1

MICROCOPY RESOLUTION TEST CHART
NATIONAL BUREAU OF STANDARDS
STANDARD REFERENCE MATERIAL 1010a
(ANSI and ISO TEST CHART No. 2)

National Library
of Canada

Bibliothèque nationale
du Canada

Canadian Theses Service Service des thèses canadiennes

Ottawa, Canada
K1A 0N4

NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

**A STUDY OF THE HEMICELLULOLYTIC AND CELLULOLYTIC ENZYMES OF
THE THERMOPHILIC FUNGUS THERMOASCUS CRUSTACEUS 235E**

by

MICHELLE E.A. ILLING, B.Sc. (Hons.)

A thesis submitted to
the Faculty of Graduate Studies and Research
in partial fulfilment of
the requirements for the degree of

Master of Science

Department of Biology

Carleton University

Ottawa, Ontario

December, 1990

© Michelle E.A. Illing, 1991

National Library
of Canada

Bibliothèque nationale
du Canada

Canadian Theses Service Service des thèses canadiennes

Ottawa, Canada
K1A 0N4

The author has granted an irrevocable non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of his/her thesis by any means and in any form or format, making this thesis available to interested persons.

The author retains ownership of the copyright in his/her thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without his/her permission.

L'auteur a accordé une licence irrévocable et non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de sa thèse de quelque manière et sous quelque forme que ce soit pour mettre des exemplaires de cette thèse à la disposition des personnes intéressées.

L'auteur conserve la propriété du droit d'auteur qui protège sa thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

ISBN 0-315-68925-0

Canada

Carleton University
Ottawa, Canada K1S 5B6

Thesis contains black & white
photographs &/or explanatory tables
which when microfilmed may lose their
significance. The hardcopy of the
thesis is available upon request from
Carleton University Library.

The undersigned recommend to the Faculty of Graduate
Studies and Research acceptance of the thesis

"A STUDY OF THE HEMICELLULOLYTIC AND CELLULOLYTIC ENZYMES OF
THE THERMOPHILIC FUNGUS THERMOASCUS CRUSTACEUS 235E"

submitted by Michelle E.A. Illing, B.Sc. (Hons.)
in partial fulfilment of the requirements for
the degree of Master of Science

Thesis supervisor

Chair, Department of Biology

Carleton University

December, 1990

ABSTRACT

The controls of hemicellulase and cellulase production were found to be closely related in the cellulolytic and xylanolytic fungus Thermoascus crustaceus 235E. It produced up to 150 IU/mL of xylanase activity when grown on steam-exploded aspenwood-water solubles (SEA-WS), currently a waste product, thereby providing an inexpensive source for enzyme production.

An "enriched-xylanase mixture" was produced from the culture filtrate by concentration of the enzymes with a 10 kDa cut-off membrane, followed by a simple ammonium sulphate precipitation step. Finally, an inexpensive heat treatment procedure was performed based on the relative thermostability of the xylanase component in comparison to the endocellulase activity. The result was less than a 25% loss in xylanase activity, whereas endocellulase activity was reduced by 85%. The "enriched-xylanase mixture" exhibited a xylanase/CMCase ratio of 104 to 1, and had pH and temperature optima of pH 4.8 and 78 °C, respectively. The mixture was extremely thermostable, having a half-life of 28 days at 65 °C.

A β -xylosidase was also purified from T. crustaceus 235E. It was homogeneous by SDS-PAGE and had a specific activity of 16.8 IU/mg.

The procedure for the "enriched-xylanase mixture" was reproducible on three batches of T. crustaceus 235E, but subsequent batches did not exhibit similar heat stabilities. The implications of this irreproducibility and the possible future for T. crustaceus were discussed.

ACKNOWLEDGEMENTS

I would like to thank the Natural Science and Engineering Research Council for supporting my research through two postgraduate scholarships. I also appreciate Dr Yamazaki for acting as my supervisor at Carleton University. In addition, I am deeply indebted to Dr Neelin for all his support, enthusiasm and input into this thesis.

I appreciate Forintek Canada Corporation for allowing me to work in their labs and I would also like to thank Dr David Senior and Paul Mayers for their support during the research.

Suzanne Porter, Jan Beardall, Colyn Steeves, and Andra Dos all made my life much more fun, and Allan was always there when I needed him. Finally, my parents provided me with what no one else could.

TABLE OF CONTENTS

	page
Abstract	i
Acknowledgements	ii
Table of Contents	iii
List of Abbreviations	iv
List of Figures	v
List of Tables	vi
 Introduction	 1
 Chapter 1 - Production of hemicellulolytic and cellulolytic enzymes of <u>Thermoascus crustaceus</u> 235E	
1.1 Introduction	30
1.2 Methods and Materials	32
1.3 Results and Discussion	37
 Chapter 2 - Differential heat stabilities and heat inactivation studies of the enzymes of <u>Thermoascus</u> <u>crustaceus</u> 235E	
2.1 Introduction	57
2.2 Methods and Materials	59
2.3 Results and Discussion	62
 Chapter 3 - Purification and characterization of the endo- β - xylanase from <u>Thermoascus crustaceus</u> 235E	
3.1.1 Introduction	79
3.1.2 Methods and Materials	81
3.1.3 Results and discussion	86
 Chapter 4 - Loss of reproducibility of the heat inactivation step and a discussion of some possible explanations	
4.1 Introduction	108
4.2 Methods and Materials	110
4.3 Results and Discussion	110
 Conclusions	 117
 References	 123
 Appendix 1: Vogel's media	 134
Appendix 2: Mandel's media	135
Appendix 3: Lowry reagent, Dinitrosalicylic acid reagent	136
Appendix 4: Statistical equations	137
Appendix 5: Standard curves for the Dinitrosalicylic acid assay	138
Appendix 6: Chromatographic profile of endo- β -xylanase from <u>Thermoascus crustaceus</u> 235E on SP- Sephadex column	139

LIST OF ABBREVIATIONS

BSA	bovine serum albumin
CMC	carboxymethyl cellulose
CMCase	endocellulase activity (CMC as the substrate)
dH ₂ O	double distilled water
DNS	dinitrosalicylic acid
FP	filter paper
FPase	filter paper activity
IEA	International Energy Agency/Bioenergy Agreement
IEF	isoelectric focusing
IU/mL	International units per milliliter
NaAc	sodium acetate
pI	isoelectric point
rpm	rotations per minute
SEA-WI	steam-exploded aspenwood - water insolubles
SEA-WS	steam-exploded aspenwood - water solubles
SDS	sodium dodecyl sulphate
SALase	salicinase activity (β -glucosidase)
TMP	thermomechanical pulp
w/v	weight per unit volume

LIST OF TABLES

TABLE	DESCRIPTION	PAGE
1	Summarizing data for wood hemicelluloses	5
2	The composition of <u>Pinus radiata</u> and oat-spelts xylan	6
3	Characterized xylanases from various microorganisms	18
4	Enzyme activities in a representative batch of <u>Thermoascus crustaceus</u> 235E culture filtrate	31
5	Variations in the enzyme activities in the culture filtrates of <u>Thermoascus crustaceus</u> 235E grown in batch on 1% oat-spelts xylan	39
6	Characteristics of various preparations from a representative batch of <u>Thermoascus crustaceus</u> 235E	64
7	Summary of the purification of <u>Thermoascus crustaceus</u> 235E culture filtrate to yield an "enriched-xylanase mixture"	74
8	Summary of the purification of an endo- β -xylanase from <u>Thermoascus crustaceus</u> 235E	88
9	Properties of a β -xylosidase partially purified from <u>Thermoascus crustaceus</u> 235E Pellicon retentate	95
10	Enzyme activity on various substrates by <u>Thermoascus crustaceus</u> 235E "enriched-xylanase mixture"	102
11	Study of a method to store and transport the "enriched-xylanase mixture" from <u>Thermoascus crustaceus</u> 235E	107
12	Residual activities after heat treatment of various batches of 1.4 M ammonium sulphate precipitated <u>Thermoascus crustaceus</u> 235E Pellicon retentate	114

LIST OF FIGURES

FIGURE	DESCRIPTION	PAGE
1	The structure of various components of wood	2
2	Structures of hemicelluloses commonly found in wood species	3
3	Site of action of xylanases and cellulases on their substrates	14
4	Zymogram technique	25
5	Enzyme activities in <u>Thermoascus crustaceus</u> 235E produced over time on Mandel's media	40
6	Maximum enzyme activities in <u>Thermoascus crustaceus</u> 235E produced on Mandel's media	42
7	Ratio of xylanase/CMCase in <u>Thermoascus crustaceus</u> 235E	43
8	Enzyme activities in <u>Thermoascus crustaceus</u> 235E grown on 0.5% Solka floc	44
9	Enzyme activities in <u>Thermoascus crustaceus</u> 235E grown on 1% oat-spelts xylan + 0.5% steam-exploded aspenwood - water solubles	47
10	Enzyme activities in <u>Thermoascus crustaceus</u> 235E grown on 1% oat-spelts xylan + 1% steam-exploded aspenwood - water solubles	48
11	Enzyme activities in <u>Thermoascus crustaceus</u> 235E grown on 1% oat-spelts xylan + 1% glucose	50
12	Enzyme activities in <u>Thermoascus crustaceus</u> 235E grown on 1% oat-spelts xylan + 1% glucose	52
13	Maximum enzyme activity in <u>Thermoascus crustaceus</u> 235E grown with varying initial pH levels	53
14	SDS-PAGE of day 12 culture filtrates from <u>Thermoascus crustaceus</u> 235E pH study	54

FIGURE	DESCRIPTION	PAGE
15	Residual enzyme activity in <u>Thermoascus crustaceus</u> 235E heated at 70 °C	66
16	Residual activities in enzyme preparations from <u>Thermoascus crustaceus</u> 235E (batch #5) heated at 72.5 °C	67
17	Ratio of xylanase/CMCase in batch #5 Pellicon retentate from <u>Thermoascus crustaceus</u> 235E heated at 72.5 °C	68
18	Residual enzyme activity in <u>Thermoascus crustaceus</u> 235E Pellicon retentate heated at various temperatures	70
19	Residual enzyme activity in <u>Thermoascus crustaceus</u> 235E Pellicon retentate after precipitation with ammonium sulphate	73
20	Residual enzyme activity in various <u>Thermoascus crustaceus</u> 235E Pellicon retentates heated at 72.5 °C	75
21	Residual enzyme activities in <u>Thermoascus crustaceus</u> 235E Pellicon retentate pre-precipitated with ammonium sulphate, then heated at 72.5 °C	76
22	IEF gel of various stages in the purification of endo- β -xylanase from <u>Thermoascus crustaceus</u> 235E	78
23	Chromatographic profile of <u>Thermoascus crustaceus</u> 235E Pellicon retentate on SP-Sephadex C50 column	83
24	IEF gel of the various stages in the purification of endo- β -xylanase from <u>Thermoascus crustaceus</u> 235E	89
25	IEF gel of the various stages in the purification of endo- β -xylanase from <u>Thermoascus crustaceus</u> 235E	90
26	Oat-spelts xylan zymogram of the IEF gel in Figure 25	91
27	Chromatographic profile of concentrated pooled fractions (48-64) from SP-Sephadex C50 column on DEAE-Sephadex A-50 column	93

FIGURE	DESCRIPTION	PAGE
28	"Native gel" of fractions from the DEAE-Sephadex column in the purification of endo- β -xylanase	94
29	Optimum temperature for the "enriched-xylanase mixture"	97
30	Optimum pH for the "enriched-xylanase mixture"	98
31	Action of the "enriched-xylanase mixture" on oat-spelts xylan over time at various temperatures	99
32	Residual enzyme activity in the "enriched-xylanase mixture" over time at various temperatures	104
33	Ratio of residual xylanase/CMCase activity in the "enriched-xylanase mixture" over time at various temperatures	105
34	Residual enzyme activity in <u>Thermoascus crustaceus</u> 235E batch #7 heated at 72.5 °C	111
35	Residual enzyme activity in <u>Thermoascus crustaceus</u> 235E batch 7; ammonium sulphate precipitated and then heated at various temperatures	113
36	IEF gel comparing various Pellicon retentates from <u>Thermoascus crustaceus</u> 235E	115

INTRODUCTION

Plant cell walls are complex materials made up of (a) cellulose, a linear polymer of glucose residues linked in the β -1,4 configuration; (b) hemicellulose, a linear or slightly branched non-cellulosic polysaccharide, possessing side chains of 4-*O*-methyl-D-glucuronic acid and L-arabinose, which is also linked in the β -1,4 configuration; and (c) lignin, which is a polymer of *p*-hydroxyphenylpropane units connected by C-C and C-O-C linkages (Figure 1; Woodward, 1984).

The most abundant renewable polysaccharide in nature is cellulose. It is a simple polymer of glucose molecules that contains amorphous and crystalline regions, the latter being less susceptible to enzymatic attack. After cellulose, hemicellulose is the most abundant renewable polysaccharide. The hemicelluloses have been defined as those polysaccharides that are soluble in alkali that are associated with the cellulose of the plant cell wall (Timmel, 1967). More recently, they have also been found to be affiliated with lignin (Eriksson *et al.*, 1980). Unlike cellulose, hemicelluloses are quite diverse and contain a variety of methylated and acetylated sugars (see Figure 2 for examples).

The hemicelluloses from vascular land plants are built up from relatively few sugar residues, the most common of which are D-xylose, D-mannose, D-galactose, D-glucose, L-arabinose, 4-*O*-methyl-D-glucuronic acid, D-galacturonic acid, and D-glucuronic acid (Timmel, 1967). These polysaccharides are

FIGURE 1 - The structure of the various components of wood
(A) cellulose polymer (B) portion of a D-xylan molecule
(C) representative portion of lignin.

The diagram illustrates the chemical structure of cellulose, a linear polysaccharide composed of glucose units. Each glucose unit is shown in its cyclic Haworth projection. The units are connected by β -1,4 glycosidic bonds, where the C1 of one glucose unit is linked to the C4 of the adjacent unit. The structure shows alternating hydroxyl groups and hydroxymethyl groups (CH_2OH) at the C2 and C6 positions, which are characteristic of the β -D-glucopyranose units in cellulose.

[illegible]

The chemical structure is a complex polycyclic molecule with the following features:

- Ring A:** A benzene ring with a hydroxyl group (OH) at the bottom position and a methoxy group (OCH₃) at the bottom-right position. It is connected to ring B via an ether linkage.
- Ring B:** A benzene ring with a methoxy group (OCH₃) at the bottom position and a vinyl group (CH=CH-CHO) at the bottom-right position. It is connected to ring A via an ether linkage and to ring C via an ester linkage.
- Ring C:** A benzene ring with a methoxy group (OCH₃) at the bottom-right position and a hydroxymethyl group (HOCH₂) at the top position. It is connected to ring B via an ether linkage and to ring D via an ester linkage.
- Ring D:** A benzene ring with a methoxy group (OCH₃) at the bottom-right position and a hydroxymethyl group (HOCH₂) at the top position. It is connected to ring C via an ester linkage and to ring E via an ether linkage.
- Ring E:** A benzene ring with a methoxy group (OCH₃) at the bottom position and a hydroxymethyl group (HOCH₂) at the top position. It is connected to ring D via an ether linkage and to a central ether bridge via an ether linkage.
- Central Ether Bridge:** A five-membered ring containing two oxygen atoms, connecting ring E to ring F.
- Ring F:** A benzene ring with a methoxy group (CH₃O) at the top-left position and a hydroxymethyl group (HOCH-CH₂OH) at the bottom position. It is connected to ring E via an ether linkage and to ring I via an ether linkage.
- Ring I:** A benzene ring with a methoxy group (OCH₃) at the bottom-right position and a hydroxymethyl group (HOCH-CH₂OH) at the bottom position. It is connected to ring F via an ether linkage and to ring J via an ether linkage.
- Ring J:** A benzene ring with a methoxy group (CH₃O) at the bottom position and a hydroxymethyl group (CH₂OH) at the top position. It is connected to ring I via an ether linkage and to ring K via an ester linkage.
- Ring K:** A benzene ring with a methoxy group (CH₃O) at the bottom-left position and a hydroxymethyl group (CH₂OH) at the top position. It is connected to ring J via an ester linkage and to ring L via an ether linkage.
- Ring L:** A benzene ring with a methoxy group (CH₃O) at the bottom-left position and a hydroxymethyl group (CH₂OH) at the top position. It is connected to ring K via an ether linkage.
- Ring G:** A benzene ring with a methoxy group (CH₃O) at the top-left position and a hydroxymethyl group (HOCH-CH₂OH) at the bottom position. It is connected to ring H via an ether linkage.
- Ring H:** A benzene ring with a methoxy group (OCH₃) at the top-right position and a hydroxymethyl group (HOCH-CO-CH₃) at the bottom position. It is connected to ring G via an ether linkage.

FIGURE 2 - Structures of hemicelluloses, commonly found in wood species (from Dekker, 1985).

β -D-Xylp	= β -D-xylanpyranose
4-O-Me- α -D-GlcpA	= 4-O-Methyl- α -D-Glucuronic Acid
β -D-Manp	= β -D-Mannopyranose
L-Araf	= L-Arabinofuranose
α -D-Galp	= α -D-Galactopyranose

$$\begin{array}{ccc} \rightarrow 4) -\beta-D-Xylp-(1 \rightarrow 4) -\beta-D-Xylp-(1 \rightarrow 4) -\beta-D-Xylp-(1 \rightarrow 4) -\beta-D-Xylp-(1 \rightarrow & & \\ | & & | \\ 3 & & 2 \\ | & & | \\ \text{Acetyl} & & 4-O-Me-\alpha-D-Glc pA \end{array}$$
$$\rightarrow 4)-\beta-D-Glcp-(1 \rightarrow 4)-\beta-D-Manp(1 \rightarrow 4)-\beta-D-Manp-(1 \rightarrow 4)-\beta-D-Manp(1 \rightarrow$$
$$\begin{array}{ccc} \rightarrow 4)-\beta-D-Xylp(1\rightarrow 4)-\beta-D-Xylp(1\rightarrow 4)-\beta-D-Xylp(1\rightarrow 4)-\beta-D-Xylp(1\rightarrow & & \\ & \begin{array}{c} 2 \\ | \\ 1 \end{array} & \begin{array}{c} 3 \\ | \\ 1 \end{array} \\ 4-(1-Me-\alpha-D-\alpha-D-GlcpA & & \alpha-1-Araf \end{array}$$
[illegible]
$$\begin{array}{ccccc}
 \rightarrow 3)\text{-}\beta\text{-D-Galp-(1}\rightarrow 3)\text{-}\beta\text{-D-Galp-(1}\rightarrow 3)\text{-}\beta\text{-D-Galp-(1}\rightarrow 3)\text{-}\beta\text{-D-Galp-(1}\rightarrow 3)\text{-}\beta\text{-D-Galp-(1}\rightarrow & & & & \\
 \downarrow 6 & \downarrow 6 & \downarrow 6 & \downarrow 6 & \downarrow 6 \\
 \beta\text{-D-Galp} & \beta\text{-D-Galp} & \beta\text{-D-Galp} & R & L\text{-Araf} \\
 \downarrow 6 & \downarrow 6 & \downarrow 6 & & \downarrow \\
 \beta\text{-D-Galp} & \beta\text{-D-Galp} & \beta\text{-D-Galp} & & \beta\text{-L-Araf}
 \end{array}$$
$$\begin{array}{c} \rightarrow 4)-\beta\text{-D-Gal}p(1\rightarrow 4)-\beta\text{-D-Gal}p-(1\rightarrow 4)-\beta\text{-D-Gal}p-(1\rightarrow 4)-\beta\text{-D-Gal}p-(1\rightarrow \\ | \\ 6 \\ | \\ \beta\text{-D-Gal}p\Lambda \end{array}$$

3

normally named for their most frequently occurring residue(s). Therefore, the hemicelluloses include non-cellulosic β -D-glucans, polygalacturonans (pectic substances), and several complex heteropolysaccharides such as those enriched in galactose (e.g. arabinogalactans), mannose (galactogluco- and glucomannans), and xylose (arabinoglucurono- and glucuronoxylans; Dekker, 1985). Table 1 contains a summary of hemicelluloses found in various types of wood.

The β -1,4-xylans have been characterized at two levels of complexity: the isolated polysaccharide and its associations with other lignocellulosic components. These polysaccharides are generally heteropolymers (with more than one type of substituent) that are highly polydisperse (i.e. varying degree of polymerization). The most common residues found on the D-xylosyl residues are acetyl, arabinosyl, and glucuronosyl residues. Table 2 indicates the chemical composition of two commonly used xylans: oat-spelts xylan and Pinus radiata xylan. The frequency and composition of the isolated xylans depend on their source and their method of isolation (c.f. Aspinall, 1980; Brice and Morrison, 1982; Fincher and Stone, 1981). Homoxylans, which consist exclusively of xylosyl residues, have been isolated from esparto grass and tobacco (Wong et al., 1988).

The two major forms of xylan in wood are the acetylated xylan of hardwoods and the arabinoxylan of softwoods. These two types of xylans have single 4-O-methyl- α -D-glucuronic acid

TABLE 1 - Summarizing data for wood hemicelluloses (adapted from Timmel, 1967)

Polysaccharide	Occurrence	Percent of wood	Composition	Parts	Linkages	Linear or branched
<i>O</i> -Acetyl-4- <i>O</i> -methyl-glucurono-xylan	Hardwoods	10...35	β -D-Xylp 4- <i>O</i> -Me- α -D-GlucA <i>O</i> -Acetyl	10 1 7	1 $\rightarrow$ 4 1 $\rightarrow$ 2	Slightly branched
Glucomannan	Hardwoods	3...5	β -D-Manp β -D-Glup	1...2 1	1 $\rightarrow$ 4 1 $\rightarrow$ 4	Undecided
Arabino-4- <i>O</i> -methyl-glucurono-xylan	Softwoods	10...15	β -D-Xylp 4- <i>O</i> -Me- α -D-GlucA 1-Araf	10 2 1.3	1 $\rightarrow$ 4 1 $\rightarrow$ 2 1 $\rightarrow$ 3	Undecided
Galacto-glucomannan (water-soluble)	Softwoods	5...10	β -D-Manp β -D-Glup α -D-Galp <i>O</i> -Acetyl	3 1 1 0.24	1 $\rightarrow$ 4 1 $\rightarrow$ 4 1 $\rightarrow$ 6	Probably branched
Galacto-glucomannan (alkali-soluble)	Softwoods	10...15	β -D-Manp β -D-Glup α -D-Galp <i>O</i> -Acetyl	3 1 0.1 0.24	1 $\rightarrow$ 4 1 $\rightarrow$ 4 1 $\rightarrow$ 6	Probably branched
Arabinogalactan	Larch wood	10...20	β -D-Galp L-Araf β -D-Arap β -D-GlucA	6 2/3 1/3 Few	1 $\rightarrow$ 3, 1 $\rightarrow$ 6 1 $\rightarrow$ 6 1 $\rightarrow$ 3 1 $\rightarrow$ 6	Heavily branched

β -D-Xylp = β -D-xylanpyranose
 4-*O*-Me- α -D-GlucA = 4-*O*-Methyl- α -D-Glucuronic Acid
O-Acetyl = *O*-Acetyl group
 β -D-Manp = β -D-Mannopyranose
 β -D-Glup = β -D-Glucopyranose
 L-Araf = L-Arabinofuranose
 α -D-Galp = α -D-Galactopyranose

TABLE 2 - The composition of Pinus radiata xylan and oat-spelts xylan (adapted from data collected by H. Brownell at Forintek Canada Corporation)

	<u>P. radiata</u> xylan ^a	oat-spelts xylan ^b g/100g substrate
Xylan	-- ^c	77.7
Xylose	71.2	--
Glucan	--	2.6
Glucose	3.1	--
Galactose	4.7	--
Arabinose	10.4	~2-12
Mannose	1.6	--
4-O-Me-Glucuronic acid	9.0	--
Lignin	--	3.7
unaccounted	0.0	~4-14

^a hardwood xylan preparation from Australia

^b commercially available xylan preparation (Sigma) used throughout the thesis

^c -- not assayed

substituents, occurring on approximately 10 and 18% of their xylosyl residues, respectively. The acidic unit is attached to the C-2 of the xylosyl residues, whereas O-acetyl and α -L-arabinofuranosyl substituents tend to occur on C-3 (Timmel, 1967). None of the xylosyl residues have more than one branch. Acetyl groups occur on approximately 70% of the hardwood xylosyl residues, whereas the arabinosyl substituents are found on approximately 12% of the xylosyl residues in softwoods. Both hardwood and softwood xylans have a reducing end consisting of a rhamnosyl, a galacturonosyl, and a xylosyl residue (Andersson *et al.*, 1983; Johansson and Samuelson, 1977). The xylans in grasses occur normally as arabinoxylans, and other xylans have branches containing various combinations of arabinosyl, galactosyl, glucuronosyl, and xylosyl residues (Aspinall, 1980; Wilkie, 1979; Wilkie and Woo, 1977).

Distribution of the xylans *in situ* is dependent on the species and on the cell growth and differentiation (Wong *et al.*, 1988). In secondary plant cell walls, xylan appears to be a major interface between lignin and other carbohydrates. It appears as a major component in many isolated phenolic-carbohydrate complexes, and probably it is linked covalently to the phenolic residues via its arabinosyl and glucuronosyl residues. The phenolic compounds isolated with the carbohydrates may be linked to lignin fragments, but they may also cross-link xylan molecules and xylan to other polysaccharides (Eriksson *et al.*, 1980).

The covalent linkages that xylans form with other molecules are often difficult to assess. During the isolation procedure, some of these bonds are selectively broken while others may be formed. The alkaline-labile acetyl substituents and the reducing end groups have been recognized in xylans, but as much as 50% of the alkaline-labile xylosidic linkages remain to be identified in some plant materials (Johansson and Samuelson, 1977). Some researchers have made use of "characterized" enzymes to hydrolyse and determine the cross-linkages between lignocellulosic components. This research often gives more reliable results than chemical hydrolysis studies, but the results are suspect because some xylanases have transferase activity; one xylanase is even known to transfer xylosyl residues to cellobiose (Wong *et al.*, 1988).

Xylan can also be affiliated with other carbohydrates in non-covalent ways. Xylan tends to adsorb onto cellulose and aggregate with other hemicellulose components. This is due likely to hydrogen-bonding interactions. Selective removal of xylan from delignified wood fibre results in a substantial increase in fibre porosity, suggesting that xylan plays a major role in cell wall cohesion (Mora *et al.*, 1986). Both covalent and non-covalent associations can contribute to the structural integrity of lignocellulose, whose current concept is that of cellulose fibres embedded in a lignin-polysaccharide matrix (Wong *et al.*, 1988).

Xylose is also found in a variety of molecules other than

xylans. For example, xyloglucans have a β -1,6-glucan backbone substituted with α -1,6-xylosyl residues. In addition, algae containing β -1,3-linked xylans and a β -1,3: β -1,4-linked xylan have been found (Aspinall, 1980). Xylosyl residues occur in many different molecules and play various structural and functional roles.

Since xylans make up part of the structural support system of plants, it stands to reason that they would be at least partially resistant to microbial attack. There is a distinct relationship between the structure and the function of the components of the cell walls. Both the substituents on the xylan molecule's backbone as well as its lignocellulosic associations prevent the access to the xylosidic residues by enzymes.

A variety of studies that demonstrate that the substituents limit the enzymatic hydrolysis of the xylan. Some purified xylanases which cleave xylotriose are unable to hydrolyse arabino-xylotriose, indicating that the arabinose group is somehow protecting the xylan backbone (Takenishi and Tsujisaka, 1975). It has been posited that the shortest acidic- and arabino-xylooligosaccharides that have been found in xylan hydrolysates are the "limit dextrans" of the xylan preparation. Thus, the residual xylooligosaccharide is protected from further enzymatic attack (c.f. Brillouet, 1987; Comtat and Joselean, 1981; Takenishi and Tsujisaka, 1975).

On the other hand, these substituents may play a

beneficial role in the formation of the enzyme-substrate complex, thereby enhancing hydrolysis of the xylan. One xylanase from Ceratocystis paradoxa and two from Aspergillus niger were found to have a preference for hydrolysing branched xylans (Dekker and Richards, 1975; Frederick et al., 1985). The validity of these conclusions is difficult to assess because the efficiency of hydrolysis increases as the solubility increases, and the solubility of the xylan is directly proportional to its degree of branching.

Enzymatic hydrolysis of lignocelluloses can be enhanced by the use of various pretreatment methods that expose more of the polysaccharide (Chang et al., 1981). Delignified wood has been selectively hydrolysed for the xylan component with purified xylanases, with an enriched xylanase preparation, and with crude enzyme in which the cellulases had been inhibited (Mora et al., 1986; Paice and Jurasek, 1984). Complete removal of the xylosyl residues was not achieved under any of these conditions. These findings suggest that the residual xylosyl residues may be inaccessible for a number of reasons, including the possibilities that the xylans may contain substituents or that they may be encased by other polysaccharides (Wong et al., 1988). When xylan or mannan was selectively removed from cellulose, through the use of enzymes, the efficiency of the cellulose hydrolysis increased. However, when the same fibres were enzymatically pre-treated for the removal of cellulose, there was no significant

increase in the efficiency of the hemicellulose hydrolysis (Sinner et al., 1979). It has been postulated that the xylan is more important in fibre cohesion and its selective removal increases the fibre porosity of the material. It has be shown that increases in fibre porosity are positively correlated with cellulose hydrolysis (c.f. Wong et al., 1988).

The hydrolysis of xylan has been studied less than that of cellulose. This is primarily due to the fact that the products of xylan breakdown are less important and more heterogeneous than the glucose produced by the breakdown of cellulose. The heterogeneous nature of the xylan breakdown products makes no yield of a single product obtainable (Reilly, 1981). However, the xylan must also be used with the cellulose to make the cost of the products competitive with the same products from other sources (Bickerstaff, 1987).

As is the case with cellulose, there are two ways to break down xylan: acidic and enzymatic hydrolysis. Acid hydrolysis was the first method to be used on an industrial scale with first cellulose and then xylan. This method is relatively fast and easy, and if the conditions are sufficiently mild, the xylan may be solubilized and separated from the cellulose without significant attack on the latter. However, acid hydrolysis is not specific and many products, difficult to separate, may be formed. In addition, acid hydrolysis is rarely complete unless the conditions are sufficiently harsh that by-products are formed (Dekker, 1985).

Enzymatic hydrolysis of polysaccharides has the advantages of being more specific and less harsh than acid hydrolysis. To date, there has not been enough research carried out on these enzymes for their use in industry. Their drawbacks are that the production costs are still high, the product yield is low, and the products are mixed. Some of the lignocellulosic enzymes yield specific products, while others have less substrate specificity. While many of these enzymes were previously thought to be too specific or too general in the nature of their hydrolysis patterns, recent research has shown that they may have other uses (c.f. Saddler et al., 1988).

The xylanases may be compared on many levels to the cellulases. While there are many similarities, there are also some differences between these families of enzymes. The differences are caused by the greater structural and chemical heterogeneity of xylan compared to cellulose, the latter being unbranched and composed completely of glucose. As most hydrolases are quite specific as to the bonds they attack, it might be expected that there would be more xylanases than cellulases. There are at least six types of xylanases that have been characterized (c.f Bailey and Poutanen, 1989; Dekker, 1985; Reilly, 1981):

(1) β -Xylosidases, which break down short xylooligosaccharides to xylose and have substantial transferase activity;

(2) Exoxylanases, producing xylose at high rates from xylan, but only at low rates from short xylooligosaccharides. These enzymes invert product configuration unlike β -xylosidase, and have little or no transferase activity. As yet there is little or no conclusive evidence of an exoxylanase that produces mainly xylobiose;

(3) Endoxylanases of four types:

(a) Those that cannot cleave L-arabinosyl initiated branch points and produce mainly xylobiose and xylose as final products. They are generally active on xylooligosaccharides as short as xylotriose, but at much lower rates than on larger substrates.

(b) Those that cannot cleave branch points and produce mainly oligosaccharide fragments larger than xylobiose. They are generally inactive on xylotetraose and smaller substrates.

(c) Those that can cleave branch points and produce mainly xylobiose and xylose.

(d) Those that can cleave branch points and produce mainly xylooligosaccharides of intermediate size.

In summary, there are three main types of xylanases and cellulases: β -xylosidases/ β -glucosidases (otherwise known as xylobioses and cellobioses); exoxylanases/exocellulases; and endoxylanases/endocellulases. A schematic diagram of their method of substrate hydrolysis is found in Figure 3. It is clear that these two families of enzymes display similar patterns of hydrolysis. It is important to note that both the

FIGURE 3 - Site of action of xylanases and cellulases on their substrates. Xylans and other hemicelluloses are not simple xylose polymers, but complex molecules made up of many different sugars and side-groups. Note that the existence of "exocellulases" is a debated issue, and as of yet, there is no direct evidence for the equivalent "exoxylanase". It is thought that the endoxylanase does the work of both enzyme functions (for discussion, see Wong et al., 1988).

method of purification used and the degree of purification of the xylan will affect its hydrolysis by various enzymatic or chemical procedures (Timmel, 1967).

Recently, there have been some revisions to the list of xylanases. Some of these include the α -glucuronidases, the α -L-arabinofuranosidases, and the acetylerases (or the acetyl xylan esterases; Biely *et al.*, 1988; Karimiri and Ward, 1989).

The occurrence of both 1,3- and 1,4- β -xylanases is reported to be widespread. They are reported to occur in bacteria from marine and terrestrial environments, fungi (the phytopathogens and the rot fungi being the most active producers of hemicellulases), rumen microorganisms (bacteria and protozoa), caecal bacteria of ruminants and nonruminants, yeasts, xylophagous insects, certain mollusca (e.g. snails), crustaceans, marine algae, and in germinating seeds of land plants (Dekker, 1985; Dekker and Richards, 1976; Woodward, 1984). No vertebrates have been found to possess hemicellulases in their alimentary canals. These animals rely instead upon the hemicellulases of microbial origin in their digestive tracts, e.g. bacteria and protozoa in the rumen (Dekker and Richards, 1976), bacteria in the caecum (Reilly, 1981), and bacteria in the human colon (Dekker, 1985).

Most of the bacteria, fungi, and yeasts producing hemicellulases secrete the enzyme extracellularly, although there are some reports of intracellular localization (Ishihara et al., 1978). Cell-bound hemicellulases have also been described (Dekker, 1985).

The xylanases play an important role in the rate of soil plant litter decomposition. The enzyme has been isolated from coniferous and deciduous leaf litters and has been shown to be largely soluble. Typical xylanase activities measured in 18 types of coniferous leaf litter range from 24 to 283 μmol of reducing sugar formed per hour per gram of leaf litter when 6% xylan is used as the substrate (Woodward, 1984).

Xylanases are also involved in wood and bark degradation. One of the most common terrestrial fungi isolated from wood chips is Aspergillus fumigatus. There are also several marine fungi which have been shown to produce cellulase, mannanase, and xylanase, which degrade the lignocellulosic material of submerged wood (Henningsson, 1978). The main function of the xylanases in biodegradation, therefore, is to provide a source of metabolizable energy (Woodward, 1984).

Xylanases have been implicated in plant diseases caused by bacterial plant pathogens. In a study of plant pathogens and saprophytic bacteria, it was shown that several bacterial species, including Achromobacter and Pseudomonas spp, were able to degrade xylan (Maino et al., 1974). Xylanase has also been implicated in bacterial leaf spot of alfalfa caused by

Xanthomonas alfalfae. However, xylanase activity does not appear to be a common characteristic of plant pathogenic bacteria (Woodward, 1984).

Xylanases have also been found in invertebrates, including crustaceans and herbivorous gastropods. The enzyme presumably functions in the digestion of dietary vegetation (Dekker and Richards, 1976). Other studies have implicated a role for hemicellulose-degrading enzymes during the over-ripening period of fruit. In addition, many germinating seeds make use of hemicellulases to proceed from one stage of growth to another (Woodward, 1984).

Xylanases are produced commercially in a number of microorganisms including bacteria and fungi species (see Table 3). Hemicellulases of microbial origin have been reported to be produced both constitutively (produced irrespective of growth substrate) and inductively (produced when grown on hemicellulose or similar inducers). Cellulases, by analogy, are inductive enzymes and are produced only when the microorganism is grown on cellulose or cellobiose (Dekker, 1985; Dekker and Richards, 1976; Woodward, 1984).

Many of the bacterial genes encoding for lignocellulosic enzymes have been cloned into various expression systems (c.f. Bertrand et al., 1989; Yang et al., 1988). This procedure often facilitates the purification of protein in question. In addition, cloning can allow for the production of much higher quantities of that enzyme (e.g. by inserting the gene next to

TABLE 3 - Characterized xylanases from various microorganisms (adapted from Wong et al., 1988)

Species	Strain	Enzyme	MW (10 ³) ^a	pI	Optimum	
					pH	Temp (°C)
<i>Bacillus</i> sp.	C-59-2			6.3	6-8	60
	11-15		56.0		4.0	80
	C-125	N	16.0		6-7	70
		A	43.0		6-10	70
	W1	I	21.5	8.5	6.0	65
		II	49.5, [48.5]	3.6	7-9	70
	W2	I	22.5	8.3	6.0	65
		II	50.0, [51.0]	3.7	7-9.5	70
<i>C. acetobutylicum</i>	ATCC 824	B	29.0, [29.5]	8.5	5.5-6	60
		A	65.0, [63.0]	4.4	5.0	50
	P262	— ^c	28.0	10.0	6.0	37-43
<i>C. stercorarium</i>		A	44.0	4.5	6.5	75
		C	62.0	4.4	6.0	75
		B	72.0	4.4	6-7	75
<i>T. harzianum</i>	ES6	A	20.0, [8-11.5]	9.4	5.0	50
		b	22.0	8.5	4.5-5	45-50
		C	29.0, [25.0]	9.5	5.0	60-65
		D ^c		5.5		
		E ^c		5.0		
<i>T. longii</i>	IMI 73022	2	17.7	7.3	4.5-5.5	50
		1	29.0	7.2	4.5-6	60
<i>T. lignorum</i>			20.0		6.5	
			21.0		3.5	
<i>T. pseudokoningii</i>	C-3		15.0, [6.0], [20-22]	9.6	5.0	
<i>T. reesei</i>	QM9414	3	[(8.5)]	10.3		
		4	[(11.7)]	8.6		
		2	[(40.7)]	8.5		
		1	[(14.4)]	6.6		
<i>Aspergillus</i> sp.	Amano ^c		[21.9]	6.7	5.0	60
<i>A. niger</i>		I			4.5-5	45-50
		II			5.5-6	45-55
		I			5.5	
		II			5.0	
		III			3.5	
	14		33.0, [24.0], [24.7]	4.2	4.0	50
			[41.0]			
	11	IIA	[31.0]		4.0	50
		IIIB/IIIC	[31.0]		4.0	50
		IID	[31.0]		6-6.5	50
		IA	[50.0]		5.5-6	65-80
		IB	[50.0]		4-4.5	65-80
	Rhozyme ^c	7II	[14.0], [13.5]	9.0	5.5	45
		7I	[12.0], [13.5]	8.6	6.0	45

^a MW estimates were by SDS-PAGE. [], Gel filtration. (), ultracentrifugation.

^c Enzyme had not been purified

an inducible promoter). Most lignocellulosic enzymes are produced extracellularly, but occasionally the cloned enzyme does not get secreted. This can cause problems in the purification procedure. Also, many fungal and yeast enzymes cannot be properly expressed in bacterial systems due to the differences between eukaryotes and prokaryotes. And finally, fungal cultures are usually used for their enzyme production due to the very high yields that can be obtained (c.f. Senior, 1989).

Xylanases are produced even when the carbon source in the medium is not xylan. This constitutive production can be enhanced by xylan as well as other compounds (Biely *et al.*, 1988). Xylobiose induces xylanase in Cryptococcus albidus and is taken up by glucose-grown cells prior to the appearance of the enzyme. The uptake of xylobiose appears to be caused by an induction of an active transport system known as β -xyloside permease (Kratky and Biely, 1980). The non-metabolizable methyl- β -D-xyloside possesses a higher inducing ability than xylan (Nakanishi *et al.*, 1987). The researchers concluded that, although uptake of this inducer by the mycelia was observed, the uptake process was one of diffusion and did not involve a specific transport system.

Studies of xylanase production in Schizophyllum commune have shown that good inducers of the enzyme are: xylan (1%); thiocellulose (0.5 mM); carboxymethyl cellulose (1%); cellobiose (1 mM); and lactose (2.5 mM; Rho *et al.*, 1982).

These compounds are closely related to naturally occurring wood polysaccharides which are used by the fungus as carbon sources. Other substrates besides sugars have been shown to stimulate xylanase production in microorganisms. For example, at low concentrations (0.1%) the surfactants sucrose monopalmitate and Tween 80 have been shown to increase xylanase concentration fourfold in Aspergillus fumigatus QM45h. In addition, fatty acids such as oleic acid have been shown to increase xylanase production 1.5 fold when used at concentrations of 0.1% (Reese and McGuire, 1969).

The endo- β -xylanases are generally extracellular enzymes, especially when xylan is used as the carbon source. However, sometimes the enzymes remain membrane-bound, specifically when the organism is grown on glucose as the sole carbon source (Woodward, 1984). Biely (1985) showed that induction of β -xylanase of C. albicans by methyl- β -D-xyloside began only after all the glucose in the medium was utilized. Glucose was not able to repress xylanase formation entirely in induced cells, and it was concluded that the repression of β -xylanase synthesis in non-induced cells is primarily due to a block by glucose in the transport of the inducer to the cells.

Xylanase enzymes are purified by using standard procedures. The culture filtrate, which is normally used as the starting medium, is usually concentrated prior to the chromatographic purification procedures. Some of these concentration techniques include freeze drying, filtration

with molecular sieves (i.e. membranes of specific porosity), or flash evaporation under reduced pressure. Alternatively, the protein component of the filtrate containing xylanase is precipitated with cold ethanol (-18 °C) or with ammonium sulphate at 55-90% saturation. The culture filtrate has also been chromatographed directly. Further purification of the xylanase activity is achieved via a combination of chromatographic procedures, including gel filtration and ion exchange chromatography. Preparative electrophoresis on polyacrylamide gel and isoelectric focusing using ampholite carriers with a pH range of 3-6 have also been used (Scopes, 1987; Wood and Kellogg, 1988).

Multiple xylanases have been reported in numerous microorganisms (Dekker, 1985). For example, five different xylanases have been purified from the culture filtrate of Aspergillus niger (Frederick et al., 1985), three from Clostridium stercorarium (Bérenger et al., 1985), and three from Streptomyces spp. (Marui, et al., 1985). The extent of xylanase multiplicity in microorganisms remains to be answered, particularly because a zymogram technique (see below) has detected five major and ten minor xylanases in the culture filtrate of A. niger 14 and three major and ten minor xylanases in Cellulysin, a commercial enzyme from Trichoderma viride (Biely et al., 1985b). The relevance of these minor xylanases may continue to be a mystery as purification techniques favour the major enzymes. Minor xylanases may have

functions that are not required in large quantities (e.g. hydrolysis of linkages which occur infrequently). They may not be produced in substantial quantities under the given growth conditions, or they may be lost from the culture filtrate due to degradation or adsorption onto insoluble growth substrates (Wong *et al.*, 1988). Alternatively, the major xylanases may be over-produced under the growth conditions.

Regulation, substrate cross-specificity, and the posttranslational modifications of the multiple xylanases must be considered because each of these factors can result in the occurrence of multiple xylanolytic enzymes in organisms. In fungi, some of the multiple xylanases may also be allozymes, products of different alleles of the same gene. At one extreme, multiple xylanases may be artifacts arising from the degradation of microbial culture filtrates. On the other hand, each of the multiple xylanases may be a distinct gene product produced by the microorganism to enhance its hydrolysis of xylan (Dekker, 1983; Tan *et al.*, 1987a; Wong *et al.*, 1988).

Most of the available information on the properties of xylanases have come from studies on fungal and bacterial enzymes. Xylanases generally possess pH optima between 4 and 7 and have stable activities between pH 2 and 9. The mesophilic microorganisms grow optimally at temperatures that range from 15 to 40 °C. These organisms produce xylanases whose temperature optima are between 40 and 50 °C with thermal stability in the same range. Thermophilic microorganisms grow

at temperatures above 40 °C and produce enzymes whose temperature optima and stability can range to 80 to 90 °C (Rambosek and Leach, 1987).

A wide range of thermophilic and thermo-tolerant fungi can cause spoilage of bagasse, grain, groundnut, hay, palm kernels, peat, wood chips and other agricultural crops. Thermophilic fungi are also reported to be involved in the processing of agricultural produce such as cocoa, guayule and tobacco, and in the biodegradation of lignocellulose, animal and other wastes in controlled conditions (Sharma, 1989).

Quantitative analysis of xylanase activity ranges from colorimetric to chromatographic techniques. Hemicelluloses from different plant sources have been used for these assays. The most commonly used substrates are arabinoxylan, arabinoglucuronoxylan, glucuronoxylan, and xylan. Soluble xylan derivatives that have been used include O-(carboxymethyl)-D-xylan and O-(hydroxyethyl)-D-xylan (Ghose and Bisaria, 1987). Assay procedures usually involve measurement of the increase in reducing end groups by Nelson-Somogyi or Dinitrosalicylic acid colorimetric reagents (Khan *et al.*, 1986). In addition, pentose analysis can be monitored based on the formation of furfural from pentoses in acetic acid containing thiourea at 70 °C, and the reaction of the formed furfural with p-bromoaniline acetate to form a pink-coloured product (Deschatelets and Yu, 1986).

HPLC analysis of hydrolysates gives the most

representative values of the hydrolytic potential of cellulases (Schwald *et al.*, 1988). However, the sugar standards for the analysis of xylanases are difficult to acquire and sample preparation is tedious and costly. A recently developed method for lignocellulolytic enzyme quantification and qualitation is known as the zymogram technique (Figure 4). This procedure is becoming more popular as the problems with the various systems are being eliminated (c.f. Biely *et al.*, 1985a; Manjrekar and Krishnan, 1987; Schwarz *et al.*, 1987; Sengupta *et al.*, 1987).

The actual and potential uses of xylanases depend on their ability to degrade xylans. They are used to liquefy coffee mucilage for the manufacture of liquid coffee (Wiseman, 1975). Other potential uses include wine manufacture, for changing the organoleptic and rheologic properties of must and wines, the clarification of fruit juices, and the isolation of protoplasts from potato leaves for their hybridization (Butenko and Kuchko, 1979). Extracellular enzymes, possessing xylanase, from *Bacillus circulans* WL-12 have been used to isolate the pigment astaxanthin (3,3'-dihydroxy-4,4'-diketo- β -carotene), which is common to marine and freshwater animals and is used as a pigment source in fish diets (Johnson *et al.*, 1978). Xylanase can also be used for the preparation of radio-labelled (1,4)- β -D-xylooligosaccharides for use in metabolism investigations (Kratky *et al.*, 1981), or for the production of monomers and oligomers for analytical use. Some hemicellulose

FIGURE 4 - Zymogram Technique (A) STEP 1: separate the enzymes on an electrophoretic gel (IEF or Native); (B) STEP 2: replica plate the gel onto substrate gels made up of an insoluble substrate (CMC or xylan) in an agar matrix; (C) remove the gels, treat with stain that binds to the insoluble substrate. Therefore, any clearing will indicate enzyme activity, where the enzyme has solubilized the substrate. The bands may be correlated to the electrophoretic ones. NOTE: The two "purified" enzymes shown here do not exhibit cross-specificity for each other's substrate, however, often these enzymes are able to act on more than one substrate.

sugars, particularly pentoses and uronic acids, are very labile in acid hydrolysis. Preservation of all sugars is achieved under the moderate conditions of enzymatic hydrolysis (Puls and Poutanen, 1988). In addition, purified xylanases have been utilized in the determination of cell wall structures in plants (Senior, 1989).

Organisms fermenting hemicellulose sugars are seldom hydrolytic and, therefore, cannot utilize oligomers as their carbon source. Enzymatic hydrolysis is one option for pre-treating hemicelluloses prior to fermentation (Puls and Poutanen, 1988). It has been estimated that the fermentation of cellulose to usable compounds (e.g. butanol) would not be economically feasible without the simultaneous fermentation of the hemicellulose (Biswas et al., 1988). The number and nature of substituents in hemicelluloses have important consequences for the physical properties of the polymer and hemicellulases can be used to change these properties. For example, high-valued locust bean gum differs from cheap guar gum only by its smaller number of galactose side-groups. An enzymatic process has been developed in which the galactose content of guar gum can be manipulated (Puls and Poutanen, 1988). An analogous process could be adapted for removal of the side-groups of xylan, e.g. to aid in its crystallization.

Utilization of hemicellulases by the pulp and paper industry could lead to consumption of this group of enzymes exceeding that of starch-degrading enzymes (Puls and Poutanen,

1988). Hemicellulases have been demonstrated to improve many properties of pulps. The benefits of these enzyme treatments include: improvement in paper properties such as strength, brightness, and opacity (Noé *et al.*, 1986; Paice *et al.*, 1988); reduction in bleaching requirements with increased yields (Chauvet *et al.*, 1987); and the potential for the production of dissolving grade pulps from Kraft pulps (Paice and Jurasek, 1984). Improvements in the bleachability of the pulps by alternative processes have been of particular interest due to recent concerns of dioxins in pulping effluents. Up to 50% reduction in chemical bleaching requirements have been reported following the xylanase treatment of pulps (Chauvet *et al.*, 1987). Pretreatment of pulps with xylanases prior to chemical bleaching, could reduce bleaching chemical requirements and possibly reduce the production of dioxins (Senior, 1989). Also, the increased pulp yield associated with enzyme treatments is economically desirable and suggests a potential reduction in the waste organic matter in pulp effluent. This reduction would lower the biochemical oxygen demand (BOD) level of the waste effluent (Parisi, 1989).

Dissolving pulps must be highly purified prior to derivatization to high value items such as rayon, cellulose acetate and cellulose nitrates. Most pulps in Canada are produced using the Kraft process rather than the sulphite process used for dissolving pulps (Saddler *et al.*, 1988). It

has been demonstrated recently that it may be possible to produce dissolving-grade pulps from Kraft pulp stock, thereby creating a new market for Kraft-pulp (Senior, 1989). Hitherto, the work on the hemicellulases and pulp improvement has had limited applications. But there are many reports of preliminary successes (c.f. Blanchette et al., 1988; Chauvet et al., 1987; Jurasek and Paice, 1988; Puls and Poutanen, 1988; Senior, 1989).

Although many fungi are prolific producers of extracellular xylanases, fungal growth and enzyme production can only be carried out at mesophilic temperature (28 °C) for most of the commercially used species. The xylanase enzymes that are produced are also thermally unstable, losing over 90% of their activities within half an hour incubation at 50 °C (Feldman et al., 1988). Industrial processes usually operate at temperatures from 60 °C and up, consequently, they would have to be cooled to 37-45 °C to make use of the mesophilic enzymes. This in turn lowers the hydrolysis efficiencies and necessitates aseptic conditions during hydrolysis, as well as preventing prolonged enzyme use without replacement (Donnison et al., 1989). The thermostable xylanase is also more conveniently stored, handled, and transported at ambient temperatures (Tan et al., 1987b).

It has been found that the fungus Thermoascus crustaceus, particularly the strain T. crustaceus 235E, is highly suitable for producing thermally stable cellulolytic and xylanolytic

enzymes (Tan et al., 1988). It has been shown to grow on a variety of inexpensive substrates, including sawdust, producing high concentrations of thermostable xylanase (Yu et al., 1987). Many of the cellulases have been purified and characterized from a closely related species T. aurantiacus (Feldman et al., 1988) and their mode of action and substrate specificity have been studied (Shepard et al., 1981). However, only one xylanase from T. crustaceus 235E (Tan et al., 1987b) and one xylanase from T. aurantiacus (Khande et al., 1989) have been purified and studied.

The enzymes of T. crustaceus 235E fill the criteria for the thermostability demands that industry is making. There remains much to be studied concerning the multiplicity of the xylanases produced by T. crustaceus 235E, as well as the properties of those enzymes. One interesting attribute of the enzymes of T. crustaceus 235E is the fact that the xylanase activity of the culture filtrate appears to be slightly more thermostable than that of the cellulase component. Thus, T. crustaceus 235E also has the potential for production of a relatively inexpensive, enriched-xylanase preparation.

Chapter 1

Production of hemicellulolytic and cellulolytic enzymes of Thermoascus crustaceus 235E

1.1 INTRODUCTION

Thermoascus crustaceus 235E contains a full complement of cellulases, hemicellulases, and their related enzymes (see Table 4). In particular, it produces large quantities of endo- β -D-xylanase. This fungus has an optimum growth temperature of about 45 °C and its enzymes are extremely thermostable at temperatures up to 80 °C (see chapter 2). Therefore, T. crustaceus 235E possesses qualities that are very attractive to industry.

A review of the literature shows no evidence of genetic studies on any Thermoascus species. The focus of the research thus far has been production and subsequent purification and characterization of the extracellular enzymes. One study found that the cellulases of T. aurantiacus A-131 were produced constitutively (Kawamori *et al.*, 1987). Feldman *et al.*, (1988) showed that in fact, only the CM-cellulase (or endocellulase) production was constitutive in T. aurantiacus, while β -glucosidase and α -amylase were not produced until autolysis became evident. Finally, Yu *et al.* (1987) screened twenty-one strains of thermophilic fungi in the Forintek culture collection and chose T. crustaceus 235E as the best producer of extracellular xylanase. This strain, formerly named T. aurantiacus C436, has since been re-evaluated and classified

TABLE 4 - Enzyme activities in a representative batch of *Thermoascus crustaceus* 235E culture filtrate (Batch #3)

	Activity ^a (IU/mL)	Specific activity ^a (IU/mg)
xylanase	239	442.6
endocellulase (CMCase)	10.3	19.1
β-glucosidase (salicinase)	0.6	1.1
filter paper activity	0.6	1.1
β-xylosidase	0.03	0.6
arabinosidase	0.08	0.1
acetyl-xylan esterase	9.0	16.7
acetyl esterase	0.07,	0.1
protein ^b	0.54 mg/mL	

^a enzyme assays were carried out as specified in section 1.2 at 65 °C for 15 minutes using the DNS method (Miller, 1959) for reducing sugar quantitation

^b protein was determined by a modified Lowry assay and using BSA as the standard (see section 1.2 for details)

^c specific activity = $\frac{\text{activity (IU/mL)}}{[\text{protein}] \text{ (mg/mL)}}$

as T. crustaceus. The researchers at Forintek have not released the change in information as there is a patent pending on the name "Thermoascus aurantiacus C436" (Forintek, unpublished data).

Data pertaining to production of xylanases in T. crustaceus 235E is obviously lacking. Indeed, a slight increase in the initial amount of xylanase and/or the ratio of xylanase/other enzyme activities would greatly enhance the purification of the xylanase activity (Wheelright, 1987). This study proposes to examine briefly enzyme production in T. crustaceus 235E.

1.2 MATERIALS AND METHODS

MICROORGANISM:

Thermoascus crustaceus 235E was obtained from the Forintek culture collection (c.f. Yu et al., 1987). This fungus is a filamentous ascomycete which produces a wide range of enzymes that break down the various components of wood. Visually, it is easily identified by the formation of "crusty" brown colonies when grown on a solid substrate. Microscopically, it is identified by the production of asci, in which ascospores are formed. Because T. crustaceus 235E is a filamentous ascomycete, its asexual reproduction is by the formation of large numbers of conidia, which are also visible microscopically. Keith Seifert, the mycologist and curator of the Forintek culture collection, examined the culture of T.

crustaceus 235E used in this study bimonthly to ensure its authenticity. He would also check the experiment's growth flasks periodically for contamination, especially by yeasts or bacteria.

GROWTH CONDITIONS:

T. crustaceus 235E was maintained on 2% (w/v) Bacto Malt Extract with 2% (w/v) Difco Bacto Agar. One slant was used to inoculate 100 mL of a 2% (w/v) glucose solution in Vogel's medium (Montenecourt and Eveleigh, 1977; Appendix 1). The inoculum was shaken at 200 rpm in a 500 mL Erlenmeyer flask at 45 °C for 72 hours, after which time 7.5 mL was used to inoculate each experimental flask. These 500 mL Erlenmeyer flasks contained 150 mL of Mandel's media (Mandels, 1977; Appendix 2) and the specified substrate. The substrates included oat-spelts xylan, D-xylose, D-glucose, Sigmacell, Solka Floc BW300, and SEA-WS (steam exploded aspenwood-water solubles; Brownell and Saddler, 1987). The experimental flasks were incubated at 45 °C and 200 rpm for 11-12 days. A 10 mL sample was removed on the days indicated. This sample was filtered through Whatman 934-AH glass microfiber filters and stored overnight at 0-4 °C for assessment of enzymatic activity. Results from all culture work were obtained from at least duplicate experiments, which were reproducible within 15 percent.

Due to the nature of T. crustaceus 235E, the inoculum

varied substantially from one run to the next, therefore, a "standard" flask was included for each run. The standard was 1% (w/v) oat-spelts xylan in Mandel's media and was performed in duplicate for each run. The enzyme levels in the experimental flasks are related to those in the standard for their respective run.

ENZYME ASSAYS:

(A) β -Xylanase (β -1,4-D-xylan xylanohydrolase, EC 3.2.1.8) activity was determined by incubating 0.5 mL of an appropriately diluted enzyme preparation with 10 mg oat-spelts xylan in 0.5 mL 50 mM sodium acetate buffer, pH 4.8 at 50 °C for 30 minutes (unless otherwise indicated). The reducing sugars released were assayed colorimetrically by the addition of 1.5 mL of 3,5-dinitrosalicylic acid reagent (Appendix 3). One unit of xylanase activity was defined as 1 μ mole of xylose equivalent released per minute.

(B) Endoglucanase (carboxymethylcellulase; 1,4-(1,3;1,4)- β -D-glucan 4-glucanohydrolase, EC 3.2.1.4) activity was determined by incubating 0.5 mL of appropriately diluted enzyme preparation with 10 mg carboxymethyl cellulose (CMC) in 0.5 mL 50 mM sodium acetate buffer, pH 4.8 at 50 °C for 30 minutes (unless otherwise indicated). The reducing sugars released were assayed colorimetrically by the addition of 1.5 mL 3,5-dinitrosalicylic acid reagent. One unit of endocellulase (CMCase) activity was defined as 1 μ mole of glucose equivalent

released per minute.

(C) β -glucosidase (salicinase; β -D-glucosidase glucohydrolase, EC 3.2.1.37) activity was determined in the same manner as CMCase activity, substituting salicin for CMC. The reducing sugars released were assayed colorimetrically by the addition of 3.0 mL 3,5-dinitrosalicylic acid reagent. One unit of β -glucosidase (salicinase) activity was defined as 1 μ mole of glucose equivalent released per minute.

(D) Filter paper activity (FPase) was determined by the method of Mandels *et al.* (1976). A 1.0 mL aliquot of appropriately diluted enzyme preparation was incubated with a 1X6 cm piece of Whatman number 1 filter paper in 1.0 mL 50 mM NaAc buffer, pH 4.8 at 50 °C for 60 minutes (unless otherwise indicated). The reducing sugars released were assayed colorimetrically by the addition of 3.0 mL 3,5-dinitrosalicylic acid reagent. One unit of filter paper activity was defined as 1 μ mole of glucose equivalent released per minute.

(E) β -Xylosidase (1,4- β -D-xylan xylohydrolase, EC 3.2.1.37) activity was determined by incubating 0.5 mL of appropriately diluted enzyme preparation with 4 mg p-nitrophenyl-xylopyranoside in 0.5 mL 50 mM sodium acetate buffer, pH 4.8 at 50 °C for 30 minutes (unless otherwise indicated). The reaction was stopped with the addition of an equal volume of 10% (w/v) sodium carbonate and the absorbance at 410 was subsequently measured. The standard curve was constructed with known amounts of p-nitrophenol (not shown).

(F) Soluble protein was determined by the method of Lowry et al. (1951) as modified by Tan et al. (1984). A 100 μ L aliquot of appropriately diluted enzyme preparation was precipitated with 7% (w/v) TCA on ice for 30 minutes. The solution was then centrifuged at 3000 rpm for 25 minutes, the supernatant decanted, and the pellet reconstituted in 100 μ L of 50 mM sodium acetate buffer, pH 4.8. A 5.0 mL aliquot of Lowry reagent C (Appendix 3) was added and after 30 minutes, 0.5 mL of 1N Folin Ciocalteu reagent (diluted 1:1 in dH_2O) was added. The samples were allowed to stand in the dark for 30 minutes, then the optical density was read at 660 nm within 10-15 minutes. The standard was bovine serum albumin.

All enzyme and protein assays were performed at least in duplicate and were reproducible within 15 per cent, under similar conditions.

ELECTROPHORETIC GELS:

Pre-cast Phast SDS gels (gradient 10-15) were used in conjunction with the Phast System (Pharmacia). Pure proteins were run at approximately 1 mg/mL and mixtures of proteins were loaded according to the "best" results. All samples were diluted with water. Samples were loaded using the 8-well loaders, which transfer approximately 1-2 μ L per well. Immediately after the run, the gels were developed using the Phast development system (Pharmacia). The proteins were detected by Phast Coomassie Blue Stain (Pharmacia).

CHEMICALS:

Oat-spelts xylan, D-xylose, D-glucose, Sigmacell, ammonium sulphate, carboxymethyl cellulose, salicin, Whatman number 1 filter paper, bovine serum albumin, and Folin Ciocalteu Reagent were obtained from Sigma Chemical Company. Solka Floc BW300 was purchased from Brown and Company.

Steam-treated aspenwood was prepared at Forintek Canada Corporation by treating aspenwood chips at 240 °C for 80 seconds with explosive decompression (Brownell and Saddler, 1987), followed by extraction with water at room temperature for 2 hours as a 5% (w/v) slurry. The solids were suction-filtered, leaving the water-insoluble fraction, termed SEA-WI. The filtrate (SEA-WS) was concentrated using a Buchi rotary evaporator to 15% (w/v) and used without further preparation.

1.3 RESULTS AND DISCUSSION

The initial experiments were an attempt at increasing the enzyme production in *T. crustaceus* 235E, by examining the culture growth conditions. In addition, because the focus of this project was on the xylanase enzymes, optimization of the ratio of xylanase/cellulase activity was investigated. Any improvements in the quality or quantity of the starting material can have a significant effect on the ease, efficiency, and cost of downstream processing (Wheelwright, 1987). The standard culture conditions of *T. crustaceus* 235E have been on 1% (w/v) substrates, in particular xyans, at 45

°C and 150-200 rpm in shake flasks (c.f. Bachman and McCarthy, 1989; Kawamori et al., 1987; Kitpreevanich et al., 1984; McCarthy et al., 1985; Merchant et al., 1988).

Due to the variability in the growth nature of *T. crustaceus* 235E, the inoculum varied substantially from one run to the next. This variance had a significant effect on the production of the cellulolytic and hemicellulolytic enzymes in the experimental flasks (Table 5). The designated standard flask was 1% (w/v) oat spelts xylan at 45 °C and 200 rpm as these have been the standard conditions for enzyme production in *T. crustaceus* 235E (c.f. Kawamori et al., 1987; Yu et al., 1987). The most variation in enzyme levels occurred in the production of β -1,4-xylanase and endocellulase activities (Table 5). Therefore, it was assumed that the enzyme production was related to the inoculum, and because one inoculum was used for each run, the levels of activity produced in the experimental flasks could be related to those in the standard flask for their respective runs.

Enzyme production over time was examined to determine the optimum period of incubation prior to harvesting. The examined enzyme activities peaked around 10-12 days after inoculation (Figure 5). Yu et al. (1987) found that optimum enzyme activities occurred at different intervals depending on the substrate used. Therefore, each experimental run was analyzed periodically to determine when the optimum enzyme activities occurred.

TABLE 5 Variations in the enzyme activities in the culture filtrates of *Thermotoga gracilis* 235E grown in batch on 1% (w/v) oat-spelts xylan and Mandel's media

Batch ^a	Xylanase ^b (IU/mL)	CMCase ^b (IU/mL)	Salicinase ^b (IU/mL)	Filter paper activity ^b (IU/mL)	Xylanase ----- CMCase
1	239	10.3	1.10	0.18	23.2
2	141	10.0	2.74	0.17	14.2
3	201	16.3	1.74	0.19	12.3
4	296	29.6	1.96	0.33	10.0
5	412	21.3	2.04	0.21	19.4
6	297	18.3	3.02	0.35	16.2
Mean ^c (X)	258 (265)	17.5 (17.6)	1.92 (2.10)	0.22 (0.24)	15.8 (15.9)
Standard Deviation ^d (s)	92.0 (93.3)	7.3 (7.4)	0.53 (0.69)	0.06 (0.08)	4.8 (4.8)

^a Batches 1-5 are those in which the xylanase was more heat stable than the CMCase activity. In batch #6, the CMCase was more heat stable than the xylanase activity

^b Enzyme assays were carried out as specified in section 1.2 at 65 °C for 15 minutes using the DNS method (Miller, 1959) for reducing sugar quantitation

^c The mean was calculated as shown in Appendix 4. The first number is the mean of batches 1-5, while the number in brackets corresponds to the mean of all the batches (i.e. 1-6)

^d The standard (sample) deviation was calculated as shown in Appendix 2. The first number is the standard deviation of batches 1-5, while the number in brackets corresponds to the standard deviation of all the batches (i.e. 1-6)

FIGURE 5 - Enzyme activities in Thermosoccus crustaceus 235E culture filtrates produced over time on Mandel's media with 1% (w/v) oat- spelts xylan. (A) ■ xylanase. (B) + CMCase; ° salicinase; △ filter paper activity.

NOTE: Individual points represent the average of data obtained from a minimum of duplicate experimental flasks and each days' activity was assayed in duplicate. The values between experimental flasks was reproducible within 15% and the individual enzyme assays were reproducible within 10%.

To determine the optimum oat spelts xylan concentration for enzyme production, *T. crustaceus* 235E was grown on varying concentrations of the substrate (see Figure 6). It was found that 1% (w/v) oat spelts xylan produced the highest levels of xylanase (240 IU/mL) after growth for 12 days at 45 °C. The highest ratio of xylanase/endocellulase activity (26.5; Figure 7) was produced on 2% (w/v) oat spelts xylan, while the ratio was 23.2 on 1% xylan (Figure 7). It was decided that the 12% increase in the ratio of xylanase/endocellulase activity, accompanied by a 3% decrease in xylanase activity would not merit the use of twice as much of the relatively expensive oat spelts xylan substrate (valued at approximately \$50 per 100 g).

In order to determine if a cheaper, more widely available substrate could be used, *T. crustaceus* 235E was grown on Solka Floc, an insoluble cellulose containing approximately 8% hemicellulose (J. Saddler and K. Wong, unpublished data). Figure 8 demonstrates that the maximum enzyme levels produced on Solka Floc are equivalent to those produced on 0.1% (w/v) oat spelts xylan (refer to Figure 6). For example, the β -1,4-xylanase activity was 38.0 IU/mL on 0.5% (w/v) Solka Floc and 49.9 IU/mL on 0.1% (w/v) oat spelts xylan. These data tend to suggest that xylanase production is directly related to the amount of xylan, or one of its breakdown products, in the media because both substrates contained approximately 0.08-0.10% (w/v) xylan. Others have found that xylans and their

FIGURE 6 - Maximum enzyme activities (day 12) in Thermoascus crustaceus 235E culture filtrates produced on Mandel's media with varying amounts of oat-spelts xylan. (A) ■ xylanase. (B) + CMCase; , salicinase; △ filter paper activity.

NOTE: Individual points represent the average of data obtained from a minimum of duplicate experimental flasks and each days' activity was assayed in duplicate. The values between experimental flasks was reproducible within 15% and the individual enzyme assays were reproducible within 10%.

FIGURE 7 - Ratio of xylanase/CMCase activity in day 12 culture filtrates of Thermoascus crustaceus 235E grown on Mandel's media with varying amounts of oat-spelts xylan. [* ratio of xylanase/CMCase activity]

NOTE: Individual points represent the average of data obtained from a minimum of duplicate experimental flasks and each day's activity was assayed in duplicate. The values between experimental flasks was reproducible within 15% and the individual enzyme assays were reproducible within 10%.

FIGURE 8 - Enzyme activities in Thermoascus crustaceus 235E culture filtrates produced over time on Mandel's media with 0.5% (w/v) Solka floc. (A) ■ xylanase. (B) + CMCase; □ salicinase; △ filter paper activity.

NOTE: Individual points represent the average of data obtained from a minimum of duplicate experimental flasks and each days' activity was assayed in duplicate. The values between experimental flasks was reproducible within 15% and the individual enzyme assays were reproducible within 10%.

breakdown products induce the production of xylanases in various microorganisms. For example, xylanase production in two species of the yeast Cryptococcus was induced at the transcriptional level by xylobiose, xylotriose, xylan, and β -methylxyloside (Morosoli et al., 1987; Yasui et al., 1984).

To determine the extent to which cellulose could induce the enzymes, T. crustaceus 235E was grown up on 1% (w/v) Sigmacell, a pure cellulose substrate. It was found that the enzyme levels produced were approximately 5-10% of the standard flask (data not shown). It was assumed that these values represent constitutive levels of enzyme production. Kawamori et al. (1987) reported that similar constitutive levels of cellulases were produced by T. aurantiacus A-131 without "inducers". In addition, they found that cellulase production was induced by amorphous polysaccharides containing β -1,4 linkages such as alkali-treated bagasse and xylan, rather than crystalline cellulose. Therefore, it was concluded that both the hemicellulase and cellulase components of T. crustaceus 235E require xylan or one of its breakdown products for enhancement of their levels. This requirement is different from those of other cellulase and hemicellulase-producing microorganisms. For example, Streptomyces species grown on cellulose produced cellulases, while growth on xylan produced both cellulases and hemicellulases (MacKenzie et al., 1987). Trichoderma harzianum E58 also produces high cellulase levels when grown on pure cellulose substrates, but produces mostly

xylanases when cultured on xylans such as those from oat spelts (Senior *et al.*, 1989). In addition, expression of maximum xylanase activity was induced in *Trichoderma reesei* by a glucose polymer (Panda *et al.*, 1987). Therefore, the mechanism of control of the production of hemicellulases and cellulases in *T. crustaceus* 235E appears to differ from mechanisms studied in other organisms which produce enzymes with similar activities.

In order to find a cheaper and more "realistic" substrate for enzyme production, the hemicellulose-rich steam-exploded aspenwood-water solubles (SEA-WS) fraction was chosen. *T. crustaceus* 235E grown on 0.5% (v/v) SEA-WS in Mandel's medium produced only constitutive levels of hemicellulases and cellulases (data not shown). However, when 0.5% (v/v) SEA-WS was added to 1% (w/v) oat spelts xylan, *T. crustaceus* 235E produced equivalent amounts of endo- β -1,4-xylanase and 27% less endocellulase than the standard flask (Figure 9). This had the effect of increasing the xylanase/endocellulase ratio by approximately 35%. There appeared to be a slight lag period in enzyme production initially, but levels were still increasing at day 12 (Figure 9) while those in the standard flask had levelled off. When *T. crustaceus* 235E was grown on 1% (w/v) oat spelts xylan with 1% (v/v) SEA-WS, all the enzyme activities were repressed (Figure 10). Other researchers (c.f. Saddler and Mes-Hartee, 1984; Yu *et al.*, 1987) found that culturing *T. crustaceus* 235E on SEA-WS resulted in poor fungal

FIGURE 9 - Enzyme activities in Thermoascus crustaceus 235E culture filtrates produced over time on Mandel's media with 1% (w/v) oat-spelts xylan + 0.5% (w/v) steam-exploded aspenwood - water-solubles (SEA-WS). (A) ■ xylanase. (B) + CMCase; ° salicinase; △ filter paper activity.

NOTE: Individual points represent the average of data obtained from a minimum of duplicate experimental flasks and each days' activity was assayed in duplicate. The values between experimental flasks was reproducible within 15% and the individual enzyme assays were reproducible within 10%.

FIGURE 10 - Enzyme activities in Thermoplasma crustaceus 235E culture filtrates produced over time on Mandel's media with 1% (w/v) oat-spelts xylan + 1% (w/v) steam-exploded aspenwood - water-solubles (SEA-WS). (A) ■ xylanase. (B) + CMCase; △ salicinase; □ filter paper activity.

NOTE: Individual points represent the average of data obtained from a minimum of duplicate experimental flasks and each days' activity was assayed in duplicate. The values between experimental flasks was reproducible within 15% and the individual enzyme assays were reproducible within 10%.

growth and enzyme production. These findings were attributed to the presence of water-soluble inhibitors associated with the SEA-WS (Saddler and Mes-Hartee, 1984). Xylanase production, but not xylanase/endocellulase ratios, was enhanced in Trichoderma harzianum E58 when it was cultured on oat spelts xylan containing 0.5% SEA-WS (Senior et al., 1989). It may be that the repression effects are only apparent at high concentrations of SEA-WS (~1%), while at lower concentrations, the SEA-WS may increase the xylanase/endocellulase level by selectively repressing the cellulase component in T. crustaceus 235E. The variability in repression effects may also be due to a variability in the amounts of inhibitors in the different batches of SEA-WS.

To investigate these "repression effects", T. crustaceus 235E was grown on 1% (w/v) oat spelts xylan with 1% (w/v) glucose (see Figure 11). The addition of glucose inhibited the production of all the hemicellulase and cellulase activities, but cellular growth was good and the extracellular protein levels were almost 50% higher than those of the standard flask (data not shown). A review of the literature shows that production of xylanase in various microorganisms is not directly associated to the amount of cellular growth (c.f. Gruninger and Fiechter, 1986). Utilization of the glucose in the medium by day 11 appears to be relieving some of the repression effects as the levels appear to be increasing (Figure 11). Feldman et al. (1988) found that CMCase

FIGURE 11 - Enzyme activities in Thelmoascus crustaceus 235E culture filtrates produced over time on Mandel's media with 1% (w/v) oat-spelts xylan + 1% (w/v) glucose. (A) ■ xylanase. (B) + CMCase; * salicinase; · filter paper activity.

NOTE: Individual points represent the average of data obtained from a minimum of duplicate experimental flasks and each days' activity was assayed in duplicate. The values between experimental flasks was reproducible within 15% and the individual enzyme assays were reproducible within 10%.

production in various strains of T. crustaceus 235E was high on glucose concentrations below 0.5% but decreased significantly when the initial glucose concentration was greater than 0.6%. The experimenters found no detectable activity at 0.9 and 1% glucose (Feldman et al., 1988).

To determine the effects of xylose on enzyme production, T. crustaceus 235E was grown on 1% oat spelts xylan containing 0.5% and 1.0% (w/v) xylose (Figure 12). The addition of 0.5% xylose had no detectable effect on the various enzyme levels compared to the standard flask (data not shown). At a concentration of 1% xylose, there was a slight lag period in the production of all the enzymes, but the levels of all the components reached those of the standard flask by day 12 (Figure 12).

Other microorganisms have similar patterns of repression by glucose and xylose. For example, Clostridium stercoarium xylanase and CMCase is totally repressed on 0.5% glucose, but unaffected on 0.5% xylose (Béranger et al., 1985). In addition, the fungus Aureobasidium pullulans xylanases were subject to glucose repression, but were induced by xylose, xylobiose, arabinose, and xylan (Leathers et al., 1986). These are the same patterns of enzyme repression observed in T. crustaceus 235E.

Finally, the initial pH of the media was varied in order to determine the effects on extracellular enzyme production (Figures 13 and 14). Although the initial pH varied, by the

FIGURE 12 - Enzyme activities in Thermoascus crustaceus 235E culture filtrates produced over time on Mandel's media with 1% (w/v) oat-spelts xylan + 1% (w/v) xylose. (A) ■ xylanase. (B) + CMCase; ∪ salicinase; △ filter paper activity.

NOTE: Individual points represent the average of data obtained from a minimum of duplicate experimental flasks and each days' activity was assayed in duplicate. The values between experimental flasks was reproducible within 15% and the individual enzyme assays were reproducible within 10%.

FIGURE 13 - Maximum enzyme activity (day 12) in culture filtrates of Thermoascus crustaceus 235E grown on Mandel's media with 1% (w/v) oat-spelts xylan and varying initial pH levels. (A) ■ xylanase. (B) + CMCase; ° salicinase; △ filter paper activity.

NOTE: Individual points represent the average of data obtained from a minimum of duplicate experimental flasks and each days' activity was assayed in duplicate. The values between experimental flasks was reproducible within 15% and the individual enzyme assays were reproducible within 10%.

FIGURE 14 - SDS-PAGE (Phast, gradient 10-15) of culture filtrates from day 12 of the pH study of enzyme production in Thermococcus crustaceus 235E (see also Fig. 13). The protein was stained with Coomassie Blue (Phast).

SAMPLES*: lane 1 - low molecular weight standards
(Biorad)
lane 2 - culture filtrate from pH 4.0 sample
lane 3 - " " " pH 4.5 "
lane 4 - " " " pH 5.0 "
lane 5 - " " " pH 5.5 "
lane 6 - " " " pH 6.0 "
lane 7 - " " " pH 6.5 "
lane 8 - empty

NOTE: the pH in lanes 2-7 represents the initial pH of the media, but the samples are from day 12 culture filtrates

* all samples were 1 mg/mL (see chapter 1.2 for details)

time the maximum enzyme activities had been reached, the pH of all the flasks had levelled at pH 5.8-6.0 (data not shown). Generally, the higher the initial pH, the greater the xylanase activity, but the cellulase activities remained fairly constant (Figure 13). At initial pH's greater than pH 6.5, all extracellular enzyme activity dropped dramatically (data not shown). The culture filtrates from day 12 (maximum activity) were analyzed by SDS-PAGE (Figure 14). The filtrates did not appear to be composed of different proteins (bands), even between those filtrates that contained highly different xylanase activities. The initial pH of the medium was altered to pH 6.4 for all subsequent enzyme production in *T. crustaceus* 235E.

A review of the recent literature found that there are few studies on the effects of pH on extracellular xylanase production. Generally, the optimum growth conditions for cellulase production in fungi have been at pH less than 6.5.

It would appear that the controls of the production of hemicellulose and cellulose degrading enzymes in *T. crustaceus* 235E are closely related. Glucose represses all the enzymes, whereas xylose has little effect up to at least 1% (w/v). The inhibition effects observed with the 1% and not with 0.5% (v/v) SEA-WS may have been due to increased glucose in the medium rather than growth inhibitors. The SEA-WS appears to have potential as an inexpensive additive for increasing xylanase production and xylanase/endocellulase ratios in *T.*

crustaceus 235E, however, more research is required to determine whether its inhibitory effects could be overcome (e.g. fed-batch production to reduce sugar levels) Lastly, the pH and indeed the temperature of the growth medium can greatly influence extracellular enzyme production in fungi (Suh et al., 1988). More information is required on these parameters regarding xylanase production in T. crustaceus 235E.

Chapter 2

Differential heat stabilities and heat inactivation studies of the enzymes of Thermoascus crustaceus 235E

2.1 INTRODUCTION

Several methods to prepare high-purity xylanases for potential industrial applications have focused on eliminating the cellulase contamination instead of purifying the xylanase components. This appears to be a very sensible approach as it precludes the need for very expensive biochemical procedures and focuses, rather, on a limited number of simple steps to remove the cellulase activity. Because the remaining materials in the preparation are often essentially inert with respect to the cellulose (or hemicellulose), their presence is simply ignored (Saddler et al., 1988).

Paice et al. (1988) have prepared a cellulase-free xylanase preparation from the Bacillus subtilis/E. coli cloned system. Although approximately 50 per cent of the xylanase was excreted extracellularly, the remaining portion of enzyme had to be released from the bacterial cells by solvent disruption of the membranes. Bacterial xylanases are not usually secreted extracellularly and, therefore, lysis of the cells is necessary. For cloned systems such as B. succinogenes, which still produce high levels of cellulases, further purification steps using traditional biochemical procedures are required (Sipat et al., 1987).

Using the high-xylanase producing filamentous fungus Trichoderma harzianum E58, Tan et al. (1987c) have developed a procedure for the bulk purification of a cellulase-free endo-xylanase of high specific activity (Tan et al., 1987c; Tan et al., 1988). The simple procedure involves ultra-filtration of the culture fluid through a 10,000 molecular weight cut-off membrane to remove the cellulase components, and further purification and concentration by ion-exchange chromatography. The combination of the enzyme's low molecular weight (20 kDa) and high isoelectric point (9.5) makes purification a rapid and effective procedure.

Selective inactivation of cellulase activities is another method which has been applied to fungal culture filtrates to effectively purify xylanases. Cellulase inactivation of a crude enzymatic complex (LaRapidase) was achieved by Barnoud et al., (1986) using a 1 mM mercuric chloride solution. In the presence of this sulphydryl-binding metal, complete inactivation of endocellulases was observed, whereas, the xylanases retained 80% of their activity.

Another method for selective inactivation of cellulase in enzyme mixtures is the use of selective heat inactivation. Thermostable organisms generally possess thermostable enzymes. The optimum temperature for activity of these enzymes is usually at least 10 °C higher than the optimum growth temperature for the organism. In many instances, the enzymes have differential heat stabilities, thereby providing a method

for purification or "enhancement" of the more thermostable activity (Saddler et al., 1988).

While establishing the thermostable properties of crude xylanases from fungal strain Y-94, Mitsuishi et al. (1987) identified the different heat labilities of the cellulase and xylanase activities in the culture filtrate. After an incubation period of 20 minutes at 65 °C, the xylanase activity was reduced by 5-10%, whereas the Avicelase (an endocellulase) and β -glucosidase activities were reduced by 100% and 60% respectively.

Similar temperature dependency patterns were noted for the enzymes of Thermoascus crustaceus 235E. An initial study carried out by Paul Mayers indicated that treatment of the Pellicon retentate at 70 °C for 20 minutes resulted in less than a 5% loss in xylanase activity, whereas cellulase activities were reduced by 40-50% (Forintek Canada Corp., unpublished data). It was proposed that heat treatment methods would be a viable technique for rapid and inexpensive purification of xylanases.

2.2 MATERIALS AND METHODS

ENZYME PRODUCTION:

Bulk production of T. crustaceus 235E enzyme was carried out in batch. A slant was used to inoculate 300 mL of Vogel's medium (Montenecourt and Eveleigh, 1977) in a 1 L Erlenmeyer flask containing 2% (w/v) glucose as the carbon source. It was

incubated at 45 °C and 200 rpm for 3 days, after which time, 30 mL was used to inoculate 2 L Erlenmeyer flasks containing 600 mL of Vogel's medium with 1% (w/v) oat-spelts xylan. The flasks were incubated at 45 °C and 200 rpm for 12 days, after which time the crude culture filtrates were pooled and filtered through Whatman 934-AH glass microfibre filters. This culture filtrate was then concentrated to approximately one tenth the volume using the Pellicon Cassette system (Millipore Ltd) fitted with 0.47 m² of polysulfone membranes with a relative molecular mass exclusion limit of 10000. The concentrated fraction was termed the "Pellicon retentate" and contained more than 95% of the enzyme activity. The Pellicon retentate was stored at 0-4 °C. Enzyme activity was determined as described in chapter 1.2.

AMMONIUM SULPHATE PRECIPITATION:

Varying amounts of ammonium sulphate were added to 1.0 mL aliquots of Pellicon retentate and stirred at 25 °C for 30 minutes. The samples were then centrifuged at 3000 rpm for 25 minutes and the supernatant decanted and saved. The precipitates were reconstituted in 10 mL of 50 mM sodium acetate, pH 4.8. The supernatants and the reconstituted precipitates were desalted with BioRad disposable-desalting-columns and were assayed for residual enzyme activities as described in chapter 1.2. The enzyme activities were expressed as a percent of the activity of the untreated solution. These

experiments were done at least in duplicate for each of the batches tested and were reproducible within 10%.

HEAT TREATMENT STABILITY STUDY:

In order to determine the relative heat stabilities of the various enzyme components of T. crustaceus 235E, 1.0 mL aliquots of Pellicon retentate and 1.4 M ammonium sulphate-precipitated-Pellicon-retentate were heated at various temperatures on a heating block in sealed, screw-topped vials and placed on ice after specified time intervals. Both "fresh" (i.e. less than 6 months old) and 1-2 year old Pellicon retentate were heat treated in this manner. After heat treatment, the resultant solutions were desalted (where necessary) and assayed for enzyme activity as described in chapter 1.2. The activities were expressed as a percent of the activity of the untreated solution. The experiments were carried out at least in duplicate for each of the batches tested and were reproducible within 15%.

"ENRICHED-XYLANASE" MIXTURE:

T. crustaceus 235E Pellicon retentate was precipitated with 1.4 M ammonium sulphate for 30 min at 25 °C and then centrifuged at 3000 rpm for 25 minutes. The supernatant was decanted and heated at 72.5 °C for 30 minutes, and subsequently desalted prior to assaying for enzymatic activity. The resultant solution had a higher

xylanase/endocellulase ratio and was termed the "enriched-xylanase" mixture. This experiment was repeated at least in duplicate on three different batches of Pellicon retentate (i.e. from three different bulk productions of T. crustaceus 235E) and was reproducible within 15%.

ELECTROPHORETIC GELS:

Pre-made Phast IEF (pI 3-9) gels were used in conjunction with the Phast System (Pharmacia). Pure proteins were run at approximately 1 mg/mL and mixtures of proteins were loaded according to the "best" results. All samples were diluted with water. The IEF gels were prefocused prior to sample application. Samples were loaded using the 8-well loaders, which transfer approximately 1-2 μ L per well. Immediately following focusing, the gels were developed using the Phast development system (Pharmacia). The proteins were detected by Phast Silver Stain (Pharmacia).

2.3 RESULTS AND DISCUSSION

A shake-flask system in incubators was used for the bulk production of Thermoascus crustaceus 235E extracellular enzymes. The preferred method of fungal growth is in liquid- or solid-state fermenters, because these methods are less labour-intensive and tend to produce higher volumes of enzyme, but not necessarily higher levels of enzyme activity (c.f. Grajek, 1987). Attempts were made to utilize a 20L liquid-

state fermenter to produce the T. crustaceus 235E enzymes; however, the fermenters are often only capable of simulating mesophilic growth temperatures. The outlet valves on the fermenter utilized were not fitted with condensers and more than half the volume of the filtrate was lost by evaporation after 6 days at 45 °C. In addition, the aeration provided by the fermenters available could not match that granted by the shake-flask method (data not shown). If T. crustaceus 235E enzymes are to be used commercially, a fermenter capable of providing thermophilic conditions and good aeration would be essential.

The enzyme activities in a typical batch of T. crustaceus 235E are shown in Table 6. A ratio of 16.9 for xylanase/CMCase activity is already quite high as far as starting material is concerned. Most cellulolytic systems produce far higher quantities of endocellulase than xylanase (Yu et al., 1987). In addition, the levels of enzyme activity are quite high (Table 6). However, concentration of the enzymes by the Pellicon Cassette System (Millipore, Ltd) increases all the enzyme activities in the retentate with better than 76% recovery of xylanase with little loss to the filtrate (Table 6). It would be beneficial if the xylanase were somehow selectively concentrated in the retentate without the cellulases, as is the case with the mesophilic fungus Trichoderma harzianum E58 (Tan et al., 1987c); however, the xylanase/CMCase ratio is improved and this concentrated enzyme

TABLE 6 - Characteristics of various preparations from a representative batch of *Thermosascus crustaceus* 235E (Batch #5)

Activity ^a	Culture filtrate	Pellicon retentate	Pellicon filtrate
xylanase (IU/mL)	320.2	2725.0	7.9
CMCase (IU/mL)	18.9	134.9	0.5
salicinase (IU/mL)	2.5	26.0	0.1
filter paper activity (IU/mL)	0.3	1.5	0.1
protein ^b (mg/mL)	0.8	7.5	0.01
volume (L)	5.0	0.45	4.55

- ^a enzyme assays were carried out as specified in section 1.2 at 65 °C for 15 minutes using the DNS method (Miller, 1959) for reducing sugar quantitation
- ^b protein was determined by a modified Lowry assay and using BSA as the standard (see section 1.2 for details)

preparation is easier to transport, store, treat and use than the more dilute culture filtrate.

The initial experiment performed by Paul Mayers at Forintek Canada Corporation showed that treatment of T. crustaceus 235E Pellicon retentate at 70 °C for 20 minutes resulted in less than a 5% loss in xylanase activity, and a 40-50% reduction in cellulase activities (data shown in Figure 15A). At this point in time, the enzyme mixture was about 6 months old. The same batch was heat treated one year later (i.e. when it was 18 months old) and the thermostabilities of the enzymes had changed remarkably (Figure 15B). The cellulase was no longer any less heat stable than the xylanase. Indeed, both of the activities appeared to be much more thermostable at 70 °C than previously found.

A new batch of T. crustaceus 235E was grown up, passed through the Pellicon system, and heat treated. It was found that this "fresh" batch of enzyme displayed the differential heat stabilities of the xylanase and the CMCase (Figure 15) that were noted in the initial experiment. Both the culture filtrate (Figure 16A) and the Pellicon retentate (Figure 16B) were treated, but only the retentate exhibited the marked differences in the thermostabilities. Figure 17 illustrates that the ratio of xylanase/CMCase in the Pellicon retentate peaked at 104:1 after 30 minutes incubation (72.5 °C).

This experiment showed that the concentration of the enzymes by the Pellicon cassette system somehow affected the

FIGURE 15 - Residual enzyme activity in *Thermosascus crustaceus* 235E Pellicon retentate (batch 5/6/86) heated at 70 °C. (A) the batch was heat treated on 9/12/86, i.e. after 6 months of storage. (B) the same batch was heat treated on 9/28/87, i.e. after 18 months of storage. [■ xylanase; + CMCase; ○ salicinase; △ filter paper activity]

NOTE: Individual points represent the average of data obtained from a minimum of duplicate experiments reproducible within 15% and the individual enzyme assays were reproducible within 10%.

FIGURE 16 - Residual activities in enzyme preparations from batch #5 of Thermoascus crustaceus 235E heated at 72.5 °C. (A) percent residual activities in the culture filtrate. (B) percent residual activities in the Pellicon retentate.
[■ xylanase; + CMCase]

NOTE: Individual points represent the average of data obtained from a minimum of duplicate experiments reproducible within 15% and the individual enzyme assays were reproducible within 10%.

FIGURE 17 - Ratio of residual xylanase/CMCase activity in batch #5 Pellicon retentate from Thermococcus crustaceus 235E heated at 72.5 °C. [■ culture filtrate; + Pellicon retentate]

NOTE: Individual points represent the average of data obtained from a minimum of duplicate experiments reproducible within 15% and the individual enzyme assays were reproducible within 10%.

2 of/de 2

MICROCOPY RESOLUTION TEST CHART
NATIONAL BUREAU OF STANDARDS
STANDARD REFERENCE MATERIAL 1010a
(ANSI and ISO TEST CHART No. 2)

thermostabilities of the xylanase and the endocellulase components. The mechanism of this effect is as yet unknown, but may be caused by the increased molarity in the solution, thereby decreasing the water shell around the individual molecules (Hochachka and Somero, 1984). This phenomenon may increase the thermostability of the molecules in solution, more so for the xylanase activity than the endocellulase activity. Himmel *et al.* (1986) found that spray-drying cellulase preparations (various commercially available and in-house mixtures) led to enhancement of CMCase and filter paper activity, while the more labile β -glucosidase activity was entirely lost. They found a correlation between the reduced moisture content of the preparations and their abilities to be stored at 25 °C without loss of activity.

Replication of the differential heat stabilities was difficult at 70 °C (note that the above results were after incubation at 72.5 °C). The Pellicon retentate was treated at a range of temperatures to try to optimize the conditions of the experiment. It was established that the differential thermostability were best expressed at 72.5 °C (Figure 18A and 18B). This very small temperature latitude (i.e. 72.5 °C $\pm$ 1 °C) has not been reported to date in the literature for any heat treatment experiments. The optimum heat treatment of 72.5 °C for 30 minutes was repeated 3 times with three different batches of Pellicon retentate and was found to be reproducible within 15 percent.

FIGURE 18 - Residual enzyme activity over time in Thermoascus crustaceus 235E Pellicon retentate (batch #5) heated at various temperatures. (A) xylanase activity. (B) CMCase activity. [• 70 °C; + 72.5 °C; ° 75 °C; 80 °C]

NOTE: Individual points represent the average of data obtained from a minimum of duplicate experiments reproducible within 15% and the individual enzyme assays were reproducible within 10%.

Only one other study has shown xylanase activity to be more thermostable than the cellulase activities. Mitsuishi et al. (1987) published that the xylanase activity of strain Y-94 had a temperature of inactivation 5-10 °C higher than that of the concomitant cellulase (CMCase). They went on to purify three xylanases from the resulting preparation. Other researchers have reported thermostability differences between cellulases and xylanases, but the cellulases were always more heat stable. For example, Margaritis and Merchant (1983) presented data that indicated that heat treatment of Thielavia terrestris culture filtrates enhanced the cellulase activities. In particular, β -glucosidase activity was much more thermostable than the other cellulase components and the xylanase activity. One study actually reported thermal activation and increased stability of cellulases upon heat treatment. Nigam et al. (1988) heat-treated culture filtrates from two basidiomycetes and found that the cellulase stability increased over short periods of time (minutes). Upon examination of their methodology, it became apparent that the "enhancement" was more than likely due to evaporation and concentration effects: the activity first increased and then dropped significantly after being heated at 65 °C for 5 minutes.

Another heat inactivation strategy makes use of the differential thermostabilities of mesophilic and thermophilic proteins. Patchett et al. (1989) cloned thermostable

cellulases and xylanases from Caldocellum saccharolyticum into Escherichia coli. The researchers then heat-treated the culture filtrates to denature the mesophilic E. coli proteins, but retained the more thermostable cloned enzymes.

Although the Pellicon retentate offers a fairly concentrated mixture of enzymes, there are still many cellulase activities present. One simple and inexpensive procedure for further purification is ammonium sulphate precipitation. Figure 19A shows that both the salicinase and the filter paper activities precipitate out of solution at lower salt concentrations than the xylanase and endocellulase components. Figure 19B, an enlarged section of the graph indicates that the xylanase and endocellulase activities co-precipitated at a concentration of 1.4 M ammonium sulphate. The ratio of xylanase/CMCase activity increased only slightly (from 20.1 to 26), but the other contaminating activities had been removed (Table 7).

The ammonium sulphate precipitation proved to be beneficial in two ways. Firstly, it removed many unwanted cellulase activities (Figure 19). Secondly, the 1.4 M salt conferred more thermostability on the xylanase component than the cellulase (Figure 20A and 20B; Figure 21). The Pellicon retentate was heated at the optimum conditions (72.5 °C for 30 minutes) after 1.4 M ammonium sulphate precipitation and with 1% (w/v) oat-spelts xylan (Figure 20A and 20B). It was thought that if the salt conferred stability to the enzymes, the

FIGURE 19 - Residual enzyme activity in the supernatant of Thermoascus crustaceus 235E batch #5 Pellicon retentate after precipitation with ammonium sulphate and desalting. (A) 0-4 M ammonium sulphate. (B) 1-2 M ammonium sulphate.
[■ xylanase; + CMCase; ° salicinase; ; filter paper activity]

NOTE: Individual points represent the average of data obtained from a minimum of duplicate experiments reproducible within 15% and the individual enzyme assays were reproducible within 10%.

TABLE 7 Summary of the purification of *Thermotoccus crustaceus* 235F culture filtrate to yield an "enriched-xylanase" mixture

	Culture filtrate	Pellicon retentate	1.4 M ammonium sulphate precipitation ^a	"enriched" mixture ^b
xylanase ^c (IU/mL)	320.2	2725	3363	2089
CMCase ^c (IU/mL)	16.9	135	132	20.1
salicinase ^c (IU/mL)	2.5	26	0	0
filter paper activity ^c (IU/mL)	0.3	1.5	0	0
protein ^d (mg/mL)	0.8	7.5	6.2	6.2
volume (mL)	140	14	13	13
specific activity ^e of xylanase (IU/mg)	400	363	542	336
purification factor	1	0.9	1.4	0.8
<u>xylanase</u> CMCase	16.9	20.1	26	104

^a activity of the supernatant from the precipitation carried out as specified in section 2.2

^b heat treatment was at 72.5 °C for 30 min. as specified in section 2.2

^c enzyme assays were carried out as specified in section 1.2 at 65 °C for 15 minutes using the DNS method (Miller, 1959) for reducing sugar quantitation

^d protein was determined by a modified Lowry assay and using BSA as the standard (see section 1.2 for details)

^e specific activity = $\frac{\text{activity (IU/mL)}}{\text{protein (mg/mL)}}$

FIGURE 20 - Residual enzyme activity in various Thermoascus crustaceus 235E batch #5 Pellicon retentates heated at 72.5 °C. (A) xylanase activity. (B) CMCase activity.
[• Pellicon retentate, untreated; + Pellicon retentate, precipitated with 1.4 M ammonium sulphate, centrifuged, heat treated, then desalted; ° Pellicon retentate, heat treated with 1% (w/v) oat-spelts xylan]

NOTE: Individual points represent the average of data obtained from a minimum of duplicate experiments reproducible within 15% and the individual enzyme assays were reproducible within 10%.

FIGURE 21 - Residual enzyme activities in Thermoascus crustaceus 235E Pellicon retentate precipitated with 1.4 M ammonium sulphate then heated at 72.5 °C and desalted.
(A) ■ xylanase. (B) + CMCase.

NOTE: Individual points represent the average of data obtained from a minimum of duplicate experiments reproducible within 15% and the individual enzyme assays were reproducible within 10%.

xylanase substrate might also confer some stability to the hemicellulase activity. The presence of the salt increased the stability of the xylanase more than that of the CMCase, providing another simple step in the "enrichment" of the xylanase activity. A summary of the purification of the culture filtrate of T. crustaceus 235E to yield an "enriched xylanase" mixture is shown in Table 7. The final ratio of xylanase/CMCase activity is quite high at 104:1. In addition, the contaminating salicinase and filter paper activities have been removed. An IEF gel (Figure 22) also demonstrates the purification.

With the success of these purification measures, an enriched-thermophilic-xylanase preparation was available that had been relatively simple and economical to prepare. The next logical experiments were to characterize the mixture for the implementation of some useful applications (e.g. treatment of pulps to remove hemicellulose).

FIGURE 22 - IEF gel (Phast, pI 3-9) of various stages in the purification of endo- β -xylanase from Thermoascus crustaceus 235E. The protein was stained with Phast Silver stain.

SAMPLES*: lane 1 - IEF standards (Biorad)
lane 2 - Pellicon retentate (batch #5), 5X diluted
lane 3 - concentrated pooled fractions (48-64) from SP-Sephadex column, 5X diluted
lane 4 - empty
lane 5 - purified endo- β -xylanase, 5X diluted
lane 6 - Pellicon retentate, 1.4 M ammonium sulphate precipitated and desalted, 5X diluted
lane 7 - Pellicon retentate, 1.4 M ammonium sulphate precipitated, heated at 72.5 °C for 30 min. and desalted, 5X diluted
lane 8 - empty

* protein concentration in the samples varied; samples were applied directly to the gel, diluted in dH₂O where indicated

1 2 3 4 5 6 7 8

Chapter 3

Purification and characterization of the endo- β -xylanase from Thermoascus crustaceus 235E

3.1 INTRODUCTION

The β -D-xylanase purified by Tan *et al.* (1987b) is the only known purified hemicellulolytic enzyme from Thermoascus crustaceus 235E. Recently, there has been much interest in the enzymes of the closely related Thermoascus aurantiacus. An endocellulase, an exocellulase, a β -glucosidase, an α -amylase, and a xylanase were purified from Thermoascus aurantiacus (Feldman *et al.*, 1988; Kawamori *et al.*, 1987; Khande *et al.*, 1989). Otherwise there has been little reported in the literature about this genus of thermophilic fungi.

Most often, enzyme activities are investigated in aqueous solution with at least partially soluble substrates. To determine enzyme specificity, zymograms can be run (Figure 4). A variety of substrates have been designed for use with them (c.f. Biely *et al.*, 1985b; Schwarz *et al.*, 1987). The substrates chosen to be used in this study were the same used for the aqueous enzyme assays, i.e. oat-spelts xylan and CMC.

The assay system employed is of obvious importance for reporting enzymatic activity. In the past few years, much attention has been paid to the way enzyme activities are described in the literature. Many of the recorded activities are not comparable to each other due to the method in which they were assayed. Many laboratories took part in a study to

describe in detail the assays used and reported by the researchers. The biggest problem seems to be the non-availability of a suitable substrate commercially which would show a linear relationship between the amount of reducing sugars produced and the time of hydrolysis (Ghose and Bisaria, 1987). In addition, there is no available standard enzyme with which to compare all other findings. These factors must be kept in mind in interpreting the results of this thesis.

Initially, researchers tried to purify all the lignocellulosic enzymes that they could identify. Then it was discovered that most of the systems displayed a high degree of synergism (Poutanen and Sundberg, 1988). For example, in cellulose degradation, the factors limiting hydrolysis efficiency are the solubility of the substrate and the mixture of the components of the cellulase enzyme system. The mode of action of cellulases has not yet been fully defined but it is thought that usually the β -glucosidase is the rate-limiting step to complete hydrolysis (Saddler, 1986).

Cellulose is a relatively simple polymer compared to the hemicelluloses. Recent results have shown xylans to be less homogeneous and xylanolytic enzymes to act more specifically than commonly anticipated (Puls and Poutanen, 1988). Many xylan-debranching enzymes have been characterized including the α -arabinosidases, the α -glucuronidases, and the acetyl-xylan esterases. For complete hydrolysis of the xylan to occur, all of these enzymes must be present in the proper

amounts. In the case of pulp treatments to remove the hemicellulose and leave the cellulose undamaged, a more purified xylanase may be desirable (Poutanen et al., 1987). It would be favourable to solubilize only the hemicellulose in this case.

The "enriched-xylanase mixture" described in chapter 2 was partially characterized and compared in this chapter to the major β -D-xylanase (also purified here). However, before the characterization could be "completed", the heat treatment procedure was no longer reproducible, compelling a study into the cause of the sudden change.

3.2 METHODS AND MATERIALS

ENDO- β -D-XYLANASE PURIFICATION:

T. crustaceus 235E Pellicon retentate was assayed for protein concentration (chapter 1.2). Then an aliquot of the retentate containing 100 mg of protein was diluted to three times its volume with 10 mM Na Acetate buffer, pH 4.4. In the first run, the sample was loaded directly onto the column. For the second and subsequent runs, the pH of the sample was adjusted to pH 4.4 prior to loading. A 2.5 X 40 cm column of SP-Sephadex C50 was equilibrated with ten column volumes of 10 mM Na Acetate, pH 4.4 (BUFFER A). The diluted protein samples were then applied to the column at 4 °C. The column was subsequently washed with 400 mL of buffer A. The bound

proteins were eluted with a linear gradient of 750 mL each of buffer A and buffer A containing 0.4 M NaCl, and 2 mL fractions were collected. The optical density (280 nm), protein concentration (chapter 1.2), and the xylanase activity of the fractions were determined. Fractions 48-64, inclusive, were pooled from the second trial and labelled the "fractions from SP-Sephadex" (peak C; Figure 23).

A 1.5 X 7.8 cm DEAE-Sephadex A50 column was equilibrated with ten column volumes of 50 mM Na acetate buffer, pH 4.8 (BUFFER B). The pH of a 5 mL aliquot of the "fractions from SP-Sephadex" containing the endo- β -xylanase activity was adjusted to pH 4.8 and was then loaded onto the column. The beads were subsequently washed with buffer B and 3 mL fractions were collected. The optical density (280 nm), protein concentration (chapter 1.2), and xylanase and CMCase activities of the fractions were determined. In addition, the fractions were run on a "native gradient gel" (see later).

This 2-column purification was performed twice with different batches of Pellicon retentate with the same results.

ELECTROPHORETIC GELS:

The procedure for IEF gels can be found in chapter 2.2. Pre-cast "Native gels" (gradient 8-25) were used in conjunction with the Phast System (Pharmacia). Pure proteins were run at approximately 1 mg/mL and mixtures of proteins were loaded according to the "best" results. All samples were

FIGURE 23 - Chromatographic profile of Thermoascus crustaceus 235E Pellicon retentate on SP-Sephadex C50 column. (A) first trial. (B) second trial, modifying sample pH to account for differences in methodology from original procedure.

(A) ● protein (optical density at 280nm). (B) +
xylanase activity (DNS assay, optical density at 575nm).

diluted with water. Samples were loaded using the 8-well loaders, which transfer approximately 1-2 μ L per well. Immediately after the run, the gels were developed using the Phast development system (Pharmacia). The proteins were detected by Phast Silver Stain (Pharmacia).

ZYMOGRAMS:

The zymograms were made with Gelbond acetate sheets on a casting unit (both from BioRad) to give a final thickness of 0.1 mm. The gel consisted of 0.5% oat-spelts xylan or CMC, 2% Bacto-agar (Difco), and 0.02% Na azide. The gels were degassed prior to pouring. A second layer of Gelbond was laid on top of the gel and allowed to harden. The zymograms were stored in a sealed container moistened with 50 mM Na acetate, pH 4.8 and used within a week.

An IEF gel was run (chapter 2.2), washed quickly in 50 mM Na acetate, pH 4.8 and laid facedown on top of the 0.5% oat-spelts xylan zymogram for 30 minutes at 65 °C. The gel was removed and the zymogram was stained in 0.1% Congo Red (BioRad) for 1 hour at 65 °C. The zymogram was then destained with 1 M NaCl until the bands were visible, and subsequently, a 5% acetic acid solution was added to fix the zymogram and give it a blue colour.

CHARACTERIZATION OF THE "ENRICHED-XYLANASE MIXTURE" (CHAPTER 2.2):

(A) Optimum pH/temperature studies

The xylanase activity of the "enriched-xylanase mixture" was measured as in chapter 1.2, but for the temperature optimum, the temperature of the assay was varied from 45 to 85 °C and the assay was 15 minutes long. For the pH study, 50 mM Na acetate was adjusted from pH 3 to pH 7.5 prior to a 15 minute assay.

(B) Enzyme activities on various substrates

The xylanase and CMCase activities of the "enriched-xylanase mixture" were determined as in chapter 1.2. The assays were carried out at 65 °C for 15 minutes. The substrates included oat-spelts xylan, larchwood xylan (Sigma), aspen xylan (isolated by Dr Harold Brownell, Forintek Canada Corporation), and Pinus radiata xylan (gift from Dr Timothy Leathers, Northern Regional Research Centre, Illinois).

(C) Study of a method to store and transport the mixture

Two 10 mL aliquots of the "enriched-xylanase mixture" were re-precipitated with 2.5 M ammonium sulphate for 30 minutes at 25 °C. The resulting solutions were centrifuged at 3000 rpm for 25 minutes. The supernatants were decanted and the pellets saved. One pellet was made up in 10 mL of dH₂O, desalted with a BioRad disposable desalting column, and assayed immediately for enzyme activity at 65 °C for 15 minutes (chapter 1.2). The second pellet was stored in a

sealed vial at 0-4 °C for 92 days, after which time it was reconstituted in 10 mL of dH₂O and assayed as above. The protein was determined as in chapter 1.2.

(D) Temperature stability over prolonged periods

Three 10 mL aliquots of the "enriched-xylanase mixture" were placed in screw-top vials and kept at 0 °C, room temperature (22-26 °C), and 65 °C for 39 days. Periodically, a sample of each of the aliquots was removed and assayed for xylanase and CMCase activity at 65 °C for 15 minutes (chapter 1.2).

All enzyme assays were performed at least in duplicate and were reproducible within 15 per cent, under similar conditions.

3.3 RESULTS AND DISCUSSION

Purification of the major β -D-xylanase enzyme was performed following the protocol outlined by Tan *et al.* (1987c). Their procedure involved a single chromatographic step; the enzyme was adsorbed on a SP-Sephadex C50 column and eluted with a NaCl gradient (Appendix 6). The purified preparation was homogeneous according to denaturing acrylamide (SDS) and isoelectric focusing (IEF) gels. When the procedure was repeated in order to have some pure β -D-xylanase to

compare to my "enriched-xylanase mixture", the results were slightly different.

No protein bound to the column in my first trial (Figure 23) and attempts to locate the xylanase were futile because no activity was recorded; the pH of my Pellicon retentate was too high for enzyme adsorption. However, when the initial pH of the enzyme concentrate was adjusted to pH 4.4, the elution profile reassembled that of Tan *et al.* (1987b). Peak "C" (Figure 23) was pooled, assayed for enzyme activity, protein concentration, and run on an IEF gel to ensure its homogeneity (Table 8; Figures 23 and 24). A specific activity of 2141 IU/mg was much higher than the specific activity of 61.3 IU/mg found by Tan *et al.* (1987b). This discrepancy can be accounted for in part by the difference in the assay temperature used.

The IEF gel of the SP-Sephadex fractions containing the β -D-xylanase activity (Figure 24) showed that the fractions from my SP-Sephadex column were not pure β -xylanase. There were at least two contaminating bands having a pI of about 3, which were not observed by Tan and co-workers. An oat-spelts xylan zymogram (described in chapter 3.2 and Figure 4) was run using various fractions from the purification process (Figures 25 and 26). Although difficult to discern, the fractions from the SP-Sephadex column had only one band of xylanase activity at about pI 7 (lane 3, Figure 25; lane 6, Figure 26). A CMC zymogram was run with the same IEF gel and was found to have a similar pattern of hydrolysis (data not shown). The purified

TABLE 8 - Summary of the purification of an endo β xylanase from *Thermosaccus crustaceus* 235E culture filtrate

	Culture filtrate	Pellicon retentate	Fractions from SP-Sephadex ^a	"Pure" endo- β -xylanase ^b
xylanase ^c (IU/mL)	320.2	2725	3468	2249
CHCase ^c (IU/mL)	18.9	135	1.6	0.9
protein ^d (mg/mL)	0.8	7.5	1.6	0.9
volume (mL)	140	14	7.5	12.4
specific activity ^e of xylanase (IU/mg)	400	363	2141	2445
<u>xylanase</u> CHCase	16.9	20.1	2168	2319
purification factor	1	0.9	5.4	6.1

^a fractions (48-64) from SP-Sephadex C50 column concentrated with a 10 KDa cut-off membrane (see section 3.2 for details)

^b fractions 3-5 from DEAE-Sephadex A-50 column (see section 3.2 for details)

^c enzyme assays were carried out as specified in section 1.2 at 65 °C for 15 minutes using the DNS method (Miller, 1959) for reducing sugar quantitation

^d protein was determined by a modified Lowry assay, using BSA as the standard (see section 1.2 for details)

^e specific activity = $\frac{\text{activity (IU/mL)}}{\text{protein (mg/mL)}}$

FIGURE 24 - IEF gel (Phast, pI 3-9) of the various stages in the purification of endo- β -xylanase from Thermoascus crustaceus 235E. The protein was stained with Phast Silver stain.

SAMPLES*: lane 1 - purified endo- β -xylanase, 5X diluted
lane 2 - concentrated pooled fractions (48-64) from SP-Sephadex column, 5X diluted
lane 3 - IEF standards (Biorad)

* protein concentration in the samples varied; samples were applied directly to the gel, diluted in dH_2O where indicated

1 2 3

FIGURE 25 - IEF gel (Phast, pI 3-9) of various stages in the purification of endo- β -xylanase from Thermoascus crustaceus 235E (see also Fig. 26 for xylan zymogram). The protein was stained with Phast Silver stain.

SAMPLES*: lane 1 - IEF standards (Biorad)
lane 2 - purified endo- β -xylanase, 5X diluted
lane 3 - concentrated pooled fractions (48-64)
from SP-Sephadex column, 5X diluted
lane 4 - Pellicon retentate (batch #6), 20X
diluted
lane 5 - Pellicon retentate (batch #6), 10X
diluted
lane 6 - Pellicon retentate (batch #6), 5X
diluted
lane 7 - Culture filtrate (batch #6)
lane 8 - IEF standards (Biorad)

* protein concentration in the samples varied; samples were applied directly to the gel, diluted in dH₂O where indicated

FIGURE 26 - Oat-spelts xylan zymogram of IEF gel in Fig. 25 of various stages in the purification of endo- β -xylanase from Thermoascus crustaceus 235E. The xylan was stained with Congo Red.

SAMPLES*: lane 1 - IEF standards (Biorad)
lane 2 - Culture filtrate (batch #6)
lane 3 - Pellicon retentate (batch #6), 5X
diluted
lane 4 - Pellicon retentate (batch #6), 10X
diluted
lane 5 - Pellicon retentate (batch #6), 20X
diluted
lane 6 - concentrated pooled fractions (48-64)
from SP-Sephadex column, 5X diluted
lane 7 - purified endo- β -xylanase, 5X diluted
lane 8 - IEF standards (Biorad)

* protein concentration in the samples varied; samples were applied directly to the gel, diluted in dH₂O where indicated

NOTE: the zymogram is a mirror image of the IEF gel in Fig. 25

xylanase is known to have cross-specificity for CMC, perhaps explaining why there is always some residual CMCase activity in the "enriched-xylanase mixture". The contaminating bands were definitely not xylanases or CMCases.

The zymogram technique is relatively new, and there is not as yet a perfect methodology; each enzyme system appears to require a different set of conditions. Some zymogram techniques have been published (c.f. Biely *et al.*, 1985a; Schwarz *et al.*, 1987) but there was not time to do an intensive study into the optimal conditions for the enzymes being studied here.

In order to remove the contaminating bands, a DEAE-Sephadex A50 (anion exchange) column was run (Figure 27). Due to its pI, the β -D-xylanase was passed through the column unadsorbed (fractions 3-5). The contaminating proteins were also eluted from the column with washing, but in a later fraction (9). The fractions were run on a "Native gel" (Figure 28) and were found to have relative molecular weights of approximately 20 and 45 kDa. The now pure β -D-xylanase has a molecular weight of 32 kDa. The contaminating proteins had no xylanase or CMCase activity, but were found to have β -xylosidase activity (Table 9). To date, no one has reported a β -xylosidase activity in any *Thermoascus* species in the literature. However, most organisms that contain xylanase activities also make β -xylosidase(s) (Wong *et al.*, 1988).

Although the new enzyme was an interesting find, the main

FIGURE 27 - Chromatographic profile of concentrated pooled fractions (48-64) from SP-Sephadex C50 column (Thermoascus crustaceus 235E Pellicon retentate) on DEAE-Sephadex A-50 column. (A) ■ protein (optical density at 280nm). (B) + xylanase activity (DNS assay, optical density at 575nm). (C) ° CMCase activity (DNS assay, optical density at 575nm).

NOTE: Individual points represent the average of data obtained from a minimum of duplicate experiments reproducible within 15% and the individual enzyme assays were reproducible within 10%.

FIGURE 28 - Native gel (Phast, gradient 8-25) of fractions from the DEAE-Sephadex column in the purification of endo- β -xylanase from Thermoascus crustaceus 235E. The protein was stained with Phast Silver stain.

SAMPLES*: lane 1 - low molecular weight standards
(Biorad)
lane 2 - fraction #3 (6.8 mL)
lane 3 - fraction #4 + #5 (mixed) (15.2 mL)
lane 4 - fraction #6 (18.1 mL)
lane 5 - low molecular weight standards
(Biorad)
lane 6 - fraction #7 (21.4 mL)
lane 7 - fraction #8 (26.0 mL)
lane 8 - fraction #9 (30.4 mL)

- *
-protein concentration in the samples varied;
samples were applied directly to the gel, diluted
in dH₂O where indicated
-the fractions refer to those collected off the
DEAE-Sephadex column, the volume in brackets
indicates the elution volume (see also Fig. 27)

TABLE 9 - Properties of a β -xylosidase partially purified^a from Thermosascua crustacea 235E Pellicon retentate

	activity ^b (IU/mL)	specific activity ^c
xylanase	0.0	0.0
CMCase	0.0	0.0
β -xylosidase	3.2	16.8
protein ^d	0.19 (mg/mL)	

^a fraction 9 (30.4 mL) from DEAE-Sephadex A50 column (see section 3.2 for details)

^b enzyme assays were carried out as specified in section 1.2 at 65 °C for 15 minutes using the DNS method (Miller, 1959) for reducing sugar quantitation

^c specific activity = $\frac{\text{activity (IU/mL)}}{\text{protein (mg/mL)}}$

^d protein was determined by a modified Lowry assay and using BSA as the standard (see section 1.2 for details)

purpose of this thesis was not to purify enzymes from T. crustaceus 235E. The major β -D-xylanase was now pure (Table 8) and could be used for comparison purposes with my "enriched-xylanase mixture".

The "enriched-xylanase mixture" was examined for its temperature and pH optima (Figures 29 and 30). The temperature optimum was found to be 78 °C (Figure 29), while that of the pure β -D-xylanase was 80 °C (Tan et al., 1987b). The pH optimum of the "enriched-xylanase mixture" was 4.8 (Figure 30), which compares to a pH optimum for the purified enzyme of pH 5.1 (Tan et al., 1987b). Minor differences between the "enriched-xylanase mixture" and the purified β -D-xylanase can probably be contributed to the relative impurity of the mixture.

In assaying the enzyme activity of the "enriched-xylanase mixture", the time, temperature, and dilution of the enzyme assay itself are very important. The mixture was assayed at three different temperatures over one hour (Figure 31). The xylanase activity is constant at 50 °C, after which activity slowly decreases. The activity is constant for only 25 minutes at 65 °C (Figure 31A). The reducing sugars produced are shown in a reaction time-course study (Figure 31B). The time of the assay must fall in the region of the curve where the initial rate is linear (Engel, 1981). Although the optimum temperature of the "enriched-xylanase mixture" is around 80 °C (Figure 29), the time-course at 70 °C is linear for only 10 minutes.

FIGURE 29 - Optimum temperature for the "enriched-xylanase" mixture^a, based on a 15 minute assay on oat-spelts xylan, as measured by the DNS method (Miller, 1959). [. xylanase specific activity (IU/mg protein)]

^a Thermoascus crustaceus 235E Pellicon retentate precipitated with 1.4 M ammonium sulphate, centrifuged, then supernatant heated at 72.5 °C for 30 minutes.

NOTE: Individual points represent the average of data obtained from a minimum of duplicate experiments reproducible within 15% and the individual enzyme assays were reproducible within 10%.

FIGURE 30 - Optimum pH for the "enriched-xylanase" mixture^a, based on a 15 minute assay on oat-spelts xylan, as measured by the DNS method (Miller, 1959). [■ xylanase specific activity (IU/mg protein)]

^a Thermoascus crustaceus 235E Pellicon retentate precipitated with 1.4 M ammonium sulphate, centrifuged, then supernatant heated at 72.5 °C for 30 minutes.

NOTE: Individual points represent the average of data obtained from a minimum of duplicate experiments reproducible within 15% and the individual enzyme assays were reproducible within 10%.

FIGURE 31 - The action of the "enriched-xylanase" mixture^a on oat-spelts xylan over time at various temperatures, as measured by the DNS method (Miller, 1959). (A) xylanase activity (B) reducing sugars produced. [■ 50 °C; + 65 °C; ° 70 °C]

^a Thermoascus crustaceus 235E Pellicon retentate precipitated with 1.4 M ammonium sulphate, centrifuged, then supernatant heated at 72.5 °C for 30 minutes.

NOTE: Individual points represent the average of data obtained from a minimum of duplicate experiments reproducible within 15% and the individual enzyme assays were reproducible within 10%.

Furthermore, short assays at high temperatures are less reliable because the water baths I used did not keep constant temperatures above 65 °C. Because the curve is linear at that temperature for at least 20 minutes, it was decided that an assay temperature of 65 °C and a time of 15 minutes would be "representative" for this "enriched-xylanase mixture".

The entire concept of a "representative" xylanase assay has been under scrutiny for more than a decade now. Whole conferences have been devoted to standardizing assay systems for lignocellulosic enzymes (e.g. IEA conference, June 1988). Khan et al. (1986) showed that the enzyme dilution prior to assay and the type of substrate affected the activity of the enzyme. There is no standard substrate for xylanase assays as there exists for filter paper, β -glucosidase, and β -xylosidase assay systems. Khan and his co-workers suggested that the definition of composition and the use of a standard method for the preparation of xylan would help in minimizing variation due to substrate. In addition, the use of enzyme dilution to give 0.9 to 1.0 mg of reducing sugars with a 1% xylan solution for the calculation of xylanase units would help in minimizing the variation in the results obtained by different laboratories using the same substrate (Khan et al., 1986).

Unfortunately, although their recommendations appear sound, there is no one source of xylan that is dependably reproducible amongst batches. In addition, not all xylanases will degrade all types of xyans. The standard assay that was

used in this thesis tended to produce reducing sugars in the order of 0.7-1.2 mg/mL, which falls close to the guidelines set by Khan et al. (1986) and the participants at the IEA Conference (June 1988, Ottawa). The activity reported for the "enriched-xylanase mixture" is important if comparisons to other enzyme systems are to be made. This is one problem that researchers in the field of lignocellulosic enzyme study face perpetually. But, the assay system now in use must do until a more standardized one can be devised.

The activity of the "enriched-xylanase mixture" was measured on a variety of xylan substrates that were available (Table 10). The assay was performed at 65 °C for 15 minutes on all the substrates after verifying that those conditions produced a linear reaction rate in the given time (data not shown). The highest activity was on oat-spelts xylan, the same substrate that is used to assay xylanase activity throughout the thesis. Therefore, the activities of the "enriched-xylanase mixture" reported here may not extrapolate well to other substrates and assay systems.

The ability of the "enriched-xylanase mixture" to degrade various xylan preparations may depend on the enzyme complement present in the mixture. Poutanen et al. (1987) compared the abilities of five different microbial xylanolytic systems to degrade steamed birchwood hemicellulose. The researchers found that Trichoderma reesei QM 9414 had the best system for complete hydrolysis of the hemicellulose. They postulated

TABLE 10 - Enzyme activity on various substrates by Thermoascus crustaceus 235E "enriched-xylanase mixture"^a

substrate ^b	activity ^c (IU/mL)	xylanase CMCase
oat-spelts xylan	6377	35.7
larchwood xylan	4360	24.4
aspen xylan	3196	17.9
<u>Pinus radiata</u> xylan	2616	14.7
carboxymethyl- cellulose (CMC)	178.4	/

- ^a Thermoascus crustaceus 235E Pellicon retentate (Batch #6) precipitated with 1.4 M ammonium sulphate, centrifuged, then supernatant heated at 72.5 °C for 30 minutes
- ^b 0.5% (w/v) final concentration in 50 mM sodium acetate buffer, pH 4.8
- ^c enzyme assays were carried out as specified in section 1.2 at 65 °C for 15 minutes using the DNS method (Miller, 1959) for reducing sugar quantitation

that, because the T. reesei filtrate contained many of the side-group cleaving activities, including acetyl esterase, α -glucuronidase, and α -arabinosidase, hydrolysis was bound to be more complete. They also found that some of its other enzymes were useful to avoid complete hydrolysis. The enzyme complement of T. crustaceus 235E was found to contain many different activities (Table 4), but it is not known how many of these survived the purification procedure and are present in the "enriched-xylanase mixture". The lack or presence of these enzymes would greatly influence the ability of the "enriched-xylanase mixture" to degrade various xylans.

A study was made of the hydrolysis potential of the "enriched-xylanase mixture" on various pulps, including bleached and unbleached aspenwood, bleached and unbleached alder (all hardwoods), and bleached and unbleached hemlock (softwood). Due to the lack of xylooligomeric standards and the little that was understood about the enzymes present in the "enriched-xylanase mixture", the results were inconclusive and difficult to interpret (data not shown). Indeed, the facilities were not available to correctly determine the effects that the mixture had on the pulps tested.

Studies were done to investigate the stability of the "enriched-xylanase mixture" under various conditions. Initially, the preparation was kept for 40 days at different temperatures (Figures 32 and 33). None of the enzymatic activity was lost when the mixture was kept at 0-4 °C or at

FIGURE 32 - Residual enzyme activity in the "enriched-xylanase" mixture^a from Thermoascus crustaceus 235E incubated over time at various temperatures. (A) xylanase activity. (B) CMCase activity. [■ 0 °C; + room temperature; ° 65 °C]

^a Thermoascus crustaceus 235E Pellicon retentate precipitated with 1.4 M ammonium sulphate, centrifuged, then supernatant heated at 72.5 °C for 30 minutes.

NOTE: Individual points represent the average of data obtained from a minimum of duplicate experiments reproducible within 15% and the individual enzyme assays were reproducible within 10%.

FIGURE 33 - Ratio of residual xylanase/CMCase activity in the "enriched-xylanase" mixture^a from Thermoascus crustaceus 235E incubated over time at various temperatures. [■ 0 °C; + room temperature; ° 65 °C]

^a Thermoascus crustaceus 235E Pellicon retentate precipitated with 1.4 M ammonium sulphate, centrifuged, then supernatant heated at 72.5 °C for 30 minutes.

NOTE: Individual points represent the average of data obtained from a minimum of duplicate experiments reproducible within 15% and the individual enzyme assays were reproducible within 10%.

room temperature (22-26 °C; Figure 32). However, the mixture had a half-life of 28 days at 65 °C. The ratio of xylanase to CMCase activity didn't vary much over any temperature range (i.e. both activities disappeared at the same rate; Figure 33). This extremely long half-life at 65 °C makes this preparation an ideal candidate for adaptation to industrial practices. A review of the literature showed that no one has reported a thermophilic "enriched-xylanase mixture" such as this one.

A final study was set up to investigate a method to store and transport the "enriched-xylanase mixture" (Table 11). The preparation was re-precipitated with ammonium sulphate and stored for 92 days at 0-4 °C. When reconstituted, the mixture had retained all its enzymatic activities, even after the long incubation (table 11). Again, this property makes the "enriched-xylanase mixture" a likely candidate for use in industrial situations.

At this point in time, a problem arose. The heat inactivation step (72.5 °C for 30 minutes) was no longer reproducible (see chapter 4 for details).

TABLE 11 - Study of a method to store and transport the "enriched-xylanase" mixture^a from Thermoascus crustaceus 235E (Batch #6)

	"enriched-xylanase" mixture ^a	resuspended pellet ^b immediately	after 92 days ^c
xylanase ^d (IU/mL)	3951	3674	3695
CMCase ^d (IU/mL)	41.2	41.4	40.2
<u>xylanase</u> CMCase	96	91	92
protein ^e (mg/mL)	5.6	5.3	5.2

- ^a Thermoascus crustaceus 235E Pellicon retentate (Batch #6) precipitated with 1.4 M ammonium sulphate, centrifuged, then supernatant heated at 72.5 °C for 30 minutes
- ^b precipitated "enriched-xylanase" mixture with 2.5 M ammonium sulphate, centrifuged, reconstituted pellet^c in equivalent amount of dH₂O (see section 2.2 for details)
- ^c stored pellet (moist) for 92 days at 0-4 °C, then reconstituted it in an equivalent amount of dH₂O and assayed immediately (see section 2.2 for details)
- ^d enzyme assays were carried out as specified in section 1.2 at 65 °C for 15 minutes using the DNS method (Miller, 1959) for reducing sugar quantitation
- ^e protein was determined by a modified Lowry assay and using BSA as the standard (see section 1.2 for details)

Chapter 4

Loss of reproducibility of the heat inactivation step and a discussion of some possible explanations

4.1 INTRODUCTION

Filamentous fungi are distinct from single-celled yeasts because they grow as branching filaments (hyphae), collectively referred to as the mycelium. The fungus grows by elongation at the tips of the hyphae, while the nuclei (usually haploid) divide mitotically in the leading cells. Many fungal species have several haploid nuclei per cell. Each nucleus is surrounded by a nuclear envelope. If these nuclei are not all genetically identical, the condition is called heterokaryosis. Heterokaryosis can arise from hyphal fusion, spontaneous mutation, or transformation by recombinant DNA techniques (Ball, 1984; Rambossek and Leach, 1987).

Many fungi can reproduce both sexually and asexually. Asexual reproduction occurs when the vegetative mycelium differentiates and produces spores (conidia) that contain nuclei from mitotic divisions. Conidia germinate and continue the asexual cycle. The sexual stage of fungi (both filamentous and non-filamentous) occurs when two haploid nuclei fuse and then undergo meiosis. In ascomycetes, such as Thermoascus crustaceus 235E, the four products of meiosis (frequently duplicated) are contained in a sac called an ascus (the sexual spores contained in the ascus are known as ascospores; Johri,

1980).

The genetic manipulation of some filamentous fungi, such as Neurospora crassa and Aspergillus nidulans, are relatively well understood. However, the genetics of most of the fungi used in industry remain uncharacterized. Industrial production strains have long been selected and improved using mutagenesis and selection as well as sexual and asexual manipulations (Bennett and Lasure, 1985; Timberlake, 1985). These techniques are usually very slow and time-consuming. For example, Trichoderma reesei has been mutated and manipulated for more than 20 years, but now extracellular enzymes are produced at levels up to 100-fold higher than in the wild-type fungus.

Researchers at Forintek, as well as other laboratories have realized that there is an important difficulty with culturing filamentous fungi, especially those that are capable of undergoing meiosis. It is often difficult to reproduce results of mycelial growth and extracellular enzyme production. Indeed, this impediment was experienced in the work of this thesis. After five batches of T. crustaceus 235E had been grown and three treated to obtain the "enriched-xylanase mixture" (chapter 2), the heat treatment step of the process was no longer reproducible. This chapter represents the concluding data of the thesis, and compares the "old" and "new" batches of the enzymes from T. crustaceus 235E.

4.2 METHODS AND MATERIALS

COMPARISON OF BATCHES OF PELLICON RETENTATE

The data in this chapter was collected through the comparison of "old" batches (batches 4 and 5) and "new" batches (batches 6-8) of *T. crustaceus* 235E Pellicon retentate. Enzyme activities were assayed as in chapter 1.2. The ammonium sulphate precipitation and heat treatment procedures are described in chapter 2.2. Finally, the IEF gel was run as reported in chapter 2.2

4.3 RESULTS AND DISCUSSION

The "new" batches of *T. crustaceus* 235E Pellicon retentate exhibited the same ammonium sulphate precipitation pattern as did the "old" batches (Figures 19 and 22; data not shown for "new" batches). However, the differential heat inactivation patterns (Figures 16-18, 20 and 21) could no longer be reproduced. When the Pellicon retentates of batches 6-8 were heat treated at 72.5 °C, the CMCase activity was actually more heat stable than the xylanase activity (Figure 34). This finding was inconsistent with previous work as the heat treatment step had been reproduced on three separate batches of the Pellicon retentate which had been grown up earlier (i.e. batches 3, 4 and 5).

Since the differential heat labilities were more pronounced after the ammonium sulphate step, the Pellicon

FIGURE 34 - Residual enzyme activity in Thermoascus crustaceus 235E Pellicon retentate (Batch #7) heated at 72.5 °C over time. (A) ■ xylanase. (B) + CMCase.

NOTE: Individual points represent the average of data obtained from a minimum of duplicate experiments reproducible within 15% and the individual enzyme assays were reproducible within 10%.

retentates were precipitated and heat treated again. After heating at 72.5 °C, the precipitated Pellicon retentates (batches 6-8) showed insignificant loss of either xylanase or CMCase activity (Figures 35A and 35B; Table 12). Preparations were heated at higher temperatures, but the inverse heat inactivation ratios were observed (Figures 35C and 35D; Table 12) compared to the earlier batches of enzymes. The CMCase activity was exhibiting a higher heat stability than that of the xylanase - completely opposite to the earlier findings. Table 12 contains a summary comparing an "old" batch (5) to a "new" batch (7) of precipitated Pellicon retentates. From the ratio of xylanase/CMCase, it is clear that the endocellulase component is now the more heat stable enzyme.

These findings are very surprising since according to the IEF gel, there were no visible differences in the protein compositions of the various "old" and "new" batches of T. crustaceus 235E Pellicon retentates (Figure 36). Unfortunately, only small amounts of the "old" batches of enzyme were treated to obtain the "enriched-xylanase mixture" and the Pellicon retentate was now too old to heat treat (see chapter 2 for discussion of the importance of the age of Pellicon retentate). The authenticity of the cultures of T. crustaceus 235E were once again verified by Keith Siefert, but no explanation for the transformation in the enzyme properties has been found.

One aspect of the industrial production of extracellular

FIGURE 35 - Residual enzyme activity in Thermoascus crustaceus 235E Pellicon retentate (batch #7): 1.4 M ammonium sulphate precipitated and then heated at various temperatures. (A) heated at 72.5 °C (B) heated at 85 °C. [■ xylanase; + CMCase]

NOTE: Individual points represent the average of data obtained from a minimum of duplicate experiments reproducible within 15% and the individual enzyme assays were reproducible within 10%.

TABLE 12 - Residual activities after heat treatment of various batches of 1.4 M Ammonium sulphate precipitated Thermoascus crustaceus 235E Pellicon retentate

	% RESIDUAL ACTIVIT		
	Batch #5 ^a	Batch #7 ^b	Batch #7 ^c
Xylanase ^d (IU/mL)	62	93	4
CMCase ^d (IU/mL)	15	101	60
Xylanase CMCase prior to heating	26	22	22
after heat treatment	104	21	2

- ^a Batch #5 Pellicon retentate, 1.4 M ammonium sulphate precipitated, centrifuged, then supernatant heated at 72.5 °C for 30 minutes
- ^b Batch #7 Pellicon retentate, 1.4 M ammonium sulphate precipitated, centrifuged, then supernatant heated at 72.5 °C for 30 minutes
- ^c Batch #7 Pellicon retentate, 1.4 M ammonium sulphate precipitated, centrifuged, then supernatant heated at 85 °C for 30 minutes
- ^d Enzyme assays were carried out as specified in section 1.2 at 65 °C for 15 minutes using the DNS method (Miller, 1959) for reducing sugar quantitation

FIGURE 36 - IEF gel (Phast, pI 3-9) comparing various Pellicon retentates from Thermoascus crustaceus 235E that were 1.4 M ammonium sulphate precipitated and heat treated at 72.5 °C for 30 minutes. The protein was stained with Phast Silver stain.

SAMPLES*: lane 1 - IEF standards (Biorad)
lane 2 - Pellicon retentate from batch 3/6/86
lane 3 - Pellicon retentate from batch #5
lane 4 - Pellicon retentate from batch #7
lane 5 - Pellicon retentate from batch #8

* protein concentration in the samples was approximately 0.7 µg per sample; samples were diluted in dH₂O

enzymes by filamentous fungi has been the problem of reproducibility. Discussions with researchers at Forintek Canada Corporation led to the conclusion that although ratios of activities produced by filamentous fungi could vary up to 100-fold from one batch to the next, no one had really studied the reproducibility of the differential heat stabilities of the enzymes.

Although the genetics of Thermoascus species has not been well documented, it is known that the nuclear genome of most filamentous fungi is organized into six to eight chromosomes (Ball, 1984). The chromosomal DNA contains mainly unique sequences (usually from 90 to 97 percent of the DNA). In addition, the repetitive DNA in most fungi has been attributed to ribosomal RNA genes (Rambosek and Leach, 1987). Therefore, one would expect that a single mutation in the gene coding for the major xylanase activity could have been the cause for the change in its properties. This matter will be discussed in more detail in the next section of the thesis.

CONCLUSIONS

One of the major expenses incurred in the application of enzymes for industrial processes is the cost of the enzyme production (Biely, 1985). The total cost of production includes the cost of fermentative production as well as downstream processing requirements. Both of these factors must be optimized and integrated for maximum cost effectiveness (Saddler et al., 1988). Use of a thermostable enzyme can significantly enhance the technical and economic feasibility of hydrolysis processes. The thermostable enzymes can be used at elevated industrial temperatures with increased activity and longevity, with decreased risk for contamination. The cellulolytic and xylanolytic fungus Thermoascus crustaceus 235E can grow on a variety of inexpensive substrates, including untreated sawdust, to produce high concentrations of thermostable xylanase (Yu et al., 1987).

The first area of application for xylanolytic enzymes involves the use of xylanases with other hydrolytic enzymes in the bioconversion of wastes such as those from the pulp and paper and agricultural industries, and in the clarification and liquefaction of juices, vegetables and fruits (Biely, 1985; Eriksson, 1988). Crude xylanase preparations have been used in the bioconversion of cellulosic materials for subsequent fermentation (Doppelbauer et al., 1987) and in the hydrolysis of pulp waste liquors and wood extractives to monomeric sugars for subsequent production of single-cell

protein (Royer and Nakas, 1989; Weckstrom and Leisola, 1981). xylose produced by the action of xylanases can be used for subsequent production of higher value compounds such as ethanol, xylitol and xylonic acid (Skoog and Hahn-Hagerdal, 1988).

The second area for application of xylanases involves the use of normally cellulase-free xylanases for removal of hemicellulose from high-grade cellulose pulps (Paice et al., 1988; Paice and Jursak, 1984) and plant fibres (Sharma, 1987). There are many examples of the purification of xylanases to homogeneity (c.f. Reilly, 1981; Woodward, 1984).

Preliminary investigations have shown that concentrations of up to 1% (w/v) xylose did not inhibit enzyme production in T. crustaceus 235E, while the same concentration of glucose had a completely inhibitory effect on the enzyme yield. It was concluded that the controls for the production of hemicellulases and cellulases in this organism are closely related. T. crustaceus 235E grows and produces relatively high quantities of xylanases on the water-soluble fraction of steam-exploded wood (SEA-WS), currently a waste product. Optimization of this growth medium could make the enzymes from this fungus a viable alternative to the currently employed mesophilic fungi (e.g. Trichoderma harzianum). Optimization of the xylanase/CMCase ratio in the starting product (i.e. the culture filtrate) would make downstream processing easier and more cost-efficient (Wheelwright, 1987).

The procedure for the creation of the "enriched-xylanase mixture" from T. crustaceus 235E Pellicon retentate involved an easy ammonium sulphate precipitation step followed by an inexpensive heat treatment process. This mixture exhibited a xylanase/CMCase ratio of 104 to 1. The presence of the residual endocellulase activity makes the preparation useful for the aforementioned bioconversion and clarification processes.

Initially it was presumed that cellulase-free xylanases were required for the treatment of pulps to remove the hemicellulose component without destroying the cellulose fibres. However, the cellulose fibres frequently restrict access of the xylanases to the hemicellulose and solubilization and subsequent removal is impossible. Therefore, elimination of the hemicellulose often requires small amounts of cellulases to degrade some of the cellulose that is obstructing the xylanases' ability to hydrolyse its substrate (K. Poutanen, unpublished data).

Synergism in the action of hydrolytic enzymes is not a new concept. It has been observed especially in the degradation of cellulose by the three cellulolytic enzymes: endocellulases, "exocellulases", and β -glucosidases (Saddler, 1986). Often the enzymes form an enzyme-complex and the rate-limiting step is most often the amount of β -glucosidase present. A similar synergism has been proposed for xylanases and their related enzymes (c.f. Johnson et al., 1989; Poutanen

and Sundberg, 1988; Wong et al., 1988). Biodegradation of the hemicelluloses requires enzyme activities that remove non-xylose substituents from the xylan backbone in addition to endoxylanases and β -xylosidases. Acetyl xylan esterases and arabinosidases are two of the better studied substituent-hydrolysing activities (Johnson et al., 1989). Less is known about the α -glucuronidases and the 4- α -methylglucuronidases. Many of these activities are actively being investigated to determine their possible roles in vivo and in industrial applications.

Recent studies have reported treatment of various pulps with xylanases to alter their physical properties. For example, Noé et al. (1986) and Mora et al. (1986) demonstrated that xylanases could modify the "beatability" of pulps thereby enhancing its paper-making properties. In another study, Jeffries and Lins (1989) illustrated that xylanase treatment of aspen kraft or thermomechanical pulp (TMP) could reduce the amount of energy required for refining the pulp, without any effect on its brightness or opacity. Indeed, enzyme treatment of recycled paper was found to improve the physical properties of the material (Pommier et al., 1989). Finally, the substituent-hydrolysing activities have been implicated for use in treating pulps for improved physical properties as well as decreasing the energy requirements and the pollution created by the existing technologies.

T. crustaceus 235E exhibits many of these substituent-

hydrolysing activities (Table 4), but the "enriched-xylanase mixture" was not tested for these enzymes. The enzyme system of T. crustaceus 235E requires more study into its hydrolytic potential as well as its production. At a conference held at Forintek Canada Corporation in June 1989, many of the researchers, particularly those from Finland and Sweden, were interested in T. crustaceus and its potential for use in the industry.

Probably the most important next step in this study of this fungus is to understand the control of enzyme production and indeed its genetic mechanisms. For example, why did the differential heat stabilities of the xylanase and CMCase reverse in nature after many reproducible trials? The level of enzyme activity in the culture filtrate of the "new" batches (e.g. batch #7, data not shown) was comparable to that found in batch #5, an "old" batch (Table 6). However, the physical properties of the xylanase and CMCase enzymes had clearly changed. Were these new enzymes, or is the fungus now producing different isozymes? Considering the genetics of most filamentous fungi, the change could also be attributed to a single base pair mutation, as usually only one gene-copy exists (Rambosek and Leach, 1987).

The amino acid composition of the proteins may offer a clue to the thermostability of the enzymes. Cysteine residues have a tendency to form disulphide bridges, which are known to confer thermostability to proteins (Johri, 1980; Sharma,

1989). If the "old" batches' enzymes were available, many experiments could be performed. For example, one could treat the "old" and "new" proteins with sulphydryl-reducing agents and measure the change in activity. In addition, more zymograms would probably help elucidate the changes that were observed.

Improvements in both the xylanase assay system (Ghose and Bisaria, 1987) and the zymogram technique (Sengupta *et al.*, 1987) would make any investigations easier to perform and more meaningful in relation to other systems. Finally, the enzymes of *T. crustaceus* 235E have a lot of potential for use in the pulp and paper industry because they are naturally very thermostable and have high activities on many substrates (Figure 33; Table 10). The substituent-hydrolysing activities are important to pulp treatments as well as being critical to the bioconversion of hemicelluloses (Poutanen *et al.*, 1987). Hopefully, researchers will choose to continue investigating this potentially useful organism.

REFERENCES

- Andersson, S.-I., Samuelson, O., Ishihara, M., and Shimizu, K. (1983) Structure of the reducing end-groups in spruce xylan. *Carbohydr. Res.* **111**: 283-288.
- Aspinall, G.O. (1980) Chemistry of cell wall polysaccharides in *The biochemistry of plants* (a comprehensive treatise) Vol. 3 Carbohydrates: structure and function (J. Preiss, ed.) Academic Press Inc, New York. pp. 473-500.
- Bachman, S.L. and McCarthy, A.J. (1989) Purification and characterization of a thermostable β -xylosidase from *Thermomonospora fusca*. *J. Gen. Microbiol.* **135**: 293-299.
- Bailey, M.J. and Poutanen, K. (1989) Production of xylanolytic enzymes by strains of *Aspergillus*. *Appl. Microbiol. Biotech.* **30**: 5-10.
- Ball, C. (1984) Filamentous fungi in Genetics and breeding of industrial microorganisms (C. Bael, ed.) CRC Press, Boca Raton, Florida.
- Barnoud, F., Comtat, J., Joseleau, J.P., Mora, F., and Ruel, K. (1986) Uses of hemicellulases in the pulp and paper industry. *Biotechnology in the Pulp and Paper Industry*, Third International Conference, Stockholm, June 16-19, pp. 360-363.
- Bennett, J.W. and Lasure, L.L. (eds) (1985) Gene manipulations in fungi. Academic Press, N.Y.
- Béranger, J.F., Frixon, C., Bigiardi, J., and Creuzet, N. (1985) Production, purification, and properties of thermostable xylanase from *Clostridium stercoreum*. *Can. J. Microbiol.* **31**: 635-643.
- Bertrand, J.-L., Morosoli, R., Shareck, F., and Kluepfel, D. (1989) Expression of the xylanase gene of *Streptomyces lividans* and production of the enzyme on natural substrates. *Biotech. and Bioeng.* **33**: 791-794.
- Bickerstaff, G.F. (1987) *Enzymes in industry and medicine*. Edward Arnold Publ., New York.
- Biely, P. (1985) Microbial xylanolytic systems. *Trends in Biotech.* **3**: 286-290.
- Biely, P., Mackenzie, C.R., and Schneider, H. (1988) Production of acetyl xylan esterase by *Trichoderma*

reesei and Schizophyllum. Can. J. Microbiol. 34: 767-772.

- Biely, P., Markovic, O., and Misclovicová, D. (1985a) Sensitive detection of endo-1,4- β -glucanases and endo-1,4- β -xylanases in gels Anal. Biochem. 144: 147-151.
- Biely, P., Misclovicová, D., and Toman, R. (1985b) Soluble chromogenic substrates for the assay of endo-1,4- β -xylanases and endo-1,4- β -glucanases. Anal. Biochem. 144: 142-146.
- Biswas, S.R., Mishra, A.K., and Nanda, G. (1988) Xylanase and β -xylosidase production by Aspergillus ochraceus during growth on lignocelluloses. Biotech. and Bioeng. 31: 613-616.
- Blanchette, R.A., Burnes, T.A., Leatham, G.F., and Effland, M.J. (1988) Selection of white-rot fungi for biopulping. Biomass. 15:93-101.
- Brice, R.E. and Morrison, I.M. (1982) The degradation of isolated hemicelluloses and lignin-hemicellulose complexes by cell-free rumen hemicellulases. Carbohydr. Res. 101: 93-100.
- Brillouet, J.-M. (1987) Mode of action of xylanase II from Polyporus tulipiferae on oat-spelts arabino-glucuronoxylan and larchwood 4-O-methylglucuronoxylan. Carbohydr. Res. 159: 165-170.
- Brownell, H.H. and Saddler, J.N. (1987) Steam pretreatment of lignocellulosic material for enhanced enzymatic hydrolysis. Biotech. Bioeng. 29: 228-235.
- Butenko, R.G. and Kuchko, A.A. (1979) Physiological aspects on procurement, cultivation, and hybridization of isolated potato protoplasts. Fiziol. Rast. (Moscow) 26: 1110-1119.
- Chang, M.M., Chou, T.Y.C., and Tsao, G.T. (1981) Structure, pretreatment, and hydrolysis of cellulose. Adv. Biochem. Eng. 20: 15-42.
- Chauvet, J.M., Comtat, J., and Noé, P. (1987) The uses of xylanases in the bleaching of pulps. Symposium on Wood and Pulping Chemistry, Paris. pp.325-327.
- Comtat, J. and Joselean, J.-P. (1981) Mode of action of a xylanase and its significance for the structural investigation of the branched L-arabino-D-glucurono-D-

xylar. from redwood (Sequoia sempervirens). Carbohydr. Res. 95: 101-112.

- Dekker, R.F.H. (1983) Bioconversion of hemicellulose: Aspects of hemicellulase production by Trichoderma reesei QM9414 and enzymic saccharification of hemicellulose. Biotech. and Bioeng. 25: 1127-1146.
- Dekker, R.F.H. (1985) Biodegradation of hemicellulose in Biosynthesis and biodegradation of wood components (T. Higuchi, ed.) Academic Press, Inc., Florida pp. 505-533.
- Dekker, R.F.H. and Richards, G.N. (1975) Purification, properties, and mode of action of hemicellulase II produced by Ceratocystis paradoxa. Carbohydr. Res. 42: 107-123.
- Dekker, R.F.H. and Richards, G.N. (1976) Hemicellulases: their occurrence, purification, properties, and mode of action. Adv. Carbohydr. Chem. Biochem. 32: 277-352.
- Deschatelets, L. and Yu, E.K.C. (1986) A simple pentose assay for biomass conversion studies. Appl. Microbiol. Biotechnol. 24: 379-385.
- Donnison, A.M., Brockelsby, C.M., Morgan, H.W., and Daniel, R.M. (1989) The degradation of lignocellulosics by extremely thermophilic microorganisms. Biotech. Bioeng. 33: 1495-1499.
- Doppelbauer, R., Esterbauer, H., Steiner, W., Lafferty, R.M., and Steinmuller, H. (1987) Enzymes for the bleaching of pulps. Appl. Microbiol. Biotechnol. 26: 485-494.
- Engel, P.C. (1981) Enzyme kinetics: the steady-state approach. (2nd Ed.) Chapman and Hall, London, England.
- Eriksson, K.-E. (1988) Recent developments in biotechnology in the pulp and paper industry. Biomass 15: 117-119.
- Eriksson, Ö., Goring, D.A.I., and Lindgren, B.O. (1980) Structural studies on the chemical bonds between lignins and carbohydrates in spruce wood. Wood Sci. Technol. 14: 267-279.
- Feldman, K.A., Lovett, J.S., and Tsao, G.T. (1988) Isolation of cellulase enzymes from the thermophilic fungus Thermoascus aurantiacus and regulation of enzyme production. Enzyme Microb. Technol. 10: 262-272.

- Fincher, G.B. and Stone, B.A. (1981) Metabolism of noncellulosic polysaccharides. *Encycl. Plant Physiol. New Ser.* 13B: 68-132.
- Frederick, M.M., Kiang, C.-H., Frederick, J.R., and Reilly, P.J. (1985) Purification and characterization of endo-xylanases from Aspergillus niger. I. Two isozymes active on xylan backbones near branch points. *Biotechnol. Bioeng.* 27: 525-532.
- Ghose, T.K. and Bisaria, V.S. (1987) Measurement of hemicellulase activities. Part I: Xylanases. *Pure and Appl. Chem.* 59: 1739-1752.
- Grajek, W. (1987) Production of D-xylanases by thermophilic fungi using different methods of culture. *Biotech Lett.* 9: 353-356.
- Gruninger, H. and Fiechter, A. (1986) A novel, highly thermostable D-xylanase. *Enzyme Microb. Technol.* 8: 309-314.
- Henningsson, M. (1978) Marine fungi as producers of extracellular enzymes. *Mater. Org.* 13: 129-168.
- Himmel, M., Oh, K., Tucker, M., Rivard, C., and Gröbmann, K. (1986) Evaluation of spray-drying methods for cellulase preservation. *Biotech. Bioeng. Symp.* 17: 413-423.
- Hochachka, P.W. and Somero, G.N. (1984) Biochemical adaptations. Princeton University Press, Princeton, New Jersey.
- Ishihara, M., Shimizu, K., and Ishihara, T. (1978) Hemicellulases of brown rotting fungus, Tyromyces palustris III. Partial purification and mode of action of an extracellular xylanase. *Mokuzai Gattaishi* 24: 108-115.
- Jeffries, T.W. and Lins, C.W. (1969) Effects of Aureobasidium pullulans xylanase on properties of aspen thermomechanical and kraft pulps. 4th Int. Conf. Biotech in Pulp Paper Ind. (Raleigh, N.C.)
- Johansson, M.H. and Samuelson, O. (1977) Reducing end-groups in birch xylan and their alkaline degradation. *Wood Sci. Technol.* 11: 251-263.
- Johnson, E.A., Villa, T.G., Lewis, M.J., and Phaff, H.J. (1978) Simple method for the isolation of Astaxanthin from the basidiomycetous yeast Phaffia rhodozyma. *Appl. Environ. Microbiol.* 35:

1155-1159.

- Johnson, K.G., Silva, M.C., MacKenzie, C.R., Schneider, H., and Fontana, J.D. (1989) Microbial degradation of hemicellulosic materials. *Appl. Biochem. and Biotech.* 20/21: 245-258.
- Johri, B.N. (1980) Biology of thermophilous fungi. *Recent Adv.in Bio. of Microorganisms* 1: 265-278.
- Jurasek, L. and Paice, M.G. (1988) Biological treatments of pulps *Biomass* 15: 103-108.
- Karimiri, S. and Ward, O.P. (1989) Comparative study of some microbial arabinan-degrading enzymes. *Society for Ind. Microb.* 4: 173-180.
- Kawamori, M., Takayama, K., and Takasawa, S. (1987) Production of cellulases by a thermophilic fungus, Thermoascus aurantiacus A-131. *Agric. Biol. Chem.* 51: 647-654.
- Khan, A.W., Tremblay, D., and LeDuy, A. (1986) Assay of xylanase activities in bacterial and fungal cultures. *Enz. Microb. Technol.* 8: 373-377.
- Khande, K.M., Vithayathil, P.J., and Murthy, S.K. (1989) Purification of xylanase, β -glucosidase, endocellulase, and exocellulase from a thermophilic fungus, Thermoascus aurantiacus. *Archives Biochem. Biophys.* 274: 491-500.
- Kitpreechavanich, V., Hayashi, M., and Nagai, S. (1984) Production of xylan-degrading enzymes by thermophilic fungus Aspergillus fumigatus and Humicola lanuginosa J. of *Ferm. Tech.* 62: 63-69.
- Kratky, Z. and Biely, P. (1980) Inducible β -xyloside permease as a constituent of the xylan-degrading enzyme system of the yeast Cryptococcus albidus. *Eur. J. Biochem.* 112: 367-373.
- Kratky, Z., Biely, P., and Vranska, M. (1981) A method for preparing lower [$U-C^{14}$]-labelled (1,4)- β -D-xylooligosaccharides. *Carb. Res.* 93: 300-303.
- Leathers, T.D., Detroy, R.W., and Bothast, P.J. (1986) Induction and glucose repression of xylanase from a colour variant strain of Aureobasidium pullulans. *Biotech Lett.* 8: 867-872.

- Lowry, O.H., Rosebrough, N.H., Farr, A.L., and Randall, R.J. (1951) Protein measurement with the Folin phenol reagent J. Biol. Chem. 193: 265-275.
- Mackenzie, C.R., Bilous, D., Schneider, H., and Johnson, K.G. (1987) Induction of cellulytic and xylanolytic enzyme systems in Streptomyces spp. Appl. Env. Microb. 53: 2835-2839.
- Maino, A.L., Schroth, M.N., and Palleroni, N.J. (1974) Pathogenic saprophytic bacteria. Phytopathology 64: 881-885.
- Mandels, M. (1977) in Bioconversion of cellulose material to energy, chemicals, and microbial protein. (T.K. Ghose, ed.) IIT, New Dehli, India pp. 249-268.
- Manjrekar, J. and Krishnan, K.S. (1987) Thermal gels: A procedure for determination of heat-inactivation temperature of enzymes. Anal. Biochem. 160: 409-413.
- Margaritis, A. and Merchant, R. (1983) Production and thermal stability characteristics of cellulase and xylanase enzymes from Thielavia terrestris. Biotech. Bioeng. Symp. 13: 299-314.
- Marui, M., Nakanishi, K., and Yasui, T. (1985) Purification and properties of three types of xylanases induced by methyl β -xyloside from Streptomyces sp. Agric. Biol. Chem. 49: 3399-3407.
- McCarthy, A.J., Peace, E., and Broda, P. (1985) Studies on the extracellular xylanase activity of some thermophilic actinomycetes. Appl. Microb. Biotech. 21: 238-244.
- Merchant, R., Merchant, F., and Margaritis, A. (1988) Production of xylanase by the thermophilic fungus Thielavia terrestris. Biotech. Lett. 10: 513-516.
- Miller, G.L. (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. 31: 426-428.
- Mitsuishi, Y., Yamanobe, T., Yagisawa, M., and Takasaki, Y. (1987) Purification and properties of thermostable xylanases from mesophilic fungus strain Y-94. Agric. Biol. Chem. 51: 3207-3212.
- Montenencourt, B.S. and Eveleigh, D.E. (1977) Preparation of mutant Trichoderma reesei with enhanced cellulase production. Appl. Environ. Microbiol. 34: 777-782.

- Mora, F., Comtat, J., Barnoud, F., Pla, F., and Noé, P. (1986) Action of xylanases on chemical pulp fibers: PART 1: investigations on cell-wall modifications. J. Wood Chem. Technol. 6: 147-165.
- Morosoli, R., Durand, S., and Letendre, E.D. (1987) Induction of xylanase by β -methylxyloside in Cryptococcus albidus. FEMS Microb. Lett. 48: 261-266.
- Nakanishi, K., Yokotsuka, K., and Yasui, T. (1987) Induction of membrane bound xylanases in a Streptomyces sp. J. Ferm. Technol. 65: 1-6.
- Nigam, P. and Prabhu, K.A. (1988) Thermal activation and stability of cellulases derived from two Basidiomycetes. Biotech. Lett. 10: 919-920.
- Noé, P., Chevalier, J., Mora, F., and Comtat, J. (1986) Action of xylanases on chemical pulp fibers: PART II: Enzymatic beating. J. of Wood Chem. Technol. 6: 167-184.
- Paice, M.G., Bernier, R., and Jurasek, L. (1988) Viscosity-enhancing bleaching of hardwood kraft pulp with xylanase from a cloned gene. Biotech. Bioeng. 32: 235-239.
- Paice, M.G. and Jurasek, L. (1984) Removing hemicellulose from pulps by specific enzyme hydrolysis. J. Wood Chem. Technol. 4: 187-198.
- Panda, T., Bisaria, V.S., and Ghose, T.K. (1987) Effect of culture phasing and a polysaccharide on production of xylanase by mixed culture of Trichoderma reesei D1-6 and Aspergillus wentii Pt2804. Biotech. Bioeng. 30: 868-874.
- Parisi, F. (1989) Lignocellulosics materials: Economic evaluation of pretreatment and hydrolysis procedures. COST 84 bis (Galway) April 12-14.
- Patchett, M.L., Neal, T.L., Schofield, L.R., Strange, R.L., Daniel, R.M., and Morgan, H.W. (1989) Heat treatment purification of thermostable cellulase and hemicellulase enzymes expressed in E. coli. Enz. Microb. Technol. 11: 113-115.
- Pommier, J.-C., Fuentes, J.-L., and Goma, G. (1989) Using enzymes to improve the process and the product quality in the recycled paper industry. Tappi Journal June: 187-191.

- Poutanen, K., Rättö, M., Puls, J., and Viikari, L. (1987) Evaluation of different microbial xylanolytic systems. *J. Biotechnol.* 6: 49-60.
- Poutanen, K. and Sundberg, M. (1988) An acetyl esterase of Trichoderma reesei and its role in the hydrolysis of acetyl xylans. *Appl. Microb. Biotech.* 28: 419-424.
- Puls, J. and Poutanen, K. (1988) Use of hemicellulolytic enzymes. IEA/BIOENERGY AGREEMENT.
- Rambosek, J. and Leach, J. (1987) Recombinant DNA in filamentous fungi: Progress and prospects. *CRC Critical reviews in Biotech.* 6: 357-393.
- Reese, E.T. and McGuire, A. (1969) Surfactants as stimulants of enzyme production by microorganisms. *Appl. Microbiol.* 17: 242-245.
- Reilly, P.J. (1981) Xylanases: structure and function in *Trends in biology of fermentations for fuel and chemicals* (A. Hoellaender, ed.) Plenum Press, New York.
- Rho, D., Desrochers, M., Jurasek, L., Driguez, H., and Defaye, J. (1982) Induction of cellulase in Schizophyllum commune: thiocellulose as a new inducer. *J. Bacteriol.* 149: 47-53.
- Royer, J.C. and Nakas, J.P. (1989) Xylanase production by Trichoderma longibrachiatum. *Enz. Microb. Technol.* 11: 405-410.
- Saddler, J.N. (1986) Factors limiting the efficiency of cellulase enzymes. *Microbiol. Sciences* 3: 84-87.
- Saddler, J.N. and Mes-Hartee, M. (1984) Production of cellulases and hemicellulases by Trichoderma reesei. *Biotechno. Adv.* 2: 161-181.
- Saddler, J.N., Senior, D.J., and Mayers, P.R. (1988) Purification and application of xylanases. *Symp. on Biosyn. and Biodegr. of Plant Cell Wall Polymers*.
- Schwald, W., Chan, M., Breuil, C., and Saddler, J.N. (1988) Comparison of HPLC and colorimetric methods for measuring cellulolytic activity. *Appl. Microbiol. Biotechnol.* 28: 398-403.
- Schwarz, W.H., Bronnenmeier, K., Gräbnitz, F., and Staudenbauer, W.L. (1987) Activity staining of cellulases in polyacrylamide gels containing mixed linkage β -glucans. *Anal. Biochem.* 164: 72-77.

- Scopes, R. (1987) Protein purification: Principles and practice. 2nd Edition. Springer Verlag, New York.
- Sengupta, S., Khowala, S., and Goswami, P.K. (1987) Assay of endo- β -xylanase activity with a soluble O-(carboxymethyl) derivative of larchwood D-xylan. Carbohydr. Res. 168: 156-161.
- Senior, D.J. (1989) Study and application of hemicellulases in the preparation of high value cellulosic materials. Progress Report for the CFS (March 1989).
- Senior, D.J., Mayers, P.R., and Saddler, J.N. (1989) Influence of the growth substrate on xylanase production by Trichoderma harzianum E58. Appl. Microbiol. 32: 137-142.
- Sharma, H.S.S. (1987) Enzymatic degradation of residual non-cellulosic polysaccharides present on dew-retted flax fibres. Appl. Microbiol. Biotechnol. 26: 358-362.
- Sharma, H.S.S. (1989) Economic importance of thermophilous fungi. Appl. Microbiol. Biotechnol. 31: 1-10.
- Shepard, M.G., Tong, C.C., and Cole, A.L. (1981) Substrate specificity and mode of action of the cellulases from the thermophilic fungus Thermoascus aurantiacus. J. Biochem. 193: 67-74.
- Sinner, M., Parameswaran, N., and Dietrichs, H.H. (1979) Degradation of delignified spruce wood by purified mannanase, xylanase, and cellulases. Adv. Chem. Ser. 181: 303-329.
- Sipat, A., Taylor, K.A., Lo, R.V.C., Forsberg, C.W., and Krell, P.J. (1987) Molecular cloning of a xylanase gene from Bacteroides succinogenes and its expression in Escherichia coli. Appl. Environ. Microbiol. 53: 477-481.
- Skoog, K. and Hahn-Hagerdal, B. (1988) The hemicellulases of various mesophilic bacteria and fungi. Enz. Microbiol. Technol. 10: 66-80.
- Suh, D.H., Becker, T.C., Sands, J.A., and Montenecourt, B.S. (1988) Effects of temperature on xylanase secretion by Trichoderma reesei. Biotech. Bioeng. 32: 821-825.
- Takenishi, S. and Tsujisaka, Y. (1975) On the modes of action of three xylanases produced by a strain of

APPENDIX 6:

Chromatographic profile of endo-β-xylanase purification from Thermoascus crustaceus 235E on SP-Sephadex column (from Tan et al., 1987c) [● protein (optical density at 280 nm); ○ xylanase activity (DNS assay, optical density at 575 nm)]

Ellis Horwood Ltd, Chichester.

- Wong, K.K.Y., Tan, L.U.L., and Saddler, J.N. (1988) Multiplicity of β -1,4-xylanase in microorganisms: Functions and applications. *Microbiol. Reviews* 52: 305-317.
- Wood, W.A. and Kellogg, S.T. (eds) (1988) *Methods in Enzymology* Vol. 160. Academic Press, New York.
- Woodward, J. (1984) Xylanases: Functions, properties and applications. *Topics in Enz. Ferm. Biotechnol.* 8: 9-30.
- Yang, R.C.A., Mackenzie, C.R., Bilous, D., Seligy, V.L., and Narang, S.A. (1988) Molecular cloning and expression of a xylanase gene from Bacillus polymyxa in E. coli Appl. *Env. Microbiol.* 54: 1028-1029.
- Yasui, T., Nguyen, B.T., and Nakanishi, K. (1984) Inducers for xylanase production by Cryptococcus flavus J. *Ferm. Technol.* 62: 353-359.
- Yu, E.K.C., Tan, L.U.L., Chan, M.K.-H., Deschatelets, L., and Saddler, J.N. (1987) Production of thermostable xylanase by a thermophilic fungus, Thermoascus aurantiacus *Enz. Microb. Technol.* 9: 16-24.

APPENDIX 1:

Vogel's Media (Montenencourt and Eveleigh, 1977)

Vogel's Salt Stock (kept at 4 °C)

Na ₃ Citrate 2H ₂ O	125 g
KH ₂ PO ₄	250 g
NH ₄ NO ₃	100 g
MgSO ₄ 7H ₂ O	10 g
CaCl ₂ 2H ₂ O	5.01 g
TOTAL VOLUME	1000 mL

Vogel's Vitamin Solution (stored at 0 °C)

Biotin	0.5 mg
Myo-inositol	120 mg
Ca-pantothenate	20 mg
Pyridoxine HCl	20 mg
Thymine HCl	15.5 mg
TOTAL VOLUME	100 mL

Vogel's Trace Minerals Solution (kept at 4 °C)

Citric acid H ₂ O	5.0 g
ZnSO ₄ 7H ₂ O	5.0 g
Fe(NH ₄) ₂ (SO ₄) ₂ 5H ₂ O	1.0 g
CuSO ₄ 5H ₂ O	0.25 g
MnSO ₄ H ₂ O	0.05 g
H ₃ BO ₃	0.05 g
Na ₂ MoO ₄ 2H ₂ O	0.05 g
TOTAL VOLUME	100 mL

Complete medium:

	per litre
Vogel's Salt Stock	20 mL
Vogel's Vitamin Solution	1.0 mL
Vogel's Trace Minerals	0.1 mL
Tween 80	1.0 mL
Bacto-peptone	1.0 g (1/10th of carbon source)
Cellulose or other carbon source	10.0 g

APPENDIX 2:

Mandel's Media (Mandel, 1977)

	g/L
$(\text{NH}_4)_2\text{SO}_4$	1.4
Urea	0.3
KH_2PO_4	2.0
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.3
CaCl_2	0.3
Trace Minerals:	
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	5.0
$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	1.56
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	1.4
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	2.0

dH₂O to 1 L

use 1.0 mL of this solution per litre of media

APPENDIX 3:

Lowry Reagent:

Reagent A	30 g	Na ₂ CO ₃
	4 g	NaOH

	dH ₂ O to 1000 mL	
Reagent B1	2 g	CuSO ₄ 5H ₂ O

	dH ₂ O to 100 mL	
Reagent B2	4 g	NaKTartrate

	dH ₂ O to 100 mL	

Lowry reagent C: mix 48 mL Reagent A
 1 mL Reagent B1
 1 mL Reagent B2

3,5-Dinitrosalicylic acid Reagent:

mix:	14.16 L	dH ₂ O
(DNS)	106.0 g	3,5-dinitrosalicylic acid
	198.0 g	sodium hydroxide
mix, then add to above:	3060.0 g	NaKTartrate
	70.0 mL	Phenol
	83.0 g	sodium meta-bisulphite

allow solution mix overnight at room temperature

* see Appendix 5 for standard curves of xylose and glucose for the 3.0 mL DNS assay for reducing sugars (chapter 1.2)

APPENDIX 4:

Sample mean:

$$\bar{x} = \frac{\sum_{i=1}^n x_i}{n}$$

Sample variance:

$$s^2 = \frac{\sum_{i=1}^n (x_i - \bar{x})^2}{n - 1}$$

Sample standard deviation:

$$s = \sqrt{s^2}$$

APPENDIX 5:

Standard curve* for the Dinitrosalicylic acid (DNS) reducing sugar assay. [xylose; + glucose]

* see chapter 1.2 for method

APPENDIX 6:

Chromatographic profile of endo-β-xylanase purification from Thermoascus crustaceus 235E on SP-Sephadex column (from Tan et al., 1987c) [● protein (optical density at 280 nm); ○ xylanase activity (DNS assay, optical density at 575 nm)]

END

04|03|9|2

FIN