Investigation of β-xylosidase,

α-L-arabinofuranosidase and acetylesterase from

Thermotoga hypogea

by

Fariha Salma

A thesis

presented to the University of Waterloo

in fulfillment of the

thesis requirement for the degree of

Master of Science

in

Biology

Waterloo, Ontario, Canada, 2008

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Signature

_____

Abstract

Hemicellulases are key components in the degradation of plant biomass and carbon flow in nature. *Thermotoga hypogea* is a bacterium that can grow anaerobically at 90°C. It utilizes carbohydrates and peptides as energy and carbon sources. Three hemicellulytic enzymes: β-xylosidase, α-L-arabinofuranosidase and acetylesterase were investigated. Xylan and xylose were the best substrates for the growth as well as for yielding high activity for all three enzymes in the cells. Glucose grown cells possessed the least amount of enzyme activity for all three enzymes. More than 87% ± 3.0 of β-xylosidase and α-L-arabinofuranosidase activities and 34% ± 11 of acetylesterase activity were associated with the cells. Arabinofuranosidase and acetylesterase were partially purified but β-xylosidase was purified to homogeneity using the Fast Performance Liquid Chromatography system. The latter enzyme has an apparent molecular mass of 75 kDa demonstrated through sodium dodecyl sulfate-polyacrylamide gel electrophoresis and a nondenatured weight of 130 kDa estimated by Gel-filtration. Its optimal temperature and pH-value for activity were 70°C and 6.0, respectively. The purified enzyme had a half life of 22 min at 70°C and pH 6.0. Among all tested substrates, the purified enzyme had specific activities of 44, 32, 4.5, 1.71 U/mg on p-nitrophenyl-β-xylopyranoside (pNβxp), 4-nitrophenyl-β-D-glucopyranoside (pNβgp), 4-nitrophenyl-α-L-arabinofuranoside (pNαLaf) and 4-nitrophenyl-α-D-xylopyranoside (pNαxp) respectively. The apparent K_m of the xylosidase with pNβxp, was 2.6 mM and V_{max} was 196 U/mg and for pNβgp the K_m and V_{max} values were 0.31 mM and 24 U/mg respectively. Based on N-terminal analysis, xylosidase showed high homology with Family 3 β-glucosidases.

Acknowledgements

I would like to take this opportunity to thank all the people who have had a significant role in this research project and who have actively encouraged me to take meaningful strides. First, I would like to thank my supervisor Dr. Kesen Ma, for his guidance, continous patience and mentorship during the project. I am grateful to Dr. Ma for giving me the opportunity to realize my full potential. I would also like to thank my committee members, Dr. Owen P. Ward and Dr. Barbara J. Butler for their time and helpful comments.

I would like to thank my lab mates for their countless hours of helpful suggestions and insight. I would like to acknowledge the help received from Seyed Mohammad Eram in the analysis for amino acid sequences and large scale growth. I am also grateful to Dr. Bernie Duncker's lab for providing me with the strains of *Saccharomyces cerevisiae* for this project.

To my husband, Faizi, I owe many thanks for love, continued understanding, and the patience to deal with my crazy life as a graduate student. I would also like to thank my parents for their blessings and for teaching me the importance of continous hard work. Again, without the guidance of these many people, this accomplishment would not have been possible.

Finally, I would like to thank God for helping me overcome every barrier in my life with great ease.

Dedication

This thesis is dedicated to Syed Faizi Ehsan Gilani.

Table of Contents

Chapter 2

List of Tables

xi

List of Figures

List of Abbreviations

CFE   Cell-free extract

CGH   Comparative genomic hybridization

DEAE   Diethylaminoethyl

DTT   Dithiothreitol

EPPS   N-(2-hydroxyethyl)piperazine-N'-(3-propanesulfonic acid)

FPLC   Fast Performance Liquid Chromatography

GF   Gel-filtration

GHs   Glycoside hydrolases

HAP   Hydroxyapatite

kDa   Kilodaltons

MES   Methylethylsulfonic acid

pNαLaf   4-nitrophenyl-α-L-arabinofuranoside

pNβgp   4-nitrophenyl-β-D-glucopyranoside

pNαgp   4-nitrophenyl-α-D- glucopyranoside

pNαxp   4-nitrophenyl-α-D-xylopyranoside

pNβxp   4-nitrophenyl-β-D-xylopyranoside

SDS-PAGE Sodium dodecylsulfate-polyacrylamide gel electrophoresis

SDT   Sodium dithionite

Chapter One

Copyright Permission Notes from the author and publisher

Fig 1. Permission from author

Date: Mon, 30 Jun 2008 12:06:04 +0200 [30/06/2008 06:06:04 EDT]
From: Pierre Beguin <beguin@pasteur.fr>
To: fsalma@uwaterloo.ca
Subject: Re: permission to print figure

Dear Ms. Salma,

You are welcome to use this Figure, and any Figure that I may have published, as far as I am concerned. You may wish to check the policy of the publisher, but I don't think there should be any problem.

Yours sincerely,
--
Pierre Béguin
Département de Biologie Structurale et Chimie
Institut Pasteur
28, rue du Dr. Roux
75724 Paris Cedex 15. France
e-mail beguin@pasteur.fr

Permission from the publisher

Date: Wed, 20 Aug 2008 07:51:59 +0100 [20/08/2008 02:51:59 EDT]
From: Journals Rights <jrights@wiley.com>
To: "fsalma@sciborg.uwaterloo.ca" <fsalma@sciborg.uwaterloo.ca>
Cc: fems <fems@wiley.com>
Subject: FW: permission to print figure

Dear Fariha

Thank you for your email request. Permission is granted for you to use the material below for your thesis, subject to the usual acknowledgements and on the understanding that you will reapply for permission if you wish to distribute or publish your thesis commercially.

Good luck!

Yours sincerely

Sue Mattingley
Senior Rights Controller

Fig. 2. Permission from the author

Date: Tue, 8 Jul 2008 10:13:20 +0200 [08/07/2008 04:13:20 EDT]
From: Garo Antranikian <antranikian@tuhh.de>
To: fsalma@uwaterloo.ca
Subject: Re: permission to print your figures
    This will be Ok.

    All the best

    G. Antranikian

Permission from the publisher

Retrieved online on August 25, 2008, 8.15 p.m.

Thesis/Dissertation Reuse Request

Taylor & Francis is pleased to offer reuses of its content for a thesis or dissertation free of

charge contingent on resubmission of permission request if work is published.

Fig 4 + 5: Permission from the author

Date: Tue, 3 Jun 2008 14:14:37 +0530 [03/06/2008 04:44:37 EDT]
From: Mundher mnoman <mnoman@nio.org>
To: fsalma@uwaterloo.ca
Subject: Re: Re: question about review paper

Dear Ms Fariha
I have no objection to adopt any fig from that article as far as my concern. However, you should know that all the copy rights was transfered to the publisher of the journal.

Best of luck
Mondher Numan

Permission from the publisher

Date: Mon, 9 Jun 2008 06:20:26 -0400 [09/06/2008 06:20:26 EDT]
From: Allen Laskin <alaskin@hughes.net>
To: fsalma@uwaterloo.ca
Subject: Re: copyright issue
Dear Fariha:

As Editor-in-Chief of JIMB, I give you permission to adopt the two figures that you refer to below. I wish you success with your Master's thesis.

Sincerely,

Allen Laskin

Fig. 7. Permission from the author

Date: Fri, 27 Jun 2008 15:39:11 -0400 [27/06/2008 15:39:11 EDT]
From: Bob Kelly <rmkelly@eos.ncsu.edu>
To: fsalma@uwaterloo.ca
Subject: Re: permission to adopt figures
OK with me. Good luck with your thesis.

rmk

Permission from the publisher

Copyright permission policy present online.

http://www.jbc.org/misc/Copyright_Permission.shtml, Retrieved on August 23, 2008.

Fig 8: Permission from the author

Date: Mon, 30 Jun 2008 10:11:51 +0200 [30/06/2008 04:11:51 EDT]
From: Bernard OLLIVIER <bernard.ollivier@univmed.fr>
To: fsalma@uwaterloo.ca
Subject: Re: permission to adopt figures

Dear Fariha,

Thanks for your interest in our work. You have my permission to introduce the figures mentionned below for your Master thesis.

Best Regards, Bernard

Permission from the publisher

Date: Mon, 16 Jun 2008 10:50:50 +0100 [16/06/2008 05:50:50 EDT]
From: Sarah Ferris <s.ferris@sgm.ac.uk>
To: fsalma@sciborg.uwaterloo.ca
Subject: RE: copyright issues

Dear Fariha

On behalf of IUMS (who has instructed SGM to act on their behalf in this regard) I am happy to grant permission for this material to be reproduced, provided that the source is acknowledged and the authors have no objection.

Best wishes

Sarah Ferris

1.0. Introduction

Hemicelluloses are non-cellulosic low molecular weight polysaccharides that are found together with cellulose in plant cell wall (Woodward, 1984). Xylan is the most common hemicellulosic polysaccharide, representing more than 30% of the dry weight in hardwoods and annual plants (Joseleau et al., 1992). The complete degradation of xylan requires the action of a battery of xylanolytic enzymes involving xylanases, xylosidases and a set of debranching enzymes. Three xylanolytic enzymes; xylosidase, arabinofuranosidase and acetylesterase are significant for two major reasons: firstly, these enzymes are collectively involved in hydrolyzing plant cell wall (hemicellulose) and releasing the monosaccharides such as D-xylose, xylitol and L-arabinose, which are also known to have medicinal relevance (Saha, 2003; Numan and Bhosle, 2006). Secondly, each of these enzymes have different commercial applications, such as biomass conversion to produce feedstock and fuel, food processing and paper and pulp processing.

Thermophilic bacteria have received considerable attention as sources of highly active and thermostable cellulolytic and xylanolytic enzymes (Bronnenmeier and Staudenbauer, 1993). Bacteria belonging to the genus *Thermotoga* are thermophilic and organotrophic. These bacteria, which are fermentative microorganisms, metabolize a wide variety of carbohydrates (Huber and Stetter, 1992). It seems reasonable to look for polysaccharide depolymerizing enzymes in these organisms in order to compare them with their mesophilic counterparts that have been studied previously.

The model organism used to purify these enzymes in this study was *Thermotoga hypogea* which belongs to the order *Thermotogales*. *T. hypogea* is a Gram-negative, rod

shaped, anaerobic thermophile that can grow at temperatures up to 90°C (Fardeau et al., 1997). Furthermore, it is known to grow on simple as well as complex carbohydrates such as glucose, fructose, xylose, xylan and starch (Fardeau et al., 1997).

This study aims to explore the different xylanolytic enzymes present in *T. hypogea*, which can utilize xylan, as a substrate for growth. A xylanase has been purified and characterized from *T. hypogea* (Dhanjoon, 2005), however, other enzymes have not been studied. It will be the goal of this study to investigate different enzymes involved in cleaving xylan, and understand the biochemical characteristics of the enzymes as well as the physiology of the organism.

1.1. Lignocellulosic biomass

Cellulose and hemicellulose are the most abundant polysaccharides on earth (Chandrakant and Bisaria, 1998). In plants cellulose and hemicellulose are in a tightly associated matrix bound in part by lignin which forms the structural components of the cell walls (Clarke, 1997) (**Fig. 1**).

Cellulose is a major structural carbohydrate of plants and is a linear homopolymer of D-glucose linked by β-(1, 4) glycosidic bonds. It is insoluble in water and indigestible in the human intestine (Farabee, 2000). The nature of the β-glycosidic linkage is such that each glucose unit is rotated 180 degrees relative to its neighbor changing the repeating unit from glucose to cellobiose (Yernool, 2000).
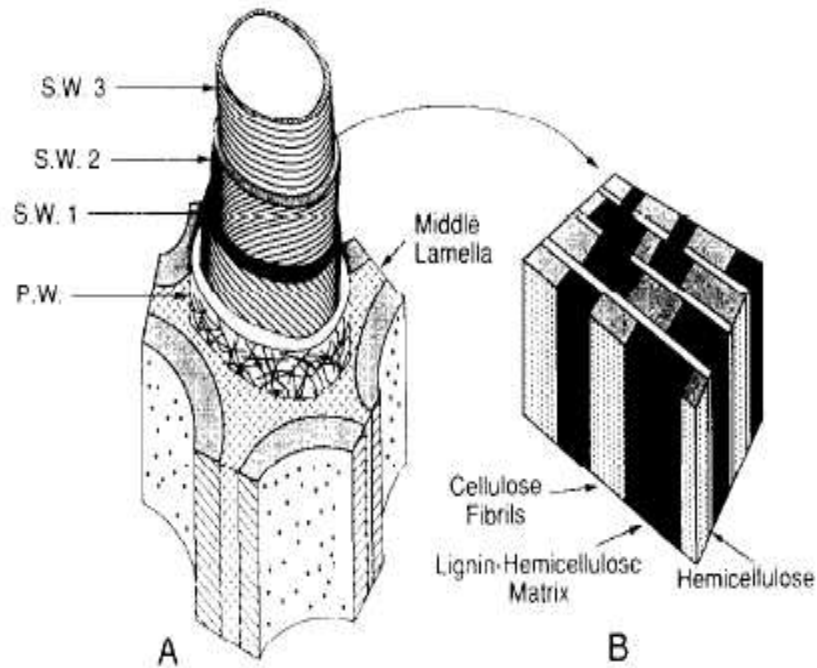
Fig 1. Hypothetical view of the arrangement of cellulose, hemicellulose and lignin in secondary cell wall of wood. A. Cellulose microfibrils are deposited at right angles to the long axis of the cell . B. Cross section of secondary wall to show localization of hemicellulose. S.W= Secondary wall., P.W= Primary wall.(Beguin and Aubert, 1994; printed with the permission from the author and publisher).

1.1.1. Hemicelluloses

Hemicelluloses are the second most abundant polysaccharides in nature after cellulose (Saha, 2003). They form part of most plant materials and appear in close association with cellulose and lignin **(Fig. 1).** Hemicelluloses, with the exception of galactoglucomannans which are water soluble, are defined as plant cell wall polysaccharides or chelating agents that are not soluble in water but are soluble in alkaline solutions (Selvendran, 1987). Hemicelluloses are heterogeneous polymers of pentoses (D-xylose, L-arabinose), hexoses (D-mannose, D-glucose, D-galactose) and

sugar acids (D-galacturonic acid, D-glucuronic acid, 4-O-methylglucuronic acid) (Aspinall, 1985). There are two main groups of hemicelluloses: xylans (β-1,4-linked D-xylose units) and mannans (β-1,4-linked D- mannose units). Only xylan will be discussed as mannan is outside the scope of this study.

1.1.2. Composition of xylans

There are characteristic differences in the composition and structure of hemicelluloses from softwood and hardwood. Xylan makes up the major hemicellulose components of hardwood cell wall polysaccharides (15-30 %) but in softwood, xylan represents only (7–10 %) (Sjostrom, 1981). In softwood glucomannans are more common, accounting for between 14-20% of the total hemicellulose. Xylan in hardwood is substituted at irregular intervals with 4-O-methyl-α-D-glucuronic acid groups joined to xylose by α-1,2-glycosidic linkages **(Fig. 2)**. On average, every tenth xylose unit has an uronic acid side group attached at carbon 2 or 3 of the xylopyranose. About 60 to 70% of the xylose residues in hardwood xylan are acetylated at the carbon 2 and/or 3 positions, whereas softwood xylan is not acetylated (Maekawa, 1976; Timell, 1967).

In addition to 4-O-methyl-glucuronic acid the xylan of softwood also contains α-L-arabinofuranoside units which are α-1,3-linked mainly to the C-2 position of xylose (Timell, 1967) and are esterified with feruloyl and coumaroyl groups (Puls and Schuseil, 1993). In hardwoods, L-arabinose residues are not common. When present, the feruloyl and p-coumaroyl groups are esterified to hydroxyl groups of the carbon 5 of arabinose bound to xylan as a side group (Mueller-Harvey et al., 1986). Xylans from different sources, such as grasses, cereals, softwood, and hardwood, differ in composition. Birch

wood xylan contains 89.3% xylose, 1% arabinose, 1.4% glucose, and 8.3% anhydrouronic acid (Kormelink and Voragen, 1993). Rice bran neutral xylan contains 46% xylose, 44.9% arabinose, 6.1% galactose, 1.9% glucose, and 1.1% anhydrouronic acid (Shibuya and Iwasaki, 1985). Corn fiber hemicellulose contains 48–54% xylose, 33–35% arabinose, 5–11% galactose and 3–6% glucuronic acid (Doner and Hicks, 1997; Hespell, 1998).
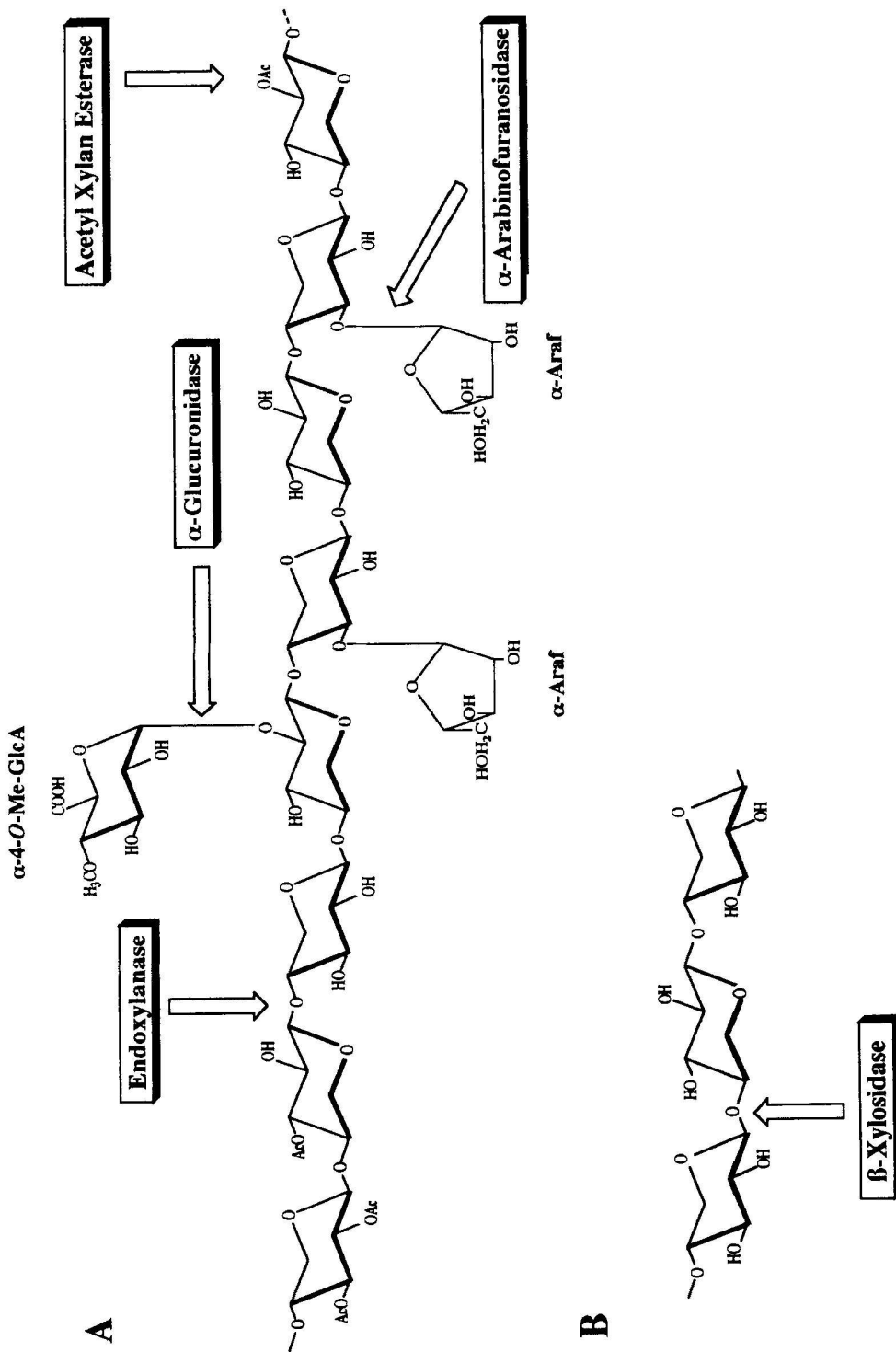
Fig 2 Hypothetical xylan showing the sites of action of the enzymes involved in hydrolysis. A. branched xylan being hydrolysed by xylanolytic enzymes. Ac: Acetyl group., α-Araf: arabinofuranosidase, α-4-O-methylglucuronic acid. B. Site of attack of β-xylosidase (Sunna and Antranikian, 1997; Reproduced with the permission of author and the publisher).

12

1.1.3. Application of xylans

Xylans are potentially very useful. The current uses of xylans on an industrial scale involve their conversion to xylose, xylitol and furfural using enzymes or acids (Biely, 1985). Wheat straw hemicelluloses have been investigated for their potential to serve as adhesives, thickeners, stabilizers and as film formers and emulsifiers (Doner and Hicks, 1997). Some important applications of xylans have been recently discovered. These include use as cholesterol depressants (Chinen and Sadoyama, 1989), HIV inhibitor and dietary fiber (Magerstaedt et al., 1991). Evidently, hemicellulosic biopolymers have a very wide variety of direct food and non-food applications. In particular, some hemicelluloses from higher plants and herbs represent a potential source of pharmacologically active polysaccharides. Glucuronic acid-containing (acidic) xylans isolated from annual plant residues such as bamboo leaves, corn stalks, as well as hard woods have been reported to inhibit markedly the growth of sarcoma-180 and other tumors (Ebringerova et al., 2002). Inhibition against tumors can be attributed to the indirect stimulation of the non-specific immunological host defenses (Ebringerova et al., 1995).

1.1.4. Hemicellulose debranching enzyme classification

Polysaccharide acting enzymes are roughly classified into three different types: endoglycanases, exoglycosidases and glycosidases (Gatenholm and Tenkanen, 2004). Endoglycanases hydrolyse internal linkages in the backbone randomly and therefore produce a set of different linear and branched oligosaccharides. Examples of endoglycanases include endoxylanases, endomannanases, endoarabinanases,

endogalactanases and endoglucanases. Exoglycosidases are often referred to as accessory enzymes as they are involved in removing side groups. These include α-arabinosidases, α-glucuronidases, α-galactosidases, β-galactosidases, and α-xylosidases (Gatenholm and Tenkanen, 2004). The last category of enzymes, glycosidases also referred to as glycoside hydrolases; are very similar to exoglycosidases in that they both remove sugar units from the nonreducing end of a polymer. However the two groups differ in their substrate requirements and pattern of hydrolysis (Reese et al., 1968). Exoglycosidases are most readily characterized by their relative rates of hydrolysis of oligosaccharides of increasing degree of polymerization (McCleary, 1982). As compared, in glycosidases, the size and stereochemistry of the noncarbohydrate region (aglycone) of the substrate affect the rate of hydrolysis of the oligosacharides (Aspinall, 1985). Secondly, hydrolysis by exoglycosidases occurs with inversion of the C1-hydroxyl, whereas in hydrolysis by glycosidases it proceeds with retention of anomeric configuration. Thirdly, exoglycosidases are specific in their hydrolysis of linkage between the dimers. Some of the exoglycosidases only hydrolyse (1→4) linkages. Glycosidases as compared are more diverse. They are known to hydrolyse dimers linked (1→ 2), (1→ 3), (1→ 4) and (1→ 6) (Aspinall, 1985). Examples of glycosidases are β-xylosidases, β-mannosidase, β-galactosidases, β-fucosidase, α-L-arabinofuranosidase.

1.2. Glycoside hydrolases

Glycoside hydrolases (also called glycosidases) are a broad group of enzymes which hydrolyse glycosidic bonds between carbohydrate moieties or between a carbohydrate and a non carbohydrate moiety (Henrissat, 1991). They are key enzymes of carbohydrate metabolism that are found in the three major domains (Archaea, Bacteria and Eukarya) (Henrissat and Coutinho, 2001). The general enzyme classification numbers of glycoside hydrolases are (EC 3.2.1.x). The E.C. inside the parenthesis refers to enzyme classification number, under the IUB Enzyme Nomenclature (1984) and is based on the type of reaction that enzymes catalyse and on their substrate specificity. For glycoside hydrolases (EC 3.2.1.x), the first three digits indicate enzymes hydrolysing O-glycoside linkages whereas the last number indicates the substrate. These hydrolases are implicated in a wide spectrum of biological processes.

Glycoside hydrolases are known to be one of the most efficient catalysts as they can accelerate the hydrolysis of the glycosidic bonds by more than 10^{17}-fold (Rye and Withers, 2000; Shallom et al, 2002). In plants, glycoside hydrolases are found within the Endoplasmic reticulum and Golgi apparatus where they are involved in processing of N-linked glycoproteins and in animals, glycoside hydrolases are found in the intestinal tract and in saliva where they degrade complex carbohydrates such as lactose, starch, sucrose and trehalose (Henrissat, 1991). Deficiency in specific lysozomal glycoside hydrolases can lead to a range of lysosomal storage disorders that result in developmental problems or death. Glycoside hydrolases play an important role in many essential steps required for sustaining life such as in defense strategies against bacteria (e.g., lysozyme), in pathogenesis mechanisms (e.g., viral neuraminidases) and in normal cellular function

(e.g., trimming mannosidases involved in N-linked glycoprotein biosynthesis). Heritable deficiencies of glycosyl hydrolases in humans result in lactose intolerance (Auricchio et al., 1963) and lysosomal storage disorder such as mucopolysaccharidosis (Itzstein, 1996; Taylor, 1996). In microorganisms, glycoside hydrolases assist in substrate (i.e., carbohydrate) processing for energy purposes. Glycoside hydrolases have also been gaining significant scientific interest as they play a critical role in the global carbon cycle and in their industrial applications.

1.2.1. Classification of glycoside hydrolases

Initially glycoside hydrolases were classified based on substrate specificities, however an alternate and complementary classification scheme for glycoside hydrolases based on amino acid sequence similarities has been proposed (Henrissat, 1991). Because there is a direct relationship between sequence and folding similarities, the modified classification is expected to: (i) reflect the structural features of these enzymes better than their major substrate specificity and also assist in revealing the evolutionary relationships between these enzymes (Henrissat, 1991). The classification has expanded from 300 sequences in 35 families in 1991, to more than 3000 sequences in 108 families today (Henrissat and Coutinho, 2001; Carbohydrate-Active Enzymes server at http://afmb.cnrs-mrs.fr/CAZY/fam/acc_GH.html). Hence enzymologists have grouped families that show related structures into "Clans"(Henrissat and Davies, 1997). So by definition, families are grouped into clans: (i) when new sequences are found to be related to more than one family, (ii) when the sensitivity of sequence comparison methods is increased or (iii)

when structural determinations demonstrate the resemblance between members of different families (Henrissat and Bairoch, 1996). So far 14 clans have been described (http://afmb.cnrsmrs.fr/CAZY/fam/acc_GH.html). The largest of these is glycoside hydrolase clan GH-A. It includes 17 families. Within the same clan, the protein fold and amino acid residues at the active site are similar.

1.2.2. Mechanism of hydrolysis via glycoside hydrolases

Glycoside hydrolases hydrolyse glycosidic bonds via two basic mechanisms at the hydrolysis site; hydrolyzing the glycosidic bond with net inversion of the anomeric configuration and hydrolyzing with net retention of the anomeric configuration (Sinnott, 1990). Retaining glycoside hydrolases operate via a double displacement mechanism that leads to the retention of the configuration at the anomeric carbon of the sugar ring undergoing catalysis (Sinnott, 1990). The catalytic machinery of these enzymes involves two catalytic carboxylates located on opposite sides of the sugar plane that perform two separate chemical steps: firstly, facilitating the departure of residual group and secondly nucleophilic attraction of a water molecule from the opposite side of the sugar rings (McCarter and Withers, 1994).

In retaining hydrolases, the top carboxylates of the sugar plane act as a general acid catalyst by donating a proton to the scissile β-1,4 oxygen of the saccharide. Protonation of anomeric carbon breaks the β-1,4 linkage formed with the other saccharide. This will result in the build-up of a positive charge at the anomeric carbon (C1). The positive charge will attract this ionic species to the second carboxylate located at the lower end of the plane which acts as a catalytic nucleophile, leading to the

formation of a covalent intermediate. The formation of this covalent species will release the R group (aglycon) and a water molecule. The water molecule through ionic reaction disrupts the covalent intermediate state of glycosyl-enzyme. This catalysis leads to retention of anomeric configuration at C1 (White and Rose, 1997).

With inverting glycosidase, hydrolysis leads to an inversion of the anomeric configuration via a single nucleophilic displacement. In *Thermotoga* the major mechanism of action of glycoside hydrolases is via retention (Henrissat and Coutinho, 2001). The preference towards the retaining mechanism at high temperatures could arise from difficulties in controlling the inverting mechanism with the formation of undesired condensation products (Driskell et al., 1999). However an issue faced by thermophiles is that high temperatures induce the non-enzymatic degradation of saccharides by the Maillard reaction and caramelization (Driskell et al., 1999). Enzymologists have speculated regarding some of the strategies used by thermophiles to reduce the exposure of saccharides to the degrading reactions. Some of the suggested strategies are i) extracellular degradation of saccharides to produce di- and oligosaccharides that are specifically transported into the cell where intracellular degradation will follow, rather than full extracellular degradation and transport of monosaccharides (Driskell et al., 1999), ii) transglycosylation reactions to yield cyclic compounds with family 13 and 77 transglycosidases, that will prevent substrate degradation in the medium and (iii) another strategy thermophiles use to preserve the integrity of the substrate is by production of the nonreducing dissacharide α-trehalose from maltooligosaccharides, as in *Sulfolobus*, by the combined use of maltosyl trehalose synthase and a maltooligosyl trehalose

trehalohydrolase (Maruta et al., 1996). In biological applications, trehalose functions as a stabilizing agent (Di Lernia et al., 1998).

1.3. Hemicellulases

Hemicellulases are a group of enzymes which belong to glycoside hydrolases. They hydrolyze the complex heteroglycans: hemicelluloses. The hemicellulases are broadly classified into D-xylanases, D-mannanases, D-galactanases, L-arabinases and esterases (Timell, 1965). Naturally occurring xylans are complex branched heteropolysaccharides with various substituent groups. There are two major types of enzymes that are involved in xylan degradation: endo-1,4-β-xylanases (E C 3.2.1.8) and β-xylosidase (EC 3.2.1.37) **(Table 1)**. Complete and fast degradation of the xylan backbone to its monomers requires the concerted action of not only xylanases but also xylosidases with "accessory enzymes" (Biely, 1983). Accessory enzymes are involved in hydrolyzing the substituent groups attached to the xylan backbone. Some of the examples of accessory enzymes are L-arabinofuranosidases, acetylesterases, feruloyl esterases, α-L-rhamnosidase and α-L-fucosidase (**Table 2**).

1.3.1. β-1,4-endoxylanases

The breakdown of xylan by xylanases and xylosidases results in the production of both unsubstituted and substituted xylo-oligomers. Complete degradation of xylan and the removal of substituents requires the action of a complex of several hydrolytic enzymes with diverse specificity and modes of action. The substituents are removed by the action of acetylesterases, arabinofuranosidases and α-glucuronidases, resulting in

unsubstituted xylo-oligomers (**Fig. 2**). The xylo-oligomers, including xylobiose are converted to their monomer, xylose by the action of β-xylosidases. Xylanases have already been characterized from several bacteria as well as from *Thermotoga hypogea* (Dhanjoon, 2005), whereas the possible existence of any of the other glycoside hydrolases from *T. hypogea* is still unknown.

β-1,4-endoxylanases (EC 3.2.1.8) catalyze the hydrolysis of the internal glycosidic linkages of a heteroxylan backbone resulting in a decreased degree of polymerization of the substrate. The most thermostable endoxylanases that have been described so far are those purified from the anaerobic thermophilic species of the genus *Thermotoga*, namely *Thermotoga* sp. strain FjSS3-B.1 (Simpson et al., 1991), *T. maritima* (Winterhalter and Liebl., 1995), *T. neapolitana* (Bok et al., 1994), and *T. thermarum* (Sunna et al., 1996). *Thermotoga* sp. strain FjSS3-B.1 produces a thermostable endoxylanase with a molecular mass of 31 kDa (Simpson et al., 1991). The endoxylanase is optimally active at 105°C and pH 5.4. The enzyme is thermostable at 95°C and exhibits a half-life of 8 min at 100°C and 2 min at 105°C. The endoxylanase hydrolyzes oat spelts xylan to mainly xylobiose and xylotriose (Sunna et al., 1997).

Table 1.Enzymes hydrolyzing the main chains of heteroxylans

Common name	Systematic name	EC Number	Description
Endoxylanase	1,4-β-xylan xylanohydrolase	3.2.1.8	Random cleavage of β-1,4 linkages of xylans with preference for unsubstituted regions. Affinity decreases with decreasing degree of polymerization with no activity on xylobiose.
Endoxylanase	1,4-β-D-arabinoxylan xylanohydrolase	3.2.1.x	Cleavage of arabinoxylans with preference at linkages in the vicinity of arabinofuranosyl substituents.
Endoxylanase	1,4-β-D-glucuronoxylan xylanohydrolase	3.2.1.x	Cleavage of glucuronoxylans with preference at linkages in the vicinity of 4-O-methyl-glucuronic acid substitutents
β -xylosidase	β-xyloside xylohydrolase	3.2.1.37	Release of β-D-xylose from the non reducing ends β-1,4 linked heteroxylans and xylo-oligosaccharides, including xylobiose
Exoxylanase	β-xylan xylohydrolase	3.1.2.x	Release of β-xylose from non reducing ends of β-1,4 heteroxylans, and xylo-oligosaccharides, excluding xylobiose

Table 2. Heteroxylan debranching enzymes

Common name	Systematic name	EC number *	Description
Acetyl xylan esterase	Same as common name	3.1.1.x	Release of acetyl groups from heteroxylans
α-L-arabinofuranosidase	1,4-α-arabinoxylan arabinofuranohydrolase	3.2.1.x	Release of 1,3- or 1,2- α-L-arabinosyl substituents from arabinoxylans
Coumaric acid esterase	Same as common name	3.1.1.x	Release of arabinose-linked coumaric acid from cereal arabinoxylans
Ferulic acid esterase	Same as common name	3.1.1.x	Release of arabinose-linked ferulic acid from cereal arabinoxylans
α -1,2-L-fucosidase	2-O-α-L-fucopyranosyl- β-D-galactoside galactohydrolase	3.2.1.63	Release of L-fucose α-1,2 linked to D-galactosyl substitutents of xyloglucans
α -D-glucuronidase	Same as common name	3.2.1.x	Release of α-1,2-linked D-glucuronic acid or 4-O-methylglucuronic acid residues from substituted xylooligomers released from endoacting enzymes

1.3.2. β-xylosidases

β-xylosidases (E.C 3.2.1.37) officially referred to as xylan 1,4-beta-xylosidases are exo-acting enzymes which preferentially cleave xylobiose and other xylo-oligomers to D-xylose (Timell, 1965). β-xylosidases are produced by both fungi and bacteria growing on xylans **(Table 3 and 4)**. These enzymes are mostly intracellular. They are divided into five glycoside hydrolase families; 3, 39, 43, 52 and 54 (Henrissat and Bairoch, 1993). However, there is still ambiguity with the identity of xylosidases, as there is no clear distinction between the types. In general there are two types of β-xylosidases;

1. True β-xylosidases hydrolyzes naturally occurring oligomers and xylans and synthetic chromogenic substrates such as p-nitrophenyl-β-xylopyranoside. Examples include β-xylosidases purified and characterized from the thermophilic bacterium *Thermotoga neapolitana* (Yernool, 2000).

2. aryl xylosidases hydrolyse synthetic chromogenic xylosides only and not naturally occurring xylo-oligomers. They may be active against other naturally occurring oligo-saccharides. Another characteristic of this group is that they have side activity with other substrates.

To date, two types of xylosidases have been reported; β-glucosidase/xylosidases and α-L-arabinofuranoside/xylosidases. There are reports of β-glucosidase/xylosidases known from *Caldocellum saccharolyticum* (Hudson et al., 1991), *Erwinia chrysamthemi* (Vroemen et al., 1995), and *Thermoanaerobacter brockii* (Breves et al., 1997). The β-xylosidase from hyperthermophilic archaea, *Sulfolobus solfataricus* is one of the β-glycosidases with broad specificity (Sunna, 1997). Enzymes containing α-L-

arabinofuranoside/xylosidase activity have been reported in *Butyrivibrio fibrisolvens* (Utt et al., 1991) as well as *Thermoanaerobacter ethanolicus* (Shao and Weigel**,** 1992).

Table 3: Properties of representative fungal β-xylosidases

Organism	Molecular mass (kDa)	Optimum pH	Optimum temp (°C)	K_m [a] (mM)	References
Aspergillus niger	122 [b] , 253 [c]	3.8-4.0	70	0.36	Rodinova et al., 1983
Emericella nidulans	116 [b], 240 [c]	4.5 – 5.0	55	6.60	Matsuo and Yasui, 1984
Sclerotium rolfsii	170 [b], 180 [c]	4.5	50	0.04	Lachke, 1988
Trichoderma reesei	100 [b]	4.0	60	0.08	Poutanen and Puls, 1988

[a] with 4-nitrophenyl-β-D- xylopyranoside
[b] estimated by SDS-PAGE
[c] estimated by gel-filtration

Table 4: Properties of representative bacterial β-xylosidases

Organism	Molecular mass (kDa)	Optimum pH	Optimum temp (°C)	K_m^a (mM)	References
Bacillus pumilis	75 [b] , 150 [c]	6.0	70	1.2	Nanmori et al., 1995
Thermoanaerobacter ethanolicus	85 [b], 150 [c]	5.0 -6.0	82	0.018	Shao and Weigel, 1992
Thermoanaerobacterium saccharolyticum	55 [b], 60 [c]	5.5	70	Na	Lee and Zeikus, 1993
Thermotoga strain FjSS3-B.1	92 [b], 174 [c]	7.0	Na	7.0	Ruttersmith and Daniel, 1993
Thermotoga maritima	Na	6.1	90	0.13	Xue and Shao, 2004
Thermotoga neapolitana	85 [c]	5.6-6.4	85	1.28	Yernool, 2000

[a] with 4-nitrophenyl-β-D- xylopyranoside

Na= Not available

[b] estimated by SDS-PAGE

[c] estimated by gel filtration

Conversion of xylose to xylulose is catalysed in *Thermotoga* by a xylose isomerase (EC 5.3.1.5) (Hallborn et al., 1991; Vielle et al., 2001). Xylulose is then phosphorylated and enters the pentose phosphate pathway (**Fig. 3**). Xylose isomerase also accepts glucose as substrate; it is extensively used to isomerize glucose to fructose in the manufacture of high fructose corn syrup (Chayen et al., 1997).

D-Xylose

↕   *Xylose isomerase*

D-xylulose

↕   *D-xylulose kinase*

Xylulose-5-P

↓

Pentose Phosphate Pathway
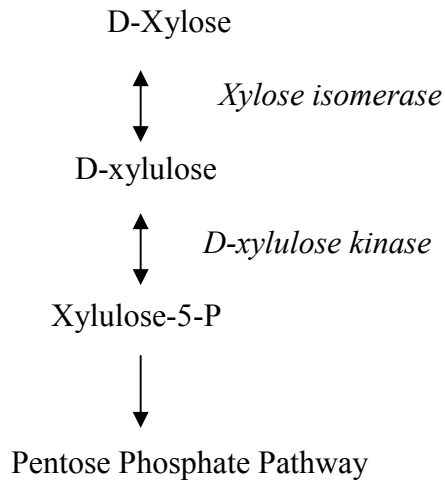
Fig 3. Mechanism of xylose breakdown in *Thermotoga* (Vieille et al., 2001)

1.3.3. α-L-arabinofuranosidases

Polysaccharides containing L-arabinose residues are important in many plant tissues. The quantities of L-arabinose and L-arabinan present in living tissues are relatively small, but L-arabinose residues are widely distributed in homoglycans, generally associated with pectins **(Fig. 4),** or as heteroglycans such as arabinoxylans **(Fig. 5),** arabinogalactans and arabinogalactan proteins (Gatenholm and Tenkanen, 2004). L-arabinose sugar is one of the components of middle lamella and cell wall of higher plants (Numan and Bhosle, 2006). Arabinans are mainly composed of α-L-arabinofuranosyl

residues that are arranged in (1,5)-linked chains with varying number of residues substituted with other α-L-arabinofuranosides at the O2 and/or O3 position.
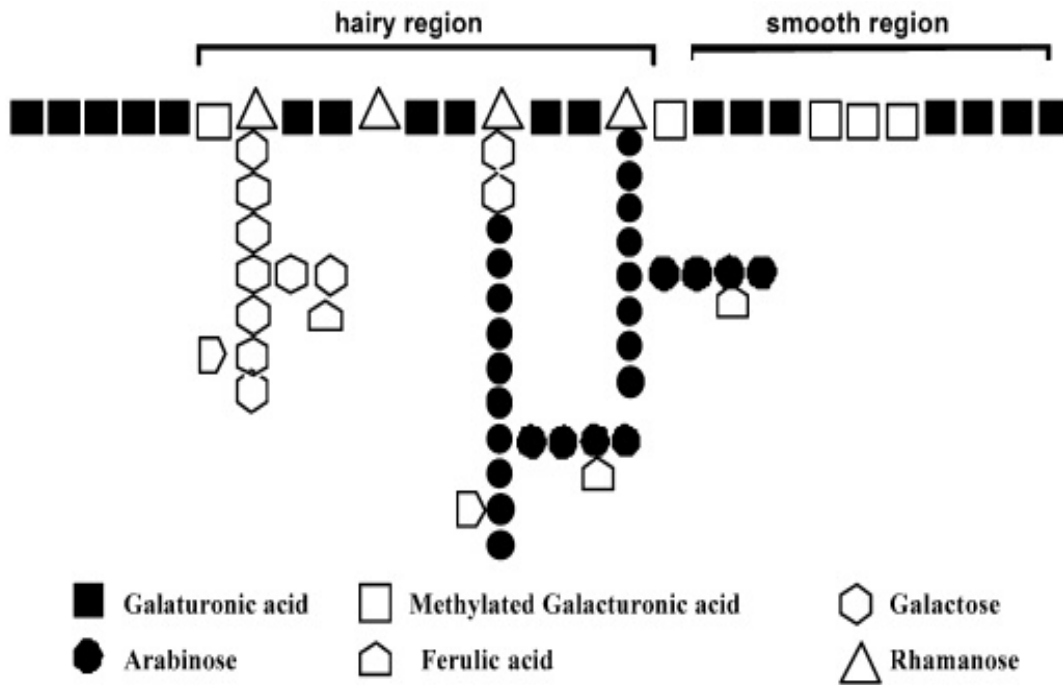
Fig. 4. Schematic diagram of pectin. Composition of different saccharides on the main chain and their side chains (Numan and Bhosle, 2006; Reproduced with the permission of the author and publisher).
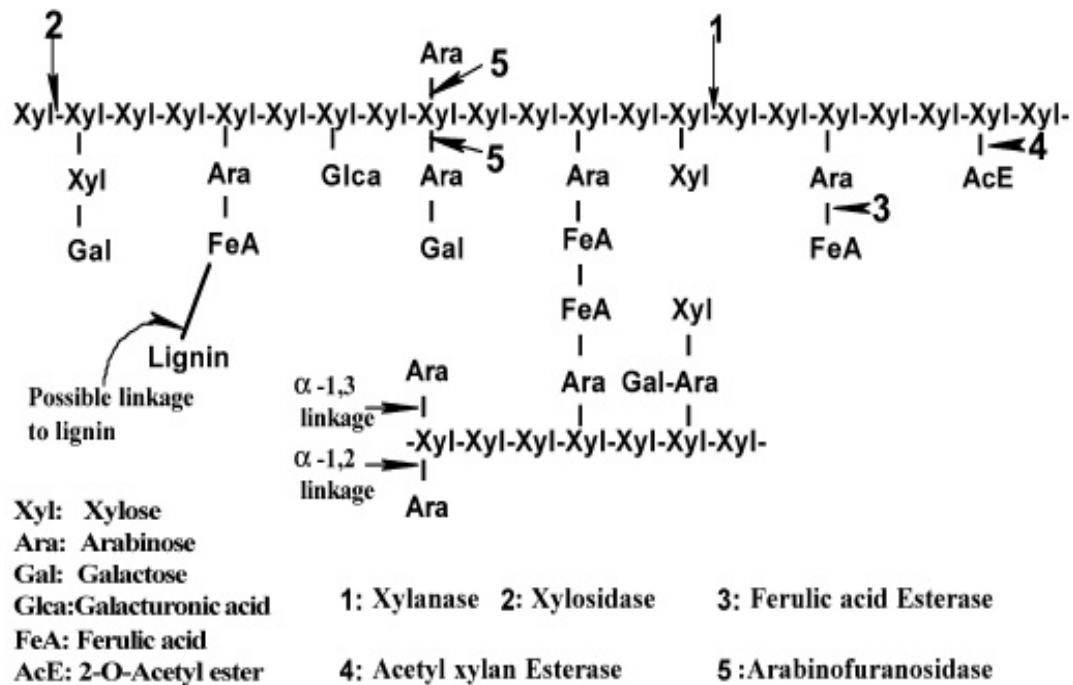
Fig. 5. The main structural features present within water soluble wheat arabinoxylans. The diagram indicates the variety of enzymes active against arabinoxylan (Numan and Bhosle, 2006; Reproduced with the permission of the author and publisher).

The enzymes hydrolyzing L-arabinose linkages have been classified into two major groups: α-L-arabinofuranosidases (EC 3.2.1.55) that hydrolyze terminal non reducing α-L-1,2 or α-L-1,3 and α-L-1,5-linked arabinofuranosyl residues, and endo-1,5-α-L-arabinases (EC 3.2.1.99) that hydrolyze only α-1,5-L-arabinofuranosidic linkages of arabinans (Kaji, 1984). Another way of differentiating between the enzymes is by hydrolysis of the colorimetric substrate p-nitrophenyl α-L-arabinofuranoside (pNαLaf). Endo-1,5-α-L-arabinases are inactive towards the substrate, whereas α-L-arabinofuranosidase activity is commonly detected using the substrate pNαLaf (Kaji, 1984). By definition, α-L-arabinofuranosidases (EC 3.2.1.55, α-L-arabinofuranoside arabinofurano-hydrolase) hydrolyze terminal nonreducing α-L-arabinofuranosyl groups of α-L-arabinofuranosides, arabinans, arabinoxylans and arabinogalactans (IUB, 1984) or from arabino-oligosaccharides, and synthetic substrates such as pNαLaf (Kaji, 1984). The most recent classification scheme based on amino acid sequences, primary structure similarities and hydrophobic cluster analysis has classified α-L-arabinofuranosidases into five glycoside hydrolase families (GHs) i.e. GH3, GH43, GH51, GH54 and GH62 (Henrissat and Davies, 2000). This classification is useful to study evolutionary relationship, mechanistic information and structural features of these enzymes. Currently, most of the known α-L-arabinofuranosidases sequences are classified into GH 51 (Coutinho and Henrissat, 1999).

1.3.3.1. The synergistic role of α-L-arabinofuranosidase

The importance of α-L-arabinofuranosidase has come from the fact that arabinose side chains on hemicelluloses and pectins participate in cross-linking within the plant cell wall structure. The presence of these side chains also affects the form and functional properties of hemicelluloses and pectins (De Vries et al., 2000). They reduce the interaction between polymer chains due to their inherently more flexible water-hungry furanose conformations. Moreover, the L-arabinofuranoside substitutions on xylan strongly inhibit the action of xylan-degrading enzymes, thus preventing the complete degradation of the polymer to its basic xylose units (Saha, 2000). The α-L-arabinofuranosidase acts synergistically with other hemicellulases and pectinases for the complete degradation of hemicellulose and pectin, respectively (De Vries et al., 2000).

The importance of α-L-arabinofuranosidase in industry has long been established. Some of the industrial uses include production of important medicinal compounds, improvement of wine flavors, bread quality, pulp treatment, fruit juice clarification, quality of animal feedstock, production of bioethanol and the synthesis of oligosaccharides (Numan and Bhosle, 2006). In terms of human health, current research shows that these are extremely significant enzymes as they produce antimetastatic and anticarcinogenic compounds by transforming ginsenosides into a compound K that exhibits antimetastatic and/or anticarcinogenic effects (Bae et al., 2000). Its significance is highlighted in another area of human health involving the production of arabinose that serves as an antiglycemic agent. Studies carried out on mice revealed that L-arabinose delays and reduces the digestion, absorption, and the net energy derived from sucrose

when both are ingested simultaneously. In this way, L-arabinose is useful in preventing postprandial hyperglycemia in diabetic patients (Sanai, 1997).

It is speculated by Lehninger et al.(1993), that L-arabinose is broken down and directed into the Pentose Phosphate Pathway by conversion of 1,5 α-L-arabinan into L-ribulose-5-phosphate through the sequence of steps shown in (**Fig. 6**).

1,5 α-L-arabinan

↓   *endo-arabinase*

Arabinan oligomers

↓   *α-L-arabinofuranosidase*

L- arabinose

↕   *L-arabinose isomerase*

L-ribulose

↕   *L-Ribulose-kinase*

L-ribulose-5-phosphate

↕   *L- Ribulose-5-P-epimerase*

D-Xylulose-5-P

↓

Pentose Phosphate Pathway
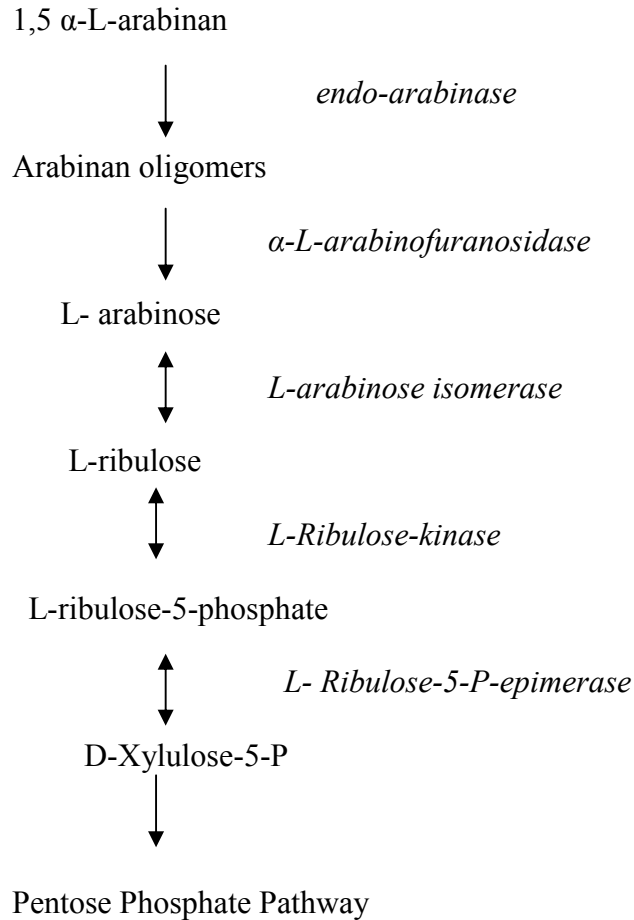
Fig. 6. L-arabinose catabolic pathway in *Thermotoga* (Kim et al., 2002; Lehninger et al., 1993).

The available information on the biochemical properties of α-L-arabinofuranosidase is mostly derived from studies carried out on the enzymes isolated from bacteria, fungi and plants. Microbial α-L-arabinofuranosidases display considerable

diversity in a number of properties. The native enzymes range from 30 to about 500 kDa in size, and several of these enzymes consist of subunits (Greve, 1984; Gilead and Shoham, 1995) **(Table 5)**. In terms of optimal pH, fungal α-L-arabinofuranosidases have pH optima of less than 5.0; whereas, those for ruminal bacteria are in the range of 6.0-7.0, which can be explained by the pH range commonly found in the rumen ecosystem. The effect of temperature and pH on the α-L-arabinofuranosidase depends on the source from which the enzyme is isolated. The highest temperature stability has been obtained for α-L-arabinofuranosidase from *T. maritima* MSB8. The recombinant enzyme* has an optimal temperature of 90°C at pH 7.0. Moreover, at this temperature (90°C) and pH 7.0, the enzyme is stable for 24 hours (Miyazaki, 2005). The other example is α-L-arabinofuranosidase from *Rhodothermus marinus*, which is stable at 85°C for 8.3 h in a pH range of 5.0–9.0 (Gomes et al., 2000). The activities of α-L-arabinofuranosidase are affected by metal ions, ionic and nonionic detergents, and chelating and reducing agents depending on the enzyme and concentration of the agent used (Hespell and O'Bryan, 1992). For instance, the activities of α-L-arabinofuranosidase from *Bifidobacterium longum* B667 (Margolles, 2003) and α-L-arabinofuranosidase from *Thermobacillus xylanilyticus* D3 were not affected by EDTA or DTT, but were affected by Cu^{2+} ions (Margolles, 2003). Metal ions such as Ag^+, Hg^{2+}, Zn^{2+}, Cd^{2+}, Co^{2+} and Ni had an inhibitory effect on some of these enzymes (Margolles, 2003).

---

[*] A putative a-L-arabinofuranosidase gene from hyperthermophilic bacterium *Thermotoga maritima* MSB8 was cloned, sequenced, and overexpressed in *Escherichia coli*.

Table 5: Properties of α-L-arabinofuranosidase from different microorganisms

Microorganisms	Molecular mass[#] (kDa)	Optimum pH	Optimum temp(°C)	GHS family	References
Fungi					
A. oryzae HL15	110	5.5	60	Na	Hashimoto and Nakata, 2003
A. kawachii	80	4	55	51	Koseki et al; 2003
P. chrysogenum	79	4	50	51	Carvallo et al., 2003
Bacteria					
B. pumilus PS213	22	7	55	51	Degrassi et al., 2003
B. breve K-110	60	Na	Na	51	Shin et al., 2003
Clostridium cellulovorans	138	6	4-50	51	Kosugi et al., 2003
S. chartreusis GS901 Afase 1	80	5.5	55	51	Matuso et al., 2000
S. chartreusis GS901 Afase 2	37	7.0	50	43	Matuso et al., 2000
Thermoanaerobacterium ethanolicus JW200	85	Na	65	3	Mai et al., 2000
Bacterium PRI-1686	350	6.0	70	51	Birgisson et al., 2004
T. maritima MSB8	332	7.0	90	51	Miyazaki, 2005

[#]Native molecular mass determined by gel-filtration; Na= Not available

The existence of α-L-arabinofuranosidase as a separate entity in thermophilic bacteria is often questioned. Several researchers have referred to its activity as overlapping activity of β-xylosidase (Ruttersmith and Daniel, 1993; Bronnenmeier et al.,

1995; Mai et al., 2000). Perhaps the similarity between both the enzymes could be due to the fact that they belong to the same clan GH-A. Another common understanding of α-L-arabinofuranosidase is that for efficient production of this enzyme, arabinose containing substrates are required for growth. De Ioannes et al. (2000), reported that monomeric compounds such as L-arabitol and L-arabinose induce the genes involved in the production of these enzymes. Moreover some researchers have also found that arabinogalactan and oat meal arabinoxylan are the best inducers for α-L-arabinofuranosidase (Degrassi et al., 2003). It is one of the aims of this project to elucidate α-L-arabinofuranosidase production and function and to investigate if the overlapping activity with β-xylosidase is due to substrate similarity or if α-L-arabinofuranosidase is an isozyme of β-xylosidase.

1.3.4. β-Glucosidases

β-Glucosidases (β-glucoside hydrolases) are enzymes that hydrolyse the β-glucosidic linkages of low molecular weight glycosides and are considered to be an important component of the cellulose enzymes (Bhat et al., 1993). For most bioconversion processes, endo(1-4)-β-glucan glucanhydrolases and exo(1-4)-β-glucan cellobiohydrolases catalyze the random hydrolysis of cellulose to produce cellobiose, which is finally hydrolysed by β-glucosidase to yield glucose. Therefore, the complete degradation of cellulose requires the synergistic action of all three enzymes. Due to their broad substrate specificity, β-glucosidases can be classified into three groups 1). Phospho-β-glucosidases and galactosidases, specific for phosphorylated substrates, 2) β-galactosidases, specific for β-galactosides, 3) β-glucosidases which contains β-

34

glycosidases, mostly microbial, with rather broad specificity towards β-glucosides, galactosides, fucosides and xylosides (Sunna et al., 1997).

1.3.5. The Family -3 Glycoside Hydrolases

Members of Glycoside Hydrolase family 1 are mainly of bacterial origin with molecular masses of about 50 kDa, and display activity on β-glucosides. In contrast, GHs family 3 consists of bacterial and fungal proteins which are larger (about 80 kDa) and are active on β-glucosides as well as on β-xylosides. The family 3 GHs is composed of glucosidase (EC 3.2.1.21); xylan 1,4-xylosidase (EC 3.2.1.37); β-N-acetylhexosaminidase (EC 3.2.1.52); glucan 1,3-glucosidase (EC 3.2.1.58); glucan 1,4-glucosidase (EC 3.2.1.74); exo-1,4-glucanase (EC 3.2.1.155); alpha-L-arabinofuranosidase (EC 3.2.1.55) (http://afmb.cnrs-mrs.fr/~pedro/CAZY/db.html). The β-glucosidases (EC 3.2.1.21), β-xylosidases (EC 3.2.1.37) and β-N-acetylhexosaminidases (EC 3.2.1.52) remove successive β-D-glucose, β-D-xylose, and β-N-acetylglucosamine residues, respectively, from the nonreducing termini **(Table 6).** Enzymes belonging to this group are versatile in function as not only do they cleave simple homodisaccharides, oligomers and polymers but also remove glycosidic units from heterogeneous molecules such as xyloglucans (Varghese, 1994), arylglycosides (Henrissat and Davies, 1997), a glucosylated antibiotic (Teeri et al., 1998), or saponins (Hrmova and Fincher, 1998). Family 3 enzymes are less well characterized than their family 1 homologues and to date only three crystal structures have been solved (Harvey et al., 2000). The three-dimensional structure of one representative member of family 3 β-

glucan hydrolase from barley was recently elucidated, and used as a reference to predict the three-dimensional structure of other family 3 GHs (Harvey et al., 2000).

Table 6. Standard substrates of family 3 GHs (Faure, 2002)

Released residue	Substrates [a]
β-D-Glucose	Cellobiose [Glc (1,4)]$_2$, gentiobiose [Glc (1,6)]$_2$, sophorose [Glc (1,2)]$_2$, laminaribiose [Glc (1,3)]$_2$, aryl- β-glucosides (such as salicin, arbutin, pNPG, etc.), 1,4- β-glucans [Glc (1,4)]$_n$, 1,3- β-glucans , [Glc (1,3)]
β-D-Xylose	Xylobiose [Xyl (1,4)]$_2$, 1,4-β-xylans [Xyl (1,4)]$_n$, pNPX, MUX

[a] pNPG, *p*-nitrophenyl-β-D-glucopyranoside; pNPX, *p*-nitrophenyl-β-D-xylopyranoside; pNPGNac, *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide; MUG, methylumbelliferyl-β-D-glucopyranoside; MUX, methylumbelliferyl-β-D-xylopyranoside.

Sequence alignments indicate that the position of the catalytic nucleophile is conserved in all family 3 glycoside hydrolases (Varghese et al., 1999). In the plant enzymes, the catalytic nucleophile is located in a highly conserved GFVISDW sequence, and is surrounded by amino acid residues with similar characteristics in other family 3 enzymes (Varghese et al., 1999). Molecular modeling and hydrophobic cluster analysis procedures indicate that the catalytic nucleophile is always found near the COOH-terminus of β strand (Varghese et al., 1999). The active site of a family 3 enzyme, barley-glucan exohydrolase was found to contain a shallow pocket that has the appearance of a coin slot (Varghese et al., 1999). The pocket was approximately 13 angstrom deep and could accommodate only two glycosyl residues of bound substrate; the remainder of the substrate was proposed to project away from the surface of the enzyme (Varghese et al., 1999). On this basis, one might predict that many of the other family 3 glycoside

hydrolases will also have broad substrate specificities. β-glucosidase from family 3 isolated from *T. neapolitana* and *T. maritima* were found to have wide specificity for β-D-chromogenic substrate: such as β-D-galactosides, β-D-xylosides and β-D-fucosides, as well as for alpha-L-arabinosides (Turner et al., 2007).

Relatively few of the enzymes have been purified and characterized in detail. Many are simply deduced from open reading frames in cloned genes or cDNAs. Those that have been partially purified have been tested against a narrow selection of potential substrates, and many are classified as glucosidases because they can hydrolyze synthetic aryl-glucosides such as 4-nitrophenyl-D-glucoside (Henrissat and Davies, 1997). Glucosidases hydrolyzing synthetic substrates also fall into the family 1 group of glycoside hydrolases (Henrissat and Davies, 1997). Family 1 β-glucosidases do have an active site pocket on their surfaces but, in contrast to the shallow pocket found in family 3 enzymes, the family 1 enzymes have a much deeper pocket that is more like a dead-end tunnel and can accommodate approximately six glucosyl residues of the substrate (Hrmova et al., 1998; Barrett et al., 1995). The deep narrow tunnel of the family 1 enzyme's active site can place much greater constraints on the conformation of substrates that could fit into the active site therefore, relatively straight (1,4)-β-linked oligoglucoside substrates would be required (Hrmova et al., 1998).

This consideration of broad substrate specificity of family 3 enzymes raises several issues regarding their functions. Since family 3 enzymes have only been identified in plants and microorganisms and not in animals, therefore it is a possibility that the enzymes may be involved in plant cell wall degradation. Family 3 glycoside hydrolases have two domains; the active site is located on domain 1 and catalytic amino

acids might be located on domain 2 (Teeri et al., 1998). It is often speculated by Teeri et al. (1998), that the second domain of these enzymes bind xyloglucans, which are polysaccharides analogous to the (1->3, 1->4)-β-glucan substrates. Based on the speculation, it is a possibility that domain 2 plays a role in attaching the enzyme to insoluble substrates or to cell walls, in a manner similar to that observed for the cellulose-binding domains of cellobiohydrolases (Teeri et al., 1998), chitinases (Watanabe, 1994), glucoamylases and related polysaccharide hydrolases (Sigurskjold, 1998).

1.3.6. Acetylesterase

Esterases are one of the accessory enzymes involved in xylan degradation required for the removal of acetyl groups. Since they act on ester bonds and not glycosidic bonds, they are classified as hydrolases and not glycoside hydrolases. In hardwoods, as many as 60 to 70% of the xylosyl residues are acetylated, whereas softwood xylans generally are not acetylated (Maekawa, 1976). Thus the total hydrolysis of xylans is accomplished by the combined action of esterases and all other hemicellulases. There are three broad types of esterases (A, B, and C) on the basis of their reactivity with organophosphorus compounds such as paraoxon [O,O-diethyl-O-(p-nitrophenyl)phosphate] and diisopropylfluorophosphate (DFP) (Aldrich, 1953). The first type is known as A-esterase or arylesterase (E.C. 3.1.1.2). They are not inhibited by organophosphorus compounds and instead they hydrolyse them as substrates. They also split aromatic esters such as phenyl acetate and therefore are referred to as arylesterases. The second group of enzymes are called B-esterases (E.C. 3.1.1.1). B-esterases which include acetylcholinesterase (EC 3.1.1.7) and nonspecific carboxylesterase (EC 3.1.1.1)

are inhibited by organophosphates (Eto, 1974). Because of their wide specificity, nonspecific carboxylesterases were formally known as "unspecific esterases". Other examples of B-esterases include cholinesterase and several important endopeptidases e.g. chymotrypsin, trypsin, elastase, thrombin and subtilisin (Krisch, 1971). The last type of esterases are known as C-esterases. They do not interact with organophosphates; these enzymes are neither inhibited by organophosphorus compounds nor do they hydrolyse them (Krisch, 1971). Recently there has been interest in using esterases in products and processes, particularly in detergent, oils, fats and dairy industries (Meghji, 1990). In terms of human health, arylesterases catalyze a transesterification reaction; hence researchers have postulated that they are involved in the transport of free fatty acids, serving as an auxiliary "coenzyme system" for lipoprotein lipase, which is an enzyme that is involved in hydrolyzing lipids in lipoproteins into fatty acids and glycerol (Pilz et al., 1966).

Acetylesterases (EC 3.1.1.6) occur mainly in higher plants, citrus fruits and fungi, and also in animal tissues (Byrde and Fielding, 1955). Since they are resistant to inhibition by organophosphorus compounds such as diethyl p-nitrophenyl phosphate, and are able to hydrolyze aromatic esters such as phenyl acetate they are related to the A-esterases (Byrde and Fielding, 1955). Acetylesterases are a broad group of enzymes which release acetic acid from various substrates. Acetyl xylan esterases are a special class of acetylesterases which deacetylate xylans and related hemicelluloses (Tenkanen and Poutanen, 1992). Acetyl groups are common substituents at the C2 and C3 positions of xylans and other hemicelluloses such as galacto-glucomannans (Puls and Schusell, 1993). There are several speculations about other functions of acetyl groups. Some

39

researchers have suggested that they stabilize the xylan structure as they form strong bonds which are resistant to acidic hydrolysis (Timell, 1965). Many cellulolytic and xylanolytic fungi and bacteria exhibit acetyl xylan esterase activity. However in the genus *Thermotoga*, it was only reported from *Thermotoga neapolitana* and *Thermotoga maritima* when the putative esterase gene was overexpressed in *E. coli* (Yernool, 2000; Levisson et al., 2007).

Table 7: Properties of representative microbial and fungal acetylesterases

Organism	Molecular mass (kDa)	Optimum pH	References
Thermoanaerobacterium Strain SL-YS485	Ae 1= 32 Ae 2 = 26	1= 7.0 2= 7.5	Shao and Weigel, 1995
Thermomonospora fusca	Ae 1= 80 Ae 2= 40	Na	Bachmann and McCarthy, 1991
Pyrococcus furiosus	Na	7.6	Ikeda and Clark, 1998
Aspergillus ficuum	29.5	7.0	Chung et al., 2002
Saccharomyces cerevisiae	40	7.0	Degrassi et al., 1999
Trichoderma reesei	Ae 1= 34 Ae 2= 34	1= 5.0- 6.0 2= 5.9-6.0	Poutanen et al., 1990
Schizophyllum commune	31	7.7	Biely et al., 1988
Thermotoga neapolitana	37	Na	Yernool, 2000
Thermotoga maritima	44.5	7.0	Levisson et al., 2007

Na = not available
Ae= acetylesterase

1.4. Thermozymes

Enzymes synthesized by thermophiles and hyperthermophiles are known as thermozymes. These enzymes are generally thermostable, resistant to irreversible inactivation at high temperatures, and thermophilic, that is optimally active at elevated temperatures between 60 and 125°C (Li et al., 2005).

Researchers have found no new amino acids, covalent modifications and structural motifs that explain the thermostability in thermozymes (Fields, 2001). In general, thermostability seems to be a property acquired by a protein through a combination of many small structural modifications that are achieved with the exchange of some amino acids for others, the modulation of interactive forces such as hydrogen bonds, ion-pair interactions, hydrophobic interactions, disulfide bonds (Scandurra et al., 1998).

Thermozymes are utilized in several industries; some of the areas which were revolutioned by the use of thermozymes are in areas of molecular biology analysis, industrial catalysts and in production of biofuels.

1.4.1. Utilization of thermozymes

One of the early successful commercialized examples was analytical use of a thermostable enzyme, *Taq*-polymerase, in polymerase chain reaction (PCR) for amplification of DNA (Satyanarayana et al., 2005). Another area of interest has been the prospects of thermozymes as industrial enzymes. Fundamental reasons to choose thermostable enzymes in bioprocessing other than thermostability are increased tolerance to organic solvents, better substrate solubility, reduced risk of contamination, as well as

low activity losses during processing (when staying below the T_m of the enzyme). Moreover, thermozymes require environmentally friendly processing instead of toxic metal ions for functionality (Comfort et al., 2004). In short, thermostable enzymes offer viable catalyst alternatives as they are able to withstand the often relatively harsh conditions of industrial processing. The detergent, food, feed, starch, textile, leather, pulp and paper and pharmaceutical industries are the major users of thermostable enzymes (Adams, 1995; Hough, 1999; Cherry, 2003; Schulein, 2000).

Amylolytic enzymes are also used in the textile, paper and baking industries. Cellulolytic enzymes are employed in the removal of polyphenolic substances from juices, in detergents for color brightening and softening of textiles, in the bio-stoning of jeans, in the pulp and paper industries and in the pre-treatment of plant biomass (Bhat, 2000). Cellulase-free thermostable hemicellulases like xylanases have offered a major step forward in the biobleaching of pulp and paper, thus lowering the environmental pollution by halogens (Viikari, 1994). Thermostable xylanases, amylases and proteases are added to animal and poultry feed in order to increase digestibility, and as additives to wheat flour for improving the quality of baked products (Viikari, 1994). Microbial pectinases account for 25% of the global food enzymes sales, and are used extensively for fruit juice clarification, juice extraction, manufacture of pectin-free starch, refinement of vegetable fibers, degumming of natural fibers, waste-water treatment, curing of coffee, cocoa and tobacco and as an analytical tool in the assessment of plant products (Alkorta, 1998).

Polysaccharide structural analysis is performed by hydrolyzing the polysaccharide to monosaccharides with acids or microbial enzymes (Scherz and Bonn, 1998). The main

advantages of using enzymes in the polysaccharide analysis are that one can choose enzymes that are selective for hydrolysis of a particular polysaccharide in a mixture. Also microbial hydrolysis occurs without the use of high temperatures or acidic or alkaline conditions, which modify low molecular weight products and/or release harmful bioproducts as well (Aspinall, 1985). The market is continuously expanding as the discovery and applications of these thermophilic enzymes increase. Global markets for industrial enzymes grew from $2 billion in 1995 to almost $4 billion in 2001 (Collins et al., 2005). Hydrolases constitute approximately 75% of the markets for industrial enzymes, with the glycosidases, including cellulases, amylases and hemicellulases constituting the second largest group of industrial enzymes (Bhat, 2000).

1.4.2. Conversion of biomass into ethanol

Another area where hemicellulytic enzymes are utilized is in the processing of agricultural residues. Agricultural residues are the parts of the crop plants for which there is no commercial productive use. Instead of disposing or burning, the cellulose in the residues is utilized for conversion into useful products such as biomass fuel and construction materials. There are two advantages to using agricultural residues: economic and environmental. By relying on domestically produced biofuel, there will be less dependence on foreign oil. Moreover, a biofuel industry would create jobs and ensure growing energy supplies to support national and global prosperity. Near the end of 2006, as per Canadian Association of Petroleum Products, 850,000 barrels per day of crude oil was imported in Canada (http://www.capp.ca/default.asp?V_Doc_ID=603); as compared in 2007, 840 million liters of ethanol was produced in Canada (Forge, 2007). Indigenous

production of biofuel through ethanol will help in eliminating the cost of importing crude oil. Alternately, these funds can be utilized in supporting ethanol-based farming industry resulting in a revitalized economy.

A mature cellulose ethanol industry based on agricultural residues has the potential to not only immensely improve the economy but also the environment. Ethanol assists by eliminating one of the biggest polluter-engine emissions. The U.S. Department of Energy estimates that 82 percent of the carbon monoxide, 43 percent of the reactive organic gases and 57 percent of the nitrogen oxides in U.S cities are emitted from petroleum-based transportation fuels (National Corn Growers Association, 2008). Furthermore, the U.S. Environmental Protection Agency (EPA) also claims that gasoline is the largest source of manmade carcinogens. In summary, an engine emission not only pollutes the environment, but also the human bloodstream with lethal compounds. In comparison, ethanol is comparatively a much environmentally friendly option. The biochemical conversion of biomass to ethanol currently involves three basic steps: (1) thermochemical treatments of raw lignocellulosic biomass to make the complex polymers more accessible to enzymatic breakdown; (2) production and application of special enzyme preparations (cellulases and hemicellulases) that hydrolyze plant cell-wall polysaccharides to a mixture of simple sugars; and (3) fermentation, mediated by bacteria or yeast, to convert these sugars to ethanol.

It is no doubt that the most harped application of biomass is cellulosic ethanol fuel, however the technology is still in its infancy. Nevertheless, conversion of biomass for energy production will be the prevalent trend in the future in order to overcome the needs of an increasing population. A more complete understanding of the enzymes and

microbes involved in biomass conversion to ethanol is needed to overcome much current inefficiency in the production process. There is also a consideration of food vs. fuel debate-which is growing crops specifically for biofuel instead of human consumption. The ultimate goal of biomass researchers is to produce biofuels from non-food crops and waste biomass, thereby saving the corn and other fuel crops for food use, and doing it without wrecking natural ecosystems (Pearce, 2006).

1.5. Thermophiles

Thermophiles are microorganisms that have optimal growth temperature between 50-64°C and a maximal temperature at which growth occurs below 70°C (Wiegel and Cangenella, 2001). Extreme thermophiles are those microorganisms whose optimal growth temperature is between 65-85°C and a maximal temperature above 70°C and lastly, hyperthermophiles are microorganisms that have an optimal temperature above 85°C, and a maximal temperature above 90°C (Wiegel and Cangenella, 2001). Microorganisms have been observed to thrive between temperatures as low as –12°C to highs of 121°C. The highest temperature presently known to support biological life is 121°C (Cowan, 2004). The hyperthermophile *Pyrolobus fumarii* isolated from a hydrothermally heated black smoker wall can grow at 112°C and survive one-hour of autoclaving at 121°C (Blochl et al., 1997). Extreme thermophiles include both Archaea and Bacteria. Among methanogens, which are anaerobic Archaea; *Methanothermus fervidus* has an optimal growth temperature of 83°C. Among aerobic thermoacidophiles, *Sulfolobus acidocaldarius* (with optimum temperature of 75°C), and also many thermoneutrophilic microorganisms, belonging to genera *Thermoproteus, Thermophilum, Desulfurococcus* and *Archeoglobus*, have optimal growth temperatures ranging between 80 and 85°C (Wiegel, 1992). It is estimated that scientists know less than 2% of the estimated number of existing microorganisms that include the extreme thermophiles (Wiegel and Cangenella, 2001). Among extreme thermophilic archaea there is a dominance of anaerobes over aerobes (Wiegel and Cangenella, 2001). One possible reason could be that at high temperatures where archaea dominate, oxygen is less soluble

than at more moderate temperature. Other examples of bacterial extreme thermophiles include species that are aerobic such as saccharolytic *Bacillus caldolyticus* and *Bacillus caldotenax (*T $_{opt}$ 72°C and 80°C, respectively), the hydrogen-oxidizing *Bacillus schlegelii* (T $_{opt}$ 70°C), and the non-spore-forming *Hydrogenobacter thermophilus* (Wiegel, 1992). Among anaerobic thermophiles, there are organisms with modified cell membrane structure; such as *Fervidobacterium pennavorans, Acetomicrobium faecalis* and *Dyctioglomus thermophilum*, which forms multicellular aggregates with a surrounding membrane; and there are some organisms that grow in chains within a surrounding sheet called a toga such as *Thermosipho africanus*, and species of genus *Thermotoga* (Wiegel and Cangenella, 2001).

1.5.1. Ecology and biodiversity of extreme thermophiles

In nature thermophilic and hyperthermophilic microorganisms are found associated with geothermal habitats as well as with artificial environments that exhibit elevated temperatures. Geothermal habitats are widely distributed and thermal activities are mainly connected with tectonic activity where the earth's plates are moving, either drifting away from each other or colliding. Geothermal areas are mainly of three types: (1) acidic solfatara fields with abundant sulfur, acidic soils, acidic hot springs, and boiling mud pots (2) freshwater hot springs and geysers with neutral-to-alkaline pH (3) deep-sea hydrothermal vents with neutral pH values and temperatures ranging between 20-350°C (Wiegel and Cangenella, 2001). The majority of extreme thermophiles have been isolated from hot springs. A few of the extreme thermophilic methanogens (e.g.

Methanobacterium spp.) have been found in sewage treatment systems and lake sediments as well (Wiegel and Cangenella, 2001).

1.6. Thermotogae as a source of thermozymes

Thermotogales are hyperthermophilic bacteria and are one of the earliest lineages of the bacterial domain (Archenbach-Richter et al., 1987). *Thermotogae* are Gram-negative rod shaped organisms (Fardeau et al., 1997). They are obligately organotrophic, strictly anaerobic; moderately thermophilic to hyperthermophilic microorganisms with rod shaped cells surrounded by a proteinaceous sheath-like structure termed the "toga" (Huber and Stetter, 1986). The toga containing genera include *Thermotoga*, *Fervidobacterium*, *Thermosipho*, *Geotoga*, *Petrotoga* and *Marinitoga* (Fardeau et al., 1997). The genus *Thermotoga* consists of 9 members: *Thermotoga maritima* (Huber et al., 1986), *Thermotoga neapolitana* (Jannasch et al., 1988), *Thermotoga thermarum* (Windberger et al., 1989), *Thermotoga subterranean* (Jeanthon et al., 1995), *Thermotoga elfii* (Rovot, 1995), *Thermotoga hypogea* (Fardeau et al., 1997), *Thermotoga petrophila* and *Thermotoga naphthophila* (Takahata et al., 2001), and *Thermotoga lettingae* (Balk et al., 2002). *T. petrophila**, T. naphthophila, T. maritima* and *T. neapolitana* grow optimally at 80°C while *T. hypogea* has an optimal growth temperature of 70°C.

Thermotoga species utilize their hydrolytic enzymes on the carbon sources derived from hemicellulose in soils and composts in their natural habitats as well as on hydrocarbon sources such as petroleum. *Thermotoga* species natural habitats are found in diverse locations: volcanic origin as well as nonvolcanically heated geothermal sites such as oil fields and hot springs (Huber et al., 1986; Fardeau et al., 1997). The ubiquity of

these species is likely the result of genomic and metabolic versatility, which includes an extraordinary array of enzymes involved in diverse carbohydrate utilization pathways in some genus members (Conners et al., 2006). *Thermotogae* are fermentative, and of huge significance because of their ability to hydrolyze simple and complex carbohydrates such as sucrose, starch, cellulose and xylan, for the industrial production of renewable carbon and energy sources (Van Ooteghem et al., 2002). They possess a mixed acid fermentation pathway and produce lactate, acetate, CO_2, and H_2 as major end products, when grown on glucose (Fardeau et al., 1997).

Among all hyperthermophiles studied to date, *Thermotoga* sp. are unique in their range of glycoside hydrolases produced which are capable of degradation of complex polysaccharides (Sunna et al., 1997). Among *Thermotoga* species, *T. maritima* was the first species whose genome was sequenced. From the genome sequence, approximately 7% of the *T. maritima* genes were predicted to be involved in carbohydrate utilization, breakdown and metabolism, consistent with observed growth of *T. maritima* on various sugars (Nelson et al., 1999). In fact, the *T. maritima* genome, despite its relatively small size, encodes the largest number of glycoside hydrolases of any bacterial or archaeal genome sequenced to date (Chabbra et al., 2003) (**Fig 7**). After the completion of the *T. maritima* MSB8 genome (Nelson et al., 1999), additional sequences from *Thermotoga* species, *T. lettingae* TMO*, T. petrophila, Thermotoga sp.* RQ2 have been made available in Genbank (http://genome.jgi-psf.org retrieved on April 25, 2008).
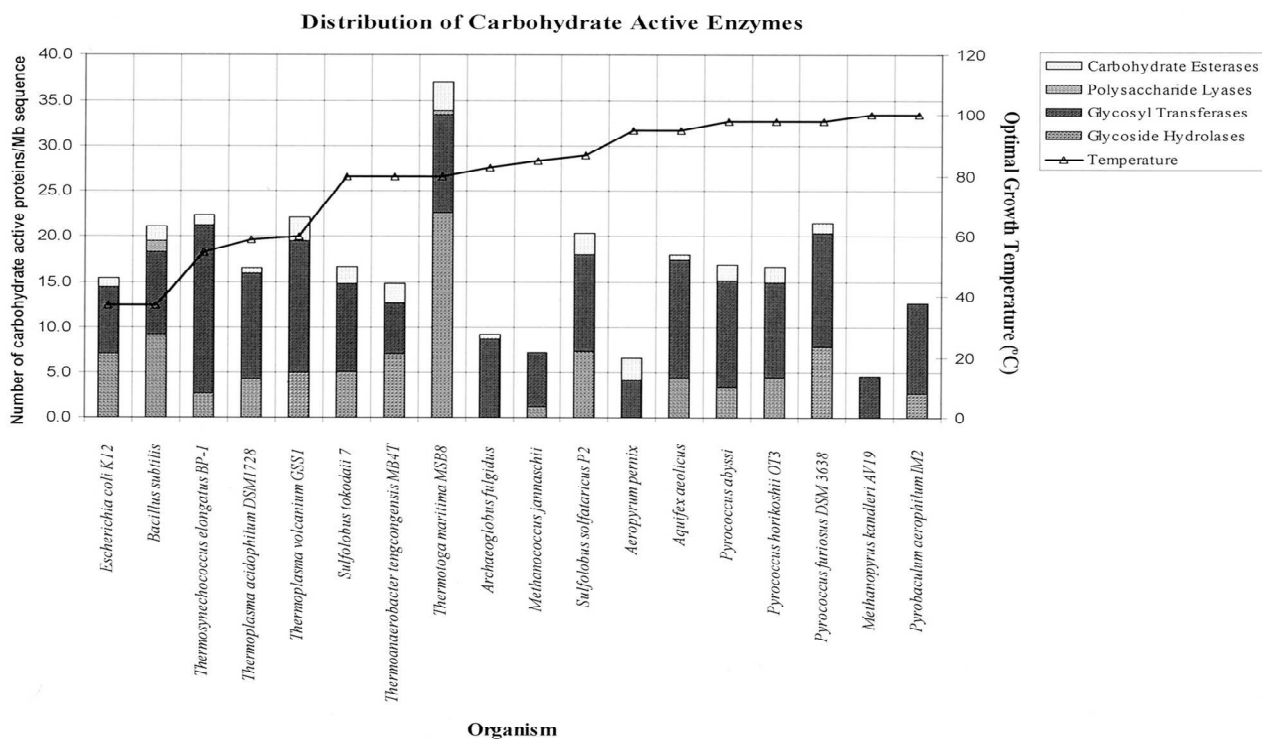
Fig. 7:Carbohydrate active enzymes in completed genome sequences of thermophilic and hyperthermophilic archaea and bacteria. *E. coli* is shown for comparison purposes (Chabbra et al., 2003; Reproduced with the permission of the author and the publisher).

1.6.1. Genetic diversity among the *Thermotogales*

With the initiation of sequencing of *Thermotoga* genomes, it is feasible to analyze genetic diversity between the species. After the completion of genome sequencing of the strain MSB8 and other *Thermotoga* species, selected sequences from *Thermotoga naphthophila*, and *Thermotoga* species KOL6, RQ7, SG1, and *Thermotoga maritima* FjSS3-B.1 have also become available (Nesbo et al., 2006). Many of the deposited sequences from other *Thermotoga* species are homologous to MSB8 genes, including

enzymes active against various carbohydrates (Ruttersmith and Daniel, 1993; Saul et al., 1995). Using comparative genomic hybridization (CGH), Mongodin and colleagues (2005) examined the genetic differences of nine *Thermotoga* species, including strain RQ2, in comparison to the sequenced *T. maritima* MSB8. Apparent differences between strains included a rhamnose utilization locus (TM1063-TM1071), putative sugar utilization genes (TM0411-TM0423), and a region containing phosphate transport genes (TM1261-TM1271), which were absent in strain RQ2 (Conners et al., 2005). Additionally, some genes which were not detected by CGH were indeed present but displayed high divergence compared to probe sequences. Many of the genes found to differ in their presence and absence patterns in the different *Thermotoga* species from the CGH study of Mongodin and colleagues (2005) seem to be related to substrate utilization, and reflect the importance of evolution and adaptation to specific habitats. For example, a large number of MSB8 carbohydrate utilization loci were found to be lacking in *Thermotoga* species PB1platt, isolated from an oil field where plant polymers are scarce (Mongodin et al., 2005). Based on these findings, it is clear that the details of carbohydrate utilization patterns in MSB8 that are gathered from functional studies cannot be automatically extended to all *Thermotoga* species and strains. However, these initial studies in *Thermotoga maritima* have been beneficial in elucidating the pathways by which *Thermotoga* strains hydrolyze, transport and utilize a variety of substrates.

1.6.2. *Thermotoga hypogea*

Thermotoga hypogea is an extremely obligate anaerobic bacterium that is able to tolerate up to 6.9 µM of dissolved oxygen in a static growth medium (Yang and Ma, 2005). This rod shaped gram negative bacterium was isolated from an African oil well (Fardeau et al., 1997). The type strain of *T. hypogea* is SEBR 7054; their growth temperature range is 56 to 90°C, optimum growth at 70°C (Fardeau et al., 1997). Growth occurs between pH 6.1-9.1 and optimum pH is 7.3-7.4. The cells are 0.5 to 1 by 2 to 3 µm and occur singly or in pairs. They can utilize a variety of carbohydrates such as glucose, fructose, maltose, mannose, sucrose, galactose and xylan. The end products of glucose and xylose fermentation are acetate, H_2, L-alanine, and traces of ethanol (Fardeau et al., 1997).
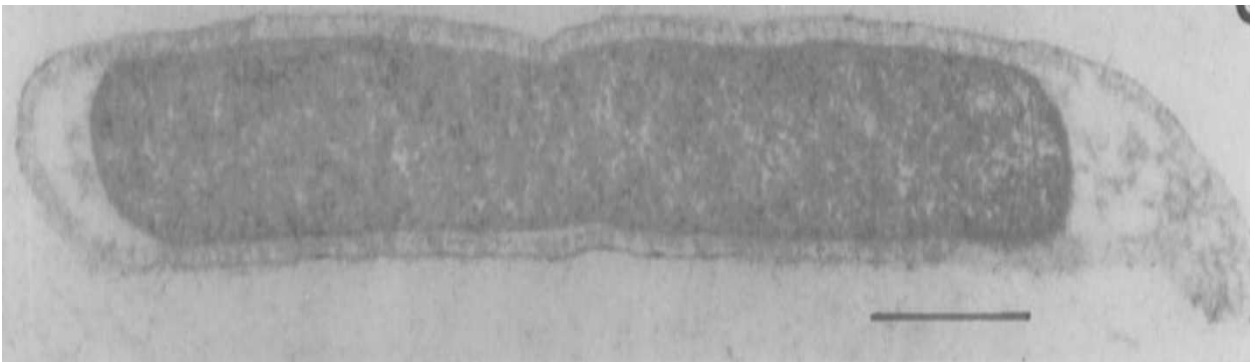
Fig. 8: Phase-contrast image of *Thermotoga hypogea* strain SEBR 7054. The typical outer sheathlike structure (toga) of the genus *Thermotoga*. Bar **=** 0.5 pm (Fardeau et al., 1997; Reproduced with the permission of the author and the publisher).

1.7. Objectives of the research

It has been reported that *Thermotogales* have the ability to metabolize a wide variety of substrates including simple sugars and complex polymers (Chabbra et al., 2003). This finding was also reported earlier by Fardeau et al. (1997), specifically for *Thermotoga hypogea. T. hypogea* is used as a model strain for the source of hydrolytic enzymes for this project, although one xylanase has been reported from this organism (Dhanjoon, 2005). Therefore, the presence of other hemicellulolytic enzymes was investigated. The specific goals of the project are listed below:

A. To determine activities of other hemicellulytic enzymes: β-xylosidase, α-L-arabinofuranosidase and acetylesterase from *T. hypogea* based on assay conditions.

B. To determine growth substrates that resulted in maximal enzyme activity for all three enzymes.

C. To purify and characterize β-xylosidase, α-L-arabinofuranosidase and acetylesterase.

Chapter 2

2.0. Materials and Methods.

2.1. Organism

T. hypogea (DSM 11164) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen, GmbH, D-38124 Braunschweig, Germany.

2.2. Chemicals and Equipment

Chromatography columns for protein purification were purchased from Amersham Biotech (Montreal, Quebec). Ultrafiltration membranes and Amicon microcentrifuge tubes for concentrating samples were purchased from Millipore (Billerica, Massachusetts). SDS-PAGE calibration proteins and enzyme substrates: 4-nitrophenyl-α-L-arabinofuranoside, 4-nitrophenyl-β-D-glucopyranoside, 4-nitrophenyl-α-D-glucopyranoside, 4-nitrophenyl-α-D-xylopyranoside, 4-nitrophenyl-β-D-xylopyranoside were purchased from Sigma (Oakville, Ontario). Bovine serum albumin (BSA), Biorad reagent, and Tetramethylethylenediamine were purchased from Biorad (Mississauga, Ontario). All chemicals used with high purities were commercially available products, unless specified.

2.3 Culturing of *Thermotoga hypogea*

The basic medium used for culturing *T. hypogea* was the same as reported previously (Fardeau et al., 1997) with some modifications. *T. hypogea* were routinely grown in a 160 ml serum bottle with 50 ml medium under anaerobic condition at 70°C unless otherwise specified. The composition of the basal medium (gram per litre) was 1 g NH_4Cl (EM Science, New Jersey), 0.3 g K_2HPO_4 (Fisher Scientific, Mississauga); 0.3 g KH_2PO_4 (Fisher Scientific, Mississauga); 0.2 g $MgCl_2 \cdot 6H_2O$ (Fisher Scientific,

Mississauga); 0.1 g $CaCl_2 \cdot 2H_2O$ (EM Science, New Jersey); 0.1 g KCl (BDH Inc, Toronto); 2.0 g yeast extract (EM Science, New Jersey), 2.0 g trypticase (BDH Inc, Toronto), 10 ml trace mineral element solution; 0.05 mg Resazurin (Sigma, Oakville) and 1 litre deionized water.

The composition of the trace mineral solution (gram per litre) was: 1.5 g Nitrilotriacetic acid (Sigma, Oakville); 3.0 g $MgSO_4 \cdot 7H_2O$ (Fisher Scientific, Mississauga); 0.5 g $MnSO_4 \cdot H_2O$ (Sigma, Oakville), 1.0 g NaCl (Fisher Scientific, Mississauga); 0.1 g $FeSO_4 \cdot 7H_2O$ (J.T.Baker, New Jersey); 0.18 g $CoSO_4 \cdot 7H_2O$ (Sigma, Oakville); 0.1 g $CaCl_2 \cdot 2H_2O$ (EM Science, New Jersey); 0.18 g $ZnSO_4 \cdot 7H_2O$ (BDH Inc, Toronto), 0.01 g $CuSO_4 \cdot H_2O$ (Sigma, Oakville); 0.01 g $Na_2MoO_4 \cdot H_2O$ (Sigma, Oakville); 0.02 g $KAl(SO4)_2 \cdot 12H_2O$ (BDH Inc, Toronto); 0.01 g H_3BO_3 (Sigma, Oakville); 0.025 g $NiCl_2 \cdot 6H_2O$ (Sigma, Oakville); 0.0003g $Na_2SeO_3 \cdot 5H_2O$ (Sigma, Oakville); 0.5 mg/ml Resazurin (Sigma, Oakville) was added to the medium as an indicator of oxygen. To prepare the trace elements solution, Nitrilotriacetic acid was added first, and then the pH was adjusted to 7.0 with 1 M NaOH (EMD Chemical Inc, New Jersey), then each component was added separately. The solution of trace minerals was stored at room temperature.

For medium preparation, all the above components were dissolved in deionized water and the pH was adjusted to 7.3-7.4 with 1 M NaOH to obtain optimal growth. A volume of 50 ml of medium was transferred into 160 ml serum bottles (Fisher Scientific, Mississauga) and subsequently autoclaved at 121°C for 20 min. The bottles were then sealed with sterile grey butyl stoppers (Snap-on, 20 mm, Fisher Scientific, Mississauga) and crimped with aluminum seals (20 mm CS/M, Fisher Scientific, Mississauga) with a

hand crimper (Wheaton, New Jersey). A 25G 5/8 disposable sterile needle fitted with a sterile 0.22 μm syringe filter (Fisher Scientific, Mississauga) was used for connecting all bottles to a manifold which was connected with a Welch Vacuum company Model 1402B-80 vacuum pump (Thomas industries Inc., IL) and an oxygen-free nitrogen tank (Praxair, Kitchener). Oxygen in the gas phase of the serum bottles was removed by degassing using the manifold for 30 min. The bottles were gassed with oxygen–free nitrogen and then degassed for 3 min repeatedly thrice to make sure there were no traces of oxygen, before being flushed with nitrogen for 10 min and then pressurized with nitrogen gas at 1.5 psi.

Prior to inoculation, 1 ml of sterilized and anaerobic 15% (w/v) xylose solution (final xylose concentration 0.3% w/v) was injected into each bottle containing 50 ml of media using a 1 mL anaerobic sterilized disposable syringe (Fisher Scientific, Mississauga) with 25G 5/8 sterilized needle. One ml of 1 M anaerobic sodium thiosulfate was added to the culture medium just prior to inoculation to make a final concentration of 20 mM. Then 140 μl of sterilized 15 % w/v cysteine-hydrochloride and 400 μl of sterilized and anaerobic 3 % Na_2S solution were added to each bottle to make final concentrations of 0.042 % (w/v) and 0.024 % (w/v), and the bottle was then incubated at 70°C in a water bath (ISO Temp 215, Fisher Scientific, Mississauga) to completely reduce any trace amount of oxygen remaining in the medium. The color of the medium was changed from pink to light yellow which confirmed that trace amount of oxygen in the medium had been reduced.

The medium was then inoculated with 2 % (v/v) fresh, late log phase, seed-culture with final cell density of 10^8 cells/ml. With a 1 ml disposable, sterile and anaerobic

syringe the new medium was inoculated with 1 mL of original seed culture. Inoculated medium was incubated at 70°C to promote the growth of the organism. Samples were removed at various times using a 1 ml anaerobic sterile gas tight syringe to analyze the growth.

2.3.1. Culturing in 500 mL medium

A one litre flask (Pyrex, Mexico) containing 500 ml of medium was autoclaved for 20 min. The flask was sealed with a black rubber stopper (size 7) that had an access port fitted with a sterile grey butyl stopper (20 mm) and aluminum cap (20 mm) (Fisher Scientific, Mississauga). The black rubber stopper was secured on the mouth of the flask using an aluminum clamp (Science Shop, University of Waterloo, Waterloo) which is a devise fastened with screws and wing nuts. The flask was connected to the manifold and degassed for 50 min, flushed with oxygen-free nitrogen for 10 min at the pressure of 1-1.5 psi and finally pressurized for 3 min at the same pressure.

Prior to inoculation, the stock solutions as well as reducing agents were added into the medium with a 5 ml disposable, sterile and oxygen-free syringe fitted with a 25G 5/8 disposable sterile needle. Then the medium was incubated at 70°C for 15 min for oxygen reduction. Inoculation was done by injecting 5 mL from a 10^8 cells/mL final seed culture, using a 5 ml syringe. Samples were withdrawn with a 1 ml syringe at different time intervals for monitoring the growth.

2.3.2. Large scale culturing (15 L)

A twenty-litre glass carboy (Corning Inc, NY) was used for the growth of *T. hypogea*. Medium was prepared by adding all the components to the 20-L carboy containing 15 L deionized water. All salts were added one by one with constant stirring, waiting between additions until the dissolving was completed. The pH was adjusted to 7.3-7.4 with 1 M NaOH. The medium was autoclaved at 121°C for 50 minutes. After autoclaving, the carboy was immediately sealed with a sterile black rubber stopper (size 10) fitted with three glass tubing inlets. The carboy was then connected to a vacuum pump/nitrogen system for degassing and flushing. The medium was subjected to five cycles of degassing and flushing with 1-1.5 psi of oxygen-free nitrogen, while kept under stirring conditions (200 rpm). Each cycle comprise 5 min degassing and 3 min flushing. The reducing agents; sodium thiosulfate, sodium sulphide and cysteine-HCl were added to the medium anaerobically by sterile silicon tubing (Dow Corning Co, Michigan) as the medium was incubated in the incubator (Lunaire, Pennsylvania). The glass carboy was placed inside the incubator and the medium was reduced at temperatures between 60-70°C. Seed culture used for inoculation was 1 % (v/v), freshly prepared late log phase culture with cell density of 10^8 cells/ml. *T. hypogea* was grown in large scale at 70°C under continuous flushing with oxygen-free nitrogen at a flow rate of 50 ml/min and stirring with a magnetic stirbar and stir plate operating at a magnitude of 6 from a range (1-10). Cell growth was monitored by cell counts, optical density readings and by measuring the pH of the medium by pH indicator strips (VWR, Toronto).

2.3.3 Cell mass collection and harvesting

For small scale (500 ml) culturing the cells were harvested using a centrifuge. Fifty ml or 250 ml Sorvall centrifugation tubes (Kendro Laboratory Products, Connecticut) were used depending on the culture volume. The Sorvall GSA (Sorvall Instruments, MN) rotor and Sorvall SS-34 rotor were used in Sorvall RC-5B refrigerated super-speed centrifuge. The centrifugation temperature was set between 4 to 10°C and the speed was 8,000 r.p.m (GSA rotor: $10444 \times g$; SS-34 rotor: $7719 \times g$) for 15 minutes.

Large scale cultures (15 litres) were harvested using a continuous centrifuge (Sharples, PA) at a maximal speed of 23,000 rpm. The culture was cooled down to 4°C with ice before transferring to the centrifuge and then it was driven from the carboy to the centrifuge through a glass cooling coil that was kept under ice. The pump used for driving the culture was a monostat economy peristaltic E-series pump EW-78221-30 (Division of Barnant, ColeParmer Instrument Company, IL). The flow rate into the centrifuge was kept at about 180 ml/min by using an aluminum clamp at the pump inlet. Phase contrast microscopy was used to ensure the absence of cells in the supernatant. The cells were harvested in late log phase of the growth (10^8 - 10^9 cells/ml) and stored at -80°C after being frozen with liquid nitrogen. The culture supernatant was collected in a glass carboy and 5 L were reserved for a cellular localization experiment by freezing to -20°C.

2.4. Preparation of stock solutions and reducing agents

All the stock solutions for medium preparations were degassed for 30 minutes and gassed for 3 minutes (with the exception of carbon substrates), according to the method described in Sec 2.3. After degassing and gassing all the stock solutions were stored at room temperature.

2.4.1. Substrate solution, 15% (w/v)

After adding 3.75 g (carbon source) to 25 ml autoclaved, de-ionized water, the solution was sealed and boiled in a 100°C water bath for 20 min for sterilization of the carbon source.

2.4.2. Sodium thiosulfate solution, 25% (w/v)

Unlike other stock solutions, sodium thiosulfate was freshly prepared for every new batch as it is not stable. The 6.25 g of sodium thiosulfate was weighed and added to a serum bottle containing 25 ml autoclaved degassed water. The transfer of solid sodium thiosulfate in the serum bottle was conducted inside a fume hood and then the bottle was immediately sealed and degassed as described above.

2.4.3. Sodium sulfide solution 3% (w/v)

The 0.6 g of sodium sulfide, based on a final concentration 3% (w/v) was added to 50 ml autoclaved and degassed water, within the fume hood. The bottle was then immediately sealed and degassed.

2.4.4. Cysteine-HCl solution, 15% (w/v)

The 3.75 g of cysteine-HCl was added inside the fume hood to 25 ml autoclaved and degassed water. The sealed bottle of cysteine-HCl solution was further degassed and flushed with nitrogen gas for 10 min.

2.5. Monitoring the growth of *T. hypogea*

T. hypogea cell numbers were monitored over time by direct cell count using a Petroff–Hausser counting chamber (0.02 mm deep, Hausser Scientific, PA) and a Nikon Eclipse E600 phase-contrast light microscope. In between the counts the optical density was also measured at 600 nm using a Genesys 10 Visible spectrophotometer (Thermospectronic Unicam, New York). Each point in the growth curve was the average of measurement values in duplicate. The growth curve was plotted as a semilogarithmic graph of the $\log_{10}$ cell density versus time.

2.6. Preparation of cell-free extracts (CFE)

All procedures for the preparation of cell-free extracts were carried out anaerobically. Depending on the anticipated volume, the frozen cells (50 g) were immediately transferred into a degassed (30 min) flask with a stir bar. The vessel was sealed by a grey butyl stopper and capped with an aluminum seal (for serum bottle), then degassed and pressurized by nitrogen gas. Lysis buffer was prepared by combining 50 mM Tris (Fisher Scientific, Mississauga) (pH 7.8), 5% glycerol (Fisher Scientific, Mississauga), 2 mM sodium dithionite (SDT, Fisher Scientific, Mississauga), 2 mM dithiothreitol (DTT, Fisher Scientific, Mississauga), 0.1 mg/ml lysozyme (Sigma,

Oakville) and 0.01 mg /ml DNAse I (Sigma, Oakville). The volume of anaerobic lysis buffer was five times the weight of dry cells. The buffer was transferred to the flask, then the cells suspension was stirred using a magnetic stirrer at 37°C for 2 hours. The lysis mixture (crude extract) was centrifuged at 13,500 r.p.m for 45 minutes at 4°C. The clear supernatant cell-free extract (CFE) was transferred back to the same flask and was degassed and gassed for 30 min/15 min respectively, to remove oxygen in gas phase. The CFE was used for further experiments.

2.7. Protein concentration determination

The Bio-Rad Protein assay was used to determine the protein concentration (Bradford 1976). The assay dye reagent sample mixture was prepared in duplicates by adding 780 µl de-ionized water, 20 µl sample and 200 µl dye reagent concentrate (Bio-Rad Laboratories, Inc) in a microcentrifuge tube. A control was also prepared, without the protein. The mixture was vortexed, and the absorbance at 595 nm was measured using a Genesys 10 Vis spectrophotometer (ThermoSpectronic) after 30 minutes incubation at room temperature. A series of protein standards prepared with bovine serum albumin at concentrations from 0.5 mg/ml to 10 mg/ml was used to obtain a standard curve. A linear relationship between protein concentration and the absorbance at 595 nm was obtained.

2.8. Enzyme assays:

The assays for all three enzymes were based on the release of p-nitrophenol from their respective chromogenic substrates, which were p-nitrophenyl derivatives. The absorbance was measured at 400 nm spectrophotometrically with plastic cuvettes using air as blank. For each enzyme assay two controls were also prepared: one without the enzyme and the other without the substrate. The sample mixture's change in absorbance was determined by subtracting with the control's (without the enzyme) change in absorbance to eliminate background interferences. Although the assay for all three enzymes was similar, reported molar extinction coefficients for the release of p-nitrophenol from the respective substrate was different for all three enzymes (Ruttersmith and Daniel, 1993; Miyazaki, 2005; Sobek and Gorisch, 1988). One unit of enzyme activity is defined as the formation of 1 μmol p-nitrophenol from the substrate per minute under the assay conditions. All enzyme assays were conducted in duplicate.

2.8.1 β-xylosidase assay

The protocol for the β-xylosidase enzyme assay was similar to previously described (Ruttersmith and Daniel, 1993) with some modifications. The substrate for β-xylosidase was p-nitrophenyl-β-D-xylopyranoside (pNβxp) (Sigma, Oakville). The standard reaction mixture, contained 0.1 M sodium acetate buffer pH 5.0 (final concentration 50 mM), 70 μl of water and 80 μl of substrate (final concentration 1 mM). The final volume of the reaction mixture was 400 μl. The reaction mixture was placed in a 70°C water bath to prewarm. The enzyme was added to start the reaction. The sample mixture was incubated for 10 min, after which 0.8 ml of 0.1 M Na_2CO_3 was added to end

the reaction. The concentration of p-nitrophenol released was calculated using the molar extinction coefficient, ϵ_{400} =18,300 M^{-1}cm^{-1} (Ruttersmith and Daniel, 1993).

2.8.2. α-L-arabinofuranosidase assay

The protocol for enzyme assay was similar to previously described for α-L-arabinofuranosidase (Miyazaki, 2005) with some modifications. The substrate for α-L-arabinofuranosidase was p-nitrophenyl-α-L-arabinofuranoside (pNαLaf) (Sigma, Oakville). The standard reaction mixture, contained 0.1 M sodium acetate buffer pH 5.0 (final concentration 50 mM), 70 μl of water and 80 μl of substrate (final concentration 1 mM) bringing the final volume of reaction mixture to 400 μl. The reaction mixture was placed in 70°C water bath to prewarm. The enzyme was added to start the reaction. The reaction mixture was incubated for 10 min, after which it was ended by adding 0.8 ml of 0.1 M Na$_2$CO$_3$. The concentration of p-nitrophenol released was calculated using the molar extinction coefficient at 400 nm, ϵ = 10,500 M^{-1} cm^{-1} (Miyazaki, 2005).

2.8.3. Acetylesterase assay

The enzyme assay for esterase was determined as described previously (Sobek & Gorisch, 1988) with some modifications. The enzyme assay was conducted by measuring the release of p-nitrophenol from a chromogenic substrate p-nitrophenyl acetate. The standard reaction mixture contained 20 mM sodium phosphate buffer pH 7.0, 750 μl of water and 100 μl of substrate (final concentration 1 mM) dissolved in dimethyl sulfoxide, bringing the final volume to 1 ml. The assay mixture was preincubated at the assay temperature of 25°C for 5 min, before the enzyme was added to

start the reaction. The mixture was mixed again, and then returned to the water bath to incubate for 10 minutes at 25°C. After 10 minutes, the reaction was stopped by placing the reaction mixture in ice. The reaction mixture was transferred into cuvettes and then the absorbance was measured. The concentration of p-nitrophenol released was calculated using the molar extinction coefficient of the substrate at 400 nm, $\epsilon=16,400$ $M^{-1}cm^{-1}$ (Sobek & Gorisch, 1988).

2.8.4. β-glucosidase assay.

The protocol for enzyme assay was similar to previously described (Ruttersmith and Daniel, 1993) with some modifications. The substrate for glucosidase was p-nitrophenyl-β-D-glucopyranoside (pNβgp). The standard reaction mixture had a final volume of 400 μl, which consisted of 0.1 M sodium acetate buffer pH 5.0 (final concentration 50 mM), 70 μl of water and 80 μl of substrate (final concentration 1 mM). The reaction mixture was prewarmed at 70°C and then the reaction was started by adding the enzyme and incubating for 10 min. After 10 minutes, 0.8 ml of 0.1 M Na_2CO_3 was added to end the reaction. The concentration of p-nitrophenol released was calculated using the molar extinction coefficient at 400 nm, (pNβgp) $\epsilon=18,300$ $M^{-1}cm^{-1}$ (Ruttersmith and Daniel, 1993).

2.9. Cellular localization of β-xylosidase, α-L-arabinofuranosidase and acetylesterase based on enzyme assay from culture supernatant and CFE

The culture supernatants reserved after cell harvesting were concentrated through ultrafiltration (Amicon Corp., Lexington Mass). The membrane size for the concentrator was 30 kDa. Concentration was conducted by applying gas pressure through cellulose tubings connected to the ultrafiltration chamber containing the supernatant. Proteins above the membrane's molecular weight cut-off are retained above the membrane, while water and solutes below the cut-off molecular weight pass into the filtrate and out of the chamber. The supernatant was washed with 50 mM Tris buffer several times. The retentate was concentrated at 11,000 x g for 10 min at room temperature in a table top Eppendorf centrifuge (VWR Canlab, Mississauga). The concentrate was collected and the pellet was discarded.

The cell free extract was processed according to Sec 2.6 and then the respective enzyme assays were conducted. Localization assays were conducted on the cell free extract, the concentrate as well as ultrafiltration passthrough to determine enzyme distribution.

2.10. Substrate source required for maximum production of β-xylosidase, α-L-arabinofuranosidase and acetylesterase

Five carbon sources: xylose, xylan, glucose, arabinose and arabinogalactan were used to grow *T. hypogea* to compare enzyme activities. The cultures were prepared in duplicate for each carbon source. The final concentration of each substrate in the 400 mL of growth medium was 0.3 % (w/v). The growth of *T. hypogea* was determined in a flask

by counting cells using the counting chamber as well as measuring optical density. The seed cultures for all the test media was grown in their respective carbon sources: i.e. glucose test medium were inoculated by seed culture that had glucose as carbon source. The original seed culture was adapted by having three successive transfers on the same carbon source. The third transferred seed culture was used to inoculate respective test media and their controls. The inoculum volume from the seed culture was 1 % v/v. The seed culture for inoculum was freshly prepared late log phase culture with final cell density of 10^8 cells/ml.

The control medium was prepared at the same time as the test medium. Except for yeast extract, no additional carbon source was added to the control medium. The test media were incubated at 70°C water bath with their control. Samples from the culture were drawn out periodically for counting cells. The test media and control were removed after 30 hours, after which cells reach stationary phase. Only test media were harvested to produce cell free extract for enzyme assays. Although there was growth in the control medium, it was not harvested. The reason for having a control was primarily to control for any growth in the absence of added C source, therefore it was not considered necessary to harvest the control cells.

2.11. Enzyme Purification

AKTA^{TM} Fast Perfomance Liquid Chromatography (FPLC), a liquid chromatography system with P-920 pump (Amersham Pharmacia Biotech) was used to purify the enzymes under anaerobic conditions using Tris buffer at room temperature. All buffers were degassed for 45 minutes and maintained under positive pressure of nitrogen.

The protocol for working with AKTA FPLC, was that all columns used were to be rinsed and then equilibrated, with Buffer A. This buffer was prepared by dissolving in one liter of water 50 mM Tris, 10% (v/v) glycerol, 2 mM dithiothreitol, 2 mM sodium dithionite and pH adjusted to 7.8, using 1 M HCl. SDT was only added in the buffer when proteins were loaded on the first column which was DEAE. Since the proteins involved in this project did not require a reducing agent therefore it was omitted in the later purification steps. Sample was loaded in the column through a needle with tubing connected to the pump in FPLC. The open end of the needle was inside the flask containing sample or buffer. Through pressure, sample was loaded through the needle into the pump, which propelled the sample inside the column. The eluted samples were collected in 10 ml serum bottles.

2.11.1. Purification of β-xylosidase

Xylosidase was purified from 100 g (dry weight) biomass grown with xylose. CFE from *T. hypogea* was prepared through large scale growth. Seven columns were run in series in order to purify the protein. The cell-free extract of *T. hypogea* was applied to a DEAE-sepharose column (5 × 10 cm) that was equilibrated with 150 mL buffer A. Buffer B was prepared by dissolving 1 M NaCl in buffer A. The bound proteins were eluted with a gradient, which is a mix of buffer A with increasing proportion of buffer B. Xylosidase eluted out as 0.24 M NaCl was applied to the column at a flow rate of 3 ml min^{-1}. Fractions containing xylosidase activity were then pooled and loaded onto a Hydroxyapatite column (2.6 × 15 cm) at a flow rate of 1.0 ml min^{-1}. The column was applied with a gradient (0.32 M potassium phosphate dibasic and 0.18 M potassium

dihydrogen phosphate in buffer A). Xylosidase started to elute from the column at the concentration of 0.07 M potassium phosphate buffer was applied to the column. Fractions containing enzyme activity were pooled and applied to a Phenyl-Sepharose column (5×10 cm) that had been equilibrated with 0.8 M $(NH_4)_2SO_4$ in buffer A at a flow rate of 2 ml min^{-1}. A linear gradient [0.82–0 M $(NH_4)_2SO_4$ in buffer A] was applied and the β-xylosidase started to elute at a concentration of 0.0 M $(NH_4)_2SO_4$. Fractions containing β-xylosidase activity were combined and then desalted with ultrafiltration using a PM-30 membrane. The concentrated sample was applied to a Q-sepharose column (1 x 10 cm). The column was eluted with a linear gradient of NaCl (0 – 0.5 M) in buffer A at a flow rate of 1 ml/min. Xylosidase was eluted out when 0.2 M NaCl was applied to the column. Since the fraction was not pure enough for gel-filtration, therefore it was loaded onto the Hydroxyapatite (HAP) column again. The elution was similar to the previous trial. Fractions with high activity were concentrated through ultrafiltration to 3 ml in preparation for loading on to a Gel-filtration column (Superdex 200, 2.6×60 cm) equilibrated with buffer A containing 100 mM KCl at a flow rate of 2 ml/min. Xylosidase activity was eluted out at an elution volume of 155 ml corresponding to molecular weight of 130 kDa.

The gel profile showed that the high activity fractions had a faint contaminant band therefore fractions were loaded on Q-sepharose HP column again. The purity of the fractions containing xylosidase activity was verified using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970). The pure fraction was stored in -20°C until future use.

2.11.2. **Purification of α-L-arabinofuranosidase**

The cell free extract was prepared from 50 g of biomass obtained by growing *T. hypogea* with xylose. A DEAE-sepharose column was equilibrated with 150 ml buffer A. After the CFE was loaded the bound proteins were eluted with a linear gradient (0–1.0 M NaCl) that was applied at a flow rate of 3 ml min^{-1}. α-L-arabinofuranosidase eluted out as 0.18 M NaCl was applied to the column. Fractions containing α-L-arabinofuranosidase activity were then pooled and loaded onto HAP column at a flow rate of 1.0 ml min^{-1}. α-L-arabinofuranosidase started to elute from the column at the concentration of 0.09 M potassium phosphate, as the gradient (0–0.5M) was applied to the column. Fractions containing enzyme activity were pooled and applied to a Phenyl-Sepharose column equilibrated with 0.8 M $(NH_4)_2SO_4$ in buffer A at a flow rate of 2 ml min^{-1}. A linear gradient [0.82–0 M $(NH_4)_2SO_4$ in buffer A] was applied and α-L-arabinofuranosidase started to elute at a concentration of 0.2 M $(NH_4)_2SO_4$. Fractions containing α-L-arabinofuranosidase activity were pooled and concentrated by ultrafiltration using a PM-30 membrane. α-L-arabinofuranosidase eluted as a single peak after each column. The concentrated sample was loaded on HAP column again. The elution profile however was different than previous trial, α-L-arabinofuranosidase started to elute from the column at the concentration of 0.15 M potassium phosphate. High activity fractions were loaded onto a Q-sepharose HP column. The column was eluted with a linear gradient of NaCl (0 – 0.5 M) at a flow rate of 1 ml/min. α-L-arabinofuranosidase was eluted out when 0.25 M NaCl was applied to the column. Fractions were concentrated and loaded onto a Gel-filtration column. α-L-arabinofuranosidase activity was eluted out at an elution volume of 167 ml corresponding to 190 kDa.

Based on SDS-PAGE profile, the fraction was still not pure as there was still a contaminant band; therefore fractions were loaded on a HAP column again. After HAP, the contaminant band was still not removed completely however due to low protein concentration the fractions were not further purified, as there was a risk of fraction being lost in the column.

2.11.3. **Purification of Acetylesterase**

Acetylesterase was purified from α-L-arabinofuranosidase's cell-free extract batch. The elution profile of acetylesterase from DEAE-sepharose column was similar to that of α-L-arabinofuranosidase. Both the proteins eluted as 0.14 M NaCl was applied to the DEAE column. After DEAE, acetylesterase recovery increased four times compared to that of CFE, which is unusual. After DEAE, the fractions were loaded on a HAP column. The column was applied with a gradient (0–0.5 M potassium phosphate in buffer A) and acetylesterase started to elute from the column at the concentration of 0.08 M potassium phosphate, until 0.10 M salt, after which it started to decrease. However near 0.17 M potassium phosphate, there was also an indication of acetylesterase elution based on enzyme activity which continued to increase until 0.5 M, after which the enzyme activity started to decrease.

Only the fractions that eluted earlier (0.17 M) and had higher activity were combined and loaded onto a Phenyl-Sepharose column equilibrated with 0.8 M $(NH_4)_2SO_4$ in buffer A at a flow rate of 2 ml min^{-1}. A linear gradient [0.82–0 M $(NH_4)_2SO_4$ in buffer A] was applied. However based on enzyme activity it appeared that acetylesterase did not bind onto the column. All the fractions starting from pass-through

to the last fraction showed activity. Therefore the salt concentration was increased due to a possibility of acetylesterase requiring a higher salt concentration to bind. However even when the gradient was increased from 0.82 to 1.21M, the scattering of activity was observed again from pass-through to the last fraction. All the fractions were concentrated and desalted through ultrafiltration, using a PM-30 membrane. The concentrated sample was applied to a Q-sepharose HP column. The bound protein was eluted with a linear gradient of NaCl (0 − 0.5 M). Acetylesterase was eluted out when 0.32 M NaCl was applied to the column. Acetylesterase eluted as a single peak. The fractions were combined and loaded on HAP column again. The elution was similar to the previous trial; however the enzyme activity distribution was different. Acetylesterase seemed to elute at two different salt concentrations again in the second trial with HAP, higher acetylesterase activity was associated with fractions eluted at 0.5 M potassium salt as compared to 0.08 M potassium salt concentration, which is different from the previous trial. All fractions containing acetylesterase activity were combined, and loaded on HAP again with stretched gradient between the two elution salt concentrations. The gradient was stretched to (0.06-0.5 M potassium phosphate in buffer A) and also covered more column volumes compared to the previous trial. In this trial, almost all the fractions started to elute at 0.5 M potassium phosphate salt concentration. The two different elution profiles from HAP trials suggest the possibility of two different types of esterases.

After HAP, fractions were almost pure except for a trace contaminant band. Fractions with high activity were concentrated through ultrafiltration to 3 ml in preparation for loading onto Gel-filtration column equilibrated with buffer A containing

73

100 mM KCl at a flow rate of 2 ml/min. Acetylesterase activity was eluted out at an elution volume of 165 ml corresponding to 200 kDa. The fraction was stored in -20°C until future use.

2.12. N-terminal analysis of β-xylosidase

The purified xylosidase enzyme sample was run on 12.5% SDS-PAGE and blotted onto Polyvinylidene Difluoride (PVDF) membrane by using Mini Trans-Blot Electrophoretic Transfer cell (Bio-Rad Laboratories, CA). The PVDF membrane was pre-wetted in 100% methanol for 1 minute right before use. The transferring buffer (25 mM Tris containing 192 mM glycine and 15% methanol) was pre-cooled at 4°C and one cooling unit was put inside the transfer cell to maintain the low temperature. Blotting was run for one and half hour at 110V and 230 mA. After that, the PVDF membrane was stained for 3 minutes by 0.1% w/v Coomassie blue R-250 in 10% methanol solution and subsequently destained by a solution containing 45% v/v methanol and 10% v/v acetic acid. After destaining, the SDS-PAGE gels were washed with Millipore-grade water (18.2 MΩ·cm) for 2 days at 4°C. The bands on PVDF membrane were cut in a flow hood by using a surgical cutter. The cut samples were transferred into a clean eppendorf tubes and stored at -20°C. The samples were sequenced by Edman degradation (Molecular Biology Core Facility, Dana Farber Cancer Institute, Boston, MA).

N-terminal analysis was done by aligning the sequences using BlastP search (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Aligned sequences from N-terminal analysis were further analyzed using the clustal W program

(http://www.ch.embnet.org/software/ClustalW.html) and box shade programs (http://www.ch.embnet.org/software/BOX_form.html).

2.13. Electrophoresis

SDS-PAGE was routinely performed using a Hoefer Mighty Small 250 mini system (Amersham Biosciences, Montreal) based on the methods of Laemmli (1970). Protein samples in SDS-PAGE loading buffer were denatured in boiling water for 5 min. The gels (12.5 % acrylamide) were run under constant current of 15 mA until the bromophenol blue marker reached the bottom of the gel. The gel was stained with Coomassie Brilliant Blue R250 after electrophoresis for one hour under shaking conditions (approximately 100-150 rpm). The gel was then destained with destaining solution, until clear protein bands start appearing on clear gel.

The molecular mass of the enzyme under denaturing conditions was estimated by using medium range markers [phosphorylase B (97.4 kDa)], BSA [66 kDa], ovalbumin [43 kDa], carbonic anhydrase [31 kDa], soybean trypsin inhibitor [20.1 kDa], and lysozyme [14.3 kDa] (Sigma, Oakville) and recombinant high molecular weight markers (Fermentas, Burlington).

2.14. Native molecular weight determination

The determination of native molecular weight was determined using size exclusion chromatography with Superdex-200 column (Amersham Pharmacia Biotech, Montreal). The mass was estimated by comparing the specific retention time of the enzyme with that of calibration run performed with four different proteins as molecular

weight standards. The column was equilibrated with buffer A, containing 100 mM KCl at a flow rate of 2 ml min^{-1}. After column equilibration, the protein standards from the protein kit (Pharmacia, New Jersey) containing: ferritin (440,000 Da), catalase (232,000 Da), aldolase (158,000 Da) and bovine serum albumin (67,000 Da) were applied to the column. A calibration curve was created by plotting Log MW of standard molecular weight proteins against the elution volume. From the calibration curve, the native molecular weight of the enzyme was determined.

2.15. Detection of esterase activity using native gel electrophoresis

The acetylesterase active band was detected using zymogram staining or activity staining. The native gel was prepared the same way as the denaturing gel except without the addition of any denaturing agent such as sodium dodecylsulfate or mercaptoethanol. Fractions containing acetylesterase activity were not heated before loading. The gel was run using the same voltage applied as the denaturing gel. Bovine serum albumin was loaded in a separated lane and served as a positive control. After the bromophenol blue marker reached the bottom of the gel, the gel was removed and cut in half to separate lanes loaded with acetylesterase and BSA.

The BSA containing gel was stained with coomassie blue. The acetylesterase containing gel slice was transferred to a Petri dish and soaked in acetylesterase assay buffer at 4°C on a shaker in an effort to change the pH of the gel from pH 8.8 at which the esterase substrate is not stable to pH 7.0. It was found that at pH 9.0, which is close to the pH of the separating gel (pH 8.8), the substrate auto-hydrolyzed (results not shown). Therefore, it was necessary to change the pH with assay buffer, 20 mM sodium phosphate (pH 7.0).

The pH of the buffer in the Petri dish was measured using pH paper strips. After two changes of buffer, the pH of the gel became constant near 7.0. The assay was started by adding 1 mM final concentration of p-nitrophenylacetate dissolved in dimethylsulfoxide, to the buffer in which the gel was soaking. The assay was conducted at room temperature and the Petri dish was gently mixed by hand. The color started to develop in 10 minutes, after which the buffer substrate solution was removed by Pasteur pipette from the Petri dish and the gel was photographed.

2.15. Data analysis

All the data are the average of duplicate values except where indicated specifically. The data were analyzed using Microsoft Excel.

Chapter 3

3.0. Results

3.1. Effect of substrates on *T. hypogea* growth

T. hypogea was able to grow on different substrates such as glucose, xylose, xylan arabinose and arabinogalactan at a concentration of 0.3% (w/v). All the carbon sources were selected for a reason; xylan and xylose were selected as some researchers have shown that xylanolytic enzymes in fungus, bacteria and actinomycete are induced by xylan, xylose and by lignocellulosic residues that contain xylan (Flores et al., 1996; Archana and Satyanarayana, 1997; Kermnicky and Biely, 1998). Glucose was chosen as it is ubiquitous in nature and therefore is available easily. Arabinose and arabinogalactan were chosen to confirm researchers' findings that arabinan or L-arabinose containing residues are necessary for the induction of arabinofuranosidases (De Ioannes et al., 2000; Degrassi et al., 2003).

The cell densities reached at 31 hours (stationary phase), were used to compare growth on different carbon sources. The experiment was done in duplicate for each carbon source. The results confirmed that *T. hypogea* can grow on simple sugars like glucose, xylose, arabinose and complex sugars such as xylan. However only slight growth was observed on arabinogalactan (**Table 8**). The highest cell density was achieved with xylan and xylose being $>2.25x\ 10^8$ cells/ml as compared to the control of $1.4x\ 10^8$ cells/ml. The size of the cells was also measured. Cells grown in xylose, glucose, arabinose and xylan were between 0.5 - 1 by 1.5 - 2 μm, whereas for arabinogalactan and carbon controls, the cells were slightly smaller, the sizes were 0.5 - 1 by 1-1.5 μm. It is possible that the smaller cell size is an indication of starvation resulting from utilization of energy to compensate for absence of preferred carbon source.

Table 8: Growth of *T. hypogea* on different carbon sources at three different time points

Carbon source	Cell density (cells/ml)		
	Zero hour	20 hour	31 hour
Xylose	5.65×10^6 $\pm 2.12 \times 10^5$	1.35×10^8 $\pm 2.21 \times 10^7$	2.32×10^8 $\pm 4.53 \times 10^7$
Xylan	3.48×10^6 $\pm 3.18 \times 10^5$	1.33×10^8 $\pm 7.00 \times 10^7$	2.25×10^8 $\pm 9.19 \times 10^7$
Glucose	4.38×10^6 $\pm 8.84 \times 10^5$	9.00×10^7 $\pm 5.66 \times 10^7$	1.80×10^8 $\pm 2.83 \times 10^7$
Arabinose	5.18×10^6 $\pm 1.06 \times 10^5$	9.80×10^7 $\pm 3.54 \times 10^7$	1.85×10^8 $\pm 3.54 \times 10^7$
Arabinogalactan	3.00×10^6 $\pm 7.07 \times 10^5$	4.30×10^7 $\pm 3.82 \times 10^7$	1.69×10^8 $\pm 3.39 \times 10^7$
Control	2.23×10^6 $\pm 1.77 \times 10^5$	2.70×10^7 $\pm 1.84 \times 10^7$	1.40×10^8 $\pm 1.41 \times 10^7$

The growth in control medium occurred due to the presence of yeast extract and trypticase. For each of carbon cultures the initial cell density was between 4.0×10^6 cells/ml to 5.5×10^6 cells/ml. The inoculum volume from seed culture was 2 % (v/v) of medium culture. The seed culture density for each respective carbon source was 2.0-3.3×10^8 cells/ml with the exception of arabinogalactan equaling 1.8×10^8 cells/ml.

3.2. Effect of growth substrate on enzyme activities

β-xylosidase, α-L-arabinofuranosidase and acetylesterase presence in growth medium and cells were measured **(Table 9).** The specific activity is the average of two different batches harvested and assayed in duplicate. Both the batches were inoculated from the same seed cultures as well as the assay conditions were also kept the same.

Compared to cells grown in glucose, the specific activity of α-L-arabinofuranosidase in the cells cultivated in the presence of xylan or xylose was 4 to 6 times greater. For xylosidase activity, the specific activity was 1.5 times greater. A trend is not clear for acetylesterase, possibly because the activity was too low; it appears that the activity in the cells grown on xylan was slightly higher compared to that on xylose and glucose.

Table 9: Cell associated enzyme activity from different growth substrates

Growth substrate	SpecificActivity*(U/mg)		
	Xylosidase	α-L-arabinofuranosidase	Acetylesterase
Glucose	0.153 ± 0.086	0.070 ± 0.051	0.004 ± 0.006
Xylose	0.256 ± 0.065	0.387 ± 0.164	0.004 ± 0.004
Arabinose	0.188 ± 0.056	0.122 ± 0.086	0.002 ± 0.002
Arabinogalactan	0.198 ± 0.130	0.121 ± 0.114	0.001 ± 0.0007
Xylan	0.261 ± 0.004	0.292 ± 0.050	0.005 ± 0.002

* values shown are average specific activity ± standard deviation from two different batches

3.3. Localization of enzymes produced by *T. hypogea* based on enzyme assays

Xylosidase, α-L-arabinofuranosidase and acetylesterase activities were determined in the CFE and culture supernatant **(Fig 9).** The majority of xylosidase, α-L-arabinofuranosidase and acetylesterase activities were associated with the cells. However acetylesterase activity in the culture medium was also significant (34 % total activity), which suggests that a significant amount of enzyme was secreted into the supernatant or there are other secreted enzymes that collectively or individually are able to hydrolyse the substrate used in the acetylesterase assay.
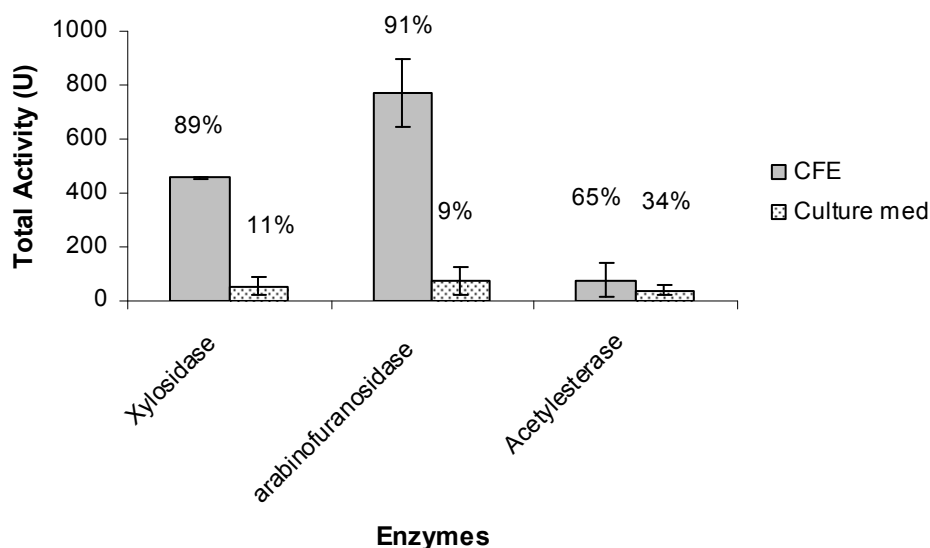
Fig. 9. Distribution of xylosidase, α-L-arabinofuranosidase and acetylesterase activity in the cells and culture medium based on enzyme activity assay. Total Activity was the average of two experiments showing standard deviation error bars.

3.4. Purification of Xylosidase

As summarized in **Table 10**, xylosidase was purified via a series of chromatography columns involving DEAE-Sepharose, Hydroxyapatite, Phenyl-Sepharose, Gel-filtration and Q-sepharose columns. The anion exchange column gave different profiles for xylosidase and α-L-arabinofuranosidase, which is an indication of both enzymes being different.

Good separation was achieved with the Q-sepharose column and Gel-filtration columns as they both yielded a purification fold increase of 25 % when compared with their preceding columns. The Gel-filtration indicated the size of the enzyme as 130 kDa. After Gel-filtration fractions were loaded on Q-sepharose column again to remove faint contaminant band observed after running SDS-PAGE gel. The Q-sepharose column successfully separated xylosidase from the contaminant protein. The purified xylosidase had a specific activity of 46 U/ mg, yielding a final purification fold of 328 and recovery of 1.33%.

The purity of the fractions containing xylosidase activity was verified using SDS-PAGE (**Fig. 10**). Pure fractions were stored at -20°C until future use.

Table 10: Purification of β-xylosidase from *T. hypogea*

Step	Total Activity (Units)	Total Protein (mg)	Specific Activity (units/mg)	Recovery (%)	Purification Fold
Crude Extract	450	3000	0.15	-	-
Cell Free Extract	378	2770	0.14	100	1
DEAE	237	584	0.39	63	2.9
HAP	125	273	0.46	33	3.3
Phenyl-Sepharose	46.1	34.3	1.34	12.2	9.5
QHP	17.7	3.42	5.20	4.7	37
HAP	12.8	2.00	6.40	3.4	46
Gel-filtration	7.31	0.24	30.5	1.9	218
QHP	5.04	0.11	46.0	1.3	328
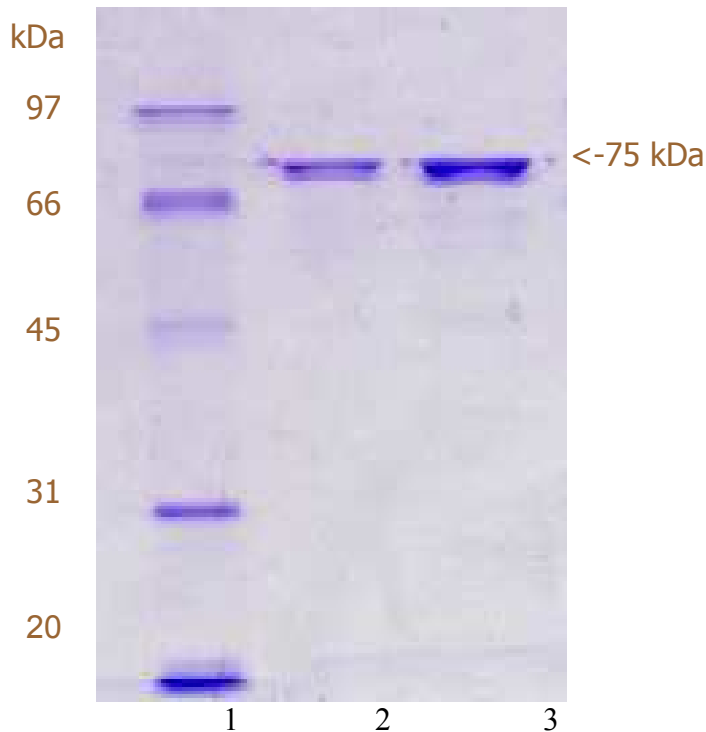
Fig. 10. SDS-PAGE image for pure β-xylosidase. 10 % gel lane 1-markers, lane 2-xylosidase (0.5 µg), lane 3- xylosidase (1.0 µg).

3.5. Purification for α-L-arabinofuranosidase

α-L-arabinofuranosidase was partially purified through a series of chromatography columns (**Table 11**). The CFE prepared from *T. hypogea* cells with 215 units of α-L-arabinofuranosidase activity was loaded on DEAE. Fractions were loaded on HAP again after Phenyl-Sepharose as the sample was still impure to be loaded onto Q-sepharose. Gel-filtration and Q-sepharose column polished the sample significantly compared with the other columns. The native molecular weight of the enzyme from Gel-filtration was calculated to be as 200 kDa. After Gel-filtration, fractions were loaded on HAP again to remove a faint contaminant band observed after running SDS-PAGE gel. However HAP was not able to remove the contaminant band as revealed by SDS-PAGE (**Fig. 11**). The partially purified α-L-arabinofuranosidase had a highest specific activity of 81 U/ mg, and final purification fold of 450 and recovery of 2%. The partially purified enzyme was stored at -20°C for future use.

Table 11: Purification table for α-L-arabinofuranosidase

Step	Total Activity (Units)	Total Protein (mg)	Specific Activity (units/mg)	Recovery (%)	Purification Fold
Crude Extract	465	1710	0.27	-	-
Cell Free Extract	215	1212	0.18	100	1
DEAE	202	440	0.46	94.0	2.6
HAP	200	67.00	3.24	93.0	18
Phenyl-Sepharose	179	28.05	6.38	83.3	35
HAP	150	8.12	18.5	70.0	103
QHP	143	3.72	38.0	67.0	211
Gel-filtration	41	0.54	76.0	18.8	422
HAP	5.0	0.062	81.0	2.3	450
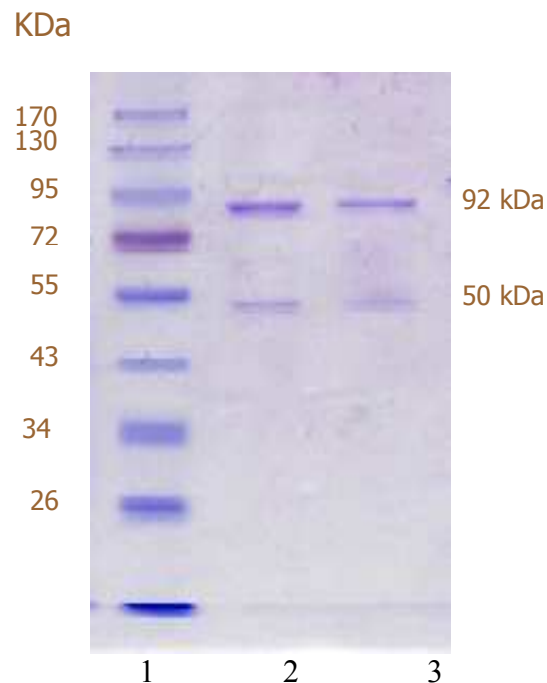
Fig. 11. SDS-PAGE gel image of partially pure α-L-arabinofuranosidase (10 % acrylamide gel) lane 1-markers, lane 2- α-L-arabinofuranosidase (2.0 μg), lane 3- α-L-arabinofuranosidase (1.0 μg)

3.6. Acetylesterase

3.6.1. Comparison of assay methods

CFE extract prepared from xylose-grown cells were used to purify esterase. To measure acetyl esterase activity, three different protocols were tested with two different substrates; 4-methylumbelliferyl acetate and p-nitrophenyl acetate. The principle of the enzyme assay and the protocol using p-nitrophenyl acetate has been described earlier (Sec 2.8.3). Acetylesterase activity using methylumbelliferyl acetate was measured by detecting release of methylumbelliferone at a wavelength of 354 nm. Esters of 4-methylumbelliferone (4-MU) fluoresce when cleaved by esterase to release the methylumbelliferone. The esterase activity with *p*-nitrophenyl acetate is determined by monitoring spectrophotometrically the hydrolysis of *p*-nitrophenyl acetate to release p-nitrophenol, which is detected at a wavelength of 400 nm (Degrassi et al., 1998). However, for enzyme assay the protocol with substrate p-nitrophenyl acetate was chosen instead of 4-methylumbelliferyl acetate, because the enzyme assay is similar to the other two enzymes' (α-L-arabinofuranosidase and xylosidase) assay since they are also analyzed by measuring release of p-nitrophenol spectrophotometrically. The assay with p-nitrophenol assay was also simpler and less time consuming than with 4-methylumbelliferyl acetate, which requires additional steps after stopping the reaction that involve centrifugation and addition of another reagent for complete release of methylumbelliferone.

3.6.2. Detection of acetyl esterase activity

It was difficult to accurately detect acetylesterase activity in *T. hypogea* CFE because enzyme activity was low. Therefore to confirm the validity of the acetylesterase assay protocol, *Saccharomyces cerevisiae* S288C CFE was borrowed from Dr. B. Dunker (Dept. of Biology, University of Waterloo). An esterase has already been purified from *Saccharomyces cerevisiae* S288C (Degrassi et al., 1999*). A comparison of acetylesterase activity was made among *S. cerevisiae*, *Thermotoga hypogea* and *Thermotoga maritima* (**Table 12**). To further validate the use of the yeast strain, a xylosidase assay was also conducted. *S. cerevisiae* are known to be unable to hydrolyze xylose or xylan sugars (La Grange et al., 2001). The yeast strain served as a positive control for acetylesterase assay and negative control for xylosidase assay. The cell free extract from *S. cerevisiae* was prepared the same way as for bacterial cultures except for the addition of SDT.

Two controls were also prepared without the substrate and the enzyme. The assay was performed in duplicate.

Table 12: Comparison of enzymatic activity between *S. cerevisiae* and *Thermotoga*

CFE	Acetylesterase Spec Act(U/mg)	Xylosidase assay Spec Act (U/mg)
S. cerevisiae	0.063 ± 0.071	0.013 ± 0.024
Thermotoga hypogea	0.014 ± 0.014	0.256 ± 0.065
Thermotoga maritima	0.009 ± 0.02	Not determined

As expected the *S. cerevisiae* sample tested negative for xylosidase assay and exhibited some activity for acetylesterase. The higher specific activity for acetylesterase assay in *S. cerevisiae* as compared to *Thermotoga* strains does not suggest that the

esterase enzyme present is similar to that of *T. hypogea* as there are many types of esterases. It merely validates the sensitivity of the assay in detecting any ester hydrolyzing activity from an organism.

3.6.3. Purification

The purification profile of acetylesterase is summarized in **Table 13**. The acetylesterase elution profile from DEAE was the same as α-L-arabinofuranosidase. Acetylesterase recovery from DEAE was 393 % as compared with the cell free extract, which was unusual. Another unusual elution profile was from Phenyl-Sepharose column. After two consecutive trials, no elution profile was determined from Phenyl-Sepharose column. Therefore all fractions were loaded on QHP column after which the protein eluted as a single peak with a recovery of 39 %. After HAP, fractions were almost pure except for a trace contaminant band as revealed by SDS-PAGE. Gel-filtration revealed native molecular size of 202 kDa. The final purification fold after Gel-filtration was 425 and recovery was 0.79%.

After Gel-filtration, only one band was observed on the SDS-PAGE gel **(Fig 12)**. However, the gel was run with less than 1 μg protein. Unfortunately, the protein could not be reassessed at higher concentration by SDS-PAGE, as the whole enzyme preparation became contaminated while concentrating possibly because the concentrator membrane was dirty. Therefore, the enzyme's purity could not be confirmed. While analyzing samples during purification, high molecular weight markers would have been appropriate for size estimation for acetylesterase, but due to convenience, medium range molecular weight markers were used. The fraction was stored at -20 °C until future use.

Table 13: Purification table for Acetylesterase

Step	Total Activity (units)	Total Protein (mg)	Specific Activity (units/mg)	Recovery (%)	Purification (fold)
Crude Extract	60	1710	0.03	-	-
Cell-free Extract	42	1212	0.04	100	1
DEAE	165	545	0.30	393	7.5
HAP	32	183	0.18	76	4.5
Phenyl-Sepharose	23.44	158	0.15	56	3.8
Phenyl-Sepharose	15	125	0.12	36	3
QHP	16.44	4.93	3.33	39	83
HAP	10.62	2.77	3.83	25	96
HAP	2.70	0.21	13	6.4	325
Gel-filtration	0.33	0.02	17	0.79	425
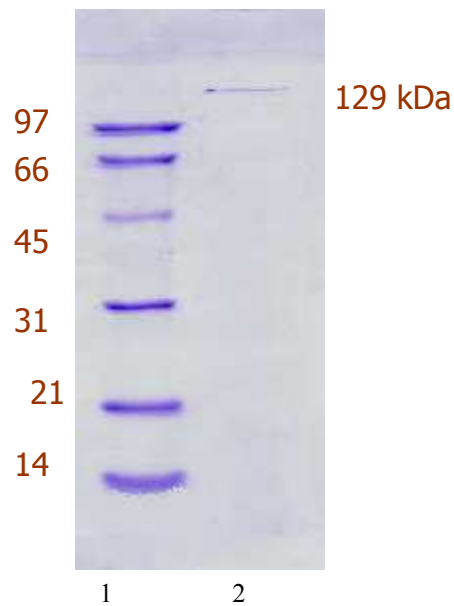
Fig 12. SDS-PAGE image for Acetylesterase. (12.5 % gel) lane 1- Markers, lane 2- Acetylesterase (0.5 µg).

3.6.4. Acetylesterase elution profile

Other researchers have reported that acetylesterase will bind to Phenyl-Sepharose and HAP columns with reproducible elution profiles (Sobek and Gorisch, 1988). Therefore an explanation was sought for the unusual elution profile exhibited by the enzyme after Phenyl-Sepharose and HAP columns. Acetylesterase enzyme assays were conducted with 2 mM DTT, SDT and ammonium sulfate (the salt for Phenyl-Sepharose chromatography) to investigate their effect on the assay substrate. The effect of each chemical on the substrate was tested separately by incubating the chemical and the substrate together in assay buffer, in the absence of any added enzyme. A control was prepared by adding substrate and assay buffer. The tested chemicals were dissolved in chromatography buffer (50 mM Tris-HCl, 10% glycerol, pH 7.8). To measure the potential for ammonium sulfate to cause hydrolysis of the substrate, two samples with concentration of 30% and 60% ammonium sulfate were prepared. The rationale was twofold: first, to see if ammonium sulfate had any effect and secondly if it had, was it concentration dependent?

Based on the results (**Table 14**), with 2 mM concentration of SDT, the absorbance from p-nitrophenol release was 9 times higher than the blank. In the 2 mM DTT assay, the absorbance from p-nitrophenol release was 10 times higher compared with the blank. This may explain the high recovery rate after DEAE for acetylesterase. While preparing to load on the DEAE column, 2 mM SDT/DTT was added to the CFE to shield the anaerobic proteins from oxygen as the CFE was shared with lab members working on other *T. hypogea* enzymes. By the time the CFE was loaded on DEAE, the reducing

agents SDT/DTT had been added twice already; once while preparing the cell free extract and a second time while preparing to load the enzyme on to the DEAE column. This raised the total concentration of reducing agents added to the DEAE fractions up to 8 mM, as compared to CFE, where the total concentration of reducing agent was 4 mM. This can explain the low specific activity observed from cell free extract prepared from xylose grown cells in (**Table 9**). In this preparation no SDT or DTT was added

Table 14: p-nitrophenol production in the presence of reducing agents.

Fractions	A_{400} at 25°C *
2 m M SDT	0.964 ± 0.012
2 m M DTT	1.024 ± 0.018
Tris Buffer (no SDT/DTT)	0.198 ± 0.0
Blank	0.173
No substrate	0.058

* absorbance values indicated are the average of duplicate trials

Regarding ammonium sulfate, it was found that the absorbance from p-nitrophenol released from both 30% and 60% ammonium sulfate solution was seven times higher than the blank (**Table 15**). The hydrolysis rate was not as high as with the reducing agents **(Table 14)**; nevertheless it assists in providing an explanation for the unusual binding profile of the Phenyl-Sepharose column. The substrate hydrolysis from ammonium sulfate can explain the false positives observed from the phenyl column, since the elution profile is determined from enzyme activity, which in turn is based on hydrolysis of substrate. It appears that the actual binding profile was not discernable due to the presence of ammonium sulfate salt in every fraction. In the future, it would be better to dialyze each fraction to remove ammonium sulfate salts for an accurate

determination of the elution profile. Moreover the substrate hydrolysis rate increase in 30% and 60% ammonium salt concentration was insignificant, suggesting that it is not concentration dependent.

Table 15: p-nitrophenol production in the presence of ammonium sulfate

Fractions	A_{400} nm at 25°C *
60% ammonium sulfate	0.760 ± 0.019
30% ammonium sulfate	0.744 ± 0.034
0% ammonium sulfate[a]	0.176 ± 0.007
Blank**	0.148
No substrate [#]	0.058

*absorbance values indicated are the average of duplicate trials

[a] 100 % 50 mM Tris-HCl, p H 7.8, 10% glycerol

** blank, no ammonium sulfate and Tris buffer were added. Only water and substrate were added

[#]No substrate indicates the reaction mixture had 50 mM Tris-HCl buffer and assay buffer only with no substrate

3.6.5. Acetylesterase activity staining on native gel

To confirm the identity of an esterase, activity staining was conducted. A yellow band was observed, however coomassie blue staining was not performed. The BSA control was used to give an estimation of molecular mass. **Fig. 13,** shows that the esterase activity stain was localized in one band. This experiment was a trial, comparing if both the activity stain and the protein stain would have the same location on the gel, but the experiment could not be repeated due to the lack of sufficient enzyme. Therefore the identity of the protein responsible for the activity stain band (**Fig 13, lane 2**) is not certain as the fraction's purity is not confirmed.
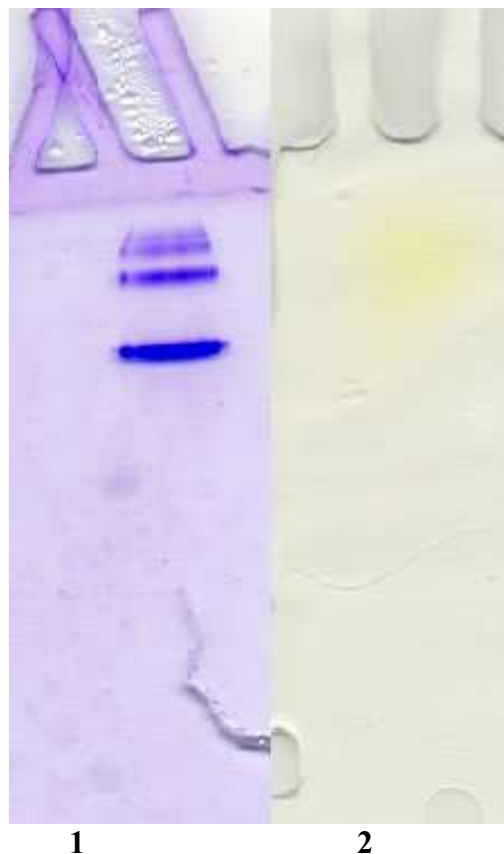

1   **2**

Fig 13. Activity stain for esterase on a native gel after Gel-filtration. The gel was cut in two pieces (Lane 1 & 2) and treated differently. Lane 1: BSA (3 µg) stained in Coomassie blue; lane 2: acetylesterase (2 µg) activity stained.

3.7.Characterization of purified xylosidase

3.7.1. Molecular weight determination

The molecular mass of the xylosidase under denaturing conditions was estimated using 12.5% SDS-PAGE using medium range molecular weight markers to be 75 kDa. The determination of the native molecular mass was performed by size exclusion chromatography using a Superdex-200 column. The enzyme was eluted with an effective volume (Ve) to be 155 ml at a flow rate of 3.0 ml/min. The native molecular mass was estimated by Gel-filtration. The molecular mass of denatured xylosidase was determined to be 130 kDa. Thus, the purified xylosidase was found to be a homodimer.

3.7.2. Optimization of the assay reaction time.

To optimize the enzymatic reaction time, CFE from xylose grown cells and purified enzyme were incubated with xylosidase substrate and assay mixture for different time periods at 70°C. Xylosidase activity was measured using the standard assay method. The time periods chosen were t= 0, 4, 8, 10, 15, 20 min. The results for CFE and pure enzyme were similar **(Fig. 14).** It was observed that the maximum enzyme activity was at 5 min and near 10 min the specific activity started to stabilize which means that the hydrolysis of p-nitrophenol was constant. Hence the optimal time was found to be 10 min. Time intervals between 5 - 8 min were not chosen since short time intervals can increase experimental errors. The same reaction profile from CFE and pure state suggests that the enzyme is stable in pure form, in contrast to xylanase from *T. hypogea*, where the
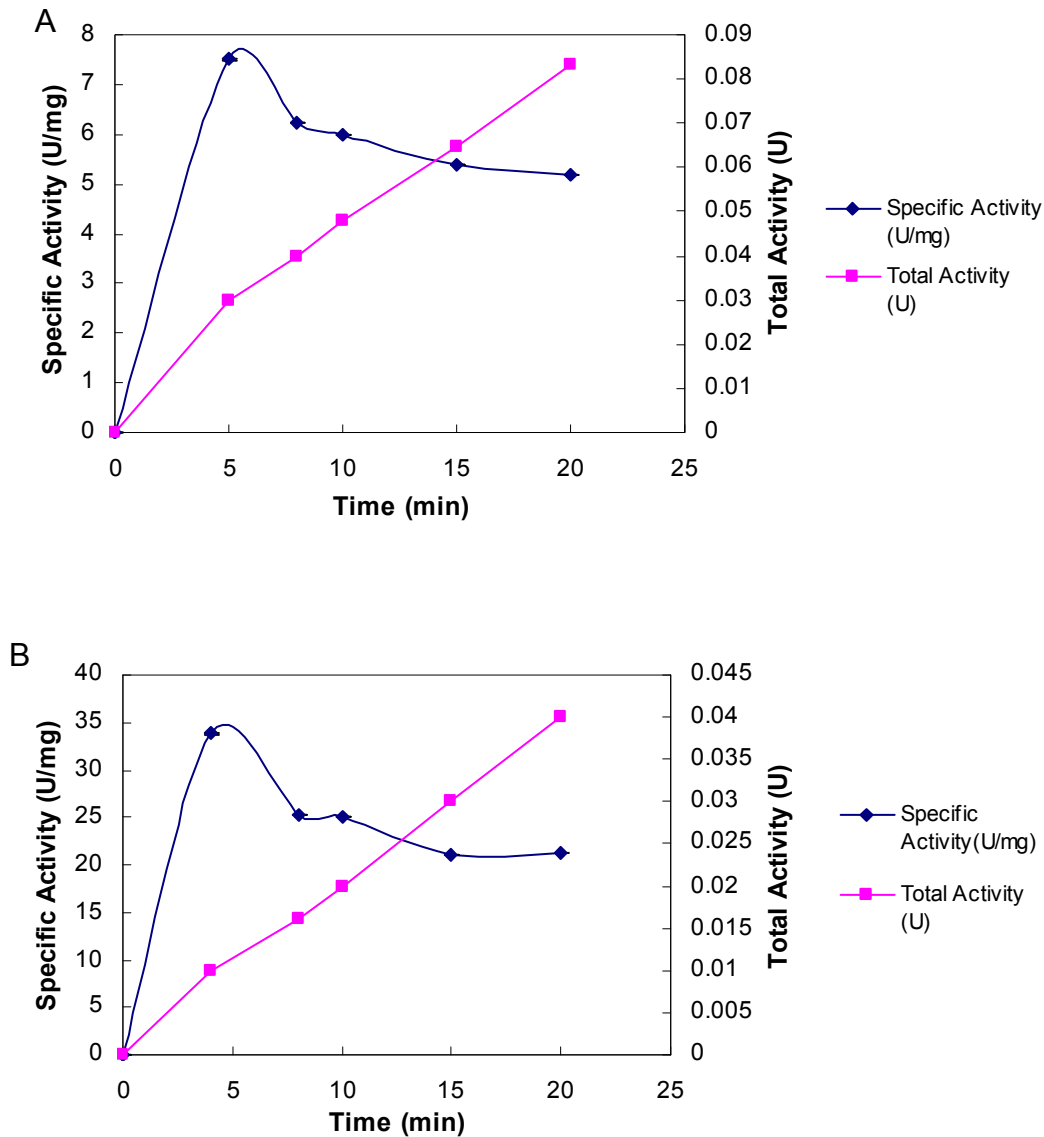
Fig 14: Optimization of assay reaction time. **A**. **CFE**. 400 ml assay mixture contained 50 mM sodium citrate buffer, 1 mM substrate (4-nitrophenyl β-D-xylopyranoside) and 0.8 µg of CFE incubated at 70°C; **B. Pure Protein**. 400 ml assay mixture contained 50 mM sodium citrate buffer, 1 mM substrate (4-nitrophenyl β-D-xylopyranoside) and 0.08 µg xylosidase and incubated at 70°C. Total activity was the activity of enzyme incubated under the specified assay condition at corresponding reaction time. One Unit is the amount of enzyme that releases 1 µmol of pNP from substrate per min under assay conditions. Specific Activity is the total amount of pNP released per milligram of enzyme per minute.

pure enzyme had a shorter optimal reaction time when compared to the CFE (Dhanjoon, 2005). This experiment is also significant as the increasing absorbance and total activity over time suggests that the substrate is being hydrolysed continuously to 20 minutes and therefore excludes the possibility of substrate being depleted during incubation time.

3.7.3. Optimization of protein concentration for xylosidase assay

To determine the appropriate protein concentration for characterization assays, different protein concentrations were incubated in the enzyme assay reaction mixture at 70°C for 10 min. Protein concentration ranged from 0.04 μg to 0.4 μg. It was found that between 0.08-0.15 μg, the specific activity was constant. Therefore, 0.15 μg of protein was selected for all characterization experiments for xylosidase.
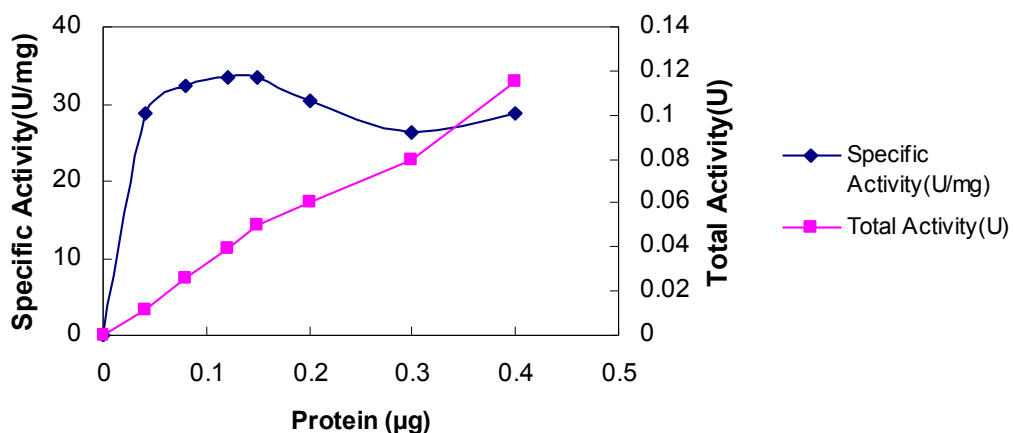
Fig 15: Optimal β-xylosidase concentration for enzyme assays. The values are the average of duplicate trials.

3.7.4. Optimal pH

Enzyme activities were assayed at pH 4.0 to 9.0 at 70°C using 0.15 μg protein with assay time of 10 minutes. 0.1 M sodium citrate buffer was used for pH 4.0-7.5, 0.1 M MES buffer was used for pH 5.5 -6.5, 0.1 M sodium phosphate buffer was used for pH 6.5-7.5, and 0.1 M EPPS was used for pH 7.0- 9.0.

The enzyme showed little activity at pH 4.0 and 9.0. Considerable activity was detected at alkaline pH with 50 % of the maximal activity measurable at pH 8.0. The pH optimum of the purified xylosidase was determined to be 6.0 at 70°C **(Fig 16).** Hence 0.1 M sodium citrate buffer pH 6.0 was used for subsequent assays.
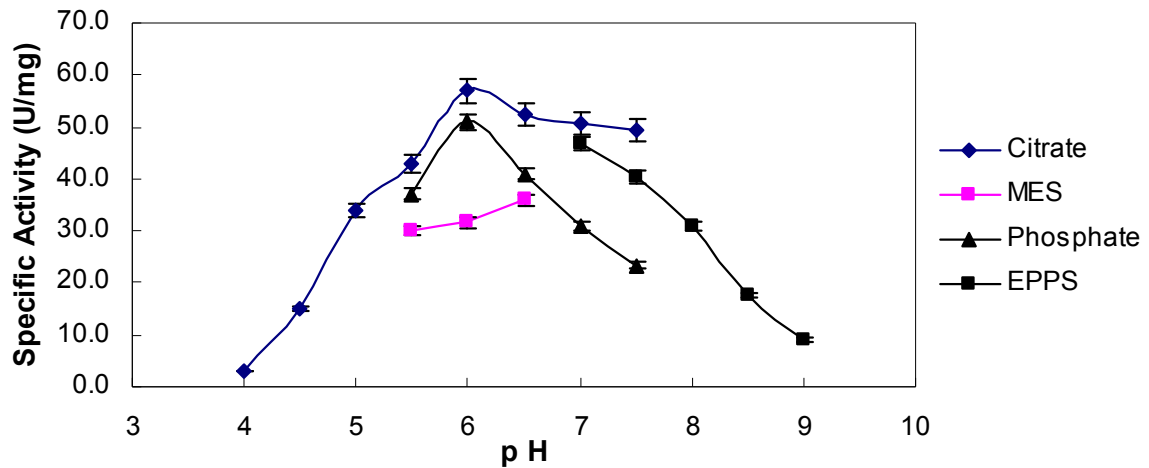
Fig 16: Optimum pH of the purified β-xylosidase. The values are the average of duplicate trials.

3.7.5. Optimum temperature

The activity of purified xylosidase was assayed over the temperature range 20-95°C using 0.15 µg protein with assay time of 10 min and pH 6.0 (50 mM sodium citrate buffer, final concentration). The enzyme had the highest activity at 70°C **(Fig 17).** At 90°C, the activity decreased to almost 45 % of its maximum activity.
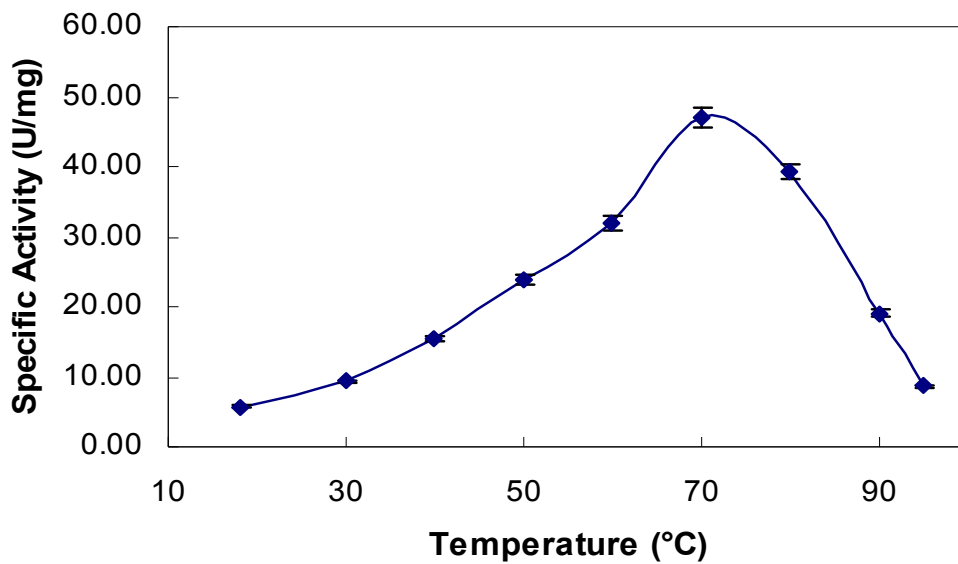
Fig 17: Temperature dependent activity of the purified xylosidase. The values are the average of duplicate trials indicated with standard deviation bars.

3.7.6. Thermostability of the purified enzyme

The thermostability of purified xylosidase was investigated at 70°C, the growth temperature of the bacterium, with pH 6.0 buffer using 0.08 μg protein. Thermal inactivation of β-xylosidase was studied by pre-incubating the enzyme preparations for different periods of time in the absence of substrate. After certain time periods, 0.08 μg of enzyme was removed and added to the xylosidase standard assay mixture and then enzyme activity was measured. About 75% of the activity was lost, after 45 min of incubation. The half life of the enzyme at 70°C was 22 minutes.

No further experiments were conducted to measure stability of the purified enzyme at different conditions such as different temperatures/pH or in the presence of stabilizing additives such as BSA.
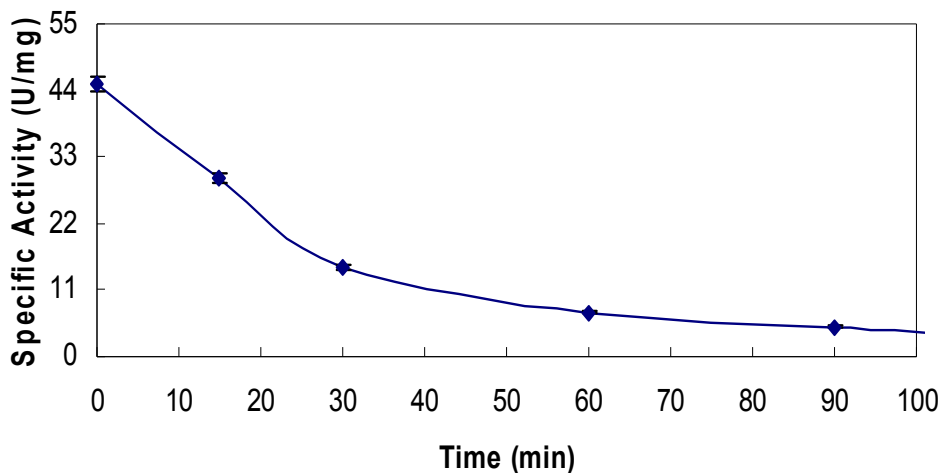
Fig 18: Stability of purified xylosidase. Enzyme was incubated at 70°C in 50 mM sodium citrate buffer, pH 6.0. Assay was conducted in duplicate.

3.7.7. Substrate specificity

The substrate specificity was determined at optimal temperature and pH (70°C, pH 6.0) by comparing other substrates relative to the assay substrate 4-nitrophenyl β-D-xylopyranoside. The other substrates were 4-nitrophenyl-α-L-arabinofuranoside, 4-nitrophenyl-α-D-xylopyranoside, 4-nitrophenyl-α-D-glucopyranoside and 4-nitrophenyl β-D-glucopyranoside. 4-nitrophenyl-α-L-arabinofuranoside was chosen to test whether the enzyme has arabinofuranosidase activity. 4-nitrophenyl β-D-glucopyranoside was selected to detect β-glucosidase activity. 4-nitrophenyl-α-D-xylopyranoside, was selected to see if the enzyme can hydrolyse the alpha configuration of its preferred substrate. 4-nitrophenyl-α-D-glucopyranoside was chosen to test if the enzyme has alpha-glucosidase (E.C. 3.2.1.20) activity. The enzyme had little or no activity on hydrolyzing 4-nitrophenyl-α-D-xylopyranoside or 4-nitrophenyl-α-D-glucopyranoside **(Table 16).** The highest activity was observed with 4-nitrophenyl β-D-xylopyranoside, which is the preferred substrate for true xylosidase (Yernool, 2000), after which the second highest activity was with 4-nitrophenyl β-D-glucopyranoside which is 30 percent lower than that of 4-nitrophenyl-β-D-xylopyranoside. Its activity with 4-nitrophenyl-α-L-arabinofuranoside was 10 times less than that of 4-nitrophenyl β-D-xylopyranoside.

Table 16: Substrate specificity for Xylosidase

Substrate	Specific Act(U/mg)
4-nitrophenyl β-D-xylopyranoside (pNβxp)	44 ± 0.001
4-nitrophenyl β-D-glucopyranoside (pNβgp)	32 ± 0.02
4-nitrophenyl-α-L-arabinofuranoside (pNαLaf)	4.45 ± 0.002
4-nitrophenyl-α-D-xylopyranoside (pNαxp)	1.71 ± 0.004
4-nitrophenyl-α-D-glucopyranoside (pNαgp)	0.0 ± 0.003

3.7.8. Determination of Michaelis-Menten constant (K_m) and maximal activity (V_{max}) using 4-nitrophenyl-β-D-xylopyranoside as substrate

Different concentrations of substrate pNβxp were added, ranging from 0.25 mM to 20 mM in the xylosidase assay. Based on assay results (**Fig 19**), the K_m calculation is 3.18 mM and V_{max} is 233 U/mg.

A

B

Fig 19: Determination of Michaelis-Menten constant of the purified xylosidase with p-nitrophenyl-β-D-xylopyranoside. **A.** pNβxp concentration dependent xylosidase activities. **B.** Lineweaver-Burk plot.

3.7.9. Determination of Michaelis-Menten constant and maximal activity using p-nitrophenol-β-D-glucopyranoside substrate

Substrate affinity tests were conducted with p-nitrophenol-β-D-glucopyranoside (pNβgp), to confirm xylosidase's identity. Different concentrations of substrate pNβgp were added, ranging from 0 mM to 10 mM. The K_m value was calculated to be 0.76 mM and V_{max} is 34 U/mg. Since the enzyme was able to hydrolyse the substrate for β-glucosidase as well, therefore it is also possible that the enzyme could be β-glucosidase.
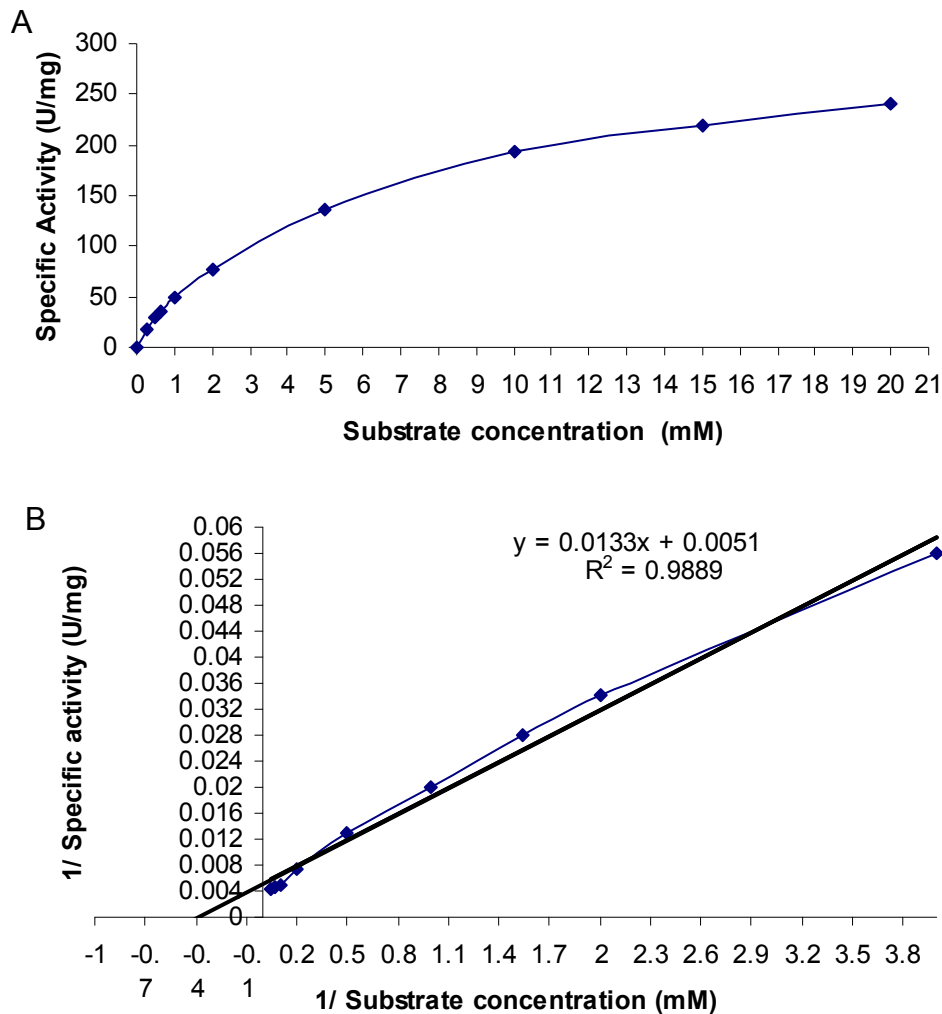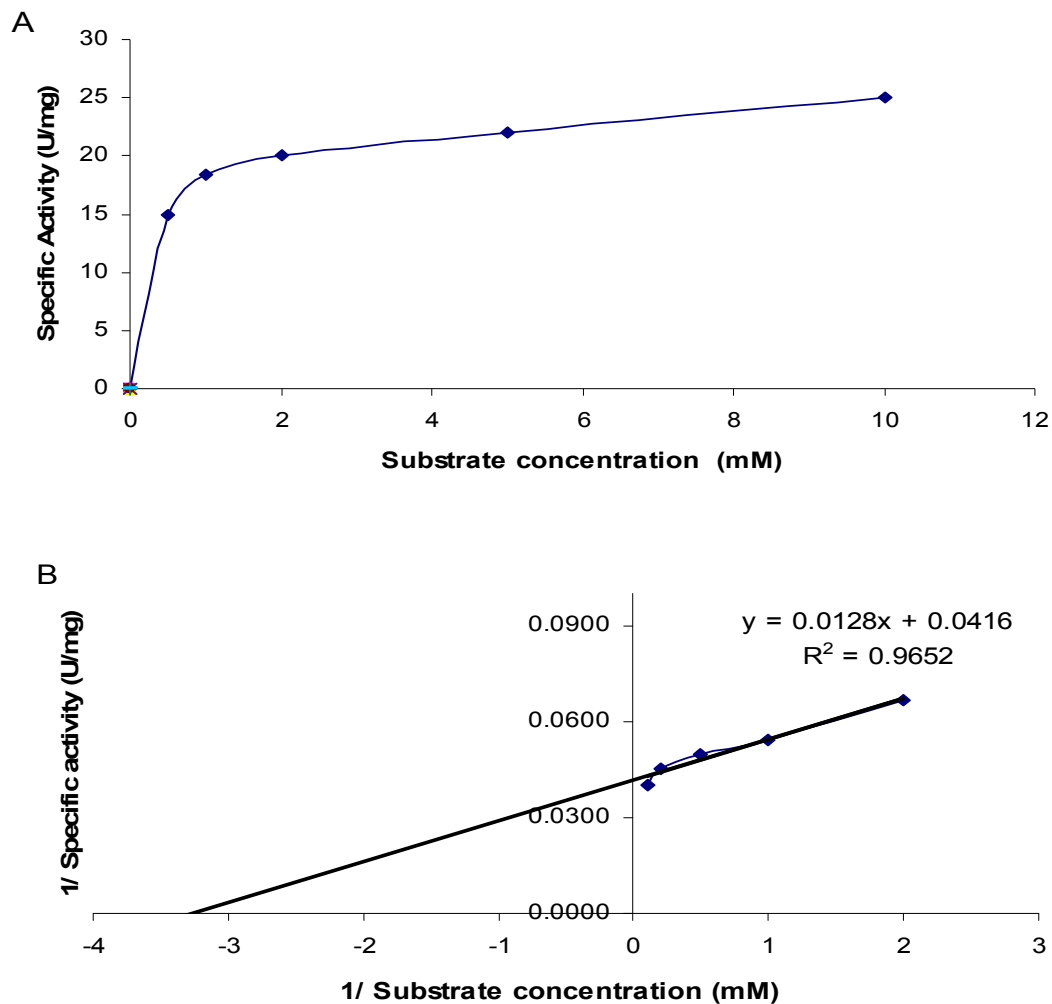

Fig 20: Determination of Michaelis-Menten constant of purified xylosidase with p-nitrophenyl-β-D-glucopyranoside. A. pNβgp concentration dependent xylosidase activities; **B.** Lineweaver-Burk plot

3.8.0: N-terminal analysis of *T. hypogea* xylosidase.

N-terminal sequence was determined by using Edman degradation (Dana Farber Cancer Institute, Boston). N-terminal sequences (fifteen amino acids) were determined to be MetIleAsnValAspGluIleValSerLysLeuThrLeuGluGlu (MINVDEIVSKLTLEE).

Table 17: Sequences from putative xylosidase from *T. hypogea*

Location	Sequences
N-terminal	MINVDEIVSKLTLEE

Sequences homology for N-terminal was sought with β-xylosidase and β-glucosidase, as either one could be the suspected identity of the enzyme. The amino acid sequences were compared with sequences from *Thermotogas* and other organisms from different genera as well **(Fig 21 and 22).**

Homology of *T. hypogea* N-terminal sequence with xylosidases from other organisms, such as *Streptomyces thermoviolaceus, Streptomyces coelicolor, Arabidopsis thaliana, Clostridium cellulolyticum* H10; ranges from 50 and 60%. Interestingly, there was also some homology with alpha-L-arabinofuranosidase/β-D-xylosidase isoenzyme from two sources; *Oryza sativa* Japonica Group (common name: rice) and *Hordeum vulgare* ranging between 50-60%. It appears that the N-terminal sequences have greater homology with β-glucosidases than with β-xylosidases from *Thermotogas* (**Fig 22**).

Alignment with β-xylosidase sequences

Figure 21- Alignment of *T. hypogea* sequences with β-xylosidases from *Thermotogas* and other microorganisms. – dash indicates no matching sequences; Residues in black boxes are identical, whereas those in grey boxes are similar, but not identical.

Alignment with β-glucosidase sequences

Figure 22- Alignment of *T. hypogea* sequences with β-glucosidases from *Thermotogas* and other microorganisms.

The presence of some amino acids in the sequence (**Table 17**) has some significance. The uncharged polar residues Gln, Asn, Ser decrease in hyperthermophilic proteins, in which the first two can easily undergo deamidation and are known to be the most temperature sensitive (Cambillau and Claverie, 2000). In contrast, hyperthermophilic and thermophilic proteins showed an increase of charged amino acid residues, especially Glutamic acid (E) as noticed from N-terminal sequences in (**Fig. 21 and 22**). The equal increase of oppositely charged residues Arginine (R) and Glutamic acid (E) in hyperthermophiles most likely derives from the increased amount of ion pairs observed at the surface of their proteins (Cambillau and Claverie, 2000). Glutamic acid composition increased significantly, which might be the structural base of rigidity of hyperthermophilic enzymes. The difference between hyperthermophile and mesophile would provide ideas to increase thermal stability of mesophilic enzymes.

3.9. Characterization of α-L-arabinofuranosidase

3.9.1. Molecular weight determination

The molecular mass of α-L-arabinofuranosidase under denaturing condition was estimated by 12.5% SDS-PAGE using medium range molecular weight markers. Since there were two bands after the last column, there are therefore two possibilities of denatured molecular weight 92 kDa and 50 kDa. The enzyme could not be purified to homogeneity. The determination of the native molecular mass was performed by size exclusion chromatography using a Superdex-200 column. Native molecular mass was estimated by comparing the specific retention volume of the enzyme with a calibration performed with four different standard proteins. α-L-arabinofuranosidase activity was detected at an elution volume of 167 ml corresponding to 200 kDa native molecular weight. The native weight suggests that the enzyme could be homodimer, if the denatured molecular weight is 92 kDa, or it could be a tetramer if the denatured molecular weight is 50 kDa.

3.9.2. Optimization of the assay reaction time

To optimize the enzymatic assay reaction time, CFE from xylose grown cells and partially purified α-L-arabinofuranosidase were incubated with enzyme assay reaction mixture for different time periods at 70°C, with 1mM substrate 4-nitrophenyl-α-L-arabinofuranoside (PNαLaf). α-L-arabinofuranosidase activity was measured using the standard α-L-arabinofuranosidase assay method. The time periods chosen were t= 0, 5, 10, 15 and 20 min. The optimal time period was between 15 min for CFE and 5-8 min for α-L-arabinofuranosidase (**Fig 23**). The shorter optimal reaction time of the partially

purified enzyme might be caused by reduced thermostability due to the removal of other proteins in the CFE.

A

B
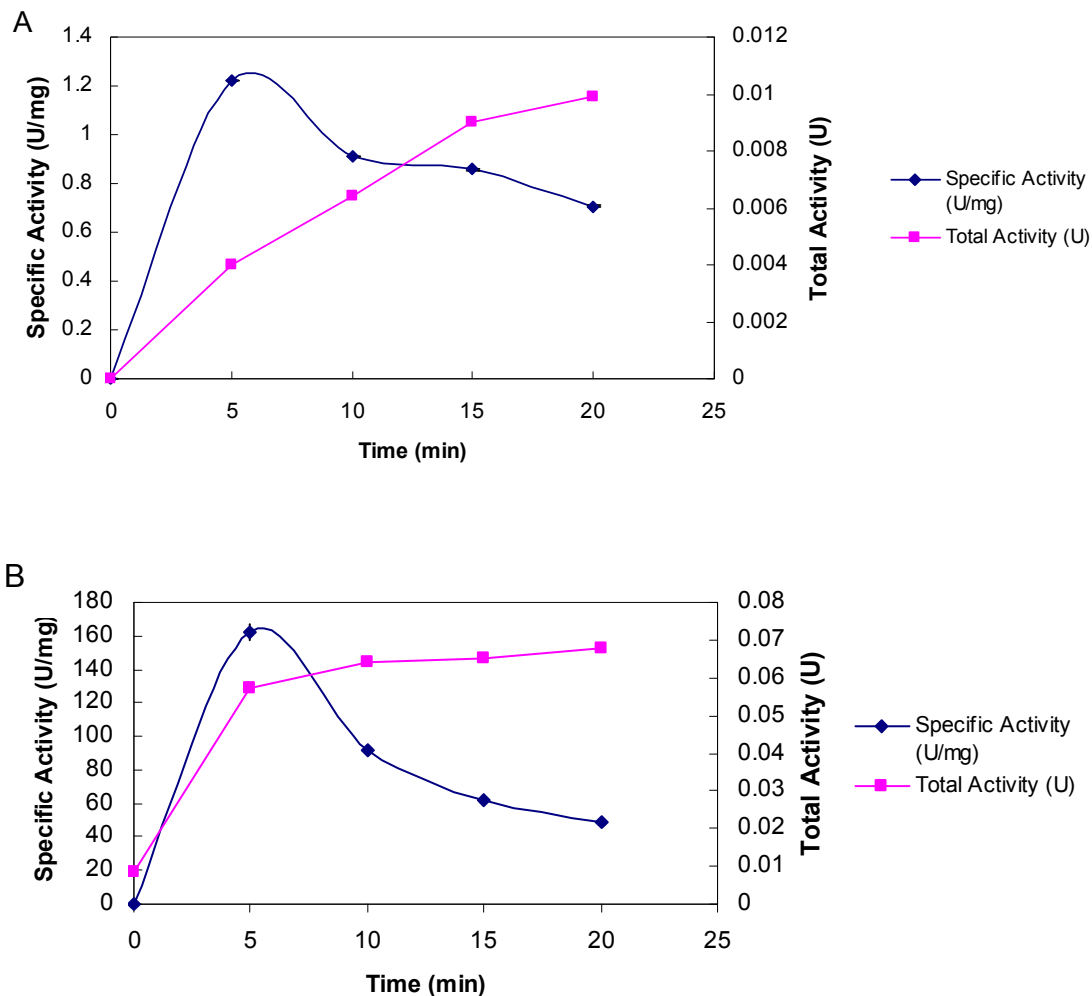
Fig 23: Optimization of α-L-arabinofuranosidase incubation reaction time. **A**. CFE. 400 ml assay mixture contained 50 mM sodium citrate buffer, 1 mM substrate (4-nitrophenyl-α-L-arabinofuranoside) and 0.7 µg incubated at 70°C; **B**. Pure protein. 400 ml assay mixture contained buffer, substrate and 0.07 µg partial pure protein incubated at 70°C. Total activity was the activity of either cell free extract or α-L-arabinofuranosidase incubated under the specified assay condition at corresponding reaction time. Specific Activity is the total amount of pNP released per milligram of enzyme per minute.

3.9.3. Thermostability

Temperature dependence of partially pure α-L-arabinofuranosidase was further tested before any other characterization assays were conducted. Thermostability of α-L-arabinofuranosidase was investigated at different temperatures ranging from 20°C, 30°C, 40°C, 50°C, 60°C, 70°C and 80°C, using 1mM substrate. The assay was done at two incubation times: 5 min and 10 min.

It was evident that the enzyme is not stable at 70°C (**Fig. 24**) for more than 5 minutes, based on specific activity. When the assay was conducted for 10 min, the specific activity increased until 50°C, after which it became constant until 70°C, which implies that near 50°C the enzyme was not stable or was beginning to inactivate. As compared with 5 min assay, the specific activity was increasing until 70°C, after which it started to decrease. Moreover, at 40°C, the specific activity for 5 min and 10 minutes assay was the same, which reflects the true activity of the enzyme irrespective of incubation time. Therefore subsequent characterization assays were conducted at 40°C with a 10 minutes incubation time.
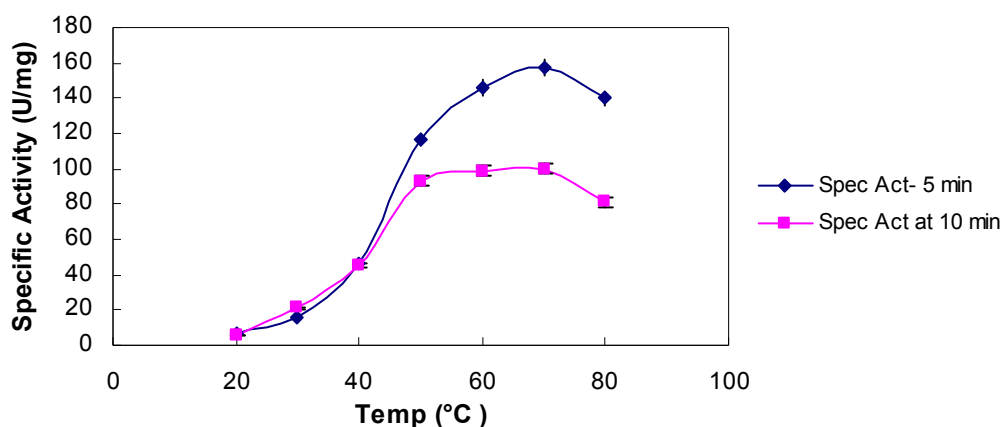
Fig 24: Comparison of 5 and 10 min assay for thermal dependence assay of partially pure α-L-arabinofuranosidase. Plotted results are the average of duplicate assays.

3.9.4. Optimal pH

Since the supply of α-L-arabinofuranosidase protein was limited, an optimal protein concentration assay was not done. The protein amount used for assays was the same as for the thermal dependence characterization, 0.06 μg. Enzyme activities were assayed from pH 4.0 to 9.0 at 40°C with assay time of 10 min. 0.1 M sodium citrate buffer was used for pH 4.0-7.5, 0.1 M sodium phosphate buffer was used for pH 6.5-7.5, and 0.1 M EPPS was used for pH 7.0- 9.0.

The enzyme showed very little activity at pH 4.0 and 9.0, 50% of the activity was measured near pH 8.0. The pH optimum of the partially purified α-L-arabinofuranosidase was determined to be 6.0 at 40°C (**Fig 25**). Hence, sodium citrate buffer pH 6.0 was selected for future assays.
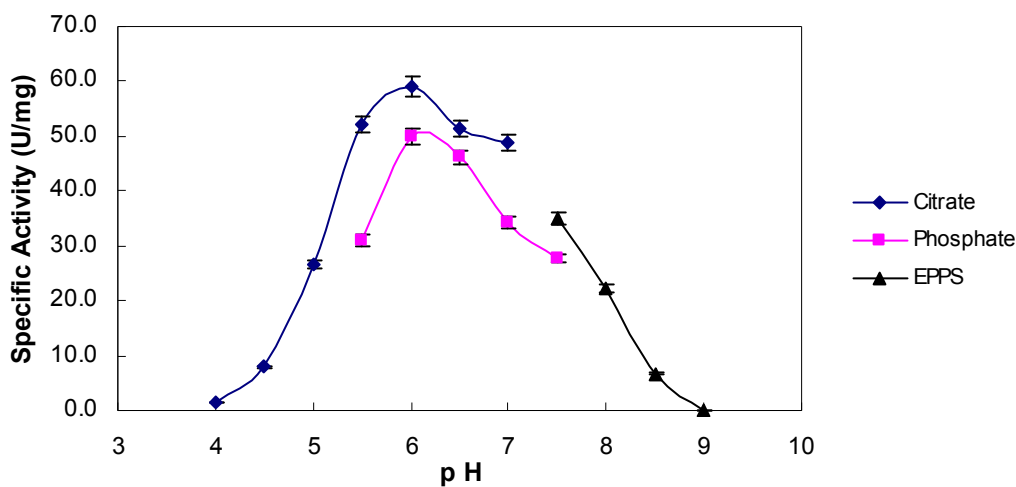
Fig 25: Optimal pH assay for α-L-arabinofuranosidase with three different buffers. Activity was determined in duplicate.

3.9.5. Substrate specificity

The substrate specificity was determined at optimal temperature and pH (40°C, pH 6.0 respectively) (**Table 18**). Final concentration of the substrate was 1 mM in the reaction mixture. Along with its assay substrate 4-nitrophenyl-α-L-arabinofuranoside, other substrates were also tested: 4-nitrophenyl-α-D-xylopyranoside, 4-nitrophenyl-α-D-xylopyranoside and 4-nitrophenyl-α-D-glucopyranoside. 4-nitrophenyl-β-D-xylopyranoside was chosen to test whether the enzyme has xylosidase activity. 4-nitrophenyl-α-D-glucopyranoside was also chosen to test if the enzyme can cleave alpha-glucosidase (E.C. 3.2.1.20) substrate. Lastly, 4-nitrophenyl-α-D-xylopyranoside was chosen to test whether the enzyme can also hydrolyse the alpha configuration of the xylopyranoside substrate. The enzyme had little or no activity with pNαxp or pNgp. The highest activity was observed with pNαLaf. Its activity with pNβxp was 16 times less than that of pNαLaf.

Table 18: Substrate affinity for α-L-arabinofuranosidase

Substrate	Specific Act(U/mg)
4-nitrophenyl α-L-arabinofuranoside(pNαLaf)	35.2 ± 0.07
4-nitrophenyl β-D-xylopyranoside(pNβxp)	2.17 ± 0.004
4-nitrophenyl-α-D-xylopyranoside(pNαxp)	0.0 ± 0.0
4-nitrophenyl-α-D-glucopyranoside(pNαgp)	0.0 ± 0.0

3.9.6. SDS-PAGE profile of α-L-arabinofuranosidase and β-xylosidase

1.5 μg of protein was loaded onto a 12.5 % SDS-PAGE gel. Based on the size, α-L-arabinofuranosidase and β-xylosidase appear to be separate entities.
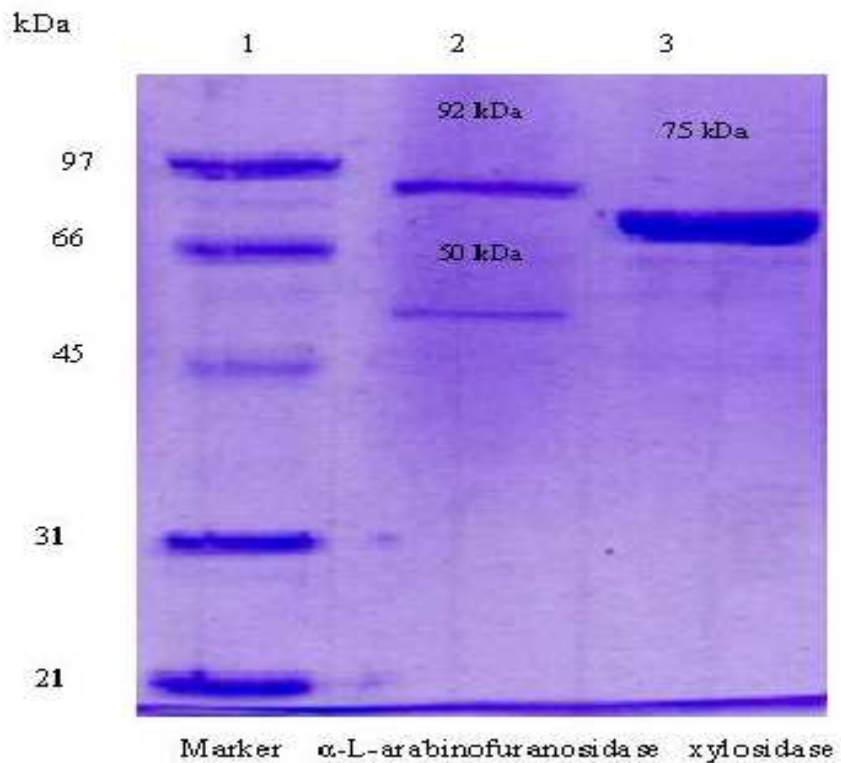
Fig 26: SDS-PAGE profile of α-L-arabinofuranosidase and β-xylosidase. (12.5% acrylamide gel) lane 1-markers, lane 2- α-L-arabinofuranosidase (1.5 μg), lane 3- xylosidase (1.5 μg).

3.10. Characterization of Acetylesterase

3.10.1. Molecular weight determination

The molecular mass of acetylesterase under denaturing conditions was estimated by 12.5% SDS-PAGE using medium range molecular weight markers. From **Fig. 12**, one band was observed that corresponds to a size of 129 kDa. The determination of the native molecular mass was performed by size exclusion chromatography using a Superdex-200 column. Fractions were eluted at an effective volume (Ve) of 164 ml at a flow rate of 2.5 ml/min. Acetylesterase activity was eluted out at an elution volume corresponding to native molecular weight of 202 kDa, which makes it a possible homodimer if the band corresponding to 129 kDa on the SDS-PAGE gel was an esterase.

3.10.2. Processing of acetylesterase

For characterization purposes, protein was concentrated through ultrafiltration using an Amicon 30 kDa membrane which was solely used for acetylesterase. A total of 0.017 mg protein with 2.1 units was concentrated. After concentration, the protein concentration actually increased to 0.025 mg, which was strange. When the protein was loaded on SDS-PAGE, it was confirmed that the protein was contaminated by protein from the membrane.

It is difficult to understand how acetylesterase could get contaminated, since care was taken to ensure cleanliness of the concentrator membrane. Before each use, the membrane was soaked in water for one hour and then washed with buffer before

concentrating esterase samples and after use the membrane was stored in 20% ethanol in 4°C fridge.
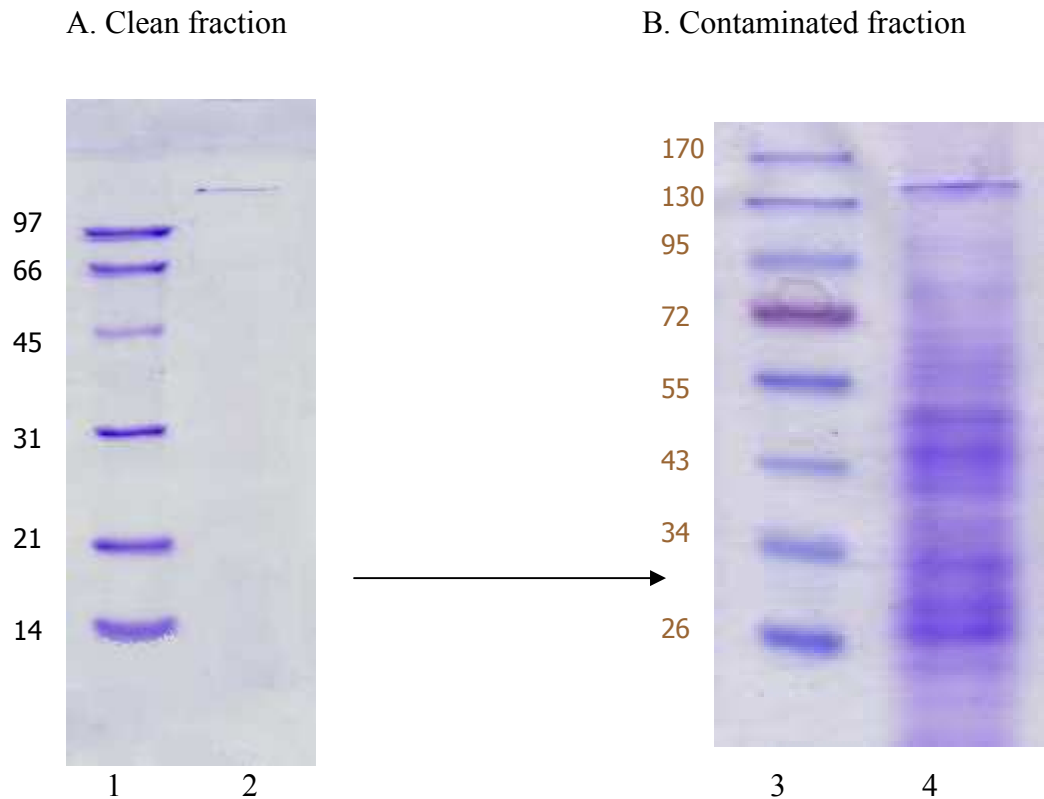
Fig 27: Comparison of Acetylesterase before and after concentrating. A. Before concentration 12.5% acrylamide gel.lane1- Marker, lane 2-acetylesterase (0.5 μg). B. After concentration 10 % acrylamide gel. lane 3 - Markers, lane 4- acetylesterase (4.0 μg).

The possibility of cross contamination with other proteins involved in the project is also excluded as acetylesterase was concentrated using the same membrane, after every column. If handling is excluded, and the membrane is considered the sole cause of the contamination, then the protein could have been contaminated even before loading on the last column which is Gel-filtration, as only 3 ml of the protein sample can be loaded on

the column. Acetylesterase was handled exactly the same way as the other two proteins, xylosidase and arabinofuranosidase. It remains a mystery why the other two proteins did not get contaminated while concentrating.

Chapter 4

4.0. Discussion

It has been reported that there is a possibility that *Thermotoga hypogea* possesses xylanolytic enzymes other than xylanase (Fardeau et al., 1997; Dhanjoon, 2005). Xylanolytic enzymes are potentially useful for various industrial processes as well as the acquisition of carbon and energy by degrading biomass. The conversion of biomass to fuels is a practical and plausible approach to combat present and future energy shortages.

The present investigation was focused on two glycoside hydrolases, xylosidase and arabinofuranosidase. The presence of an esterase hydrolyzing enzyme was also detected. The availability of these enzymes enables a better understanding of the physiology of *T. hypogea* and its habitat by suggesting sources of energy in the organism's extreme natural environment. However, there are still other side-chain releasing enzymes such acetyl-glucomannan, p-coumaroyl and feruloyl esterases which have to be investigated from *T. hypogea*.

4.1. Growth of *T. hypogea* on monosaccharides/polysaccharides

To determine the influence of carbon and energy source on the production of each enzyme, *T. hypogea* was grown on a variety of saccharides including monosaccharides such as glucose, xylose, L-arabinose and polysaccharides such as arabinogalactan and xylan *T. hypogea* was able to grow on all the carbon sources with cell densities reaching as high as 4×10^8 cells/ml. *T. hypogea* was able to grow best on xylan and xylose **(Table 8),** with the least growth and enzyme production with glucose. Amongst all 5 carbon sources investigated all three enzymes had highest activity when grown on xylan or

xylose **(Table 9).** Arabinogalactan did not support growth strongly, maybe due to lack of another enzyme such as (E.C.3.2.1.89) arabinogalactan endo-1,4-β-galactosidase, which is also involved in arabinogalactan hydrolysis and needed in synergy with arabinofuranosidase to break down arabinogalactan. To date, arabinogalactan endo-1,4-β-galactosidase is not known from any *Thermotogae*.

It was shown by Chabbra et al. (2003), that glucose is not a preferred substrate for growth for any *Thermotoga* species, perhaps due to its thermolability at high temperatures. The results showed that growth of *T. maritima* on glucose was slower than growth on other polysaccharides (Chabbra et al., 2003). Another finding from *Pyrococcus furiosus,* showed that some hyperthermophilic strains can preferentially use polymeric substrates such as starch rather than glucose or maltose (Brown and Kelly, 1993). It is highly likely that small sugars such as glucose and maltose at low pH and high temperature are not suitable substrates due to the Maillard reaction at high temperature and low pH values. In a study conducted with *Thermotoga neapolitana*, similar results were found, where xylosidase, β-mannanase and acetylesterase expression, were investigated in 4 carbon sources (xylan, cellobiose, glucose and cellulose) (Yernool, 2000). The researcher found xylan and cellobiose to be good inducers and glucose lead to a moderate level of expression. The researcher attributed the low induction of xylan debranching enzymes to catabolite repression by glucose (Yernool, 2000); i.e., when glucose is present in the medium then the enzymes required for the utilization of alternative carbon sources are synthesized at low concentrations, the phenomenon referred to as "carbon catabolite repression".

Based on higher activity detected it is concluded that *T. hypogea* cells grown on xylose and birchwood xylan had the highest activities of xylosidase and arabinofuranosidase. Similar results were also reported (Nanavati et al., 2006; Lee et al., 1993). Moreover this work also showed that L-arabinose containing residues in the substrate are not necessary to induce α-L-arabinofuranosidase as reported previously (Lee et al., 1993; Sunna and Antranikian, 1996; Numan and Bhosle, 2006).

The notable aspect of this project is the purification of the native enzymes from the bacterium and not by overexpression in a mesophilic host. Overexpression in mesophilic hosts such as *E. coli* is attractive due to simpler requirement of culture conditions, easier purification steps and higher yields of target protein. Low production of biomass is one of the biggest hurdles of working with thermophiles. Researchers have purified xylosidase and α-L-arabinofuranosidase through expression in *E. coli* from *Thermotoga* strains (Xue and Shao, 2004; Xue et al., 2006) **(Table 20 and 23)**. Moreover, acetylesterase has only been known to be purified through recombinant methods and characterized from only two species of *Thermotoga* (Yernool, 2000; Levisson et al., 2007). There has been one paper that reports the purification of native β-xylosidase and β-glucosidase from *Thermotoga* FJSS, however, no homology was found with N-terminal sequences of the native protein against any other β-xylosidase and β-glucosidase or similarity with properties with any of the reported xylosidase (Ruttersmith and Daniel, 1993).

It is possible that proteins characterized from this work may not be completely identical with the recombinant proteins isolated from the same genus. One reason of minor difference can be due to different strains. Any other reason can be attributed to

differences in expression in native vs. mesophilic host. Unless demonstrated otherwise, it cannot be assumed that recombinant and native protein will have the same characteristics. The reason that assumption cannot be made that the recombinant and native proteins are the same is that sometimes the recombinant protein cannot be folded properly in the organism as they may require specific chaperones. Also some times the post translational modifications are missing in the host and the recombinant protein cannot turn into a mature protein (Vielle and Zeikus, 2001). Lastly, some organisms use some codons that are rarely in use in expression hosts and it may cause some problems in function of the recombinant protein. There have been cases in which the recombinant protein showed different characteristics from the native one, such as alcohol dehydrogenase purified from *Thermococcus hydrothermalis*, in the thermal stability reaction at 60°C, the activity of native protein was found to be lower by 50 %, as well as had a different optimal salt concentration, as compared with recombinant protein (Antoine et al., 1999). Similar results have been reported from other proteins in hyperthermophiles exhibiting reduced thermostability in recombinant form (Cacciapuoti et al., 1999; Lebbink et al., 1995).

To date there has been no study conducted that compared a native glycosyl hydrolase protein with a recombinant protein from genus *Thermotoga*. An extension of the present project could involve comparing the native xylosidase with recombinant xylosidase prepared from *T. hypogea*.

4.2. Xylosidase and α-L-arabinofuranosidase activity

Xylan is a major component of hemicellulose, the second most abundant polysaccharide in nature after cellulose. Its degradation requires the action of several different enzymes such as endoxylanase, β-xylosidase, α-L-arabinofuranosidase activity and esterase (**Fig. 2**).

T. hypogea appears to regulate the synthesis of xylanolytic enzymes in response to the presence of xylan or xylose in the culture medium. Compared to cells grown in glucose, the specific activity of α-L-arabinofuranosidase in the cells grown in the presence of xylose or xylan was 4 to 6 times higher **(Table 9)**. For xylosidase activity, the specific activity was 2 times higher. With respect to acetylesterase the trend is not clear, as the activity was too low **(Table 9)**.

The majority of the activity of xylosidase and α-L-arabinofuranosidase was associated with the cells (**Fig 9**). Since many hyperthermophilic organisms utilize complex carbohydrates as carbon and energy sources, it is apparent that multienzyme systems are needed to hydrolyze polysaccharides that are too large to be transported across the cell membrane (Bauer et al., 1998; Driskill et al., 1999). As a result, many endo-acting glycosidases are cell membrane associated or completely secreted (Devos et al., 1998). Many xylan-degrading microorganisms have been reported to produce mainly cell-associated xylanolytic enzymes. The thermostable xylanases produced by these microorganisms are mainly cell-associated and most probably localized within the toga (Winterhalter and Liebl, 1995; Sunna and Antranikian, 1996). The thermoanaerobe *Thermoanaerobacterium saccharolyticum* B6A RI produces mainly a cell-associated

endoxylanase complex when grown on insoluble xylan (Lee et al., 1993). More than 95 % of the cellulolytic and hemicellulolytic enzymes of *Thermotoga sp.* strain FjSS-B.1 are cell associated (Ruttersmith et al., 1992). A small but significant enzyme activity from α-L-arabinofuranosidase and xylosidase were detected in the culture medium fractions. Since polymeric xylan cannot penetrate the cells, therefore, it is possible that short xylo-oligosaccharides such as xylobiose, xylotriose, cleaved by constitutively present xylanase, may act as immediate inducers for xylanase and xylosidase biosynthesis (Sunna and Antranikian, 1997). Oligosaccharides with various degrees of polymerization produced by endo-acting enzymes are transported inside the cell for further processing (Devos et al., 1998). Once the oligosaccharides are inside the cell, other endo-acting and exo-acting glycoside hydrolases within the cytoplasm play essential roles in assimilation and catabolism of these compounds to provide saccharides (e.g., glucose and galactose) to metabolic pathways.

It is highly likely that the xylanolytic enzymes are localized within the togas. Similarly, the high-molecular weight xylanase and amylase purified from *T. maritima* were found to be mainly toga associated (Winterhalter and Liebl, 1995; Schumann et al., 1991). From sequence data, the mode of attachment to the outer membrane of at least one amylase appears to be based on a bacterial lipoprotein modification which is different from xylanase attachment which is attached at the cell surface via a hydrophobic peptide anchor (Liebl et al., 1997; Liebl et al., 2008). The hyperthermophile *T. maritima* has various polysaccharide hydrolases anchored via different mechanisms in the outermost cell layer. The retention of these enzymes at the cell surface is a plausible strategy to avoid the rapid loss of secreted enzymes in an extremely hot marine environment. It has

been suggested that perhaps the unique ballooning of the outer membrane over the ends of *Thermotoga* cells serves to enlarge the surface area which becomes an attachment site for different polysaccharide hydrolases (Liebl et al., 2008).

In comparison, multiple esterases have been found to be secreted in the culture filtrate of some fungi such as *T. reesei* (Biely et al., 1987) and *P. purpurogenum* (Egana et al., 1996). However their localization varies in bacteria as in *Thermoanaerobacterium* sp., two distinct esterases known as acetyl xylan esterase (AXE) have been purified and characterized (Shao and Weigel, 1995), which were found to be cell associated, with less than 25% in the culture supernatant, whereas, for *B. pumilus* over 60% of the AXE is secreted. The versatility in localization could aid in breakdown of acetyl residues from bigger molecules that otherwise cannot enter the cells. The frequent attachment of uncommon residues such as acidic or acetyl units on a main polymeric chain makes the main chain harder to degrade as their removal require specialized enzymes, which are not expressed constitutively such as aceyl xylan esterase, ferrulic acid esterase and methyl-D-glucuronidase (Shao and Weigel, 1995).

4.3. Purification of β-xylosidase

Xylosidase was purified 328 fold, resulting in 1.3% yield with specific activity of 46 (U/mg) **(Table 10).** The cell free extract was prepared from 100 gram biomass obtained from growing *T. hypogea* with xylose. Xylosidase composition was estimated to be 0.31% of 2770 mg total protein loaded on DEAE. With regards to degree of purification, xylosidase from *T. maritima* was purified 3 fold with specific activity of 280 U/mg, using 20 mM final concentration of substrate (Xue and Shao, 2004). From *T.*

hypogea, using the same concentration of substrate, the specific activity increases up to 233 U/mg. In comparison from *T. neapolitana*, the yield was 36.5% and the specific activity was 24.14 U/mg (Yernool, 2000). Based on purification profile, xylosidase from *T. hypogea* is more similar to that of *T. neapolitana*.

T. maritima* xylosidase was purified in two steps, heat treatment and metal affinity chromatography (Xue and Shao, 2004). In comparison, *T. neapolitana* xylosidase was purified by three step protocol involving heat treatment followed by anion-exchange and cation exchange chromatography (Yernool, 2000). The higher yield of enzyme from *T. maritima* could be due to the method of purification. It is obvious that shorter purification steps would result in lower protein losses and hence higher specific activity. It is possible that the final yield of *T. hypogea* xylosidase may have been underestimated because of uncertainty in the purification steps. All xylosidase activity detected in CFE cannot be considered as resulting solely from activity of the purified enzyme. It is possible that synergistically acting enzymes such as arabinosidase and glucuronidase contribute. As the purification protocol continues, these are being separated away from xylosidase, and this could be one of the possible reasons for lower recovery of xylosidase.

4.4. Purification of α-L-arabinofuranosidase

α-L-arabinofuranosidase was partially purified 450 fold, resulting in 2% yield with specific activity of 81 U/mg (**Table 11**). α-L-arabinofuranosidase represented an estimated 0.22 % of the total protein (1212 mg) present in the CFE. The purification profile for *T. maritima* recombinantly expressed α-L-arabinofuranosidase showed purification fold of 14 with specific activity of 148 U/mg, using 2 mM final concentration

of substrate (Xue et al., 2006). As compared the highest specific activity observed from *T. hypogea* is 81 U/mg with 1 mM final concentration of substrate; it is possible that if the protein was further purified it may have higher activity. The purification steps from *T. maritima* were in two steps; heat treatment and immobilized metal affinity chromatography (Xue et al., 2006).

4.5. Purification of acetylesterase

Acetylesterase was purified 425 fold, resulting in 0.79% yield with specific activity of 17 U/mg (**Table 13**). Acetylesterase represented an estimate of 0.23 % of total protein (1212 mg) present in CFE. From *T. neapolitana*, a 37 kDa esterase was purified which is different from *T. hypogea* esterase which has a molecular weight of 129 kDa (Yernool, 2000). Recombinant esterase purified from *T. maritima* was purified 115 fold with a yield of 66%. The molecular weight was 44.5 kDa (Levisson et al., 1997) (**Table 24**).

The acetylesterase elution profile was most interesting; starting from its induction. There could be several reasons for detecting low activity of acetylesterase. Christov and Prior (1993), reported that low levels of acetylesterase activity were produced on low molecular weight substrates such as glucose, xylose and cellobiose. The highest activities were obtained from cultures grown on polymeric substrates such as xylan and cellulose. Moreover even though it was not within the scope of the study to investigate regulation of gene encoding esterase, a study of the expression of gene with strong promoter could assist in increased protein induction. Another reason for low activity detection could be that the substrate used for the assay is not the best one for this enzyme. Among the p-

nitrophenol esters tested with *T. maritima* esterase, Levisson and colleagues (1997), found that based on catalytic efficiency p-nitrophenyl valerate and p-nitrophenyl octanoate were the best substrates for esterase and p-nitrophenyl acetate was the least favorable. Besides p-nitrophenyl acetate and its other derivates, currently, there are other acetyl esterase substrates such as α-naphthyl acetate, methylumbelliferyl derivatives as well as natural substrates as cellulose acetate, xylose tetraacetate and acetylated xylan. However p-nitrophenyl acetate and methylumbelliferyl acetate are the apparent common choices. p-nitrophenyl acetate was chosen because the assay was simpler and also the assay principle of measuring release of p-nitrophenol was similar to the other two enzymes' assay.

However in the course of the project it was realized that working with p-nitrophenyl acetate brings its own challenges. The substrate was found to be labile. Even though the model organism was a thermophile, the assay temperature could not be more than 30°C as the substrate started to disintegrate (results not shown). Also the substrate was hydrolyzed in the presence of reducing agents and ammonium sulfate salts (**Tables 14 and 15**). Perhaps the reducing agents disrupt bonding between the structure and thereby collapsing the substrate. With ammonium sulfate salt it is possible that the presence of sulfate or ammonium in the medium creates an either acidic or basic environment which results in hydrolyzing the substrate. Instability of p-nitrophenyl acetate has been reported (Shao and Weigel, 1995), but only mentioned when discussing choice of substrates. There is no report of the p-nitrophenol hydrolysis due to reagents, such as DTT/SDT or ammonium sulfate salts. The reason could be that most of the time the enzyme under study is a recombinant one which does not require elaborate

purification steps and therefore hydrophobic column (which uses ammonium sulfate) is often skipped. In some studies where hydrophobic column was used to purify esterase, a different substrate was chosen to conduct enzyme assays (Yernool, 2000). Therefore the instability of p-nitrophenyl acetate is not well documented.

In terms of the elution profile of acetylesterase, the instability of the substrate helps to explain unusual phenomenon such as an apparent 4 times greater recovery after the DEAE column compared with the enzyme preparation before loading onto DEAE (**Table 13**). The higher recovery rate is due to the addition of twice the volume of reducing agents DTT and SDT added to the preparations for loading on DEAE (**Table 14**). The results from the **Table 14**, showed that in 2 mM SDT and DTT solutions, the absorbance from p-nitrophenol release was 10 times higher than the blank. Another possibility of higher recovery could be that the elution profile of acetylesterase from DEAE overlaps with that of other esterases as well. It is possible that the combined activities of more than one type of esterase were able to hydrolyse the substrate. However, then one might expect that the same trend would be apparent in the cell-free extracts as well, which was clearly not the case.

Based on the enzyme activity recovered from HAP there were two peaks observed. The major activity was associated with 0.08 M salt and the minor peak was associated with 0.5 M salt. This suggests the possibility of two types of esterases. Two different types of esterases have already been reported from *Thermoanaerobacterium* (Shao and Weigel, 1995). It was experimentally determined that phosphate salts do not hydrolyse the substrate, so the possibility of salt in the HAP eluting buffer interfering with the assay was ruled out. Only the fraction with major activity was retained and

loaded onto the next column. However when loaded on HAP again, almost all the esterase eluted at 0.5 M potassium salt as compared to 0.08 M potassium salt concentration, which is in contrast to the previous trial. The shift of the enzyme's elution profile from hydroxyapatite column is still not clear.

4.6. Properties– β-xylosidase

Since the activity of the putative xylosidase overlaps significantly with that of a β-glucosidase, therefore the characteristic properties of the enzyme from *T. hypogea* will be discussed in comparison with both β-xylosidase and β-glucosidase.

The native molecular mass of the enzyme as determined by size exclusion chromatography on a Superdex-200 column was 130 kDa. Since the molecular mass estimated by SDS-PAGE was calculated to be 75 kDa, the enzyme is deduced to be a homodimer (**Fig. 10**). The molecular mass of 75 kDa is similar to β-glucosidase purified from *Thermotoga* strains **(Table 19).** The β-xylosidases from other *Thermotoga* are slightly higher in molecular weight (**Table 20**). Most xylanases have native molecular weights of less than 100 kDa, whereas the molecular weights of most β-xylosidases are more than 100 kDa (Shao et al., 1995) (**Tables 3, 4 and 20**).

It should be noted that the native molecular mass estimated by Gel-filtration for β-xylosidase and α-L-arabinofuranosidase could be underestimations. This is due to the fact that both enzymes had greater elution volumes than expected on the basis of SDS-PAGE derived size estimates, presumably due to interaction of the enzyme with the column matrix (Winterhalter and Liebl, 1995). Investigators have reported the abnormal

behaviour of xylanolytic enzymes on Gel-filtration columns (Winterhalter and Liebl, 1995).

Table 19: Properties of representative β-glucosidases from *Thermotoga* species.

Organism	Molecular mass (kDa)	Optimum pH	Optimum temp (°C)	K_m[a] (mM)	References
Thermotoga strain FjSS3-B.1	75	7.0	80	0.1	Ruttersmith and Daniel, 1993
Thermotoga maritima	81	5.0	85	0.004	Goyal et al., 2001
Thermotoga neapolitana	81	5.5	90	0.1	Zverlov et al., 1997.
Thermotoga hypogea	75	6.0	70	0.76	This work

[a] K_m determined using pNP-β-D-glucopyranoside as substrate

Table 20: Properties of representative bacterial β-xylosidases

Organism	Molecular mass (kDa)	Optimum pH	Optimum temp (°C)	K_m[a] (mM)	References
Thermoanaerobacter ethanolicus	85[b], 150[c]	5.0 -6.0	82	0.018	Shao and Weigel, 1992
Thermotoga strain FjSS3-B.1	92[b], 174[c]	7.0	95	Substrate inhibition	Ruttersmith and Daniel, 1993
Thermotoga thermarum	Nd	6.0	90	Nd	Sunna and Antranikian, 1996
Thermotoga maritima	Nd	6.1	90	0.13	Xue and Shao, 2004
Thermotoga neapolitana	85[c]	5.6-6.4	85	1.28	Yernool, 2000
Thermotoga hypogea	75[b], 130[c]	6.0	70	3.18	This work

[a] with 4-nitrophenyl-β-D- xylopyranoside   [c] estimated by Gel-filtration
[b] estimated by SDS-PAGE   Nd: not determined

The activity range of the putative xylosidase of *T. hypogea* is comparable with those enzymes produced by bacteria and fungi (**Tables 3 and 4**). Putative xylosidase was most active in the slightly acidic pH range with optimal activity at pH 6.0 (**Fig 16**). The enzyme showed very little activity at acidic and alkaline pH 4.0 and 9.0 respectively. At pH 8.0, 50% of the xylosidase activity measured was about half of that at pH 6.0. The pH optimum was similar to *T. maritima* xylosidase, which also exhibited optimum pH of 6.1. It would be expected for xylanolytic enzymes to have a pH optimum of around 6.0, as the end products of fermentation are acidic, therefore the enzyme has to be functional at acidic range. The pH optima from *Thermotoga* strain FJSS and of β-xylosidases from other bacteria are between 5.7-7.0 (Lee and Zeikus, 1993), whereas fungi produce enzymes with neutral or basic pH optima (Shao and Weigel, 1992). It is also interesting to note for the pH optimum assay that the enzyme had highest specific activity near pH 6.0 when the buffer had sodium in it (**Fig. 16**). For instance, with sodium citrate buffer (range used pH 4-7.5) the highest activity was observed near pH 6.0, similarly with sodium phosphate buffer the highest activity was at 6.0. In contrast when MES buffer was used in the range of 5.5-6.5, the activity profile was different, the highest activity was at pH 6.5. Therefore it appears that the activity is affected by different buffers and salt. More specifically, it appears that sodium may have a stabilizing affect on the enzyme, although more tests need to be conducted to confirm this.

One of the most useful properties of enzymes from *Thermotoga* is their thermostability. The putative xylosidase from *T. hypogea* displayed maximum activity at 70°C. The optimal temperature for glucosidases and xylosidases from *Thermotoga* strain

FJSS, *T. maritima* and *T. neapolitana*, was in the range of 80-90°C (**Fig. 19**) and (**Fig. 20),** respectively. The optimal temperature for putative xylosidase is lower than other xylosidases from *Thermotoga* species (**Table 20**). One reason could be that *T. hypogea* and *T. thermarum* have lower optimal growth temperatures, compared with *T. maritima*, *T. neapolitana* and *T.* strain FJSS (Fardeau et al., 1997). It was also interesting to note that not all xylanolytic enzymes from *T. hypogea* have optimum temperatures close to the organism's growth temperature, for example, xylanase purified from *T. hypogea* had an optimum temperature of 90°C (Dhanjoon, 2005). This phenomenon was also reported by Ruttersmith and Daniel (1993), who found that in *Thermotoga* strain FJSS, purified β-xylosidase and β-glucosidase were less stable than the cellobiohydrolases and xylanases from the same strain. In nature, it would make sense for the main chain hydrolysing enzymes, such as cellobiohydrolases and xylanase, to be more stable than the internal side chain hydrolyzing enzymes.

The thermostability of the putative xylosidase was also investigated at 70°C, by measuring its half life. The $t_{1/2}$ of the enzyme at 70°C was found to be at 22 min (**Fig. 18**). About 75% of the activity was lost, after 45 min of incubation. The half life was similar to *T. maritima*'s β-xylosidase half life which was also 22 min at 95°C (Xue and Shao, 2004). As compared, β-xylosidase and β-glucosidase from *Thermotoga* FJSS were found to be highly thermostable with a halflife of 8 hr at 95°C and of 8 hr at 90°C respectively (Ruttersmith and Daniel, 1993). The half life from *T. neapolitana* β-xylosidase was determined to be 5 hours at 90°C (Yernool, 2000). From *Thermoanaerobacterium ethanolicus*, the β-xylosidase half life was determined to be 15 min at 86°C. Further experiments that would test effect of additives on thermostability were not conducted.

However for future experiments, it would be good idea to measure the thermostability in the presence of BSA and/or sodium. The addition of BSA would serve as a negative control. The reason sodium is considered is because the pH range results showed an apparent stabilizing effect of sodium when it was present in assay buffers (**Fig. 16**).

The putative enzyme had the highest activity towards xylosidase substrate, 4-nitrophenyl β-D-xylopyranoside with a specific activity of 44 U/mg **(Table 16).** Its next highest activity was with 4-nitrophenyl β-D-glucopyranoside 32 U/mg. Its activity with pNαLaf was 10 times less than that of pNβxp, suggesting that overlapping activity could be a side activity of the β-xylosidase. The enzyme had little or no activity with other α-L configuration substrates: (pNαLaf), (pNαxp) and (pNαgp). It seems that the enzyme is able to cleave only β linkages efficiently. It was also interesting to note that the enzyme had very little activity with the alpha configuration of its xylosidase substrate.

The apparent K_m of the putative xylosidase determined from the Lineweaver Burk plot with 4-nitrophenyl β-D-xylopyranoside was 3.18 mM and V_{max} was 233 U/mg **(Fig. 19)** and for 4-nitrophenyl β-D-glucopyranoside the K_m and V_{max} value was 0.76 mM and 34 U/mg respectively **(Fig. 20).** The K_m values for both the substrates is very puzzling, as the high K_m value for xylosidase substrate can indicate that it is not a true xylosidase, however the comparatively low K_m with glucosidase indicates that it could be glucosidase. At the same time the low V_{max} of glucosidase compared with xylosidase suggests otherwise.

Broad specificity with regards to the polysaccharide substrates is a common feature of the family 3 glycosidases. As mentioned earlier, family 3 glycosidases, share active site homology with other members of family 3 (Henrissat, 1991). Other examples

132

of β-glucosidases with high β-xylosidase activities are from *Aspergillus wentii* A_3 (Bause and Legler, 1980) and *Clostridium thermocellum* bg1b (Gräbnitz, 1989). Such a feature complicates the identification of their natural substrates as well as their denomination since the latter is based on the hydrolytic capacities of these enzymes. The K_m profiles for xylosidases from other *Thermotoga* species are also very unusual (**Table 21**).

Table 21: K_m for Xylosidase from *Thermotoga* species

Substrate	*T. neapolitana* K_m (mM) [*]	*T.* FJSS K_m (mM) [**]	*T. maritima* K_m (mM) [#]	*T. hypogea* K_m (mM) [##]
pNP-β-D-glucopyranoside	0.80	2.75	Nd	0.31
pNP-β-D-xylopyranoside	1.28	Substrate inhibition	0.13	3.18
pNP-α-L-arabinofuranoside	3.14	3.00	Nd	Nd

Nd; Not determined

[*] **Reference:** Yernool, 2000

[**] **Reference:** Ruttersmith and Daniel, 1993

[#] **Reference:** Xue and Shao, 2004

[##] **Reference:** This work

Table 22: Specific activity for β-xylosidase from *T. maritima* (Xue and Shao, 2004).

Substrate	Spec Act
pNP-β-D-glucopyranoside	29 U/mg
pNP-β –D-xylopyranoside	275 U/mg
pNP-α-L-arabinofuranoside	50 U/mg

It appears that xylosidase from *Thermotoga* strain FJSS has higher affinity for pNP-β-D-glucopyranoside and for pNP-α-L-arabinofuranoside than for pNP-β-D-xylopyranoside, which is very confusing (**Table 21**). Similarly, *T. neapolitana* also has a higher affinity for pNP-β-D-glucopyranoside than for pNP-β-D-xylopyranoside. However from *T. maritima* the profile is as expected. The highest specific activity for xylosidase is with pNP-β-D-xylopyranoside **(Table 22)** and then with the other two substrates it is very low, which suggests that the hydrolysis of those substrates is a side activity of the enzyme.

Yernool (2000) reported several criteria for true β-xylosidases:

1. The enzyme is active towards xylo-oligomers and naturally containing xylans isolated from birchwood, esparto grass and oatspelts. The hydrolysis product will be xylose.

2. The enzyme does not hydrolyse cellobiose or other cello-oligosaccharide and polymeric glucans, to yield glucose, thereby differentiating it from β-glucosidase.

3. The enzyme also does not release arabinose from arabinan and oat spelt xylan. Even though β-xylosidase can cleave pNP-α-L-arabinofuranoside, it cannot cleave its natural substrate to release arabinose, differentiating it from α-L-arabinofuranosidase.

It is difficult to judge the putative xylosidase from *T. hypogea* with the above criteria as end product analysis was not conducted. The BLASTP search to compare the N-terminal sequence of the putative xylosidase with showed high similarity with β-glucosidases from *Thermotogas*; as compared the homology is much lower with other organisms (**Fig. 22**). In contrast, the similarity of xylosidase with other *Thermotoga* xylosidases is lower (**Fig 21**). But, in comparison with the situation for glucosidase, the

homology of xylosidase with other organism is moderately higher. The higher similarity with glucosidase may indicate that the enzyme is a glucosidase, however, the moderate similarity of xylosidase with that of other organisms may also suggest that this protein from *T. hypogea* may be an usual protein. Obviously, the identity of the enzyme is not clear at this point; however, based on its similarity with Family 3 glucosidases and xylosidase from *Thermotoga* as well as other organisms, there is a high possibility that this enzyme also belongs to family 3. All the proteins that had $\geq$ 50% homology with xylosidase/glucosidase belonged to family 3. Structural analysis and further functional analysis need to be conducted to confirm its identity.

Although substrate specificity will remain a cornerstone of enzyme classification, additional criteria are clearly needed to identify evolutionary and functional relationships between individual glycoside hydrolases. From **Table 21**, we can see that it is misleading to rely solely on enzyme assays. Some of the other methods of identification are complete sequencing of amino acids and end product analysis. Future experiments with Xylosidase can involve analyzing resultant end products from natural substrates such as cellobiose, xylobiose and arabinotriose. Xylosidase will not cleave cellobiose or arabinotriose but will only cleave xylobiose (Yernool, 2000), where as glucosidase is not known to cleave xylobiose or arabinotriose (Ruttersmith and Daniel, 1993), it will only cleave cellobiose. Based on the experiments at this point, it can only be concluded that the enzyme can cleave β-linkages and has the highest activity with xylosidase substrate, and based on homology with N-terminal it is a member of family 3 glycoside hydrolases. As mentioned earlier it has been frequently observed that any family-3 GHs exhibit a combination of different activities, exemplified by the frequent association of β-

glucosidase and β-xylosidase activities (Watt et al., 1998; Vroemen et al., 1995), which not only suggests broad specificity for substrate but also similarity in amino acid sequences. Based on its activity, *T. hypogea* xylosidase can be regarded as both an aryl-xylosidase and an aryl-glucosidase.

4.7. Properties of α-L-arabinofuranosidase

The native molecular mass for α-L-arabinofuranosidase was determined to be 200 kDa. The enzyme was not purified successfully, as there was a faint band of 50 kDa. Microbial α-L-arabinofuranosidases are generally large molecules, with the highest molecular weight of 495 kDa reported from *S. purpuracens* (Komae et al., 1982) (**Tables 5 and 23**).

The partially purified α-L-arabinofuranosidase was partly characterized. Thermostability is one of the attractive features of enzymes from thermophiles, however with arabinofuranosidase, it was not the case. In the presence of extra proteins in the CFE, the enzyme was more stable, however, in almost pure form; the enzyme had reduced thermal stability at 70°C. The optimal enzyme activity range during the reaction was between 10-20 minutes for CFE, and 5-10 minutes for α-L-arabinofuranosidase (**Fig 23**). Also from thermostability assays, conducted with α-L-arabinofuranosidase, it was evident that the enzyme is not stable at 70°C for more than 5 minutes (**Fig. 24**). Based on the comparison of hydrolysis of substrate at the two time periods at different temperatures, the enzyme showed similar hydrolysis up to 40°C, after which the activity for 10 minute assay became constant. The decrease of activity implies that near 70°C the enzyme was not stable. Moreover, at 40°C the specific activity for 5 min and 10 min of

assay time were the same (**Fig. 24**), which reflects the true activity of the enzyme irrespective of incubation time. The low activity above 50°C is not typical of a thermophilic enzyme. However the results are not unexpected, α-L-arabinofuranosidase activities detected from crude extracts of *T. maritima, T. neapolitana and T. thermarum* were the least thermoactive amongst three xylanolytic enzymes studied; endoxylanase, β-xylosidase and α-arabinofuranosidase (Sunna et al., 1997). The α-L-arabinofuranosidase of *T. thermarum* was less than 30% active at the organism's growth temperature. From crude extract, optimal α-L-arabinofuranosidase activity from *T. maritima* and *T. neapolitana* was at 80°C and at pH 5.0 and 6.0, respectively. However when α-L-arabinofuranosidase from *T. maritima* was overexpressed in *E. coli*, the stability profile changed. The purified recombinant enzyme had an optimal activity at 90°C, and at pH 7.0. Furthermore, at this temperature (90°C) the enzyme was stable for 24 hours. To date, the most thermostable α-L-arabinofuranosidase has been obtained from *Thermotoga maritima* MSB8 (Miyazaki, 2005).

Another contributor to low activity could be that the protein in its almost pure form is not stable. This phenomenon has been described in *Thermotoga* strain FJSS (Ruttersmith and Daniel, 1993). The time interval between the end of purification and beginning of characterization was more than 8 months. The time interval for xylosidase was greater than that for α-L-arabinofuranosidase, however xylosidase was found to be more stable protein than α-L-arabinofuranosidase. Nevertheless, the long storage may explain the short half life of xylosidase compared with β-xylosidases from other *Thermotoga* species.

The pH optimum of the partially purified α-L-arabinofuranosidase was determined to be 6.0 at 40°C. About 50 % of the activity was measured near pH 5.0 and pH 8.0 (**Fig. 25**). The optimal pH profile was similar to that of xylosidase from *Thermotoga hypogea* as well as most of the other thermophiles.

From the substrate affinity assay, the trend is not very clear unlike xylosidase. α-L-arabinofuranosidase had the highest activity with 4-nitrophenyl α-L-arabinofuranoside but very little activity with xylosidase substrate, which was 16 times lower than α-L-arabinofuranosidase substrate (**Table 18**). No activity was observed with other α- linked substrates. Moreover, it was able to cleave to some extent one form of xyloside substrate (4-nitrophenyl-β-D-xylopyranoside) but not the other form (4-nitrophenyl-α-D-xylopyranoside), which is also interesting. It is possible that the spatial similarity between D-xylopyranose and L-arabinofuranose leads to bifunctional xylosidase-arabinosidase enzymes, found mainly in families 3, 43 and 54 (Mai et al., 2000).

Overlapping of substrate activity amongst glycoside hydrolases is not uncommon. This phenomenon may explain why xylosidase was considered a bifunctional enzyme Bronnenmeier et al, 1995; Xue and Shao, 2004). However, based on band location on the SDS-PAGE gel (**Fig. 26**), it is shown by this work that xylosidase and α-L-arabinofuranosidase are separate enzymes, with separate thermostabilities and different substrate affinities.

Table 23: Properties of representative bacterial α-L-arabinofuranosidase

Organism	Molecular mass (kDa)	Optimum pH	Optimum temp (°C)	References
Bacillus stearothermophilus	256 [a] , 64 [c]	5.5 -6.0	70	Gilead and Shoham, 1995
Butyrivibrio fibrosolvens	240 [a], 31 [c]	6.0- 6.5	45	Hespell and O'Bryan, 1992
Clostridium acetobutylicum	94 [a]	5.0 -5.5	Nd	Lee and Forsberg, 1987
Thermomicrobia PRI-1686	350 [a], 57 [c]	6.0	70	Birgisson et al., 2004
Thermomonospora fusca	92 [a], 46 [c]	6.0	60	Bacchman and McCarthy, 1991
Thermotoga maritima	332 [a], 55 [c]	7.0 (clone) 5.0 (native)	90 (clone) 80 (native)	(Miyazaki, 2005), (Xue et al., 2006)
Thermotoga thermarum	Nd	6.0	70	Sunna and Antranikian, 1996
Thermotoga hypogea	200 [a], 92 [c] / 50 [c]	6.0	40	This work

Nd: Not determined
a: determined from gel-filtration
c: determined from SDS-PAGE
clone: purified from a recombinant *E. coli* strain carrying *T. maritima* gene

4.8. Properties of Acetylesterase

A p-nitrophenyl acetate hydrolysing enzyme was identified but due to technical difficulties encountered, little characterization was possible. The purified fraction was loaded on the SDS-PAGE only once, but because the protein concentration was low, conclusions are tentative. The denatured molecular mass estimated was 129 kDa. The native molecular mass as estimated by size exclusion chromatography was 202 kDa, a homodimer was suggested for the enzyme. Since enzyme purification was followed by measuring hydrolysis of p-nitrophenol acetate, the enzyme is considered to be an esterase. Such a large molecular weight is unusual, as it is uncommon to find an esterase larger than 80 kDa (**Tables 7 and 24**). For future assays, it may be a good idea to use a different substrate as based on characterization assays from thermophiles, it was found that esterases exhibit significant esterase activity with a preference for short acyl chain esters containing substrates; such as *p*-nitrophenyl (*p*-NP) esters with butyrate (C4), caproate (C6), caprylate (C8) (Levisson et al., 2007). From *T. maritima* the maximum activity was observed with substrates with acyl chain length of (C4-C8) (Levisson et al., 2007) whereas, maximal activity for *G. stearothermophilus* esteras*e* was towards p-NP-(C4-C6) (Ewis et al., 2004).

Table 24:Properties of representative thermophilic microbial acetylesterases

Organism	Molecular mass (kDa) *	Optimum pH	References
Thermoanaerobacterium strain SL-YS485	Est 1, 32 Est 2, 26	1= 7.0 2= 7.5	Shao and Weigel, 1995
Thermomonospora fusca	Est 1, 80 Est 2, 40	Nd	Bachmann and McCarthy, 1991
Sulfolobus acidocaldrius	32	7.5-8.5	Sobek and Gorisch, 1988
G. stearothermophilus	Est 1. 42	7.0	Matsunaga et al., 1974
G. stearothermophilus	Est 2.; 28 Est 3: 55	Est 2: 9 Est 3: 8-9	Ewis et al., 2004
P. furiosus	Nd	7.6	Ikeda and Clark, 1998
Thermotoga neapolitana	37	Nd	Yernool, 2000
Thermotoga maritima	44.5	7.0	Levisson et al., 2007
Thermotoga hypogea	129 [*]	Nd	This work

- denatured weight determined by SDS-PAGE
- Nd ; not determined
- Est ; esterase
- [*] ; as predicted by (**Fig 12**)

The conversion of hemicellulose into useful value-added products by enzymatic routes holds strong promise for the use of a variety of unutilized and underutilized agricultural residues for practical purposes. Currently, the conversion of hemicellulosic substrates to fermentable sugars is problematic. Some of the emerging pretreatment

methods generate solubilized and partially degraded hemicellulosic biomass that needs to be treated further with enzymes or other means in order to produce fermentable sugars. With the development of a suitable pretreatment method, minimizing the formation of inhibitory compounds for fermentative organisms, and with the proper mix of hemicellulases (enzyme cocktail) tailored for each biomass conversion; this vast renewable resource can be utilized for production of fuels such as ethanol and hydrogen. Much research needs to be done to develop efficient and cost-effective pretreatment methods, enzymes for cellulose and hemicellulose conversion at an industrial scale, robust efficient microorganisms to ferment hemicellulosic sugars simultaneously in a cost-competitive way, and methods for cost-effective recovery of fermentation products.

Thus, extreme thermophiles seem to be a good candidate for the production of thermostable enzymes such as β-xylosidase, especially towards a possible application in biotechnological industrial processes. In time, when the structures of thermophilic glycoside hydrolases become available, their comparison with those of mesophilic homologous enzymes would certainly shed some light on the origins of thermal stability and activity. Additionally, hemicellulolytic enzymes also hold great promises in future by helping produce biofuels such as ethanol and hydrogen.

4.9. Future work

This work was significant as it attempted to identify major hemicellulolytic enzymes from a thermophile, *Thermotoga hypogea*. Future work would require assays that will assist in further identification of enzymes. The most important assay is conducting end product analysis through thin paper liquid chromatography for all three proteins. The other important work includes locating the gene and sequencing for all three proteins to compare the whole sequence with already sequenced xylosidase, arabinofuranosidase and acetyl esterases. Furthermore, other hemicellulolytic enzymes should also be investigated from this organism.

Chapter 5

Conclusion

The major conclusions of this research can be made as follows:

1. Three enzyme activities of β-xylosidase, α-L-arabinofuranosidase, acetylesterase were present in *T. hypogea.*

2. Xylosidase and α-L-arabinofuranosidase activities were mainly associated with the cells, and only a little activity was detected in the culture supernatant.

3. Putative xylosidase had been purified to homogeneity with a subunit of 75 kDa.

4. N-terminal analysis of β-xylosidase showed higher similarity to *Thermotoga* β-glucosidase than β-xylosidases. The β-xylosidase is speculated to be a member of family 3 glycoside hydrolases.

5. α-L-arabinofuranosidase was partially purified.

6. β-xylosidase and α-L-arabinofuranosidase were found to be two separate enzymes.

7. An esterase was found to be associated mainly with the cells with a significant activity in the remaining culture supernatant. It was partially purified.

References

Adams, E. L., Kroon, P.A., Williamson, G., Gilbert, H.J., Morrisa, V.J. 2004. Inactivated enzymes as probes of the structure of arabinoxylans as observed by atomic force microscopy. Carbohydr. Res. 339:579–590.

Adams, M. W. W., Perler, F. B., Kelly, R. M. 1995. Extremozymes: Expanding the limits of biocatalysis. Bio/technology. 13:662–668.

Aldrich, W.N. 1953. Serum esterase. I. Two types of esterases (A and B) hydrolyzing *p*-nitrophenylacetate propinate and butyrate and a method of their determination. Biochem. J. 53:110-117

Alkorta, I., Garbisu, C., Llama, M.J., Serra, J.L. 1998. Industrial applications of pectic enzymes: a review. Process Biochemistry. 33: 21–28.

Antoine, E., Rolland, J-L., Raffin, J-P., Dietrich, J. 1999. Cloning and over-expression in *Escherichia coli* of the gene encoding NADPH group III alcohol dehydrogenase from *Thermococcus hydrothermalis*. Eur. J. Biochem. 264:880- 889.

Archana, A., Satyanarayana, T. 1997. Xylanase production by thermophilic *Bacillus licheniformis A99* in solid-state fermentation. Enzyme Microbiol.Technol. 21:12-17.

Archenbacter, R., Gupta, R., Stetter, K.O., Woese, C.R. 1987. Were the original eubacteria thermophiles? System. Appl. Microbiol. 9:34-39.

Aspinall, G.O. 1985. The Polysaccharides. Vol .3. Academic Press, New York.

Auricchio, S., Rubino, A., Landolt, M., Semenza, G., Prader, A. 1963. Isolated intestinal lactase deficiency in the adult. Lancet. 17(2):324–326.

Baba, T., Shinke, R., Nanmori T. 1994**.** Identification and characterization of clustered genes for thermostable xylan-degrading enzymes, β-xylosidase and xylanase, of *Bacillus stearothermophilus* 21. Appl. Environ. Microbiol. 60(7): 2252-2258.

Bachmann, S., McCarthy, A. 1991. Purification and cooperative activity of enzymes constituting the xylan-degrading system of *Thermomonospora fusca*. Appl. Environ. Microbiol. 57: 2121-2130.

Bae, E-A., Park, S-Y., Kim, D-H. 2000. Constitutive β-glucosidase hydrolysing ginsenoside Rb1 and Rb 2 from intestinal bacteria. Biol. Pharm. Bull. 23:1481-1485.

Balk, M., Weijma, J., Stams, A.J.M. 2002. *Thermotoga lettingae* sp. nov., a novel thermophilic methanol-degrading bacterium isolated from a thermophilic anaerobic reactor. Int. J. Syst. Evol. Microbiol. 52:1361–1368.

Barrett, T., Suresh, S.G., Tolley, S.P., Dodson, E.J., Hughes, M.A. 1995. The crystal structure of cyanogenic, β-glucosidase from white clover, a family 1 glycosyl hydrolase. Structure 3: 951-960.

Bauer, M.W., Driskill, L.E., Kelly, R.M. 1998. Glycosyl hydrolases from hyperthermophilic microorganisms. Curr. Opin. Biotechnol. 9:141-145.

Bause, E., Legler, G. 1980. Isolation and structure of a tryptic glycopeptide from the active site of β-glucosidase A_3 from *Aspergillus wentii*. Biochim. Biophys. Acta. 626: 459–465.

Beguin, P., Aubert, J.P. 1994. The biological degradation of cellulose. FEMS Microbiol. Rev. 13: 25-58.

Beldman, G., Searle-van Leeuwen, M.F., Rombouts, F.M., Voragen, F.G.J. 1985. The cellulase of *Trichoderma viride.* Purification, characterization and comparison of all detectable endoglucanases, exoglucanases and β-glucosidases. Eur. J. Biochem. 146(2):301-8.

Bertoldo, C., Antranikian, G. 2002. Starch-hydrolyzing enzymes from thermophilic archaea and bacteria. Curr. Opin. Chem. Biol. 6: 151–160.

Bhat, K.M., Gaikwad, J.S., Maheshwari, R. 1993. Purification and characterization of an extracellular β-glucosidase from the thermophilic fungus *Sporotrichum thermophile* and its influence on cellulase activity. J. Gen. Microbiol. 139:2825-2832.

Bhat, M. K. 2000. Cellulases and related enzymes in biotechnology. Biotech. Adv. 18: 355-383.

Biely, P. 1985. Microbial xylanolytic systems. Trends in Biotechnology 3: 286-290.

Biely, P., MacKenzie, C. R., Schneider, H., Puls, J. 1987. The role of fungal acetyl xylan esterases in the degradation of acetyl xylan by fungal xylanases. In: Wood and cellulosics. Kennedy, J. F., Phillips, G.O., Williams, P. A. (Editors). Ellis Horwood, Ltd, New York. pp. 283–289.

Birgisson, H., Fridjonsson, O., Bahrani-Mougeot, F. K., Hreggvidsson, G., Kristjansson, J. K., Mattiasson, B. 2004. A new thermostable alpha-L-arabinofuranosidase from a novel thermophilic bacterium. Biotechnol. Lett. 26(17):1347-51.

Blöchl, E., Rachel, R., Burggraf, S., Hafenbradl, D., Jannasch, H. W., Stetter, K.O. 1997. *Pyrolobus fumarii*, gen. and sp. nov., represents a novel group of archaea, extending the upper temperature limit for life to 113°C. Extremophiles 1:14–21.

Bok, J.D., Goers, S.K., Eveleigh, D.E. 1994. Cellulase and xylanase systems of *Thermotoga neapolitana*. ACS Symp. Ser. 566:54–65.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248–254.

Breves, R., Bronnenmeier, K., Wild, N., Staudenbauer, W. L., Hofemeister, J. 1997. Genes encoding two different β-glucosidases of *Thermoanaerobacter brockii* are clustered in a common operon. Appl. Environ. Microbiol. 63(10): 3902–3910.

Bronnenmeier, K., Kern, A., Liebl, W., Staudenbauer, W.L. 1995. Purification of *Thermotoga maritima* enzymes for the degradation of cellulosic materials. Appl. Environ. Microbiol. 61:1339-1407.

Brown, S.H., Kelly, R.M. 1993. Characterization of amylolytic enzymes, having both α-1,4 and α-1,6 hydrolytic activity from the thermophilic archaea *Pyrococcus furiosus* and *Thermococcus litoralis*. Appl. Environ. Microbiol. 59:2614-2621.

Burt, A. 2006. Is corn stover a waste? Economic and agronomic considerations of corn residue removal (online). http://www.plant.uoguelph.ca/courses/plnt-6250/pdf/A_Burt.pdf , Retrieved on 16 January 2007.

Byrde, R.J.W., Fielding, A.H. 1955. Studies on acetyl esterase of *Schlerotina laxa*. Biochem. Journal. 61: 337-341.

Cacciapuoti, G., Fusco, S., Caiazzo, N., Zappia, V., Porcelli, M. 1999. Heterologous expression of 5'-methylthioadenosine phosphorylase from the archaeon *Sulfolobus solfataricus*: characterization of the recombinant protein and involvement of

disulfide bonds in thermophilicity and thermostability. Protein Expression Purif. 16:125-135.

Cambillau, C., Claverie, J.M. 2000. Structural and genomic correlates of hyperthermostability. J. Biol. Chem. 275: 32383-32386.

Canadian association of petroleum producers.

http://www.capp.ca/default.asp?V_Doc_ID=603, retrieved on August 16, 2008.

Carvallo, M., de Ioannes, P., Navarro, C., Chavez, R., Peirano, A., Bull P., Eyzaguirre, J. 2003. Characterization of an α-L-arabinofuranosidase gene (abf1) from *Penicillium purpurogenum* and its expression. Mycol Res. 107:388–394.

Chandrakant, P., Bisaria, V.S. 1998. Simultaneous bioconversion of cellulose and hemicellulose to ethanol. Crit. Rev Biotechnol. 18(4): 295-331.

Chayen, N. E., Conti, E., Vielle, C., Zeikus, G. J. 1997. Crystallization and initial X-ray analysis of xylose isomerase from *Thermotoga neapolitana.* Acta Crystallographica Section D. Volume 53 (2):229-230.

Cherry J. R., Fidantsef, A. L. 2003. Directed evolution of industrial enzymes: an update, Curr. Opin. Biotechnol. 14:438–443.

Chhabra, S. R., Shockley, K. R., Conners, S. B., Scott, K. L., Wolfinger, R. D., Kelly, R.M. 2003. Carbohydrate-induced differential gene expression patterns in the hyperthermophilic bacterium *Thermotoga maritima*. J. Biol. Chem. 278:7540-52.

Chhabra S. R., Kelly R. M. 2005. An expression-driven approach to the prediction of carbohydrate transport and utilization regulons in the hyperthermophilic bacterium *Thermotoga maritima*. J. Bacteriol.187:7267-82.

Chinen, I., Sadoyama, K. 1989. Japanese patent. JP 89 40, 502.

Christov, L.P., Prior, B.A. 1993. Xylan removal from dissolving pulp using enzymes of *Aureobasidium pullulans*. Biotechnol. Lett. 15:1269-1274.

Christov, L.P., Prior, B.A. 1993. Esterases of xylan-degrading microorganisms: production, properties, and significance. Enzyme Microb. Technol.15:460–475.

Chung, H-J., Park, S-M., Kim, H-R., Yang, M-S., Kim D-H. 2002. Cloning the gene encoding acetyl xylan esterase from *Aspergillus ficuum* and its expression in *Pichia pastoris*. Enz. Microbiol. Technol. 4:384-391.

Clarke, A.J. 1997. Biodegradation of Cellulose: Enzymology and Biotechnology. Technomic Publishing, Lancaster, PA.

Collins, T., Gerday, C., Feller, G. 2005. Xylanases, xylanase families and extremophilic xylanases. FEMS Microbiology Reviews. 29:3-23.

Comfort D.A., Chhabra, S.R., Conners, S.B., Johnson, M.R., Jones, K.L., Sehgal, A.C., Kelly, R.M. 2004. Strategic biocatalysis with hyperthermophilic enzymes. Green Chemistry. 6: 459- 465.

Conners, S. B., Montero, C. I., Comfort, D. A., Shockley, K. R.,. Johnson, M. R.,

Cournoyer, B., Faure, D. 2003. Radiation and functional specialization of the family-3 glycoside hydrolases. J. Mol. Microbiol. Biotechnol. 5(3):190-8.

Conners, S.B., Mongodin, E.F., Johnson, M.R., Montero, C.I., Nelson, K.E., Kelly, R.M. 2006. Microbial biochemistry, physiology, and biotechnology of hyperthermophilic *Thermotoga* species. FEMS Microbiol. Rev. 30:872-905.

Cowan, D.A. 2004. The upper temperature for life – where do we draw the line? Trends Microbiol. 12(2):58-60.

Dalboge, H., Lange, L. 1998**.** Using molecular techniques to identify new microbial biocatalysts. Trends in Biotechnol. 16:265-272.

Dalia, S., Belakhov, V., Solomon, D., Sara, G-G., Baasov, T., Shoham, G., Shoham, Y. 2002. The identification of the acid-base catalyst of alpha-arabinfuranosidase from *Bacillus stearothermophilus* T-6, a family 51 glycoside hydrolase. FEBS Lett. 514:163-167.

Degrassi, G., Okeke, B.C., Bruschi, C.V., Venturi, V. 1998. Purification and characterization of an acetyl xylan esterase from *Bacillus pumilus.* Appl. Environ. Microbiol. Feb; 64(2):789-92.

Degrassi, G., Vindigni, A., Venturi, V.A. 2003. Thermostable α-L-arabinofuranosidase from xylanolytic *Bacillus pumilis*: purification, and characterization. J. Biotechnol. 101:69-79.

Demirjian, D. C. Moris-Varas, F., Cassidy, C.S. 2001. Enzymes from extremophiles. Curr. Opin. Chem. Biol. 5:144–151.

De Vries, R.P, Kester, H.C.M., Poulsen, C.H., Benen, J.A.E., Visser, J. 2000. Synergy between enzymes from *Aspergillus* involved in the degradation of plant cell wall polysaccharides. Carbohydr. Res. 327:401–410.

De Vries, R.P., Visser, J. 2001. *Aspergillus* enzymes involved in degradation of plant cell wall polysaccharides. Microbiol. Mol. Biol. Rev. 65:497–522.

Devos, W. M., Kengen, S. W. M., Voorhorst, W. G. B., der Oost, J.V. 1998. Sugar utilization and its control in hyperthermophiles. Extremophiles 2:201-205.

Dhanjoon, J. 2005. Growth on biopolymers and catalytical properties of xylanase from *Thermotoga hypogea*. Master's thesis. Dept of Biology. University of Waterloo, Waterloo.

Dhaval, M., Nanavati, K., Noll, K. M. 2006. Several archaeal homologs of putative oligopeptide-binding proteins encoded by *Thermotoga maritima* bind sugars. Appl. Environ. Microbiol. 72 (2):1336–1345.

Di Lernia, I., Morana, A., Ottombrino, A., Fusco, S., Rossi, M., De Rosa, M. 1998. Enzymes from *Sulfolobus shibatae* for the production of trehalose and glucose from starch. Extremophiles. 2: 409-416.

Doner, L.W., Hicks, K.B. 1997. Isolation of hemicellulose from corn fiber by alkaline hydrogen peroxide extraction. Cereal Chem. 74:176–81.

Driskill, L. E., Kusy, K., Bauer, M.W., Kelly, R.M. 1999. Relationship between glycosyl hydrolase inventory and growth physiology of the hyperthermophile *Pyrococcus furiosus* on carbohydrate-based media. Appl. Environ. Microbiol. 65: 893-897.

Deutscher, M.P. 1990. Guide to protein purification. Methods Enzymol. 182: 588–604.

Ebringerová, A., Hromádková, Z., Hribalová, V. 1995. Structure and mitogenic activities of corn cob heteroxylans. Int. J. Biol. Macromol. 17(6): 327-331.

Ebringerová, A., Heinze, T. 2000. Xylan and xylan derivatives. Macromol. Rapid Commun. 21. 542-556.

Ebringerová, A., Kardosová, A., Hromádková, Z., Malovíková, A., Hríbalová, H. 2002. Immunomodulatory activity of acidic xylans in relation to their structural and molecular properties. Int. J. Biol. Macromol. 30(1):1-6.

Egana, L., Gutierrez, R., Caputo, V., Peirano, A., Steiner, J., Eyzaguirre, J. 1996. Purification and characterization of two acetyl xylan esterases from *Penicillium purpurogenum*. Biotechnol. Appl. Biochem. 24:33–39.

Eichler, J. 2001. Biotechnological uses of archaeal extremozymes. Biotechnol. Adv. 19: 261–278.

Elander, R.T., Putsche, V.L. 1996. Ethanol from corn: technology and economics. In: Handbook on bioethanol: production and utilization. Wyman, C.E. (Editor). Taylor and Francis, Washington DC. pp.329-349.

Eto, M. 1974. Organophosphorus Pesticides: Organic and Biological Chemistry. CRC Press, Cleveland, OH.

Ewis, H. E., Abdelal, A. T., Lu, C-D. 2004. Molecular cloning and characterization of two thermostable carboxyl esterases from *Geobacillus stearothermophilus*. Gene 329:187-195.

Farabee, M. J. 2000. http://www.emc.maricopa.edu/faculty/farabee/BIOBK/BioBookglossC.html#cell %20wall, Retrieved on January 15, 2008.

Fardeau, M. L., Ollivier, B., Patel, B.K., Magot, M., Thomas, P., Rimbault, A., Rocchiccioli, F., Garcia, J.L. 1997. *Thermotoga hypogea* sp., a xylanolytic, thermophilic bacterium from an oil-producing well. Int. J. Sys. Bacteriol. 47(4): 1013-1019.

Faure, D. 2002. The family-3 glycoside hydrolases: from housekeeping functions to host-microbe interactions. Appl. Environ. Microbiol. 68(4):1485-1490.

Fields, P.A. 2001. Protein function at thermal extremes: balancing stability and flexibility. Comp. Biochem. Physiol. 129:417– 31.

Flores, M.E., Perea, M., Rodriguez, O., Malvaez, A., Huitron, C. 1996. Physiological studies on induction and catabolite repression of β-xylosidase and endoxylanase in *Streptomyces sp*. J. Biotechnol. 49:179-187.

Forge, F. 2007. Biofuels-An energy, environmental or agricultural policy? Parliamentary Information and Research Service.

Fujiwara, S. 2002. Extremophiles: Development of their special functions and potential resources. J. Biosci. Bioeng. 94: 518-525.

Gatenholm, P., Tenkanen, M. 2004. Hemicelluloses: Science and Technology..: American Chemical Society. Oxford University Press, Washington, D.C.

Gebler, J., Gilkes, N.R., Claeyssens, M., Wilson, D.B., Beguin, P., Withers, S.G. 1992. Stereoselective hydrolysis catalyzed by related β-1,4-glucanases and β-1,4-xylanases. J. Biol. Chem. 267: 12559-12561.

Gilead, S., Shoham, Y. 1995. Purification and characterization of α-L-arabinofuranosidase from *Bacillus stearothermophilus* T-6. Appl. Environ. Microbiol. 61(1):170-174.

Gomes, J., Gomes, I., Terler, K., Gubala, N., Ditzelmuller, G., Steinera, W. 2000. Optimisation of culture medium and conditions for α-L-arabinofuranosidase production by the extreme thermophilic eubacterium *Rhodothermus marinus*. Enzyme Microb. Technol. 27:414–422.

Goyal, K., Selvakumar, P., Hayashi, K. 2001. Characterization of a thermostable β-glucosidase (BglB) from *Thermotoga maritima* showing transglycosylation activity. J. Mol. Catalysis B: Enzymatic. 15(1-3): 45-53.

Gräbnitz, F., Rücknagel, K.P., Seib, M., Staudenbauer, W.L. 1989. Nucleotide sequence of *Clostridium thermocellum* b1B gene encoding thermostable β-glucosidase B: homology to fungal β-glucosidases. Mol. Gen. Genet. 217: 70–76.

Grassia, G. S., McLean, K. M., Glenat, P., Bauld, J., Sheehy. A. J. 1996. A systematic survey for thermophilic fermentative bacteria and archaea in high temperature petroleum reservoirs. FEMS Microbiol. Ecol. 21:47–58.

Greve, J. C., Labavitch, J. M., Hungate, R. E. 1984. α-L-arabinofuranosidase from *Ruminococcus albus* 8: purification and possible role in hydrolysis of alfalfa cell wall. Appl. Environ. Microbiol. 47:1135-1140.

Hallborn, J., Walfridsson, M., Airaksinen, U., Ojamo, H., Hahn-Hägerdal, B., Penttila, M., Keranen, S. 1991. Xylitol production by recombinant *Saccharomyces cerevisiae*. Bio/technology. 9:1090–1095.

Harvey, A.J., Hrmova, M., DeGori, R., Varghese, J.N., Fincher, G.B. 2000. Comparative modeling of the three-dimensional structures of family 3 glycoside hydrolases. Proteins. Nov 1;41(2):257-69.

Hashimoto, T., Nakata, Y. 2003. Synergistic degradation of arabinoxylan with α-L-arabinofuranosidase, xylanase and β-xylosidase from soy sauce koji mold, *Aspergillus oryzae*, in high salt condition. J. Biosci. Bioeng. 95:164–169.

Hazelwood, G. P., Romaniec, M. P. M., Davidson, K., Grepinet, O., Beguin, P., Millet, J., Raynaud, O., Aubert, J. P. 1988. A catalogue of *Clostridium thermocellum*

endoglucanase, β-glucosidase and xylanase genes cloned in *Escherichia coli*. FEMS Microbiol. Lett. 51: 231-236.

Henrissat, B. 1991. A classification of glycosyl hydrolases based on amino-acid sequence similarities. Biochem. J. 280:309-316.

Henrissat, B., Bairoch, A. 1993. New families in the classification of glycosyl hydrolases based on amino-acid sequence similarities. Biochem. J. 293:781-788.

Henrissat, B., Bairoch, A. 1996. Updating the sequence-based classification of glycosyl hydrolases. Biochem. J. 316: 695-696.

Henrissat, B., Davies, G. 1997. Structural and sequence-based classification of glycoside hydrolases. Curr. Opin. Struct. Biol. 7: 637-644.

Henrissat, B., Davies, G. 2000. Glycoside hydrolases and glycosyltransferases. Families, modules, and implications for genomics. Plant Physiol. 124:1515-1519.

Henrissat, B., Coutinho, P.M. 2001. Classification of glycoside hydrolases and glycosyltransferases from hyperthermophiles. Methods Enzymol. 330:183-201.

Hespell, R.B., O'Bryan, P. 1992. Purification and characterization of an α-L-arabinofuranosidase from *Butyrivibrio fibrisolvens* GS113. Appl. Environ. Microbiol. 58:1082–1088.

Hongpattarakere, T. 2002. Hyperthermostable cellulolytic and hemicellulolytic enzymes and their biotechnological applications. Songklanakarin J. Sci. Technol 24(3): 481-491.

Hough, D. W., Danson, M. J. 1999. Extremozymes. Curr. Opin. Chem. Biol. 3: 39–46.

Hrmova, M., Fincher, G.B. 1998. Barley β-D-glucan exohydrolases: substrate specificity and kinetic properties. Carbohydr. Res. 305: 209-221.

Hrmova, M., MacGregor, E.A., Biely, P., Stewart, R.J., Fincher, G.B. 1998. Substrate binding and catalytic mechanism of a barley β-D-glucosidase (1->4)-β-D-glucan exohydrolase. J. Biol. Chem. 273: 11134-11143.

Huber, R., Langworthy, T.A., König, H., Thomm, M., Woese, M., Sleytr, U.B., Stetter, KO. 1986. *Thermotoga maritima* sp. nov. represents a new genus of unique extremely thermophilic eubacteria growing up to 90°C. Arch. Microbiol. 144: 324-333.

Huber, R., Stetter, K.O. 2001. Genus I. *Thermotoga*: Bergey's Manual of Systematic Bacteriology, second edition, vol. 1 (The *Archaea* and the deeply branching and phototrophic *Bacteria*), Springer-Verlag, New York, pp. 370-377.

Hudson, R. C., Schofield, L. R., Coolbear, T., Daniel, R. M., Morgan, H. W. 1991. Purification and properties of an aryl β-xylosidase from a cellulolytic extreme thermophile expressed in *Escherichia coli*. Biochem. J. 273(1): 645–650.

International Union of Biochemistry. Nomenclature Committee. Enzyme nomenclature. 1984. Recommendations of the Nomenclature Committee of the International Union of Biochemistry on the nomenclature and classification of the enzyme catalyzed reactions. Academic Press, Orlando. Florida.

Ioannes, P. D., Peirano, A., Steiner, J., Eyzaguirre, J. 2000. An α-L-arabinofuranosidase from *Penicillium purpurogenum*: production, purification and properties. J. Biotechnol. 76: 253-258.

Jannasch, H.W., Huber, R., Belkin, S., Stetter, K.O. 1988. *Thermotoga neapolitana* sp. nov. of the extremely thermophilic eubacterial genus *Thermotoga*. Arch. Microbiol. 150: 103-104.

Jeanthon, C., Reysenbach, A.L., Haridon, S., Gambacorta, A., Pace, N.R., Glenat, P., Prieur, D., 1995. *Thermotoga subterranean* sp. nov., a new thermophilic bacterium isolated from a continental oil reservoir. Arch. Microbiol. 164: 91-97.

Joseleau, J. P., Comtat, J., Ruel, K. 1992. Chemical structure of xylans and their interaction in the plant cell walls. In: *Xylans and Xylanases.* Visser, J., Beldman, G., Kusters-van Someren, M. A., and Voragen, A. G. J. (Editors). Elsevier, Amsterdam. pp.1-15.

Kato, Y., Nevis, P.J. 1985. Isolation and identification of O-(5-O-feruloyl-α-arabinofuranosyl)-O-B-xylopyranosyl-(1,4)-D-xylopyranose as a component of *Zea* shoot cell walls. Carbohydr. Res. 137:139-150.

Kermnicky, L., Biely, P. 1998. Disaccharide permeases: constituents of xylanolytic and mannanolytic systems of *Aureobasidium pullulans*. Biochim. Biophys. Acta 1425. 560-566.

Kim, B-C., Lee, Y-H., Lee, H-S., Lee, D-W., Choe, E-A., Pyun, Y-R. 2002. Cloning and expression and characterization of L-arabinose isomerase from *Thermotoga neapolitana*: Bioconversion of D-galactose to D-tagatose using the enzyme. FEMS Microbiol. Letters 212:121-126.

Komae, K., Kaji, A., Sato, M. 1982. An α-L-arabinofuranosidase from *Streptomyces purpuascens* IFO 3389. Agric. Bio. Chem. 46:1899–1905.

Kormelink, F.J.M., Gruppen, H., Voragen, A.G.J. 1993. Mode of action of (1, 4)-β-D-arabinoxylan arabinofuranohydrolase (AXH) and α-L-arabinofuranosidases on alkali-extractable wheat-flour arabinoxylan. Carbohydr. Res. 249:345–53.

Kormelink, F.J.M., Voragen, A.G.J. 1993. Degradation of different glucurono arabinoxylans by a combination of purified xylan-degrading enzymes. Appl. Microbiol. Biotechnol. 38:688–95.

Koseki, T., Okuda, M., Sudah, S., Kizaki, Y., Iwano, K., Aramaki, I., Matsuzawa, H. 2003. Role of two α-L-arabinofuranosidases in arabinoxylan degradation and characteristics of the encoding genes from shochu koji molds, *Aspergillus kawachii* and *Aspergillus awamori*. J. Biosci. Bioeng. 96:232–241.

Kosugi, A., Murashima, K., Doi, R.H. 2002. Characterization of two noncellulosomal subunits, ArfA and BgaA, from *Clostridium cellulovorans* that cooperate with the cellulosome in plant cell wall degradation. J. Bacteriol. 184:6859–6865.

Kuhad, R.C., Singh, A., Eriksson, K.E. 1997. Microorganisms and enzymes involved in the degradation of plant fiber cell walls. In Advances in Biochemical Engineering: Biotechnology in the Pulp and Paper Industry. Eriksson, K.E.(Editor). Springer-Verlag, New York. pp. 45-125.

Kumar, S., Nussinov, R. 2001. How do thermophilic proteins deal with heat? Cell Mol. Life Sci. 58:1216–1233.

La Grange, D. C., Pretorius, I. S., Claeyssens, M., Van Zyl, W. H. 2001. Degradation of xylan to d-xylose by recombinant *Saccharomyces cerevisiae* coexpressing the *Aspergillus niger* β-xylosidase *(*xlnD*)* and the *Trichoderma reesei* xylanase II *(*xyn2*)* genes. Appl. Environ. Microbiol. 67(12): 5512–5519.

Lachke, A.H. 1988. 1,4-β-D-xylan xylohydrolase of *Sclerotium rolfsii*. Methods Enzymol. 160: 679-684.

Laemmli, U.K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature 227: 680–685.

Lebbink, J. H., Eggen, R. I., Geerling, A. C., Consalvi, V., Chiaraluce, R., Scandurra, R.. De Vos, W. M. 1995. Exchange of domains of glutamate dehydrogenase from the hyperthermophilic archaeon *Pyrococcus furiosus* and the mesophilic bacterium *Clostridium difficile*: effects on catalysis, thermoactivity and stability. Protein Eng. 8:1287-1294.

Lee, S. F., C. W. Forsberg. 1987. Purification and characterization of an α-L-arabinofuranosidase from *Clostridium acetobutylicum* ATCC 824. Can. J. Microbiol. 33:1011-1016.

Lee, Y-E., Lowe, S. E., Zeikus, J.G. 1993. Regulation and characterization of xylanolytic enzymes of *Thermoanaerobacterium saccharolyticum* B6A-RI. Appl. Environ. Microb. 59:763-771.

Lehninger, A.L., Nelson, D.L., Cox, M. M. 1993. Principles of Biochemistry, 2nd ed. Worth Publishers, New York.

Leuschner, C., Antranikian, G. 1995. Heat-stable enzymes from extremely thermophilic and hyperthermophilic microorganisms. World J. Microbiol. Biotechnol. 11: 95-114.

Levisson, M., Oost, J.V.D., Kengen, S.W.M. 2007. Characterization and structural modeling of a new type of thermostable esterase from *Thermotoga maritima*. FEBS J. 274(11): 2832-42.

Li, W.F., Zhou, X.X., Lu, P. 2005. Structural features of thermozymes. Biotech.Advances 23: 271-281.

Liebl, W., Stemplinger, I., Ruile, P. 1997. Properties and gene structure of the *Thermotoga maritima* alpha-amylase AmyA, a putative lipoprotein of a hyperthermophilic bacterium. J. Bacteriol. 179:941-948.

Liebl, W., Winterhalter, C., Baumeister, W., Armbrecht, M., Valdez, M. 2008. Xylanase attachment to the cell wall of the hyperthermophilic bacterium *Thermotoga maritima*. J. Bacteriol. 190(4):1350-1358.

Lin, L.L., Thomson, J.A. 1991. Cloning, sequencing and expression of a gene encoding a 73 kDa xylanase enzyme from the rumen anaerobe *Butyrivibrio fibrisolvens* H17c. Mol. Gen. Genet. 228:55–61.

Maekawa, E. 1976. Studies on hemicellulose of bamboo. Wood Res. 59:153-179.

Magerstaedt, M., Meichsner, C., Schlingmann, M., Schrinner, E., Walch, A., Weisner, M., Winkler, I. 1991. German Patent DE 3,921,761 A1.

Mai, V., Wiegel, J., Lorenz, W.W. 2000. Cloning, sequencing, and characterization of the bifunctional xylosidase-arabinosidase from the anaerobic thermophile *Thermoanaerobacter ethanolicus*. Gene 247:137-143.

Margolles, A., Reyes-Gavilan, C.G. 2003. Purification and functional characterization of a novel α-L-arabinofuranosidase from *Bifidobacterium longum* B667. Appl Environ Microbiol. 69:5096–5103.

Maruta, K., Mitsuzumi, H., Nakada, T., Kubota, M., Chaen, H., Fukuda, S., Sugimoto, T., Kurimoto, M. 1996. Cloning and sequencing of a cluster of genes encoding novel enzymes of trehalose biosynthesis from thermophilic archaebacterium *Sulfolobus acidocaldarius*. Biochimica et Biophysica Acta. 1291(3):171-181.

Matsunaga, A., Koyama, N., Nosh, Y. 1974. Purification and properties of esterase from *Bacillus stearothermophilus.* Arch. Biochem. Biophys. 160: 504–513.

Matsuo, M., Yasui, T. 1988. β-xylosidases from several fungi. Methods Enzymol. 160: 684-695.

Matuso, N., Kaneko, S., Kuno, A., Kobayashi, H., Kusakabes, I. 2000. Purification, characterization and gene cloning of two α-L-arabinofuranosidases from *Streptomyces chartreuses* GS901. Biochem. J. 346: 9–15.

McCarter, J.D., Withers, S.G., 1994. Mechanisms of enzymatic glycoside hydrolysis. Curr. Opin. Struc. Biol. 4:885-892.

McCleary, B.V. 1982. Purification and properties of a mannohydrolase from guar**.** Carbohydrate Res. 101:75-92.

Meghji, K.1990. Production, purification and characterization of extracellular esterases from *B. subtilis* NRRL 365. Master's thesis. Dept. of Biology. University of Waterloo.Waterloo.

Miyazaki, K. 2005. Hyperthermophilic α-L-arabinofuranosidase from *Thermotoga maritima* MSB8: molecular cloning, gene expression, and characterization of the recombinant protein. Extremophiles 9 (5):399-406.

Mongodin, E. F., Hance, I. R., Deboy, R. T., Gill, S. R., Daugherty, S., Huber, R., Fraser, C. M., Stetter, K., Nelson, K. E. 2005. Gene transfer and genome plasticity in *Thermotoga maritima*, a model hyperthermophilic species. J. Bacteriol. 187: 4935-44.

Morris, D.D., Gibbs, M.D., Chin, C.W.J., Koh, M.H., Wong, K.K.Y., Allison, R.W., Nelson, P.J. 1998. Cloning of the *xyn*B gene from *Dictyoglomus thermophilum*

Rt46B.1 and action of the gene product on kraft pulp. Appl. Environ. Microbiol. 64:1759-1765.

Mueller-Harvey, I., Hartley, R.D., Harris, P.J., Curzon, E.H. 1986. Linkage of p-coumaroyl and feruloyl groups to cell wall polysaccharides of barley straw. Carbohydrate Res. 148:71-85.

National Corn Growers Association,

http://www.ncga.com/ethanol/environment/biggestPolluter.asp, Retrieved on

August 16, 2008.

Nelson, K.E., Clayton, R., Gill, S.R., Gwinn, M.L., Dodson, R.J., Haft, D.H., Hickey, E.K., Peterson, J., Nelson, W.C., Ketchum, K.A., McDonald, L., Utterback, T.R., Malek, J.A., Linher, K.D., Garrett, M.M., Stewart, A.M., Cotton, M.D., Pratt, M.S., Phillips, C.A., Richardson, D., Heidelberg, J., Sutton, G.G., Fleischmann, R.D., White, O., Salzberg, S.L., Smith, H.O., Venter, J.C., Fraser, C.M. 1999. Evidence for lateral gene transfer between archaea and bacteria from genome sequence of *Thermotoga maritima*. Nature 399:323–329.

Nesbo, L.C., Dlutek, M., Doolittle, F.W. 2006. Recombination in *Thermotoga*: Implications for species concepts and biogeography. Genetics. 172: 759- 769.

Numan, M.T., Bhosle, N.B. 2006. α-L-Arabinofuranosidases: the potential applications in biotechnology. J Ind Microbiol Biotechnol. 33: 247–260.

Okamoto, Y., Kawashima, M., Hatada, K. 1984. Chromatographic resolution. Useful chiral packing materials for high-performance liquid chromatographic resolution of enantiomers: phenylcarbamates of polysaccharides coated on silica gel. J. Amer. Chem. Soc. 106: 5357-5359.

Pearce, F. 2006. Fuels gold: Big risks of the biofuel revolution. New Scientist. 191(2570):36-41.

Pilz, W., Horlein, H., Stelzl, E. 1966. Research on the ferments of human blood. XII. The role of an arylesterase fraction from the liver in human fat metabolism. Physiol. Chem. 345:65-79.

Puls, J., Schuseil, J. 1993. Chemistry of hemicelluloses: relationship between hemicellulose structure and enzymes required for hydrolysis. In: Hemicellulose and hemicelluloses. Coughlin M.P and Hazelwood, G.P. (Editors). Portland Press, London.

Poutanen, K. 1988. An α-L-arabinofuranosidase of *Trichoderma reesei*. J. Biotechnol. 7: 271-281.

Poutanen. K., Puls, J. 1988. Characterization of *Trichoderma reesei* β-xylosidase and its use in hydrolysis of xylans. Appl. Microbiol. Biotechnol. 28: 425-432.

Ravot, G., Magot, M., Fardeau, M.L., Patel, B.K.C., Prensier, G., Egan, A., Garcia, J.L., Ollivier, B. 1995. *Thermotoga elfii* sp. nov., a novel thermophilic bacterium from an African oil-producing well. Int. J. Syst. Bacteriol. 45:308-314.

Ravot, G., Ollivier, B., Fardeau, M.L., Patel, B.K.C., Andrews, K.T., Magot, M., Garcia, J.L. 1996. L-alanine production from glucose fermentation by hyperthermophilic members of the domains *Bacteria* and *Archaea*: a remnant of an ancestral metabolism? Appl. Environ. Microbiol. 62: 2657–2659.

Reese, E.T., Maguire, A.H., Parrish, F.W. 1968. Glucosidases and exo-glucanases. Can. Journal Biochem. 46:25-34.

Rodinova, N.A., Tavobilov, I.M., Bezborodov, A.M. 1983. β-xylosidase from *Aspergillus niger* 15: purification and properties. J. Appl. Biochem. 5:300-312.

Ruttersmith, L.D., Daniel, R.M. 1991. Thermostable cellobiohydrolase from the thermophilic eubacterium *Thermotoga* sp. strain FjSS3-B.1. Biochem. J. 277: 887-890.

Ruttersmith L.D., Daniel, R. M., Simpson, H. D. 1992. Cellulytic and hemicellulolytic enzymes functional above 100°C. Ann. NY. Academy Sci. 672:137-141.

Ruttersmith, L.D., Daniel, R.M. 1993. Thermostable β-glucosidase and β-xylosidase from *Thermotoga* sp. strain FjSS3-B.1. Biochim. Biophys. Acta. 1156: 167-172.

Saha, B.C. 2000. α-L-Arabinofuranosidases: biochemistry, molecular biology and application in biotechnology. Biotech. Adv. 18: 403–423.

Saha, B.C. 2003. Hemicellulose bioconversion. J. Ind. Microbiol. Biotechnol. 30:279–291.

Sanai, K., Seri, K., Inoue, S. 1997. Inhibition of sucrose digestion and absorption by L-arabinose in rats. Nihon Eiyou Syokuryougaku Kaishi. 50:133-137.

Satyanarayana, T., Raghukumar, C., Shivaji, S. 2005. Extremophilic microbes: Diversity and perspectives. Current Science 89:78–90.

Scandurra, R., Consalvi, V., Chiaraluce, R., Politi, L., Engel, P.C. 1998. Protein thermostability in extremophiles. Biochimie 80: 933– 41.

Scherz, H., Bonn, G. 1998. Analytical Chemistry of Carbohydrates. Georg Thieme Verlag Press, Stuttgart, Germany.

Schulein, M. 2000. Protein engineering of cellulases. Biochimica et. Biophysica Acta – Protein Structure and Molecular Enzymology 1543:239–252.

Schumann, J., Wrba, A., Jaenicke, R., Stetter, K.O. 1991. Topographical and enzymatic characterization of amylases from the extremely thermophilic eubacterium *Thermotoga maritima*. FEBS lett. 282:122-126.

Selvendran, R.R. 1987. Chemistry of plant cell walls and dietary fibre. Scandinavian Journal of Gastroenterology 32:33-41.

Shao, W., Weigel, J. 1992. Purification and characterization of a thermostable β-xylosidase from *Thermoanaerobacter ethanolicus*. J. Bacteriol. 174 (18):5848-5853.

Shao, W., Deblois, S., Wiegel, J. 1995. A high molecular weight, cell-associated xylanase isolated from exponentially growing *Thermoanaerobacterium* sp. strain JW/SL-YS485. Appl. Environ. Microbiol. 61(3):937-940.

Shibuya, N., Iwasaki, T. 1985. Structural features of rice bran hemicellulose. Phytochemistry 24:285–9.

Shin, H-Y., Park, S-Y., Sung, J-H., Kim, D-H. 2003. Purification and characterization of α-L-arabinopyranosidase and α-L-arabinofuranosidase from *Bifidobacterium breve* K-110, a human intestinal anaerobic bacterium metabolizing ginsenoside Rb2 and Rc. Appl. Environ. Microbiol. 69:7116–7123.

Sigurskjøld, BW., Christensen, T., Payre, N., Cottaz, S., Driguez, H., Svensson, B. 1998. Thermodynamics of binding of heterobidentate ligands consisting of spacer-connected acarbose and β-cyclodextrin to the catalytic and starch-binding domains of glucoamylase from *Aspergillus niger*. Biochemistry 37:10446-10452.

Silver, A. 1974. The biology of cholinesterase. American Elsevier, New York.

Simpson, H.D., Haufler, U.R., Daniel, R.M. 1991. An extremely thermostable xylanase from the thermophilic eubacterium *Thermotoga*. Biochem. J. 277: 413–417.

Singh A., Hayashi, K. 1995. Construction of chimeric β-glucosidases with improved enzymatic properties. J. Biol. Chem. 270 (37):21928-21933.

Sinnott. M.L. 1990. Catalytic mechanism of enzymic glycosyl transfer. Chem. Rev 90(7): 1171 – 1202.

Sjostrom, E. 1981. Wood Chemistry. Fundamentals and Applications. Academic Press, New York.

Sjostrom, J. 1990. Detrimental substances in pulp and paper production- approaches to chemical analysis of deposits and dissolved organic material. PhD. Thesis . Abo, Finland: Abo Akademi University.

Sobek, H., Gorisch, H. 1988. Purification and characterization of a heat-stable esterase from the thermoacidophilic archaebacterium *Sulfolobus acidocaldarius*. Biochem. J. 250: 453-458.

Sterner, R., Liebl, W. 2001. Thermophilic adaptation of proteins. Crit. Rev. Biochem. Mol. Biol. 36: 39–106.

Stetter, K. O., Huber, R., Blochl, E., Kurr, M., Eden, D., Fielder, M., Cash, H.,Vance, I. 1993. Hyperthermophilic Archaea are thriving in deep North Sea and Alaskan reservoirs. Nature 365: 743–745.

Sunna, A., Antranikian, G. 1996. Growth and production of xylanolytic enzymes by the extreme thermophilic anaerobic bacterium *Thermotoga thermarum*. Appl. Microbiol. Biotechnol. 45:671–676.

Sunna, A., Puls, J., Antranikian, G. 1997. Characterization of the xylanolytic enzyme system of the extreme thermophilic anaerobic bacteria *Thermotoga maritima, T. neapolitana* and *T. thermarum*. Comp. Biochem. Physiol.118: 453-461.

Sunna, A., Antranikian, G. 1997. Xylanolytic enzymes from fungi and bacteria. Critical Rev. Biotechnol. 17:39-67.

Sunna, A., Gibbs, M.D., Bergquist, P. L. 2000. The thermostabilizing domain, XynA, of *Caldibacillus cellulovorans* xylanase is a xylan binding domain. Biochem. J. 346: 583–586.

Taiz, L., Zeiger, E. 1998. Plant Physiology. Sinauer Associates, Inc. Publishers, Sunderland. Massuchesetts. pp. 420-425.

Takahata, Y., Nishijima, M., Hoaki, T., Maruyama, T. 2001. *Thermotoga petrophila* sp. nov. and *Thermotoga naphthophila* sp. nov., two hyperthermophilic bacteria from the Kubiki oil reservoir in Niigata, Japan. Int. J. Syst. Evol. Microbiol. 51:1901-1909.

Takasaki, Y., Kosugi, Y. 1969. *Streptomyces* glucose isomerase. In: Fermentation advances. Perlman, D. (Editors). Academic Press Inc., New York. pp 5561-570.

Taylor G. 1996. Sialidases: Structures, biological significance and therapeutic potential. Curr. Opin. Struct. Biol. 6: 830-837.

Teeri, T.T., Koivula, A., Linder, M., Wohlfahrt, G., Divne, C., Jones, T.A. 1998. *Trichoderma reesei* cellobiohydrolases: why so efficient on crystalline cellulose. Biochem. Soc. Trans. 26: 173-178.

Tenkanen, M., Poutanen, K. 1992. Significance of esterases in the degradation of xylans. In: Xylans and Xylanases. Visser, J., Beldman, G., VonSomeren, M.A.K., Voragen, A.G. J. (Editors). Elsevier, Amsterdam. pp 203- 212.

Timell, T. E. 1965. Wood hemicellulose: Part ll. Adv. Carbohydrate Chemistry. 20:409-483.

Timell, T. E. 1967. Recent progress in the chemistry of wood hemicelluloses. Wood Sci. Technol. 1:45-70.

Tomme, P., Warren, R.A., Gilkes, N.R. 1995. Cellulose hydrolysis by bacteria and fungi. Adv. Microb. Physiol. 37:1–81.

Tsumara, N., Hagi, M., Sato, T. 1967. Enzymatic conversion of D-glucose to D-fructose. Agric. Biol. Chem. 31:902-907.

Utt, E. A., Eddy, C. K., Keshav, K.F. 1991. Sequencing and expression of the *Butyvibrio fibrisolvens* xylB gene encoding a novel bifunctional protein with β-D-xylosidase and α-L-arabinofuranosidase activities. Appl. Environ. Microbiol. 57:1227-34.

Van der Maarel, M.J.E.C., Van der Veen, B.A., Uitdehaag, J. C. M., Leemhuis, H., Dijkhuizen, L. 2002. Properties and application of starch-converting enzymes of the amylase family. J. Biotechnol. 94:137–155.

Van Ooteghem, S.A., Beer, S.K., Yue, P.C. 2002. Hydrogen production by the thermophilic bacterium *Thermotoga neapolitana*. Appl. Biochem. Biotechnol. 98: 177–189.

Varghese, J.N., Garrett, T.P.J., Colman, P.M., Chen, L., Hoj, P.B., Fincher, G.B. 1994. Three-dimensional structures of two plant-glucan endohydrolases with distinct substrate specificities. Proc. Natl. Acad. Sci. USA. 91: 2785-2789.

Varghese, J.N., Hrmova, M., Fincher, G.B. 1999. Three-dimensional structure of a barley β-D-glucan exohydrolase; a family 3 glycosyl hydrolase. Structure 7:179-190.

Vieille, C., Sriprapundh, D., Kelly, R.M., Zeikus, J.G. 2001. Xylose isomerases from *Thermotoga*. Methods Enzymol. 330: 215-216.

Vieille, C., Zeikus, G.J. 2001. Hyperthermophilic enzymes. Microbiol. Mol. Bio. Rev. 65:1-43.

Viikari, L., Kantelinen, A., Buchert, J., Puls, J. 1994. Enzymatic accessibility of xylans in lignicellulosic materials. Appl. Microbiol. Biotechnol. 41: 124-129.

Viikari, L., Kantelinen, A., Sundquist, J., Linko, M. 1994. Xylanases in bleaching: from an idea to the industry. FEMS Microbiol. Rev..13:335–350.

Von Itzstein, M., Colman, P. 1996. Design and synthesis of carbohydrate-based inhibitors of protein-carbohydrate interactions. Curr. Opin. Struc. Biol. 6: 703-709.

Vroemen, S., Heldens, J., Boyd, C., Henrissat, B., Keen, N.T. 1995. Cloning and characterization of the bgxA gene from *Erwinia chrysanthemi* D1 which encodes a β-glucosidase/xylosidase enzyme. Mol. Gen. Genet. 246(4):465–477.

Watanabe, T., Ito, Y., Yamada, T., Hashimoto, M., Sekine, S., Tanaka, H. 1994. The roles of the C-terminal domain and type III domains of chitinase A1 from *Bacillus circulans* WL-12 in chitin degradation. J. Bacteriol. 176:4465-4472.

Watt, D. K., Ono, H., Hayashi, K. 1998. *Agrobacterium tumefaciens* β-glucosidase is also an effective β-xylosidase, and has a high transglycosylation activity in the presence of alcohols. Biochim. Biophys. Acta. 1385:78-88.

White, A., Rose, R.D. 1997. Mechanism of catalysis by retaining β-glycosyl hydrolases. Curr. Opin. Struct. Biol. 7:645-651.

Wiegel, J. 1992. The anaerobic thermophilic bacteria. In: Thermophilic Bacteria. Kristjansson, J.K (Editor). CRC Press, Boca Raton. pp.105-184.

Wiegel J., Canganella. F. 2000. Extreme thermophiles. In: Encyclopedia of Life Sciences. Article 392. http://www.els.net.elsonline/html/A0000392.html.

Windberger, E., Huber, R., Trincone, A., Fricke, H., Stetter, K.O. 1989. *Thermotoga thermarum* sp. nov. and *Thermotoga neapolitana* occurring in African continental solfataric springs. Arch. Microbiol. 151: 506-512.

Winterhalter, C., Liebl, W. 1995. Two extremely thermostable xylanases of the hyperthermophilic bacterium *Thermotoga maritima* MSB8. Appl. Environ. Microbiol. 61:1810–1815.

Wong, K.K.Y., Tan, L.U.L., Saddler, J .N. 1988. Multiplicity of β-1-4-Xylanases in microorganisms: functions and applications. Microbiol. Rev. 305-317.

Woodward, J. 1984. Xylanases: functions, properties and applications. Enzyme Ferment. Biotechnol. 8:9-30.

Xue, Y., Shao, W. 2004. Expression and characterization of a thermostable β-xylosidase from the hyperthermophile, *Thermotoga maritima.* Biotechnol. Lett. 26(19): 1511-1515.

Xue, Y., Wu, A., Zeng, H., Shao, W. 2006. High-level expression of an α-L-arabinofuranosidase from *Thermotoga maritima* in *Escherichia coli* for the production of xylobiose from xylan. Biotech. letters 28(5):351-356.

Yang, X., Ma, K. 2005. Purification and characterization of an NADH oxidase from extremely thermophilic anaerobic bacterium *Thermotoga hypogea*. Arch. Microbiol. 183: 331-337.

Yernool, A. D. 2000. Molecular and functional analysis of hemicellulase gene cluster from *Thermotoga neapolitana*. PhD thesis. The State University of New Jersey, New Brunswick. Rutgers.

Zverlov, V.V., Volkov, I.Y., Velikodvorskaya, T.V., Schwarz, W. H. 1997. *Thermotoga neapolitana* bglB gene upstream of lamA, encodes a highly thermostable β-glucosidase that is a laminaribiase. Microbiology 143: 3537–3542.