus pumilus* SB-M13 AF was determined as 4.75 by IEF (Figure 43).

Microbial AFs, having multiple forms and variable $M_W$s, show pI values in the range of 3.3 to 8.8 (Table 12 and 13). Degrassi and coworkers (2003) also reported that *B. pumilus* PS213 AF has $M_W$ of 220 kDa and 60 kDa determined by gel filtration chromatography and SDS-PAGE. Isoelectric point of enzyme was found to be 5.2 as well. Having the native molecular weight of 210 kDa with four subunits of $M_W$ 53 kDa and pI of 4.8, *Bacillus pumilus* SB-M13 AF shows similar physicochemical characteristics to *B. pumilus* PS213.

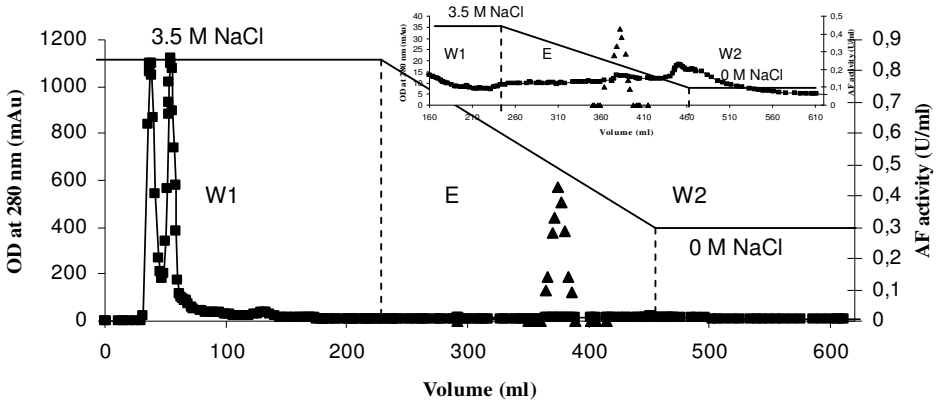
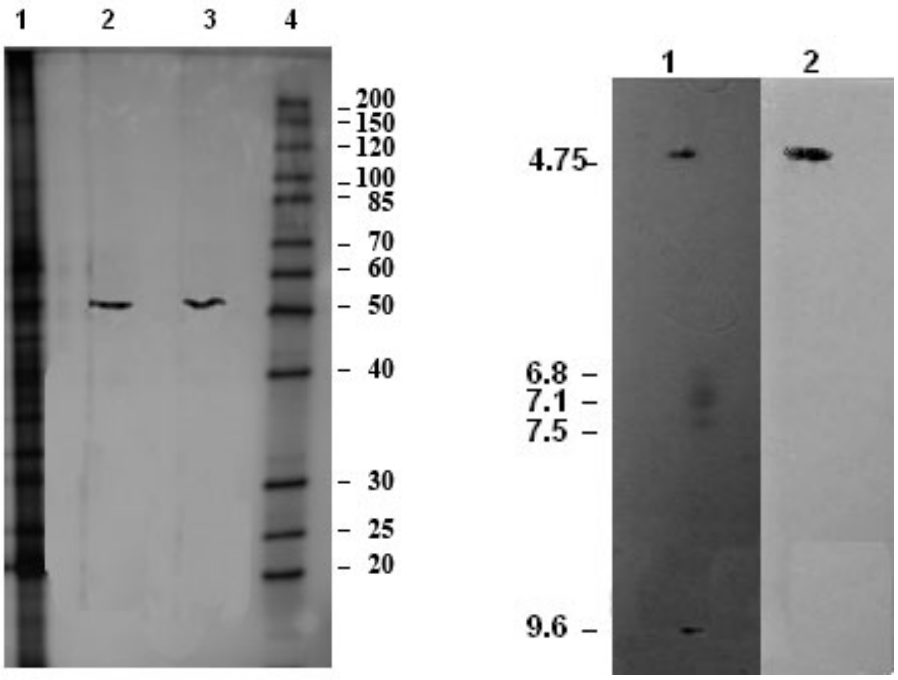



Figure 42. *B. pumilus* SB-M13 AF (BAF) purification by using phenyl sepharose high performance column at pH 6.3 and 3.5 M NaCl. W1: sample injection and wash out in 3.5 M NaCl, E: elution in 3.5 M to 0 M NaCl gradient, W2: wash out in 0 M NaCl. (■: protein concentration, ▲: AF activity).




A B

Figure 43. SDS-PAGE (12%) of the *Bacillus pumilus* SB-M13 AF (BAF). Lanes: 1- crude enzyme, 2-pure BAF from hydrophobic interaction chromatography, 3-pure AF from hydrophobic interaction chromatography, 4-molecular weight markers, B- IEF and activity zymogram of the *Bacillus pumilus* SB-M13 pure BAF from hydrophobic interaction chromatography; Lanes: 1- visible IEF markers, 2- activity zymogram of the pure BAF.

Table 12. Comparative physicochemical properties of bacterial AFs

| Microorganisms | $M_W$ of the AFs (kDa) (SDS-PAGE) | pI of the AFs | Optimum temperature (°C) | Optimum pH |
|---|---|---|---|---|
| *Bacillus subtilis* 3-6 (Kaneko *et al*., 1994) | 61 (monomer) | n.d | 60 | 7.0 |
| *Bacillus stearothermophilus* T-6 (Gilead and Shoham, 1995) | 256* (tetramer) 64 | 6.5 | 70 | 5.5-6.0 |
| *Streptomyces purpurasences* (Komae *et al.,* 1982a) | 495* | 3.9 | n.d | 5.0-6.5 |
| *Clostridium acetobutylicum* ATCC 824 (Lee and Forsberg, 1897) | 94 (monomer) | 8.2 | n.d | 5.0-5.5 |
| *Butyvibrio fibrosolvens* GS 113 (Hespell and O'Bryan, 1992) | 240* (octomer) 31 | 6.0 | 55 | 6.0-6.5 |
| *Bacillus pumilus* PS213 (Degrassi *et al*., 2003) | 220* (tetramer) 60 | 5.2 | 55 | 7.0 |
| *Bacillus pumilus* SB-M13 (Our study) | 210* (tetramer) 53 | 4.8 | 70 | 7.0 |

* *Mw was determined using gel filtration chromatography, n.d: Not defined*

Table 13. Comparative physicochemical properties of fungal AFs

| Microorganisms | $M_W$ of the AFs (kDa) (SDS-PAGE) | pI of the AFs | Optimum temperature (°C) | Optimum pH |
|---|---|---|---|---|
| *Penicillium purpurogenum* (De Ionnes *et al*., 2000) | 58 (monomer) | 6.5 | 50 | 6.5 |
| *Aspergillus awamori* IFO 4033 (Kaneko *et. al*., 1998) | 81 and 62 (monomer) | 3.3 and 3.6 | 60 | 4.0 |
| *Aspergillus niger* 5-16 (Kaneko *et al*., 1994) | 67 (monomer) | n.d | n.d | n.d |
| *Rhizomucor pusillus* (Rahman *et al*., 2001) | 88 (monomer) | 4.2 | 65 | 4.0 |
| *Aspergillus terreus (Luonteri et al., 1995)* | 39 and 59 (monomer) | 7.5, 8.3, and 8.5 | n.d | 3.5-4.5 |
| *Penicillum chrysogenum* 31B (Sakamoto and Kawasaki, 2003) | 78 and 52 (monomer) | 3.3 and 5.0 | 50 | 4.0-6.5 |
| *Aurobasidium pullulans* (Saha and Bothast, 1998) | 210* (dimer) | n.d | 75 | 4.0-4.75 |
| *Fusarium oxysporum f. sp. Dianthi* (Mártinez *et al*., 2004) | 58 (fusion protein) | n.d | 50 | 4.0 |
| Scytalidium thermophilum (Our study) | 38 and 160* (tetramer) | 6.8 | 70 | 7.0 |

* *Mw was determined using gel filtration chromatography, n.d: Not defined*

### 3.1.3 BAF substrate specifity

The activity of the pure BAF towards synthetic *p*-nitrophenol glycosides and birchwood xylan were tested. Although, BAF showed activity towards *p*-nitrophenol-α-L-arabinofuranoside, it did not hydrolyze the other glycosides and the birchwood xylan, even in the presence of the excess enzyme. Results were summarized in Table 14. BAF, lack of other polysaccharide degrading enzyme contamination, was used in subsequent hydrolysis experiment.

Table 14. The activity of pure *B. pumilus* SB-M13 α-L-arabinofuranosidase (BAF) against various substrates.

| Substrate | α-L-Arabinofuranosidase activitiy (U/ml) |
|---|---|
| p-NP-α-L-arabinofuranoside [a] | 0.4 |
| p-NP-α-L-arabinopyranoside [b] | 0.0 |
| p-NP-β-D-xylopyranoside [b] | 0.0 |
| p-NP-β-D-galactopyranoside [b] | 0.0 |
| p-NP-β-D-glucopyranoside [b] | 0.0 |
| p-NP-β-D-galacturonide [b] | 0.0 |
| p-NP-β-D-mannopyranoside [b] | 0.0 |
| p-NP-β-D-fucopyranoside [b] | 0.0 |
| Birchwood xylan [c] | 0.0 |

[a] 2mM, [b] 10 mM, [c] 1% (w/v) in 50 mM phosphate buffer at pH 7.0.

The progress of polysaccharide hydrolysis by BAF was followed using wheat (low viscosity), rye flour arabinoxylan, sugar beet arabinan (general introduction part, Figure 9), beet debranched arabinan, and larchwood arabinogalactan (general introduction part, Figure 10), (1%; w/v) as the substrates.

After 24 h incubation at pH 7.0 and 40°C, BAF (0.4 U/ml) released 22, 57 and 23% of arabinose from wheat bran arabinoxylan, rye flour arabinoxylan and branched sugar beet arabinan, respectively (Figure 44, Table 15). However, BAF did not hydolyse the linear α-1,5-L-arabinan (debranched) and arabinogalactan. Therefore, rye flour arabinoxylan was the best substrate for BAF and enzyme hydrolyses side chain arabinosyl residues rather than backbone.

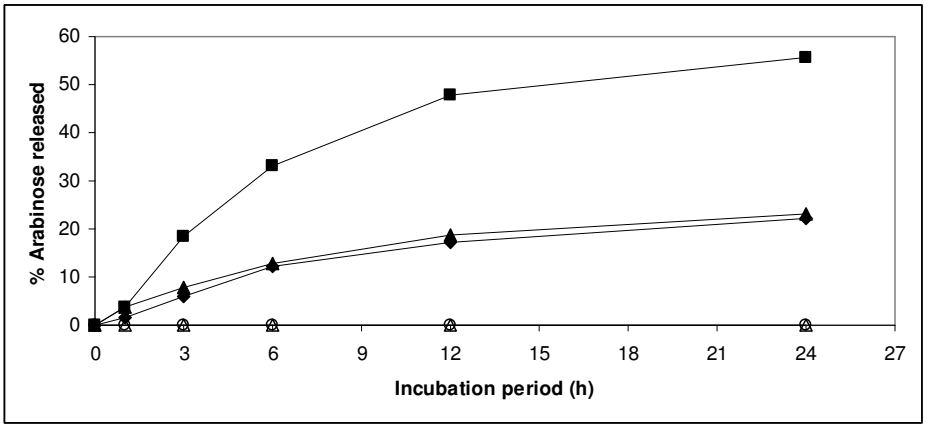



Figure 44. Degradation of arabinose containing 1.0 % (w/v) polymers by BAF at pH 7.0 and 40°C.■: rye flour arabinoxylan, ♦: wheat arabinoxylan, ▲: branched sugar beet arabinan, Δ: linear sugar beet arabinoxylan, and ○: larchwood arabinogalactan.

Table 15. Release of arabinose from arabinose containing polysaccharides by BAF. (24 h incubation at pH 7.0 and 40°C).

| Substrate (1%, w/v) | % Arabinose release after 24 h incubation |
|---|---|
| Wheat arabinoxylan (low viscosity) | 22 |
| Rye flour arabinoxylan | 57 |
| Branched sugar beet arabinan | 23 |
| Linear sugar beet arabinan | 0 |
| Arabinogalactan | 0 |

Arabinose releasing enzymes have been classified into four families of gylcanases (families 43, 51, 54, and 62). The two families (51 and 54) have also been classified further depending on their mode of action and substrate specifity (Beldman *et al*., 1997).Type A AFs, inactive towards arabinosyl linkages present in polysaccharides, preferentially degrade α-1,5-L- arabinofurano-oligosaccharides to monomeric arabinose. Type B AFs debranches L-arabinose residues from side chains of arabinan and arabinoxylan. Both types of AFs attack on synthetic p-nitrophenyl-α-L-arabinofuranoside. The third type of AFs, called α-L-arabinofuranohyrdolases, act on arabinosidic linkages in oat spelt, wheat and barley arabinoxylan, but do not show any activity towards p-nitrophenyl-α-L-arabinofuranoside, arabinans, and arabinogalactans (*Aspergillus awamori* α-L-arabinofuranohyrdolases, Kormelink *et al.*, 1991a).

Arabinose residues are found as α-1,2/or- and α-1,3 linked mono/di substituted side chains on wheat and rye flour arabinoxylan backbones which were hydrolyzed by BAF at varying level.

Moreover, sugar beet arabinan consists of a 1,5-a-linked backbone to which 1,3-a-linked (and possibly some 1,2-a-linked) L-arabinofuranosyl residues are attached.

Approximately 60% of the main-chain arabinofuranosyl residues are substituted by single 1,3-linked arabinofuranosyl groups. Unlike debrached arabinan, branched arabinan was hydrolysed by BAF, which indicated the affinity of BAF towards side chain arabinose (α-1,3 linked) rather than backbone arabinose (α-1,5 linkage).

Arabinogalactan from larchwood was not hydrolysed by BAF, which might have been due to high degree of galactose side chain substitution.

Overall, BAF preferentially removed side chain arabinoses and showed activity against synthetic p-nitrophenyl-α-L-arabinofuranoside, consequently enzyme was considered as Type B AF.

Microbial AFs have vide substrate specifity. Having no activity towards internal arabinosyl residues, *Bacillus pumillus* SB- M13 AF (BAF) is similar to AFs of *Aspergillus pullulans* (Saha and Bothast, 1998), *Stpretomyces* sp. Strain 17-1 (Kaji *et al*., 1981), *B. subtilis* 3.6 (Komae *et al*., 1982), *Trichoderma reesei* (Kaneko *et al*., 1998).

### 3.1.4 The effect of pH on BAF activity and stability

The effect of pH on *Bacillus pumilus* SB-M13 AF (BAF) was tested in buffer within the pH range of 5.0-10.0 at 40°C (Figure 45). Enzyme was active between the pH ranges from 6.0 to 9.0. The pH, at which the enzyme was the most active, was found to be 7.0, but at pH 7.5, enzyme retained 86% of its activity. The activity reduced to 43 and 29% at pH 8.0 and 9.0, respectively. Complete AF activity loss occurred at pH 5.0 and 10.0.

The effect of pH on the AF stability was also determined over the pH range of 4.0 to 10.0 (Figure 45). After incubation at 4°C and room temperature, sample pH was brought to 7.0, and then the residual AFs activity was measured at 40°C.

According to the pH stability data, after 2 h incubation at 4°C, the enzyme was 100% stable within the pH range of 5.0 to 10.0. When the incubation period was increased to 24 h, stable pH range decreased to pH 6.0-10.0. In another set of experiment, the enzyme was incubated at room temperature for 2 h, and it was found that the enzyme was also stable between the pH ranges of 5.0-10.0.

Furthermore, when the treatments for 2 h were considered, in the pH range of 6.0-10.0 enzyme retained almost 90-100% of its original activity at both 4 °C and room temperature. However, at pH 4.0 and 5.0, the effect of incubation at room temperature was more detrimental than that of at 4°C. Indeed, at 4°C, the enzyme maintained its 20% and 100% of its original activity at pH 4.0 and 5.0, but at room temperature, enzyme activity was completely lost at pH 4.0, and only 80% of the original activity retained at pH 5.0. Additionally, after the long term incubation (24 h) at 4°C, enzyme preserved almost 80% of its original activity between the pH 7.5 to 10.0. Then, at pH 7.0, the enzyme retained all of its activity, and only 60% of its activity was maintained at pH 5.0 and complete enzyme activity lost was observed at pH 4.0.

Kaneko *et al*. (1994) reported that AF from *B. subtilis* 3-6 has the maximum activity at pH 7.0 and the enzyme turned out to be inactived quickly at pHs above 10.0 and slowly at pHs below 7.0. The pH stability of AF from *B. stearothermophilus* T-6 was also assessed by Gilead and Shoham (1995) in pH range of 4.0 to 10.0 for 10 min incubation at 55°C. At pH 4.0 and 10.0, the enzyme retained only 9% of its activity and they accepted this pH profile typical for bacterial AFs

Degrassi and coworkers (2003) also tested pH stability of the AF of *B. pumilus* PS213 in the buffers ranging from pH 3.0 to 9.0 at 37°C after 1h incubation. When compared to AF of *B. pumilus* PS213 analyzed by Degrassi and coworkers (2003), BAF was rather more alkaline stable. In fact, at pH 9.0 and 10.0, AF of *B. pumilus* PS213 preserved respective 75 and 70% of original activity, but BAF was able to

conserve almost all of its activity after 2 h incubations at both 4°C and room temperatures, even after 24 h incubation at 4°C, it retained 80% of its original activity. Thus, BAF was considered to be alkaline resistant and potential enzyme in bleaching pulp.

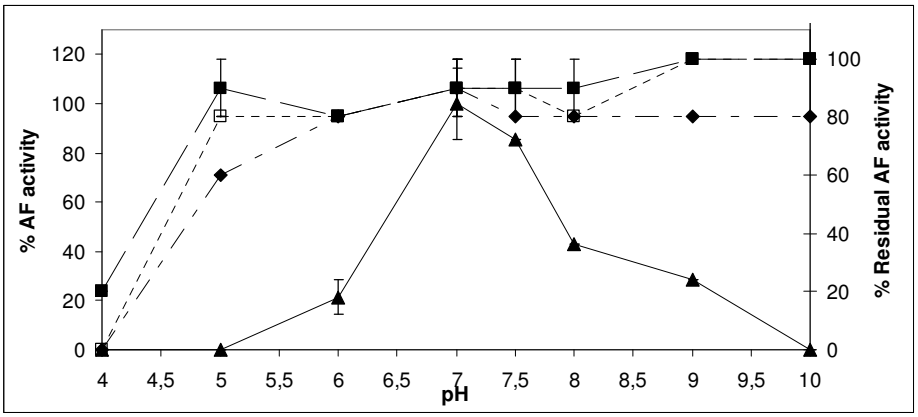



Figure 45. pH-dependance of activity and stability of *Bacillus pumilus* SB-M13 arabinofuranosidase (BAF). (▲: %BAF activity, ■: residual BAF activity after 2 h incubation at 4 °C, □: residual BAF activity after 2 h incubation at room temperature, and, ♦: residual BAF activity after 24 h incubation at 4°C). BAF activities were measured at $40^0$C and BAF activity was 1.19 U/ml at the beginning.

### 3.1.5 The effect of temperature on BAF activity and stability

The effect of temperature on xylanase activity was measured over the temperature range of 30-80°C (Figure 46). The highest xylanase activity was obtained at 70°C. AF activity measured at 65°C was 98% of the activity obtained at 70°C. Moreover, relative activities at 60 and 80°C were 85 and 68%, respectively.

Moreover, thermostability of the BAF was studied in the temperature range of 30-

90°C in 50 mM phosphate buffer at pH 7.0 (Figure 46). Enzyme samples were incubated for 2 h at given temperature range and assayed for AF activity at 40°C. About 83% of AF original activity was maintained within the temperature range of 30°C to 70°C after 2h incubation and the enzyme activity was decreased to 50% at 75°C and completely lost at temperature 80°. Hence, although *B. pumilus* SB-M13 was mesophilic microorganism, BAF was found to be thermostable. In addition, when compared to *B. pumilus* PS213 AF thermostability data, AF of both *B. pumilus* PS213 and *B. pumilus* SB-M13 maintained roughly 50% of original activity at 75°C after 2 h treatment, and enzymes showed similar thermostability profile.

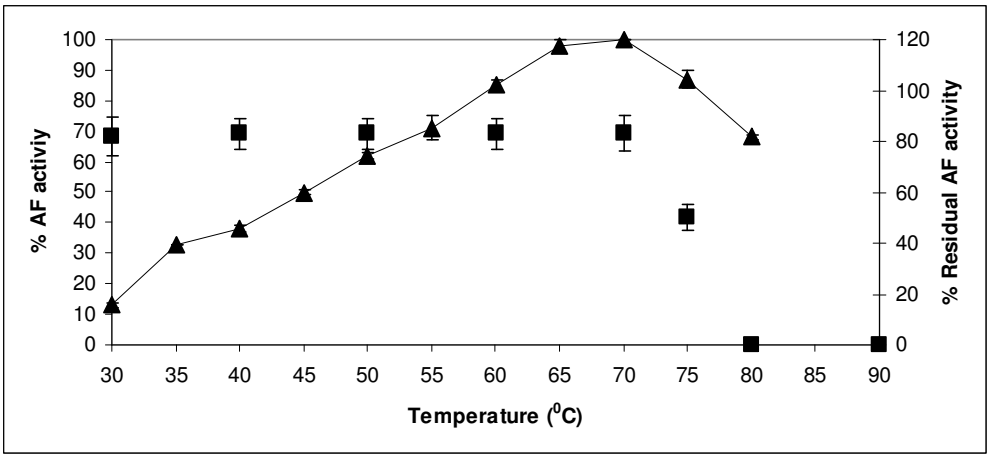



Figure 46. Temperature-dependance of activity and thermal stability of *Bacillus pumilus* SB-M13 AF (BAF). (▲: BAF activity, ■: residual BAF activity after 2 h incubation). BAF activities were measured at pH 7.0 and BAF activity was 1.32 U/ml at the beginning.

*B. stearothermophilus* T-6, a thermophilic bacterium, produces AF which was most active at 70°C. At 75°C and 80°C enzyme retained 66 and 25% of its original

activity (Gilead and Shoham, 1995). Kaneko and coworkers (1994) worked on *B. subtilis* 3-6 AF which exhibited the maximum activity at pH 7.0 and 60°C. Eubacterium *Rhodothermus marinus* produces AF which has maximum activity between pH 5.5 to 7.0 and at 85°C. Two AFs from *Aspergillus awamori* IFO 4033 were most active at pH 4.0 and 60°C (Kaneko *et al*., 1998) and the enzymes showed stability at pH values from 3.0 to 7.0 and at temperatures up to 60°C. *Penicillum purpurogenum* secreted AF with the highest activity at pH 6.0 and 50°C (De Ionnes *et al.,* 2000). Two AFs, purified from *P. chrysogenum* (Sakamoto and Kawasaki, 2003), had the highest activity at 50°C and enzyme activities of AFQ1 and AFS1 were found to be maximum at pH 4.0 to 6.5 and pH 3.3 to 5.0, respectively.

In conclusion, microbial AFs are most active in the temperature range of 30-80°C (Kaneko *et al*., 1998; Sakamoto and Kawasaki, 2003; Rahman *et al*., 2003; Degrassi *et al*., 2003; Gilead and Shoham, 1995; Kaneko *et al*., 1994), and bacterial AF show the highest activity in the pH range of 5.5 to 7.0 (Degrassi *et al*., 2003; Gilead *et al*., 1995; Kaneko *et al*., 1994). Unlike bacteria, fungi produce AF having more acidic pH optimum (Kaji, 1984 cited by Gilead and Shoham, 1995).

Having highest activity at pH 7.0 and 70°C, *Bacillus pumilus* SB-M13 AF was similar to other microbial AFs (Table 13 and 14). Due to the differences in the assay conditions and the purity of the enzyme, direct comparison of the temperature at which AFs are most active does not sound healthy. However, BAF with maximum activity at 70°C was remarkable and similar to values reported for pure AF of thermophilic *Bacillus* species (Gilead and Shoham, 1995).

Degrassie and coworkers (2003) also studied with Bacillus *pumilus* PS213 pure AF and they showed that enzyme was most active at pH 7.0 and 55°C. In our study the highest AF activity for *B. pumilus* SB-M13 was obtained at pH 7.0 and at 70°C, and at 55°C enzyme comprised only 71% of the activity obtained at 70°C. In

conclusion, these two AFs show the similar physicochemical properties except the optimum temperature which was higher for AF of *B. pumilus* SB-M13. Accordingly, they may have slight differences in their structure.

### 3.1.6 Kinetic studies

BAF activity was determined by measuring the initial reaction rates at 40°C in 50 mM phosphate buffer at pH 7.0 in various substrate concentrations ranging from 0.1-2.0 mM p-NPAraf. The enzyme followed the Michaelis-Menten kinetics and the constants were determined by using non-linear regression analysis (Sigma-Plot) (Figure 47). The $K_m$, $V_{max}$ and $k_{cat}$ values obtained by using *p*-nitrophenyl-α-L-arabinonofuranside (p-NPAraf) as a substrate were 0.3 mM, 1.28 U/ml and 2,6 U/mg protein, respectively.

AFs from various microorganisms showed $K_m$ values of 0.08-9.1 mM and $V_{max}$ values of 36.0-749 U/mg using p-NPA (Komae *et al*., 1982a; Lees and Farsberg, 1987; Hespel and O'Bryan, 1992; Uesaka *et al*., 1978; Gilead and Shoham *et al*., 1995). Gilead and Shoham (1995) indicated that $V_{max}$ value of 749 U/mg obtained from *Bacillus stearothermophilus* T-6 AF was the highest value reported to date for AFs. Besides, Degrassi and coworkers (2003) reported that at 25°C and 37°C the $K_m$ value for the *Bacillus pumilus* PS213 pure AF was 1.96±0.16 and 1.70±0.7 mM, while the $V_{max}$ value was 41±1.8 and 52.9±14.5 U/mg with p-NPAraf, respectively. They concluded that maximal reaction rate obtained for *Bacillus pumilus* was lower than most of the $V_{max}$ calculated for other microbial AFs. However, in our study $V_{max}$ value of 2,6 U/mg obtained for *Bacillus pumilus* SB-M13 AF (BAF) is remarkable and the highest $V_{max}$ value reported to date for AFs. However, although comparison cannot give sound results because of the different conditions used in enzyme assays, higher $V_{max}$ (U/mg; $k_{cat}$) value obtained from pure AF of *B. pumilus* SB M-13 determined its higher catalytic power. Moreover, $K_m$ value of 0.329 mM obtained from BAF was also lower than $K_m$ value of 1.96±0.16 and 1.70±0.7 mM obtained for *Bacillus pumilus* PS213 (Degrassi et al.,

2003). Accordingly, low $K_m$ value of pure AF of *B. pumilus* SB M-13 (BAF) also pointed to its high substrate affinity.

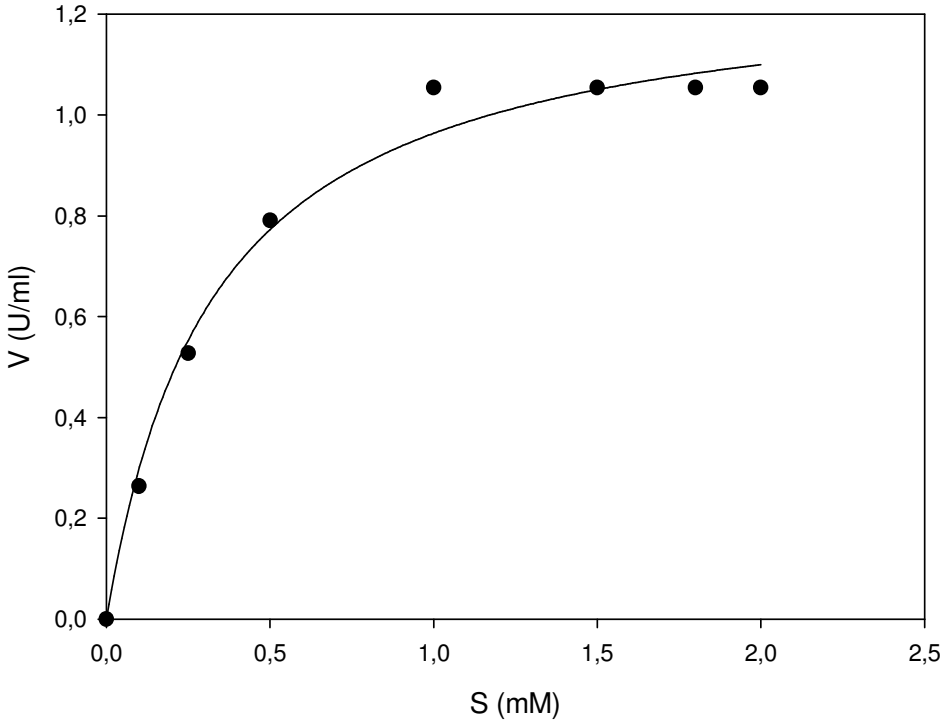



Figure 47. Michaelis-Menten plot for the pure *Bacillus pumilus* SB-M13 AF (BAF).

### 3.2 *Scytalidium thermophilum* α-L-arabinofuranosidase (STAF)

#### 3.2.1 STAF purification

STAF was purified at room temperature using a single-step hydrophobic interaction chromatography on HiLoad phenyl sepharose high performance mini column. The best purification profile was obtained at 3.5 M NaCl and pH 6.3, in

which STAF bound to matrix selectively while the majority of the contaminating proteins passed through the column without binding (Figure 48). The bound proteins were eluted with varying degrees as NaCl gradient decreases within a range of 3.5 to 0 M. Binding strongly to the column; STAF was eluted nearly at 0 M NaCl and purified by 700 fold with 66% activity recovery. Purity of the concentrated AF positive fractions was checked by SDS-PAGE (Figure 49) on which STAF was appeared as a single protein band. Therefore, using a single-step hydrophobic interaction chromatography, STAF was purified successfully.

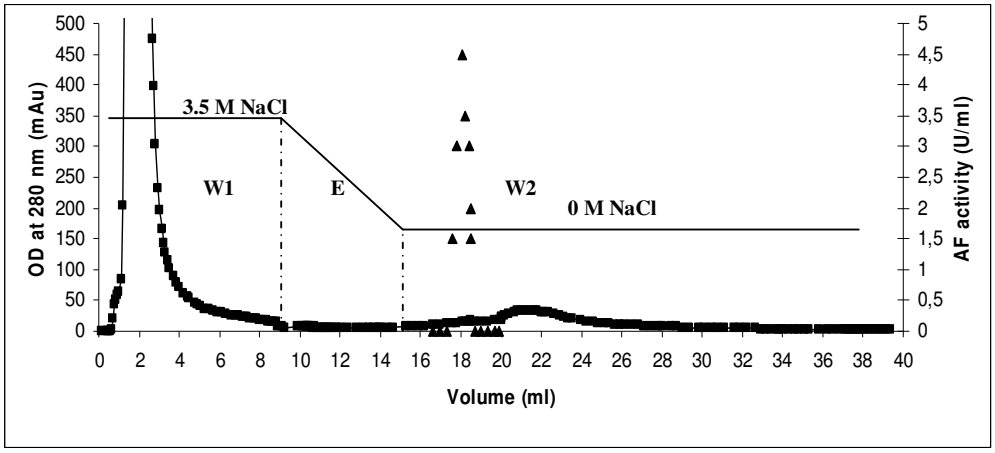



Figure 48. *S. thermophilum* AF (STAF) purification by using phenyl sepharose high performance mini column at pH 6.3 and 3.5 M NaCl. W1: sample injection and wash out in 3.5 M NaCl, E: elution in 3.5 M to 0 M NaCl gradient, W2: wash out in 0 M NaCl. (■: protein concentration, ▲: AF activity).

### 3.2.2 Molecular weight and isoelectric point determinations

*S. thermophilum* AF (STAF) molecular weights of 38 kDa and 160 kDa were obtained by SDS-PAGE and gel filtration chromatography (Appendix E), respectively. Having a native molecular weight of 160 kDa and single denaturized $M_W$ of 38 kDa, STAF demonstrated similar properties to other microbial AFs which have multiple forms and variable $M_W$s (Table 13 and 14). As of being a single protein band on SDS-PAGE gel, STAF was purified successfully by single-step hydrophobic interaction chromatography.

Moreover, the pI value of the STAF was determined as 6.8 by IEF which is also similar to other microbial AFs. They show pI values in the range of 3.3 to 8.8 (Table 13 and 14).

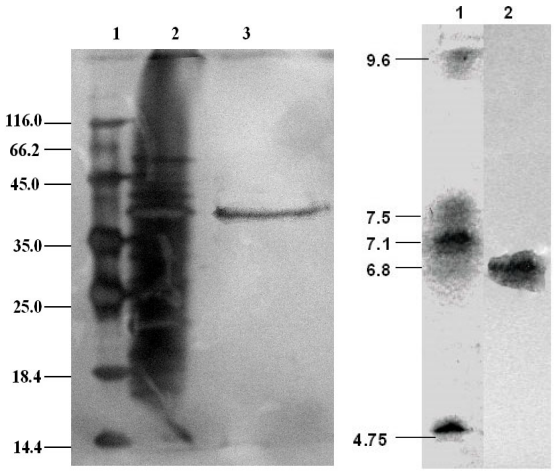



Figure 49. A- SDS-PAGE (12%) of the *Scytalidium thermophilum* AF (STAF). Lanes: 1- molecular weight markers, 2- crude enzyme, 3-pure STAF from hydrophobic interaction chromatography, B- IEF and activity zymogram of the *Scytalidium thermophilum* pure AF (STAF) from hydrophobic interaction chromatography; Lanes: 1- visible IEF markers, 2- activity zymogram of the pure STAF.

### 3.2.3 The effect of pH on STAF activity and stability

The effect of pH on AF was assessed in buffer within the pH range of 4.0-10.0 at 60°C (Figure 50). The enzyme was most active at pH 7.0, but at pH 8.0 and 9.0, enzyme had almost 76 and 61% of the activity obtained at pH 7.0, relatively. The enzyme activity was significantly low at pH lower than 5.0 and higher than 10.0 and complete AF activity loss occurred at pH 4 and 11.

Both fungal and bacterial AFs are most active in the temperature range of 30-75°C. However, when compared to fungal AFs which are most active in pH range of 2.0 to 5.8, bacterial AFs show the highest activity in pH range of 5.0 to 7.0. *S. thermophilum* AF activity was the highest at 70°C and pH 7.0 and very stable in a pH range up to 10.0 and temperature range up to approximately 75°C. Being active and stable at alkaline pHs, enzyme is different from other fungal AFs, which is an advantage in paper and pulp applications performed at high pHs.

The effect of pH on STAF stability was measured over the pH range of 4.0 to 10.0 (Figure 50). After incubations for 4 and 24 h at room temperature, sample pH was brought to 7.0, and then residual AF activities were measured at 60°C. The enzyme was stable within the pH range of 4.0 to 10.0. Respective 18 and 32% of original AF activity retained after incubations of 4 and 24 h at pH 4.0. AF activity retained after 4 h period was almost the same as that after 24 h in the pH range of 5.0-10.0. The enzyme retained more than 85% of its original activity in pH range of 6.0-10.0. Additionally, AF was more stable at alkaline pHs (8.0-10.0). Therefore, the enzyme is more pH stable than other fungal AFs which were reported to be stable at pH 3.0-7.0 (Gomes *et al*., 2000). When alkaline stability is considered, *S. thermophilum* AF seems a potential enzyme in bleaching pulp. Additionally, the effect of long term incubation at 4°C on STAF stability was more deleterious than that of at room temperature. In fact, complete STAF activity loss was defined at pH 4.0 and 5.0 and only 40 % of original STAF activity retained within pH range of 6.0-10.0 after 24 h preincubations at 4 °C. This is an expected

outcome for thermophilic enzymes produced by thermophilic microorganisms.

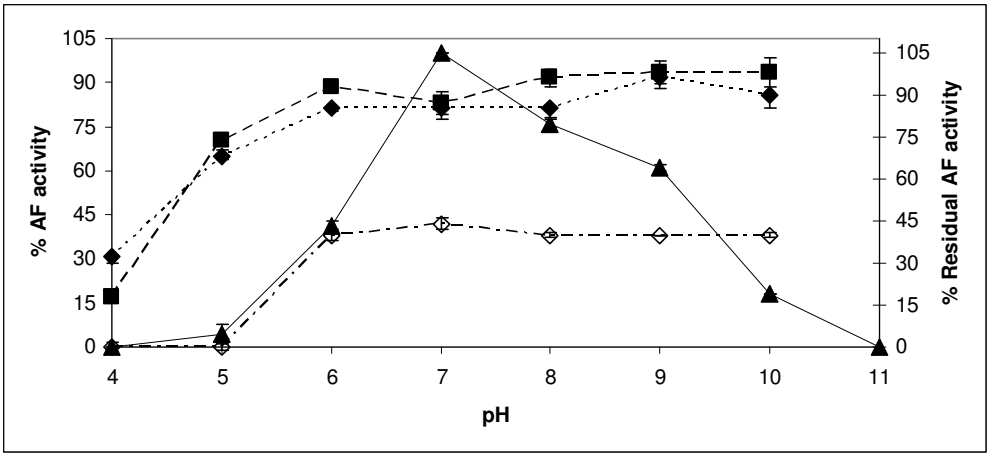



Figure 50. pH-dependence of activity and stability of *S. thermophilum* arabinofuranosidase (STAF). (▲: %STAF activity, ■: residual STAF activity after 4 h incubation at room temperature, ◆: residual STAF activity after 24 h incubation at room temperature, and, ◊: residual STAF activity after 24 h incubation at 4°C). STAF activities were measured at $60^0$C and STAF activity was 2.3 U/ml at the beginning.

### 3.2.4 The effect of temperature on STAF activity and stability

Additionally, the effect of temperature on AF activity was tested between the temperature ranges of 40-90°C (Figure 51). The highest activity of STAF was obtained at 70°C, whereas its relative activities at 65 and 75°C were calculated as 96 and 92 %, respectively. Moreover, enzyme activity sharply decreased after 75°C and at 80°C enzyme activity was 68% of the AF activity obtained at 70°C. Then, at 90°C, enzyme had 35% of the activity obtained at 70°C.

Thermostability of the pure STAF was also studied over the temperature range of 30-90°C after 15 min and 2 h incubations (Figure 51). When samples were incubated for 15 min, enzyme retained almost all of its original activity within the temperature range of 30-80°C. However, at 90°C % residual enzyme activity sharply decreased to 46.

When compared to 15 min incubations, effect of 2 h incubations on AF activity was more effective. Hence, when incubation period was increased to 2 h, % residual AF activity of 80 was obtained between the temperatures of 30 to 70°C. However, at 75°C % residual enzyme activity decreased to 55 and at 80 °C 22% of the enzyme original activity retained. Complete activity loss was observed at 90°C.

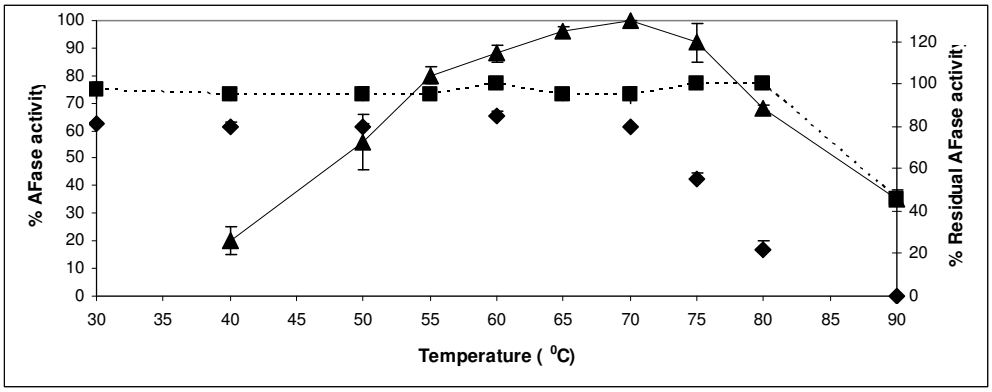



Figure 51. Temperature-dependance of activity and thermal stability of *S. thermophilum* arabinofuranosidase (STAF). (▲: STAF activity, ■: residual STAF activity after 15 min incubation, ◆: residual AF activity after 2 h incubation). STAF activities were measured at pH 7.0 and AF activity was 2.3 U/ml at the beginning.

*S. thermophilum* is a thermophilic fungus and AF produced by this fungus (STAF) shows thermophilic enzyme characteristics. Pure STAF retained 80% of the original enzyme activity after 2h incubation at 70°C; on the other hand, partially pure AF (8 fold pure) from *Bacillus stearothermophilus* T-6 (Gilead and Shoham, 1995) maintained 50% of its activity at 70°C after 1 h incubation. Moreover, STAF retained 55% of its original activity at 75°C after 2 h incubation. AF of *Thermomonospora fusca* BD25 (Tuncer, 2000) in crude enzyme extract and pure AF of *B. pumilus* SP213 (Degrassi, 2003) sustained 50% of their original enzyme activities after 1h and 135 min incubations at 75°C, respectively. AF of *B. pumilus* SB-M13 (our study) retained 75% of its activity at 70°C after 2 h incubation, as well. In general, it may be concluded that AF of *S. thermophilum* is thermostable enzyme; whereas, as a consequence of the possible protein concentration differences in each AF sample, and its influence on enzyme stability, thermostability of the AFs by different source cannot be compared efficiently.

### 3.2.5 Kinetic studies

STAF initial reaction rates were determined at 60°C in 50 mM phosphate buffer at pH 7.0 in various substrate concentrations ranging from 0.25-3.0 mM p-NPAraf. According to Lineweaver-Burk plot (Figure 52), it was found that enzyme followed the Michaelis-Menten kinetics and the $K_m$, $V_{max}$ and $k_{cat}$ values obtained by using *p*-nitrophenyl-α-L-arabinonofuranside (p-NPAraf) as a substrate were 3.7 mM, 0.67 U/ml and 80 U/mg protein, respectively.

$K_m$ and $V_{max}$ values of AFs from various microorganisms showed variation in the ranges of 0.08-9.1 mM and 36.0-749 U/mg using p-NPA, respectively (Komae *et al*., 1982a; Lees and Farsberg, 1987; Hespel and O'Bryan, 1992; Uesaka *et al*., 1978; Margolles and Reyes-Gavilán, 2003; Gilead and Shoham *et al*., 1995). The maximal velocity obtained for *S. thermopilum* (80U/mg protein) was lower than most of the $V_{max}$ calculated for other microbial AFs but it is still in the range of $V_{max}$ values of microbial AFs. Indeed, Degrassi and coworkers (2003) reported

that at 25°C and 37°C the $K_m$ value for the *Bacillus pumilus* PS213 pure AF was 1.96±0.16 and 1.70±0.7 mM, while the $V_{max}$ value was 41±1.8 and 52.9±14.5 U/mg with p-NPAraf, respectively. Moreover, the AF of *Streptomyces chartresius* (Matsuo *et al*., 2000) showed a $V_{max}$ values of 10.4 U/mg protein which is lower than $V_{max}$ values of both *S. thermophilum* (STAF, our study) and *Bacillus pumilus* PS213 (Degrassie *et al*., 2003) AFs.

Moreover, STAF $K_m$ value of 3.7 mM was also acceptable and falls in the $K_m$ values range of microbial AFs.

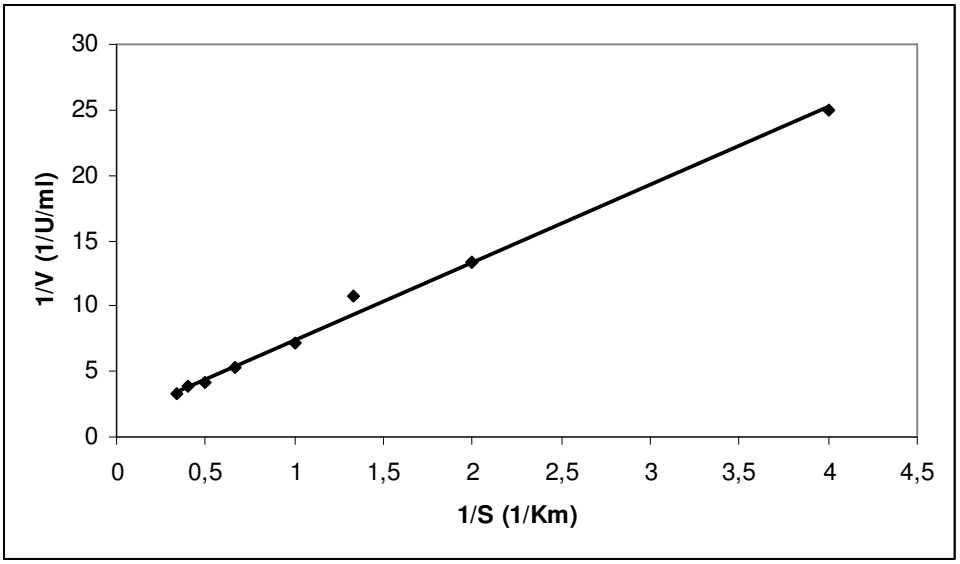



Figure 52. Lineweaver-Burk plot for the pure *Scytalidium thermophilum* AF.

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### 3.2.6 An assessment of physicochemical properties of BAF and STAF

Both of the AFs are multisubunit enzymes. When optimum pH and temperatures were considered, although BAF produced by mesophilic *B. pumilus* SB-M13, it showed similar properties to thermophilic STAF produced by *S. thermophilum*. Indeed, both enzymes were the most active at pH 7.0 and 70°C (Table 16). However, they had distinct kinetic parameters. $V_{max}$ value of the BAF was the highest value up to date, but that of STAF was remarkably low. Therefore, BAF has more catalytic power than STAF. Moreover, having the lower $K_m$ value, BAF also has more substrate specifity than STAF.

Both enzymes were alkaline tolerant, but STAF were more susceptible to 4°C incubation than BAF. After 24 h incubation at 4 °C, STAF retained almost 40% of its original activity between the pH ranges of 6-10, whereas, BAF retained almost 80% of its original activities within the same range. Moreover, unlike STAF, BAF was stable at pH 5.0. On the other hand, at room temperature STAF was more stable than BAF. After incubation at room temperature for 24 h, STAF retained almost all of its activity within the pH range of 6 to 10, and it was still stable at pH 4 and 5. However, after 2 h incubations, BAF retained almost 90% of its original activity within the pH range of 7 to 10. It retained 80% of original activity at pH 5 and 6, but it was not stable at pH 4.0.

Table 16. Comparative physicochemical properties of BAF and STAF.

| Microorganism | $M_W$ SDS-PAGE | pI | Optimum temperature (°C) | Optimum pH | $k_{cat}$ U/mg protein | $K_m$ mM | $k_{cat}/K_m$ |
|---|---|---|---|---|---|---|---|
| *B. pumilus* SB-M13 | 210* (tetramer) 53 | 4.8 | 70 | 7.0 | 2,6[a] | 0.3[a] | 8,7 |
| *S. thermoplium* | 160* (tetramer) 38 | 6.8 | 70 | 7.0 | 80[b] | 3.7[b] | 216 |

* *Mw was determined using gel filtration chromatography. Kinetic paratmertes were determined using p-NPAraf at* [a] *40* °C and [b] 60 °C.

When thermostability was considered, although BAF produced by mesophilic *B. pumilus* SB-M14, it was thermostable. Its thermostability profile was similar to STAF. Indeed, both STAF and BAF retained almost 80% of their enzyme activity between the temperature range of 30 to 70°C, after 2 h incubation. However, unlike BAF, STAF was active at 80°C and it retained 22% of original activity after 2 hr incubation. Possible protein concentration difference between BAF and STAF samples might play critical factor in temperature stability measurements.

## REFERENCES


Bachmann, S.L., and McCarthy A.J., 1991, "Purification and cooperative activity of enzymes constituting the xylan-degrading system of *Thermomonaspora fusca*", *Applied Environmental Microbiology*, Vol. 57, pp. 2121-2130.

Beldman, G., Schols, H.A., Piston, S.M., Searle-van Leeuwen M.J.F., and Voragen, A.G.J., 1997, "Arabinan degrading enzymes", *Adv Macromol Carbohyr Res*.,Vol 1,pp. 1-64.

Biran, S., Ersayin, A., Kocabas, A., and Bakir, U., 2006, "A novel xylanase from a soil isolate, *Bacillus pumilus*: Production, purification, characterization and one-step separation by aqueous-two-phase system" *Journal of Chromatography*, in rewiev.

Blum, H., Beier, H., and Grass, H.J., 1987, "Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels", *Electrophoresis*, Vol. 8, pp. 93-99.

Bradford, M.M., 1976, "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding", *Analitical Biochemistry*, Vol. 72, pp. 248-254.

Crous, J. M., Pretorius, I.S., and Van, Z. WH., 1996, "Cloning and expression of the α-L-arabinofuranosidases gene (ABF2) of *Aspergillus niger* into *Saccharomyces cerevisiae",Applied. Microbiology and Biotechnology,* Vol. 46, pp. 256-160.

De Ioannes, P.; Peirano, A.; Steiner, J.; Eyzaguirre, J., 2000, " An α-L-arabinofuranosidase from *Penicillum purpurogenum*: production, purification and properties". Journal of Biotechnology, Vol. 76, pp. 253-258.

Degrassi, G., Vindigni, A., and Venturi, V., 2003, "A thermostable α-arabinofuranosidase from xylanolytic *Bacillus pumilus*: purification and characterization", *Journal of Biotechnology*, Vol. 101, pp. 69-79.

De Vries, R.P., 1999, "Accessory enzymes from Aspergillus involved in xylan and pectin degredation", PhD Thesis, Wageningen University, Netherlands.

Gilead, S.; Shoham, Y., 1995, " Purification and characterization of α-L-arabinofuranosidases from *Bacillus stearothermophilus* T-6". *Applied Environmental Microbiology*, Vol. 61, pp. 170-174.

Gomes, J.; Gomes, I.; Terler, K.; Gubala, N.; Ditzelmüller, G.; Steiner, W., 2000, " Optimization of culture medium and conditions for α-L-arabinofuranosidases production by the extreme thermophilic eubacterium *Rhodotermus marinus*", *Enzyme Microb Technol*., Vol. 27, pp. 414-422.

Greve, L.C.; Labavitch, J.M., Hungate, R.E., 1994, "α-L-Arabinofuranosidase from Ruminococcus albus 8: purification and possible roles in hydrolysis of alfalfa cell wall", *Applied Environmental Microbiology*, Vol. 47, pp. 1135-1140.

Gunata, Z.Y., Brillouet, J.M., Voirin, S., Baumes, R., and Cordonnier, R., 1990, "Purification and some properties of an α-L-arabinofuranosidase from *Aspergillus niger.* Action on grape monoterpenyl arabinofuranosyl-glucosides", *Journal of Agric. Food. Chem*. Vol. 38, pp. 772-776.

Hespell, R.B.; O'Bryan, P.J., 1992, " Purification and characterization of α-L-arabinofuranosidases from *Butyrivibrio fibrosolvens* GS13", *Applied Environmental Microbiology*, Vol. 58, pp.1082-1088.

Kaji, 1984, cited by Gilead, S.; Shoham, Y., 1995, " Purification and characterization of α-L-arabinofuranosidases from *Bacillus stearothermophilus* T-6". *Applied Environmental Microbiology*, Vol. 61, pp. 170-174.

Kaneko, S.T.; Shimasaki, T.; Kusakabe, I., 1994, "Purification and some properties of intracellular α-L-arabinofuranosidases from *Aspergillus niger* 5-16", *Biosci. Biotechnl. Biochem*, Vol. 57, pp.1161-1165.

Kaneko, S.; Arimoto, M.; Ohba, M.; Kobayashi, H.; Ishii, T.; Kusabeke, I, 1998, "Purification and substrate specifities of two α-L-arabinofuranosidases from *Aspergillus awamori* IFO 4033", *Applied Environmental Microbiology*, Vol. 64, pp. 4021-4027.

Komae, K.; Kaji, A.; Sato, M., 1982, " An α-L-arabinofuranosidases from *Streptomyces purpurascens* IFO 3389", *Agri Biol Chem*., Vol. 46, pp. 1899-1905.

Konno, H.; Tanaka, R., Katoh, K. An extracellular α-L-arabinofuranosidase secreted from cell suspension cultures of carrot. Physiol Plant, 1994, 454-460.

Laemmli, U.K., 1970, "Cleavage of structural proteins during the assembly of the head of bacteriophage T4", *Nature (London)*, Vol. 227, pp. 680-685.

Lee, S.F., Forsberg, C.W., 1987, " Purification and characterization of an α-L-arabinofuranosidase from *Clostridium acetobutylicum* ATCC 824", Canadian Journal of Microbiology", Vol. 33, pp. 1011-1016.

Luonteri, E., Siika-aho, M, Tenkanen, M., and Viikari, L., 1995, "Purification and chrarcterization of three α-arabinosidases from *Aspergillus terraus*", *Journal of Biotechnology*, Vol. 38, pp. 279-291.

Makkonen, H.P.; Nakas, J.P., 2005, "Use of xylanase and arabinofuranosidase for arabinose removal from unbleached kraft pulp", *Biotechnology Letters*, Vol. 27, pp. 1675-1679.

Martínéz, C.A.C, Anzola, J.M., Rojas, A., Hernández, F., Junca, H., Ocampo, W., and Partillo, P.D., 2004, "Identification and characterization of the α-L-arabinofuranosidase B of *Fusarium oxysporum* f. sp. dianthi", *Physiological and Molecular Plat Physiology*, Vol. 64, pp. 201-208.

Rahman, A.K.M.S., Kawamura,S., Hatsu, M., Hoq, M., and Takamizawa, K., 2001, "Physicochemical properties of a novel alpha-L-arabinofuranosidase from *Rhizomucor pusillus* HHT-1", *Canadian Journal of Microbiology*, Vol. 47, pp. 767-772.

Rahman, A.K.M.S, Kato, K., Kawaki, S., Takamizawa, K., 2003, "Substrate specifity of the α–L-arabinofuranosidase from *Rhizomucor pusillus* HHT-1", *Carbohydrate Research*, Vol.338, pp.1469-1476.

Rémond, **C.,** Plantier-Royon, R., Aurby, N., Maes, E., Bliard, C., and O'Donohue, M.J., 2004, "Synthesis of pentose containing disaccharides using a thermostable α–L-arabinofuranısidase" *Carbohydrate Research*, Vol. 339, pp. 2019-2025.

Saha, B.C., and Bothast R.J., 1998, "Purification and characterization of a novel thermostable α-L-arabinofuranosidase from a color-variant strain of *Aureobasidium pullulans", Applied Environ Microbiology*, Vol. 64, pp. 216-220.

Saha, B.C., and Bothast R.J., 1999, "Pretreatment and enzymatic saccharification of corn fiber", *Applied Biochemitry Biotechnology*., Vol. 76, pp. 65-77.

Sakamoto, T., and Kawasaki, H., 2003, "Purification and properties of two type-B α-L-arabinofuranosidases produced by *Penicillium crysogenum", Biochimica et Biophysica Acta*, Vol. 1621, pp. 204-210.

Spagna**,** G.; Ramaognoli, D.; Angela, M.; Bianchi, G.; Pifferi, P.G., 1998, " A simple method for purifying glycosidases: α-L-arabinofuranosidase and β-D-glucopyranosidase from *Aspergillus niger* to increase aroma of wine. Part I", *Enzyme and Microb Technol*", Vol. 22, pp. 298-304.

Tajana, E.; Fiechter, A.; Zimmermann, W. Purification and characterization of two α-L-arabinofuranosidases from *Streptomyces diastaticus.* Appl Environ Microbiol.1992, 58, 1447-1450.

Tuncer. M., 2000, "Characterization of β-xylosidase and α-L-arabinofuranosidase activities from *Thermomonaspora fusca* BD25", *Turkish Journal of Biology*, Vol. 24, pp. 753-767.

Uesaka, E., Sato, M., Raiju, M., Kaji, A., 1978, "α-L-arabinofuranosidases from *Rhodotorula flava"*, *Journal of Bacteriology*., Vol. 133, pp. 1073-1077.

# CHAPTER 4

# CONCLUSIONS

In the first part of the study, xylanolytic systems of *B. pumilus* SB-M13 and *S. thermophilum* were investigated. Accordingly, microorganisms were grown on agricultural by-products; such as corn cobs, wheat bran, and rice bran and their xylanolytic enzyme production profiles were assessed. Moreover, the effect of L-arabinose on xylanolytic enzyme production level was also investigated in fermentation media containing 3% corn cobs.

Xylanolytic enzyme production was found to be sequential for both microorganisms. When grown on 3% of corn cobs, wheat bran, and rice bran containing fermentation cultures, *B. pumilus* produced extracellular, AF, XYN, and GAL at varying levels. However, there were no XYL and GLU activities measured in culture filtrate. Absence of extracellular XYL and GLU activities in culture filtrate is an advantage in xylooligosaccharide production and bleaching pulp, and culture filtrate can directly be used without any enzyme purification.

When *B. pumilus* SB-M13 xylanolytic enzyme induction efficiency of corn cobs, wheat bran, and rice bran were compared, wheat bran was the best carbon source by which maximum levels of AF (5 U/ml), GAL (0.3 U/ml), and XYN (105 U/ml) were produced. It should also be noted that corn cobs were also able to induce same level of XYN (108 U/ml) as wheat bran did.

When utilizing 3% of corn cobs, wheat bran, and rice bran, *S. thermophilum* produced four xylanolytic (XYN, AF, XYL, and GAL) and one cellulolytic (GLU)

extracellular enzymes at various degrees. Corn cobs were the best carbon source among the others and consequently, the highest enzyme activities were attained with corn cobs. Accordingly, The XYN with maximum activity of 266 U/ml was the main enzyme in the fermentation culture, followed by GLU (80 U/ml), XYL (40 U/ml), GAL (10 U/ml), and AF (3.3 U/ml).

Presence of extracellular XYL is a limitation in xylooligosaccharide production, because it hydrolyzes xylooligosaccharides into xylose monomer. Therefore, *S. thermophilum* crude enzyme extract can not be directly used in xylooligosaccharide production. However, it can be used for xylose production more efficiently than *B. pumilus* SB-M13.

*S. thermophilum* XYN production level (266 U/ml) is almost 2.5 times higher than that of *B. pumilus* SB-M13 (108 U/ml). On the other hand, production of high level of GLU (80 U/ml) restricts direct use of crude enzyme extracts in bleaching pulp.

Besides, *B. pumilus* SB-M13 AF (5 U/ml) production degree was higher than *S. thermophilum* (3.3 U/ml). Moreover, both enzymes can be used in in food industry for L-arabinose production. Recently, interest in L-arabinose has been increasing, because of its low uptake by body, sweet taste, and food additive potential.

When effect of arabinose on *B. pumilus* SB-M13 and *S. thermoplium* growth and xylanolytic enzyme production was considered, it was found that both *S. thermophilum* and *B. pumilus* SB-M13 xylanolytic enzymes were under the control of carbon catabolite repression. However, when compared to *S. thermophilum*, due to efficient and rapid consumption, suppression effect of arabinose on *B. pumilus* SB-M13 xylanolytic enzyme synthesis was relieved quickly.

In the second part of the study, *Bacillus pumilus* SB-M13 β-1,4-endoxylanase (xylanase, E.C. 3.2.1.8) was purified using a single-step hydrophobic interaction

chromatography and biochemically characterized. It has a molecular weight of 24.8 kDa and pI of 9.2. Xylanolytic activity is stable at alkaline pH and highest activity is observed at 60°C and pH 7.5. Enzyme $K_m$ and $k_{cat}$ values were determined as 1.87 mg/ml and 43,000 U/mg, respectively. Therefore, pure enzyme has high substrate affinity and catalytic power. Additional properties like, being a small molecule, having high stability at alkaline pH and almost cellulase free activity make xylanase of *B. pumilus* SB M-13 promising in paper and pulp applications.

In the last part of the study, the α-L-arabinofuranosidases (AF, EC 3.2.1.55), BAF and STAF, were produced on 3% corn cobs by soil isolate *Bacillus pumilus* SB-M13 and thermophilic fungus *Scytalidium thermophilum*, respectively. Both BAF and STAF were purified using a single step hydrophobic interaction chromatography and biochemically characterized.

Accordingly, BAF was purified 700 fold with 66% recovery. The enzyme had native $M_W$ of 210 kDa and subunit $M_W$ 53 kDa determined by gel filtration chromatography and SDS-PAGE, respectively. Enzyme had a pI of 4.75 and was most active at 70°C and pH 7.0. Although BAF produced by mesophilic *B. pumilus* SB-M14, it was thermostable. It was stable at both acidic and alkaline pHs (pH 5.0-10.0). BAF was considered to be alkaline resistant and potential enzyme in bleaching pulp.Enzyme $K_m$ and $k_{cat}$ values of 0.3 mM and 2,6 U/mg were at 40°C using p-nitrophenylα-L-arabinofuranoside, respectively. $V_{max}$ value of 2,6 U/mg obtained for *Bacillus pumilus* SB-M13 AF (BAF) is remarkable and the highest $V_{max}$ value reported to date for AFs.. ,.Although, pure BAF showed activity towards *p*-nitrophenol-α-L-arabinofuranoside, it did not hydrolyze the other glycosides and the birchwood xylan, even in the presence of the excess enzyme. Moreover, unlike debranched arabinan, arabinose from wheat bran arabinoxylan, rye flour arabinoxylan and branched sugar beet arabinan was released by BAF at varying degree. Accordingly, enzyme was considered as Type B AF.

STAF was purified 700-fold with 66% recovery and the native molecular weight of the enzyme was determined as 160 kDa by gel filtration chromatography. Enzyme had subunit $M_W$ of 38 kDa on SDS-PAGE. pI of the enzyme was determined as 6.8 and it was most active at 70°C and pH 7.0. STAF was more stable at alkaline pHs. Kinetic analysis at 60°C using p-nitrophenyl$\alpha$-L-arabinofuranoside resulted in $K_m$ and $k_{cat}$ values of 3.7mM and 80 U/mg, respectively.

Utilizing cheap agricultural by-products as a sole carbon source, *S. thermophilum* produced high level of AF which was first investigated and reported in this study. Pure enzyme shows similar physicochemical properties to other fungal AF except for the pH at which the enzyme was most active. Although originated from fungus, *S. thermophilum* AF activity was the highest at pH 7.0 which is rather higher for other reported fungal AF. Moreover, long term (24 h) pH stability of the enzyme in the pH range of 6.0-10.0 is remarkable. Considering physicochemical properties, *S. thermophilum* AF (STAF) sound feasible enzyme in pulp and paper industry. In addition, after obtaining a detailed understanding of the substrate specifity of the STAF, its potential application areas can be investigated.

## APPENDIX A

## CH-API 50 CHB/E MEDIUM KIT STRIP COMPOSITION

Table The composition of the 50 CH-API 50 CHB/E medium kit strip

| Tube | Test | Active ingredients | QTY (mg/cup.) |
|---|---|---|---|
| 0 | | Control | - |
| 1 | GLY | GLYcerol | 1.64 |
| 2 | ERY | ERYthritol | 1.44 |
| 3 | DARA | D-ARAbinose | 1.4 |
| 4 | LARA | L-ARAbinose | 1.4 |
| 5 | RIB | RIBose | 1.4 |
| 6 | DXYL | D-XYLose | 1.4 |
| 7 | LXYL | L-XYLose | 1.4 |
| 8 | ADO | D-ADOnitol | 1.36 |
| 9 | MDX | Methly- D Xylopyranoside | 1.28 |
| 10 | GAL | D-GALactose | 1.4 |
| 11 | GLU | D-GLUcose | 1.56 |
| 12 | FRU | D-FRUctose | 1.4 |
| 13 | MNE | D-MaNnosE | 1.4 |
| 14 | SBE | L-SorBosE | 1.4 |
| 15 | RHA | L-RHAmnose | 1.36 |
| 16 | DUL | DULcitol | 1.36 |
| 17 | INO | INOsitol | 1.4 |
| 18 | MAN | D-MANnitol | 1.36 |
| 19 | SOR | D-SORbitol | 1.36 |
| 20 | MDM | Methly- D-Mannopyranoside | 1.28 |

Table cont'd. The composition of the 50 CH-API 50 CHB/E medium kit strip

| Tube | Test | Active ingredients | QTY (mg/cup.) |
|---|---|---|---|
| 21 | MDG | Methly- D-Glucopyranoside | 1.28 |
| 22 | NAG | N-AcetylGlucosamine | 1.28 |
| 23 | AMY | AMYgdalin | 1.08 |
| 24 | ARB | ARButin | 1.08 |
| 25 | ESC | ESCulin, ferric citrate | 1.16, 0.152 |
| 26 | SAL | SALicin | 1.04 |
| 27 | CEL | D-CELlobiose | 1.32 |
| 28 | MAL | D-MALtose | 1.4 |
| 29 | LAC | D-LACtose (bovine origin) | 1.4 |
| 30 | MEL | D-MELibiose | 1.32 |
| 31 | SAC | D-SACcharose (sucrose) | 1.32 |
| 32 | TRE | D-TREhalose | 1.32 |
| 33 | INU | INUlin | 1.28 |
| 34 | MLZ | D-MeLeZitose | 1.32 |
| 35 | RAF | D-RAFfinose | 1.56 |
| 36 | AMD | AmiDon (starch) | 1.28 |
| 37 | GLYG | GLYcoGen | 1.28 |
| 38 | XLT | XyLiTol | 1.4 |
| 39 | GEN | GENtibiose | 0.5 |
| 40 | TUR | D-TURanose | 1.32 |
| 41 | LYX | D-LYXosE | 1.4 |
| 42 | TAG | D-TAGotosE | 1.4 |
| 43 | DFUC | D-FUCose | 1.28 |
| 44 | LFUC | L-FUCose | 1.28 |
| 45 | DARL | D-ARbitoL | 1.4 |
| 46 | LARL | L-ARbitoL | 1.4 |
| 47 | GNT | Potassium GlucoNaTE | 1.84 |
| 48 | 2KG | Potassium 2-KetoGluconate | 2.12 |
| 49 | 5KG | Potassium 5-KetoGluconate | 1.8 |

The composition of the 50 CH-API 50 CHB/E medium kit strip

## APPENDIX B

## CH-API 50 CHB/E MEDIUM KIT RESULTS EVALUATION

| Test number | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Result * (-/+) | - | + | - | - | + | + | + | - | - | - | + | + | + | + | - | - | - | - | + | - | - | + | + | + | + | + |
| Test number | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 | 36 | 37 | 38 | 39 | 40 | 41 | 42 | 43 | 44 | 45 | 46 | 47 | 48 | 49 | X | X |
| Result * (-/+) | + | + | + | - | - | + | + | - | - | - | - | - | - | + | + | - | + | - | - | - | - | - | - | - | | |

*Results were interpreted on the basis of ability of microorganism to ferment various carbohydrate present in the kit at 30 °C.

## APPENDIX C

## REAGENTS AND GEL PREPARATION FOR SDS-PAGE SLAB GEL (LAEMMLI BUFFER SYSTEM)

### Stock Solutions

A. Acrylamide/bis (30% T, 2.67%C)
   87.6g acrylamide (29.2g/100ml)
   2.4g N'N'-bis-methylene-acrylamide (0.8g/100 ml)

Make to 300 ml with distilled water. Filter and store at 4°C in the dark (30 days maximum). Since acrylamide is a neuro toxin, precautions should be taken by wearing gloves and mask during preparation of this solution.

B. 1.5 M Tri-HCI, pH 8.8
   27.23 g Tris base
   ~80 ml distilled water

Adjust to pH 6.8 with 1N HCl. Make to 100 ml with distilled water and store at 4°C.

C. 0.5 M Tri-HCI, pH 6.8
   6 g Tris base
   ~60 ml distilled water

Adjust to pH 6.8 with 1N HCI. Make to 100 ml with distilled water and store at 4°C.

D. 10% SDS

Dissolve 10 g SDS in distilled water with gentle stirring and bring to 100 ml with distilled water.

E. Sample Buffer (SDS reducing buffer) (store at room temperature)

| | |
|---|---|
| Distilled water | 4.0 ml |
| 0.5 M Tris-HCI, pH 6.8 | 1.0 ml |
| Glycerol | 0.8 ml |
| 10% (w/v) SDS | 1.6 ml |
| 2-b-mercaptoethanol | 0.4 ml |
| 0.05% (w/v) bromophenol blue | 0.2 ml |
| | 8.0 ml |

Dilute the sampl at least 1:4 with sample buffer and heat 95°C for 4 minutes

F. 5X Electrode (Running) Buffer, pH 8,3 (enough for 10 runs)

| | |
|---|---|
| Tris base | 9.0 g |
| Glycine | 43.2 g |
| SDS | 3.0 g |

Bring to 600 ml with distilled water. Store at 4°C. Warm to 37°C before use if precipitation occurs. Dilute 60 ml 5X stock with 240 ml distilled water for one electrophoretic run.

F. 10% Ammonium Presulfate (APS)

Dissolve 100 mg APS in 1 ml of distilled water in an eppendorf by vortexing. This solution should be prepared fresh daily.

G. TEMED (N,N-tetramethylene-ethylenediamine)

Use TEMED neat from the bottle.

**Procedure**

A. Preliminary Preparation

Clean the glasses and spacers with ethanol. Assemble the gel sandwich on a clean surface. Lay the longer rectangular glass plate down first, the place two spacers of equal thickness along the short edges of rectangular plate. Next, place the shorter glass plate on top of the spacers. Install the clamps and fasten the screws. Transfer the clamp assembly to one of the casting stand. To check whether the glasses are properly sealed, pour distilled water between glasses. If water level does not decrease, glasses are properly sealed. Then pour out the water and let the glasses to dry.

B. Preparation of SDS-PAGE Gel Solution

**Separating Gel**

Add the followings into a smaller beaker.

Table F.1 : Preparation of %12 SDS-PAGE separating gel

| | |
|---|---|
| Monomer Concentration (30% T, 2.67% C) | 12% |
| Acryamide/bis (30% T, 2.67% C Stock) | 4.0 ml |
| Distilled Water | 3.35 ml |
| 1.5 M Tris-HCI, pH 8.8 | 2.5 ml |
| 0.5 M Tris-HCI, pH 6.8 | - |
| 10% SDS | 100 µl |
| 10% Ammonium persulfate (fresh) | 50 µl |
| TEMED (N',N'-tetramethylene-ethylenediamine) | 100 µl |

Prepare the monomer solution by combining all reagents except ammonium persulfate and TEMED. Dearate the solution under vacuum for at least 15 minutes. Add the two catalysts just prior to casting the gels.

After adding two catalysts immediately pour the solution between glasses up to 5 cm below the upper edge of small glass. In order to avoid air contact, pour distilled water onto gel.

Allow to stand to complete the polymerization.

**Stacking Gel**

Add the following into a small beaker.

Table F. Preparation of %4 SDS-PAGE separating gel

| | |
|---|---|
| Monomer Concentration (30% T, 2.67% C) | 4% |
| Acryamide/bis (30% T, 2.67% C Stock) | 1.3 ml |
| Distilled Water | 6.1 ml |
| 1.5 M Tris-HCI, pH 8.8 | - |
| 0.5 M Tris-HCI, pH 6.8 | 2.5 ml |
| 10% SDS | 100 µl |
| 10% Ammonium persulfate (fresh) | 50 µl |
| TEMED (N',N'-tetramethylene-ethylenediamine) | 100 µl |

Dry the area above the separating gel with filter paper before pouring the stacking gel. Immediately pour the gel solution between glasses. Place a comb in the gel sandwich and tilt it so that the teeth are at a slight (~10°) angel. This will prevent air from being trapped under the comb teeth while the monomer solutions are poured. Allow the gel to polymerize 30-45 minutes.

After polymerization is completed remove the comb by pulling it straight up slowly

and gently and fill the wells with 1x loading buffer. Load the standard and the sample (diluted at least 1:4 with sample buffer and heated at 95°C for 5 minutes) into the wells in an order and keep note for them.

SDS-PAGE molecular weight marker was a mixture of proteins given in Table F.3.

Table F.3: SDS-PAGE molecular weight markers

| Protein | Source | Approx. MW (kDa) |
|---|---|---|
| β-galactosidase | *E.coli* | 116.0 |
| Bovine serum albumin | Bovine plasma | 66.2 |
| Ovalbumin | Chicken egg white | 45.0 |
| Lactate dehydrogenase | Porcine muscle | 35.0 |
| Restriction endonuclease *Bsp*981 | *E.coli* | 25.0 |
| β-lactoglobulin | Bovine milk | 18.4 |
| Lysozyme | Chicken egg white | 14.4 |

After the gels are cast, the clamp assemblies are snapped onto the inner cooling core to from the upper buffer chamber. The upper buffer is in direct contact with the inner glass plate of the gel sandwich to provide even heat distribution over the entire gel length, preventing thermal band distortion during electrophoretic separations. Fill the chamber with 1x loading buffer. Gently place the cooling core into the electrophoresis tank.

Place the lid on the top of the buffer chamber to fully enclose the cell. Attach the electrical leads to a suitable power supply with the proper polarity. The recommended power condition for optimal resolution with minimal thermal band distortion is 200 volts, constant voltage setting. The usual runtime is approximately 45 minutes. The electrophoresis cell is for rapid separation and is not recommended for runs over 60 minutes long.

## APPENDIX D

## ISOELECTRIC FOCUSING

Always use the high quality distilled or deionized water to prepare stock solution for electrofocusing.

A. Monomer-ampholyte solution

The following volumes produce sufficient reagents for two 125x65x0.4 mm gels.

| Ingredients | Volume (ml) |
|---|---|
| dH2O | 5.5 |
| Monomer concentrate; 3% C | 2.0 |
| Glycerol; 25% (w/v) | 2.0 |
| Ampholyte | 0.5 |

A.1 Monomer concentrate (3% C)

| Ingredients | Amount (w/v) |
|---|---|
| Acrylamide | 24.25 |
| N, N'-Methylene-bis-acrylamide | 0.75 |

Dissolve 24.25 g of acrylamide and 0.75 g od bis-acrylamide in distilled water and bring the final volume to 100 ml. After filtering through a 0.45 μm filter, store the solution in dark bottle at 4°C. This solution may be stored up to 1 month.

A.2 Glycerol; 25% (w/v)

Add 25 g of glycerol into 50 ml of distilled water and dilute to 100 my adding distilled water.

A.3 Ampholyte

Use Bio-Lyte 3/10 ampholytes (Bi-Rad)

B. Catalyst solution

The following volumes produce sufficient reagents for two 125x65x0.4 mm gels.

| Ingredients | Volume (μl) |
|---|---|
| Ammonium persulfate; 10% (w/v) | 15 |
| FMN; 0.1% (w/v) | 50 |
| TEMED | 3 |

B.1 Ammonium persulfate; 10% (w/v)

Dissolve 10 mg of ammonium persulfate in 100 μl of distilled water. Prepare solution fresh before use.

B.2 Riboflavin-5'-phosphate (FMN); 0.1% (w/v)

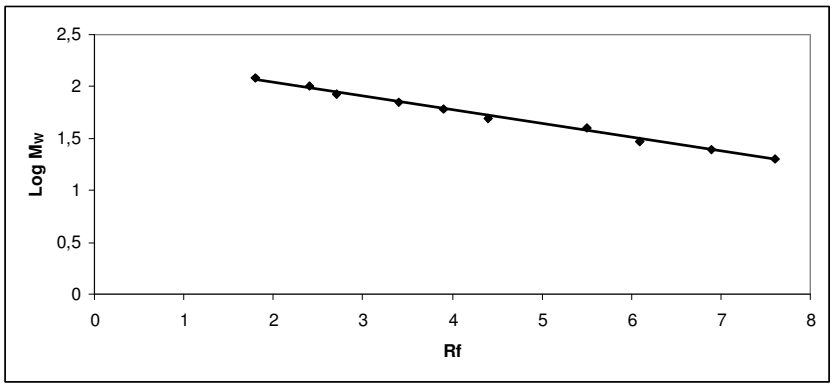
Dissolve 50 mg of FMN in 50 ml of distilled water. This solution may be stored up to 1 month at 40 °C in dark bottle.

B.3 N,N'-tetramethylene-ethylenediamine (TEMED)

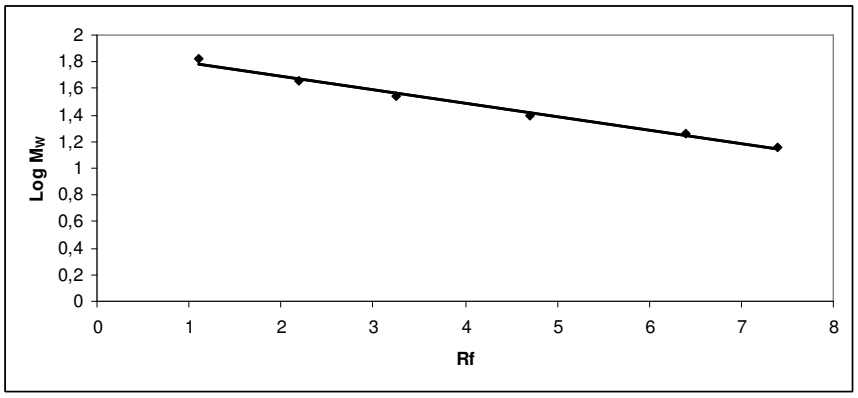
Use commercial TEMED directly from bottle. Use only pure TEMED and store it dry, cool, and protected from light.

## APPENDIX E

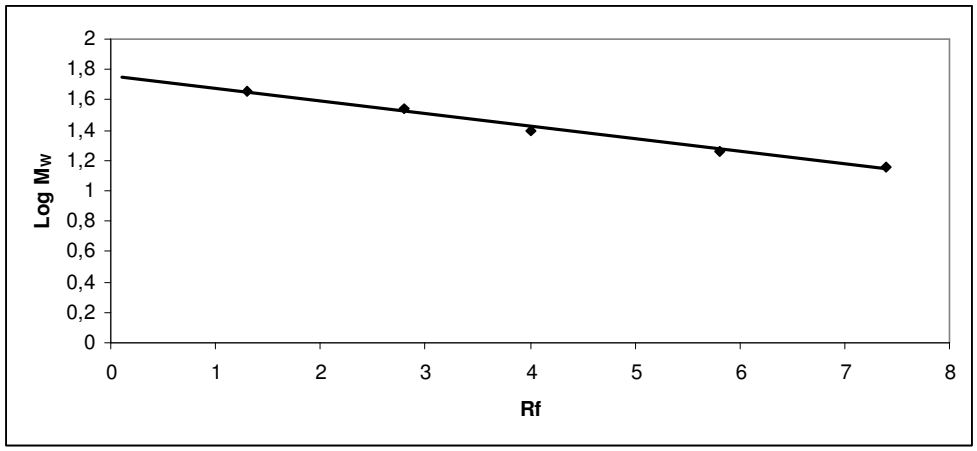
## SDS-PAGE MOLECULAR WEIGHT STANDART CURVES




Standard curve for BAF molecular weight determination by SDS-PAGE.




Standard curve for BAX molecular weight determination by SDS-PAGE.




Standard curve for STAF molecular weight determination by SDS-PAGE.

# APPENDIX F

# DNSA METHOD

## Composıtıon of DNSA reagent

36g $C_4H_4KnaO_6.4H_2O$ (Potassium-sodium tartrate), 1g NaOH, 1g DNSA (dinitrosalicylic acid), 0.2g phenol, and 0.05g $Na_2SO_3$ (Sodium sulfite) are dissolved in 80-ml distilled water and diluted with distilled water to 100 ml. The reagent must be stored in dark bottle at 4 °C in refrigerator.

## Procedure of DNSA method

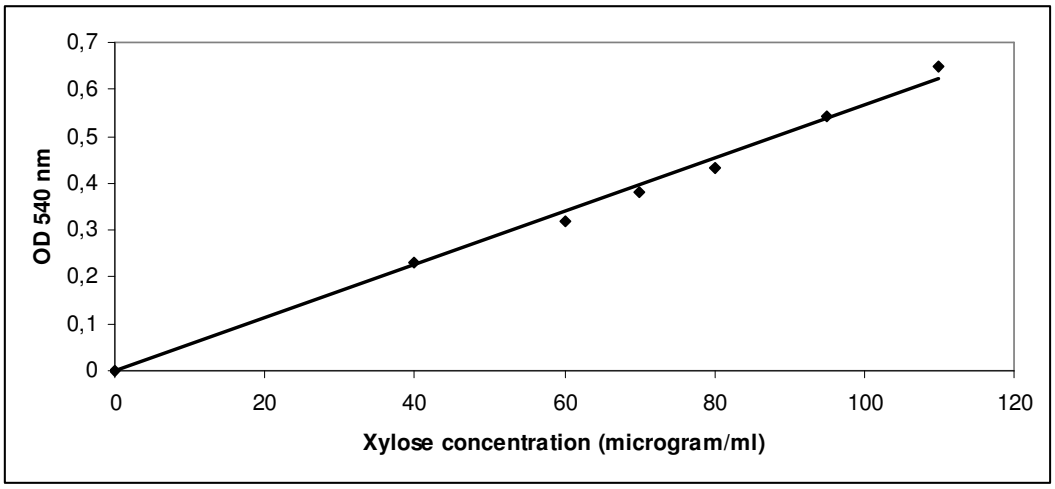
The reaction mixtures contained 1 ml of properly diluted enzyme and 10 ml of substrate solution. The reaction mixtures were incubated at suggested temperature and the 1ml of samples were mixed with 1.5 ml of DNSA reagent at 15 second intervals up to 1 min to. Then, the tubes are boiled for five minutes and cooled immediately under tap water. Finally, tubes are equilibrated to room temperature and read against a buffer blank at 540 nm**.**

## Preparation of xylose standard

100 µg/ml of xylose stock solution is prepared in buffer solution and dilutions are made for 1 ml reducing sugar sample according to the following table.

Table Dilutions for xylose standard curve

| Xylose (µg/ml) | 0 | 40 | 60 | 80 | 100 |
|---|---|---|---|---|---|
| Xylose sol'n (µl) | 0 | 400 | 600 | 800 | 1000 |
| Buffer (µl) | 1000 | 600 | 400 | 200 | 0 |




Standard curve for DNSA method

# APPENDIX G

# BRADFORD METHOD

## Preparatıon of Bradford reagent

To prepare 5x concentrated stock solution 250 ml 95% ethanol (spectroscopic), 500 mg Brilliant Blue G dye (SERVA), and 500 ml 85% ortho-posphoric acid are mixed and then is diluted to 1L with distilled water. The stock solution must be stored in a dark bottle at refrigeration temperature.

The working solution is prepared by mixing 1 volume of concentrate with 4 volumes of distilled water. Then, solution is filtered and left at room temperature for at least 24 hour before use.

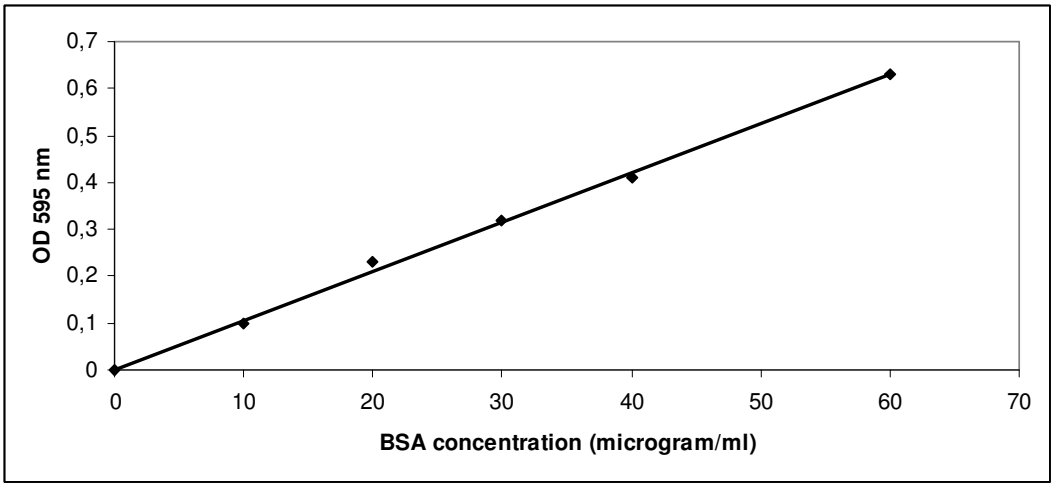
## Preparation of protein standard

BSA is diluted in buffer solution to obtain a stock solution with concentration of 100 µg/ml.

Table Dilutions for protein standard curve

| Protein (µg/ml) | 0 | 20 | 40 | 60 | 80 | 100 |
|---|---|---|---|---|---|---|
| BSA stock (µl) | 0 | 100 | 200 | 300 | 400 | 500 |
| Buffer (µl) | 500 | 400 | 300 | 200 | 100 | 0 |

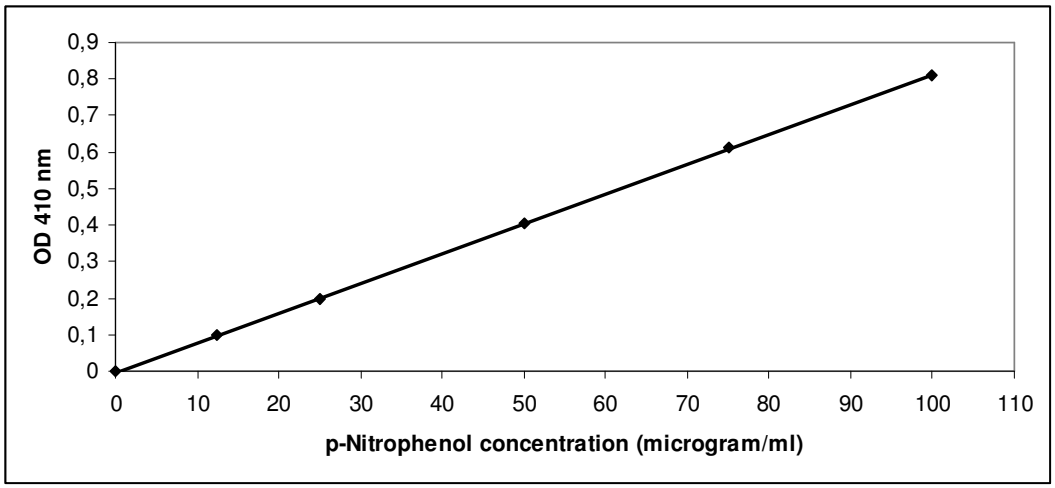
After preparations of the samples, 1 ml of Bradford reagent is added to each the tube. After incubating at room temperature for10 minutes, the optical density of the samples are measured against blank at 595 nm.




Standard curve for bradford method

## APPENDIX H

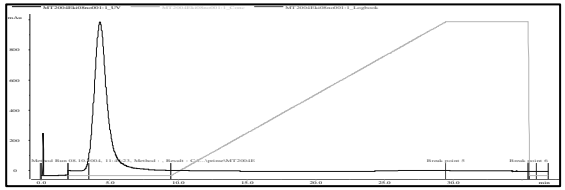
## STANDARD CURVE FOR SYNTHETIC *p*-NITROPHENOL GLYCOSIDES



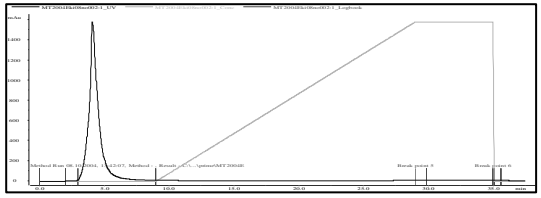
## APPENDIX I

## HYDROPHOBIC MINI COLUMN XYLANASE PURIFICATION CHROMATOGRAMS

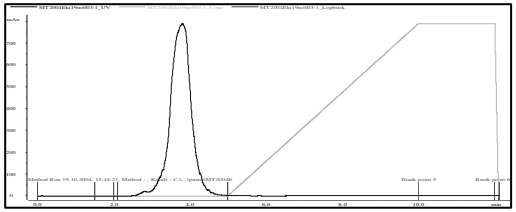
### 1. Butyl sepharose fast flow mini column chromatograms



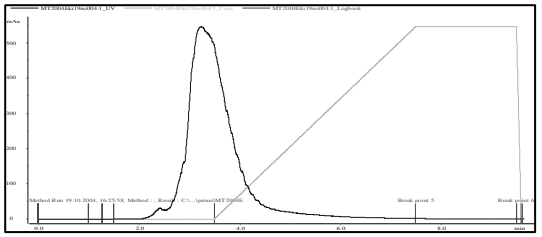
A



B



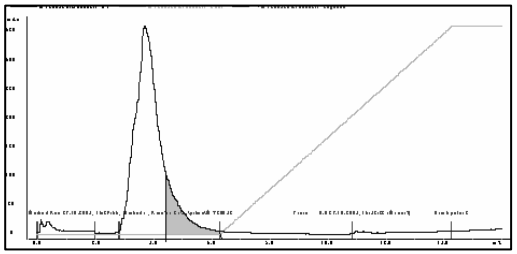
C



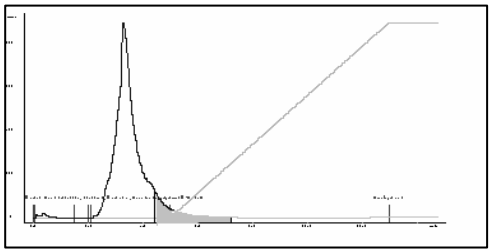
D

*Bacillus* M-13 xylanase purification by butyl sepharose fast flow mini column. Purifications were performed at; A- 1 M NaCl and pH 7.0, B- 1.5 M NaCl and pH 7.0, C- 1.5 M NaCl and pH 7.0, and D- 1.5 M NaCl and pH 8.0.

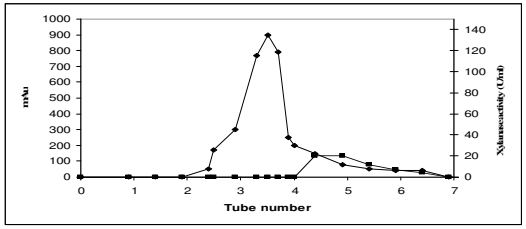
**2. Octyl sepharose fast flow mini column chromatograms**



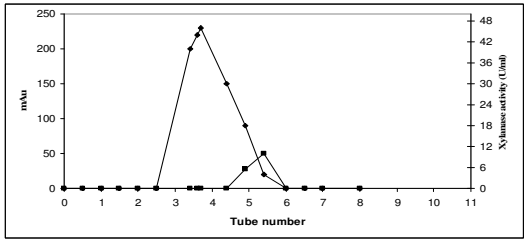
A



B



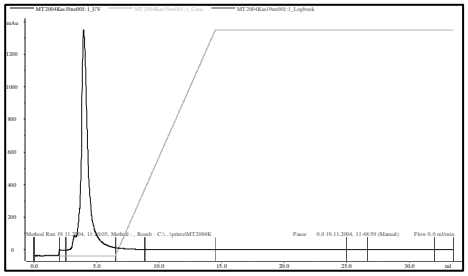

A1



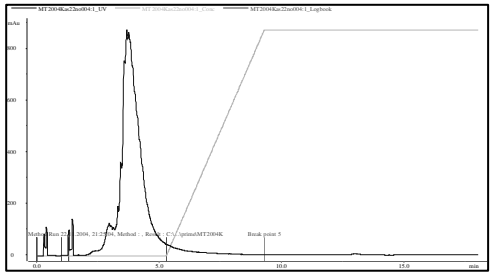

B1

*Bacillus* M-13 xylanase purification by octyl sepharose fast flow mini column. Purifications were performed at; A- 1 M NaCl and pH 7.0 (A1- activity profile of A) and B- 4 M NaCl and pH 7.0 (B1- activity profile of B).( ♦: mAu, ■: xylanase activity).
*Shaded area shows xylanase positive fractions.*

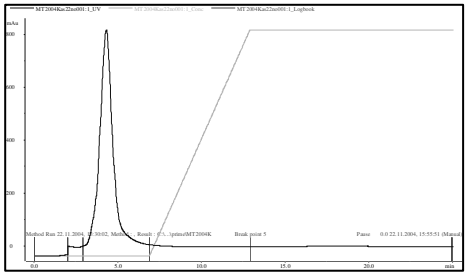
**3. Phenyl sepharose fast flow high binding mini column chromatograms**



A



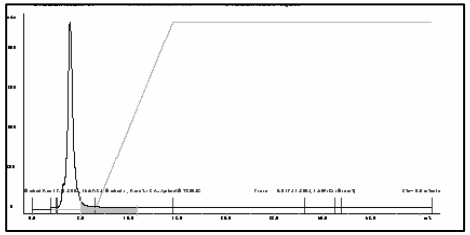
B



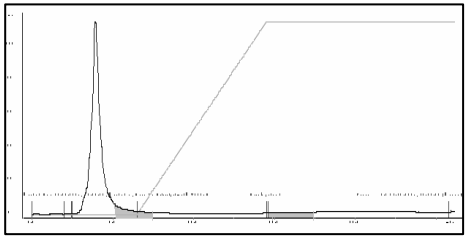
C

*Bacillus* M-13 xylanase purification by phenyl sepharose fast flow high binding mini column. Purifications were performed at; A- 3 M NaCl and pH 6.0, B- 3 M NaCl and pH 8.5, C- 4 M NaCl and pH 7.0.

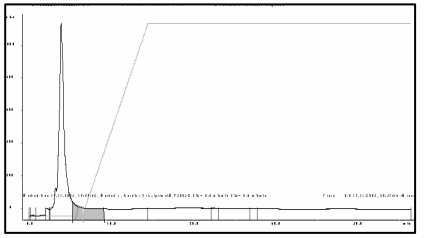
**4. Phenyl sepharose fast flow low binding mini column chromatograms**



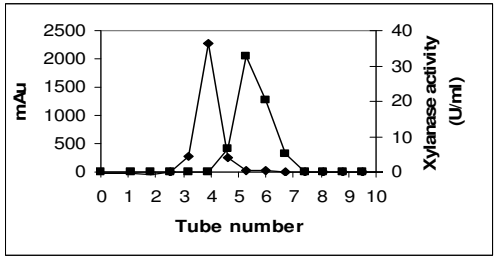
A



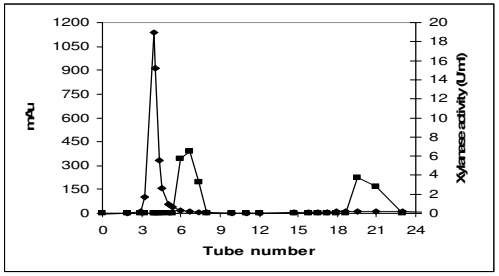
B



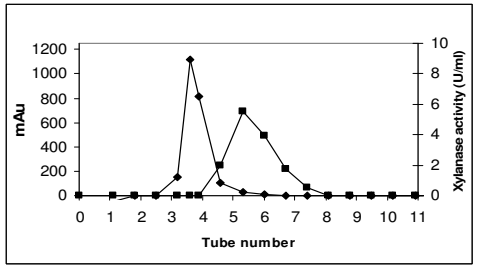
C




A1



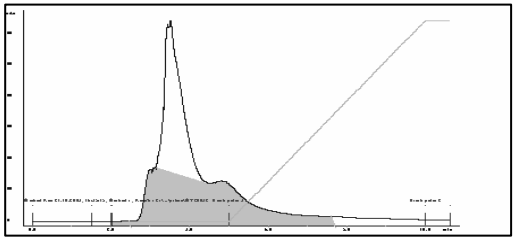

B1



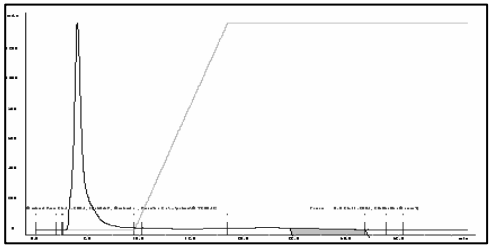

C1

*Bacillus* M-13 xylanase purification by phenyl sepharose fast flow low binding mini column. Purifications were performed at; A- 3 M NaCl and pH 6.0 (A1- activity profile of A), B- 3 M NaCl and pH 7.0 (B1- activity profile of B), and C- 3 M NaCl and pH 8.5(C1- activity profile of C). (♦: mAu, ▲: xylanase activity). 1ml/tube. *Shaded area shows xylanase positive fractions.*

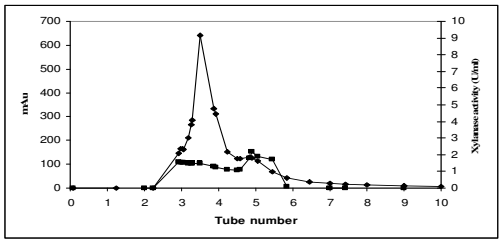
**5. Phenyl sepharose high performance mini chromatograms**



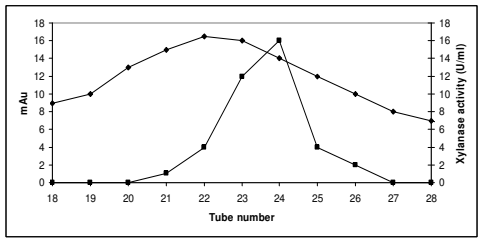
A



B




A1

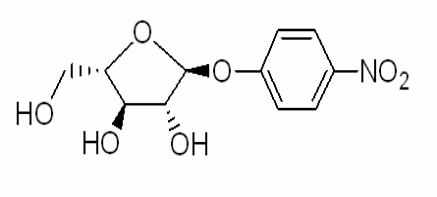



B1

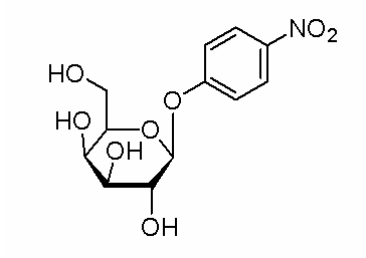
*Bacillus* M-13 xylanase purification by phenyl sepharose high performance mini column. Purifications were performed at; A- 3M NaCl and pH 7.0 (A1- activity profile of A), B- 3.5M NaCl and pH 6.0 (B1- activity profile of B).( ♦: mAu, ■: xylanase). *Shaded area shows xylanase positive fractions.*

## APPENDIX J

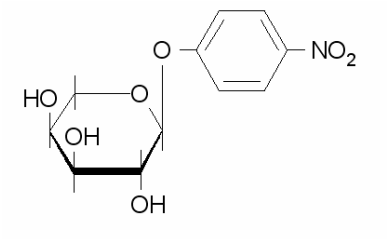
## SYNTHETIC p-NITRPHENOL GLYCOSIDES



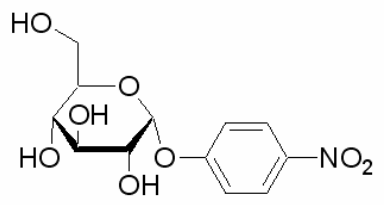

*p*-Nitrophenyl-α-L- arabinofuranoside



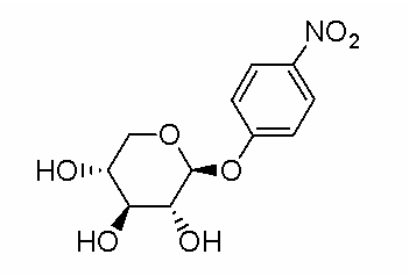

*p*-Nitrophenyl-β-D- galactopyranoside



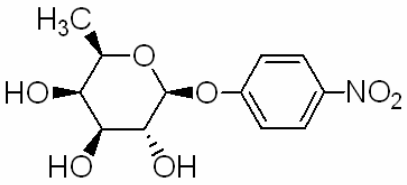

*p*-Nitrophenyl-α-L- arabinopyranoside




*p*-Nitrophenyl-β-D- glucopyranoside




*p*-Nitrophenyl-β-D- xylopyranoside




*p*-Nitrophenyl-β-D- fucopyranoside

## CURRICULUM VITAE

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### EDUCATION

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### WORK EXPERIENCE

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| 1998-2006 | METU Graduate School of Natural and Applied Sciences | Research Assistant |

**FOREIGN LANGUAGES**

Advanced English.

**PUBLICATIONS**

1. Bakir, U., Yavascaoglu, S., and Guvenc, F, and Ersayin, A., 2001, "An endo-β-1,4-xylanase from *Rhizopus oryzae*: production, partial purification and biochemical characterization", *Enzyme Microbial Technology*, Vol. 29(6-7), pp. 328-334.

2. Biran, S., Ersayin, A., Kocabas, A., and Bakir, U., 2006, "A novel xylanase from a soil isolate, *Bacillus pumilus*: Production, purification, characterization and one-step separation by aqueous-two-phase system" Journal of Chromatography, in rewiev.

**POSTER PRESENTATIONS**

1. Ersayin, A., Yavascaoglu, S., Guvenc, F., and Bakir, U. "Xylanase Production, Purification and Characterization, and Xylanase Gene Isolation from *Rhizopus oryza*". Chemical Engineering Conference for Collaborative Research in Eastern Meditterranean, Middle East Technical University, Ankara, May 20-24, 2001.

2. Ersayin, A., Bakir, U., and Ögel, B.Z. "Xylanase Production, Purification and Characterization from Soil Isolate, *Bacillus* M-13". The 12th European Congress on Biotechnology, Copenhagen, Denmark, August 21-24, 2005.

3. Ersayin, A., Bakir, U., and Ögel, B.Z. " Xylanase Production, Purification and Biochemical characterization from soil isolate *Bacillus spp*". XIV National Biotechnology Congress, Osmangazi University, Eskişehir, August 31-September 2, 2005.

4. Ersayin, A., Bakir, U., and Ögel, B.Z. "Production and Purification of α-L-Arabinofuranosidase from *Scythalidium thermophilum*". The 7th National Chemical Engineering Congress, Anadolu University, Eskişehir, December 5-8, 2006.

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