Helsinki University of Technology, Department of Chemical Technology
Laboratory of Bioprocess Engineering
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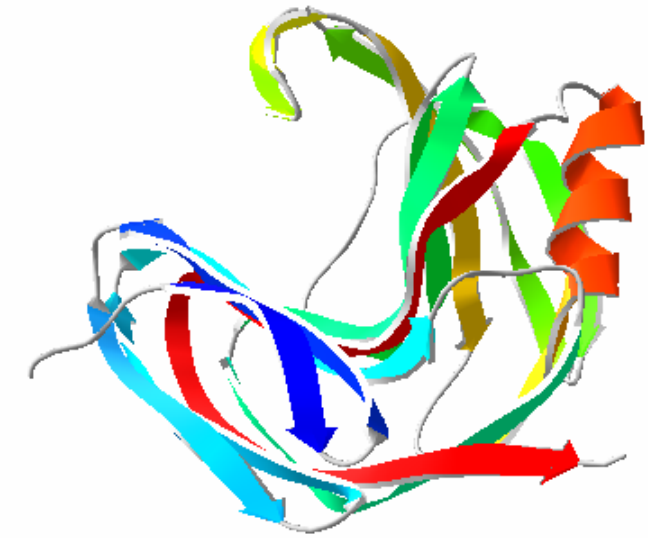

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PRODUCTION AND CHARACTERIZATION OF *TRICHODERMA REESEI* AND *THERMOMYCES LANUGINOSUS* XYLANASES

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Keywords: Xylanase, Cellulase, *Trichoderma reesei, Thermomyces lanuginosus*, pH, L-arabinose, Oligosaccharides, Characterization, Cultivation, Induction, Purification, Thermostability.

ABSTRACT

This thesis describes the production and properties of xylanases from *Trichoderma reesei* Rut C-30 and *Thermomyces lanuginosus* DSM 10635. The thermostability of several *T. reesei* xylanase II mutants was also studied. *T. reesei* Rut C-30 responds to the pH of the growth environment by modifying its enzyme production patterns. The production of the xylanases I, II and III by *T. reesei* at different pH conditions correlates with the pH-dependent activity pattern of the enzymes. The xylanase was preferentially produced when it is most active in that particular pH environment. The highest total xylanase production with *T. reesei* Rut C-30 was achieved at pH 6 on a lactose-based medium. Among the pentoses and hexoses tested, L-arabinose was the most effective inducer of the xylanases. Furthermore, in co-metabolism of L-arabinose and D-glucose, the addition of the former relieved the repression of D-glucose on xylanase production. The replacement of part lactose with L-arabinose resulted in significant improvement in xylanase production. Small amounts of bulky L-arabinose-rich plant materials, such as sugar beet pulp and oat husk hydrolysates, stimulated the xylanase production. While xylanase production was improved by these approaches, the cellulase production was not enhanced. In addition, the stability and activity of *T. reesei* xylanase II mutants containing different combinations of disulphide bridges were studied. The most stable combination mutant showed about 5000-fold half-life at 65 ^{o}C compared to the wild type xylanase II. The molecular system controlling xylanase induction was fundamentally different in *T. reesei* and *T. lanuginosus*. Unlike *T. reesei* Rut C-30, *T. lanuginosus* DSM 10635 xylanase was not induced by L-arabinose or lactose. The amino acid sequence of DSM 10635 xylanase was most likely the same as that of *T. lanuginosus* DSM 5826 xylanase. The temperature-dependent inactivation curve of the DSM 10635 xylanase decreased slowly at neutral or slightly alkaline pH, whereas at low pH, the inactivation was fast. The thermostabilizing effect of the substrate, birchwood xylan, on DSM 10635 xylanase was observed to be significant only under acidic conditions.

PREFACE

This work was carried out during the years 2001-2004 in the Laboratory of Bioprocess Engineering, Department of Chemical Technology, Helsinki University of Technology.

Firstly, I wish to express my warmest thanks to Professor Matti Leisola for his fruitful supervision and patient encouragement during this work. This thesis continues the work he started in his dissertation decades ago. I am grateful for the opportunity to continue this extensive work.

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Finally, I send my best regards to my family and friends, and my pretty son.

謹以此書獻給我摯愛的親人和朋友，和我可愛的小兒。

Hairong Xiong

September, 2004

LIST OF PULICATIONS

This thesis is based on the following publications, which are referred to as **I** to **V** in the text.

I. Xiong H, von Weymarn N, Leisola M, Turunen O. Influence of pH on the production of xylanases by *Trichoderma reesei* Rut C-30. *Proc. Biochem.* 2004; 39 (6): 729-733

II. Xiong H, Turunen O, Pastinen O, Leisola M, von Weymarn N. Improved xylanase production by *Trichoderma reesei* grown on L-arabinose and lactose or D-glucose mixtures. *Appl. Microbiol. Biotechnol.* 2004; 64 (3): 353-358

III. Xiong H, von Weymarn N, Turunen O, Leisola M, Pastinen O. Xylanase production by *Trichoderma reesei* Rut C-30 grown on L-arabinose-rich plant hydrolysates. *Biores. Technol.* In press; online 01/10/2004

IV. Xiong H, Fenel F, Leisola M, Turunen O. Engineering the thermostability of *Trichoderma reesei endo*-β-1,4 xylanase II by combination of disulphide bridges. *Extremophiles*. 2004; 8 (5): 393-400

V. Xiong H, Nyyssölä A, Jänis J, Pastinen O, von Weymarn N, Leisola M, Turunen O. Characterization of the xylanase produced by submerged cultivation of *Thermomyces lanuginosus* DSM 10635. *Enzyme Microb. Technol.* 2004; 35 (1): 93-99

The author has carried out most of the experiments except the site-directed mutagenesis experiments in the article **IV** which was done by Dr. Ossi Turunen and coworkers and the MS analysis in the article **V** which was done by Dr. Janne Jänis. The author has written the first version of all the manuscripts except the article **IV** was written by Dr. Ossi Turunen.

ABBREVIATIONS

CMC	Carboxymethyl cellulose
CSTR	Continuous-flow stirred-tank reactor
Da	Dalton
DEAE	Diethyl amino ethyl
DNS	3, 5 – dinitrosalicylic acid
DOT	Dissolved oxygen tension
E_a	Activation energy
E_d	Deactivation energy
FPA	Filter paper activity
GPC	Gel permeation chromatography
HIC	Hydrophobic interaction chromatography
HPLC	High performance liquid chromatography
IEF	Isoelectric focusing
IPTG	Isopropyl-thio-β-D-galactopyranoside
MS	Mass spectrometry
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
p*I*	Isoelectric point
RBB-Xylan	Remazol Brilliant Blue – Xylan
SDS	Sodium dodecyl sulfate
SSF	Solid-State Fermentation
XYN	Xylanase

CONTENTS

Cover picture: The Ribbon structure of *Thermomyces lanuginosus* xylanase. The picture was created by Swiss-Pdb Viewer.

1. INTRODUCTION

1.1 BACKGROUND

There are several applications of xylanases in industry (Prade, 1996; Kulkarni et al., 1999; Subramaniyan & Prema, 2002). Currently, the major applications of xylanases are in pulp and paper, feed, and baking industries.

Xylanases are used in the prebleaching of kraft pulp to reduce the use of harsh chemicals in the subsequent chemical bleaching stages. The enzymatic treatments improve the chemical liberation of lignin by hydrolysing residual xylan. This reduces the need for chlorine-based bleaching chemicals, which is beneficial for the environment (Viikari et al., 1994; Suurnäkki et al., 1997; Christov et al, 1999; Viikari et al., 2001; Beg et al., 2001). In feed formulations, cooperation of xylanases, glucanases, proteinases and amylases reduces viscosity of the feed and increases the adsorption of nutrients. Enzymes liberate nutrients either by hydrolysis of non-degradable fibres or by liberating nutrients blocked by these fibres (Leisola et al., 2004). In the food industry, xylanases are used to improve the dough properties and baking quality of bread and other baked goods by breaking down the polysaccharides in the dough. The enzyme treatment has favourable effects on dough handing, bread volume, texture and stability (Li et al, 2000; Bhat, 2000). In combination with pectinases and other enzymes, xylanases have also been used in other processes such as clarification of juices, extraction of coffee, and extraction of plant oils and starch. Other potential applications include the conversion of agricultural waste and the production of fuel ethanol (Eriksson et al., 2002; Sorensen et al., 2003; Damaso et al., 2003).

Although xylanases produced by thermophilic eubacteria and archaea have considerably longer half-life ($T_{1/2}$) at 80 ^{o}C or higher temperatures than those from thermophilic fungi, the levels of xylanase activity produced by these bacteria are considerably lower than those of fungi (Singh et al., 2003). Filamentous fungi are particularly useful producers of xylanases from the industrial point of view, due to the high production level and extracellular secretion of enzymes, as well as relative ease of cultivation. In general, xylanase activity levels from fungal cultures are typically much higher than those from yeasts or bacteria (Bergquist et al., 2002; Paloheimo et al., 2003).

1.2 XYLANASE PRODUCING STRAINS

1.2.1 *Trichoderma reesei* Rut C-30

Trichoderma reesei (also known as *Hypocrea jecorina*) is a mesophilic fungus which is one of the most efficient xylanase and cellulase producers. Industrial strains of *Trichoderma reesei* can achieve protein production levels of up to 100 g/l (Cherry & Fidantsef, 2003). The efficient secretory ability and the cheap and easy cultivation of *T. reesei* make it a useful organism for the large-scale production of enzymes for a variety of industrial applications. *T. reesei* can also be used for the large-scale production of heterologous proteins (Hui et al., 2001). Among the many *T. reesei* mutants (Table 1), Rut C-30 is a widely studied strain (Montenecourt & Eveleigh, 1979; Bader, 1993). It grows on a single carbon source, such as cellulose or xylan, and secretes both cellulases and xylanases. In addition, it produces

enzymes more efficiently than the wild-type *T. reesei*. The cellulase expression in Rut C-30 is not repressed by glucose to the same extent as in some other strains (Tangnu et al., 1981; Domingues et al., 2000). The modern production strains are genetically engineered to increase the enzyme production and often to remove the expression of unwanted enzymes, like cellulases during the production of xylanases (Paloheimo et al., 2003; Verdoes et al., 1995).

Table 1. The genealogy of different high-cellulase producing *T. reesei* mutant strains isolated worldwide (Persson et al., 1991; Nevalainen et al., 1994; Xu et al., 2000).

	Rut series, Rutgers University, USA (Montenecourt & Eveleigh, 1977, 1979)		
	Rut M-7	**Rut NG14**	**Rut C 30** (ATCC 56765)
			Rut EPI series
Trichoderma reesei			**CL series**, Societe Cayla, France (Durand et al., 1984)
QM6a	**QM9123**	**QM9414** (ATCC 26921)	**Kyowa series,** (PC-3-7), Kyowa Hakko Kogyo Co., Japan (Kawamori et al., 1985,1986)
wild type, (Mandels & Reese, 1957)	Natick, USA (Mandels et al., 1971)	Natick, USA (Mandels et al., 1971)	**D1 series,** Indian Institute of technology, India (Mishra et al., 1982)
			MHC series, Slovak Academy Sciences, Slovakia (Farkas et al., 1981)
			L series, Cetus Corporation, USA (Shoemaker et al., 1981)
			VTT-D series, Technical Research Centre, Finland (Nevalainen et al., 1980; Ilmén et al., 1996)
			MG series, Gulbenkian Institute of Science (Beja da Costa & van Uden, 1980)
			MCG series, Natick, USA (Gallo et al., 1979)

Note: the references in this table can be found in Nevalainen et al., 1994.

1.2.2 *Thermomyces lanuginosus* DSM 10635

Thermomyces lanuginosus (formerly known as *Humicola lanuginosa*) is a widely distributed thermophilic ascomycete fungus. By definition, a thermophilic fungus is one that thrives at temperatures above 60 ^{o}C and fails to grow below 20 ^{o}C (Singh et al., 2003). *T. lanuginosus* has attracted considerable interest due to its production of thermostable enzymes, especially the xylanase belonging to family 11 of glycosyl hydrolases (Henrissat & Davies, 1997). Furthermore, the xylanase production in *T. lanuginosus* is not accompanied by cellulase production. In addition very low levels of other hemicellulases are found in the culture media of this fungus (Gomes et al., 1993; Singh et al., 2003).

The lack of cellulases is important, since the treatment of paper pulp with xylanase preparations containing cellulases results in a reduction in the degree of polymerisation of the cellulose fibers and a drop in product quality (Beg et al., 2001). In the current industrial production systems, cellulases are removed genetically. However, *T. lanuginosus* was earlier thought to be a potential xylanase producer due to the lack of cellulases and lignocellulolytic enzymes in the native strains (Singh et al., 2003; Purkarthofer et al., 1993b).

T. lanuginosus DSM 5826 strain was isolated from Bangladesh (Purkarthofer et al., 1993a, 1993b; Cesar & Mrsa, 1996). SSBP, ATCC 46882 and other *T. lanuginosus* strains were isolated from different geographic locations (SSBP isolated from South Africa) (Lin et al., 1999; Bennett et al., 1998; Singh et al., 2000a, 2000b). DSM 10635 strain was isolated from the Czech Republic. No reports about the DSM 10635 xylanase, studied in this thesis, have previously been published.

1.3 ENZYMES PRODUCED BY *T. REESEI* AND *T. LANUGINOSUS*

T. reesei produces a number of extracellar enzymes (Table 2, 3, 4). It produces at least four endo-1,4-β-xylanases (XYN I, II, III and IV, EC 3.2.1.8), two β-xylosidases (EC 3.2.1.37), two endo-1,4- β -D-glucan cellobiohydrolases (CBH I and II, EC 3.2.1.91), five endo-1,4-β-D-glucan-4-glucanohydrolases (EG I, II, III, IV and V, EC 3.2.1.4) and two β-D-glucosidases (BGL I and II, EC 3.2.1.21) (Bailey et al., 1993b; Zeilinger et al., 1996; Xu et al., 1998; Nogawa et al., 2001, Karlsson et al., 2001). Several other enzymes are also produced by *T. reesei*: β–mannanase (EC 3.2.1.78), β–mannosidase (EC 3.2.1.25), α-L-arabinofuranosidase (EC 3.2.1.55), α–galactosidase (EC 3.2.1.22), acetylxylan esterases (EC 3.1.1.72) and laccases (benzenediol:oxygen oxidoreductases, EC 1.10.3.2) (Stålbrand et al., 1993; Roche et al., 1995; Shabalin et al., 2002; Hakulinen, 2003).

Several strains of *T. lanuginosus* have been found to produce extracellular xylanases, but no cellulolytic enzymes are produced simultaneously. Other hemicellulases are produced in low levels (Singh et al., 2003). This is different from many other xylan-degrading organisms, which often secrete complex mixtures of xylanases and cellulases.

1.3.1 Xylanase

Many bacterial and fungal species can produce a mixture of xylanase, β-xylosidase and accessory side-group cleaving enzymes in order to utilize xylan, a complex polymer which is the major component of hemicellulose in the plant cell wall. Xylan found in nature consists of a β-1,4-linked xylopyranose backbone substituted with acetyl, arabinosyl and glucuronosyl side chains (Gregory et al., 1998). Enzymatic hydrolysis of xylan to xylose is catalyzed by endo-1,4-β-xylanase and β-xylosidase, the former randomly hydrolyzing xylan to xylooligomers and the latter producing xylose from xylooligomers. The side chain groups are liberated by α-L-arabinofuranosidase, α-D-glucoronidase, α-galactosidase and acetyl xylan esterase (Subramaniyan & Prema, 2002). β-xylosidase shows high activity toward xylobiose but no activity toward xylan (Bajpai, 1997). However, some xylanases may also have an ability to hydrolyze xylooligomers to xylose, especially in the cross-linked enzyme crystal form (Finell et al., 2002).

The xylanase activity of *T. reesei* is composed of xylanases I, II, III and IV, and xylan-digesting cellulases. Xylanases I and II (p*I* 5.5 and 9, respectively) are approximately 20 kDa proteins belonging to the family 11 of glycosyl hydrolases (Törrönen & Rouvinen, 1997). Xylanase III (p*I* 9.1, 32 kDa) is a family 10 glycosyl hydrolase, first characterized from *T. reesei* PC-3-7 (Xu et al., 1998). The pH optimum for xylanase I is at pH 4.0-4.5, for xylanase II at pH 4.0-6.0 and for xylanase III at pH 6.0-6.5. Of the total xylanase activity in *T. reesei* PC-3-7 produced on a cellulose-based growth medium, xylanase III accounted for over 25% (Xu et al., 1998). Xylanase IV (p*I* 7.0, 43 kDa) was described in a recent patent (Clarkson et al., 2001). Its pH optimum is at pH 3.5-4.0. The activity of xylanase IV increases efficiently when it is combined with other xylanases. The different properties of *T. reesei* xylanases are summarized in Table 2. The xylanases of different *T. lanuginosus* strains have been characterized, and most of them have very similar molecular weights and p*I* values (25.5 kDa and 4.1, respectively) (Lin et al., 1999; Cesar & Mrsa, 1996; Bennett et al., 1998).

The crystal structures of different family 11 xylanases have been resolved, including the structures of *T. reesei* xylanases I and II and *T. lanuginosus* xylanase (Hakulinen et al., 2003). The protein structure is composed of two β-sheets and a single α-helix forming a right hand-like structure (See cover picture of this book). Based on the structural information, a large number of protein engineering studies have been performed with family 11 xylanases utilizing site directed mutagenesis, and also random mutagenesis techniques (Turunen et al., 2004).

Table 2. Biochemical properties of purified *T. reesei* xylanases.

Xylanase	MW* (kDa)	pI	Optimum pH	Stability	Specific activity¤ (IU/mg)	pH stability	Reference
XYN I	19	5.5	4.0-4.5	24 h, 40 °C	70	2.5-4.5	Tenkanen et al., 1992
XYN II	20	9.0	5.0-5.5	24 h, 45 °C	231	4.0-7.5	Tenkanen et al., 1992
XYN III	32	9.1	6.0-6.5	24 h, 50 °C	258	5.0-8.0	Xu et al., 1998
XYN IV	43	7.0	3.5-4.0	-	-	-	Clarkson et al., 2001

*MW: Molecular weight in SDS-PAGE; ¤Specific activity was measured at 50 °C, pH 6.0, in 1% birch wood xylan solution by Xu et al., 1998. The specific activity of XYN I and II was reported to be 120 IU/mg and 810 IU/mg, respectively, at 60 °C by Lappalainen et al., 2000.

1.3.2 Cellulase

Cellulose is degraded by three major classes of hydrolases (Table 3). Endoglucanases digest the amorphous regions of cellulose, cellobiohydrolases cut the cellulose to cellobiose from the free chain end and β-glucosidases degrade small soluble oligosaccharides and cellobiose to glucose. Efficient enzymatic degradation of insoluble polysaccharides often requires a tight interaction between the enzymes and their substrates. In the case of cellulose degradation, many cellulases are known to bind to crystalline and/or amorphous cellulose via cellulose-binding domains (CBDs) which are distinct from the catalytic domains (Ohmiya et al., 1997).

Table 3. Physical properties of *T. reesei* cellulases.

	Number of residues		Molecular weight* (kDa)	Isoelectric point (pI)	Position of the CBD	Reference
	Total	Core				
CBH I (Cel 7A)	497	430	59—68	3.5—4.2	C	Reinikainen, 1994
CBH II (Cel 6A)	447	367	50—58	5.1—6.3	N	Reinikainen, 1994
EG I (Cel 7B)	437	368	50—55	4.0—6.0	C	Reinikainen, 1994
EG II (Cel 5A)	397	327	48	5.5	N	Reinikainen, 1994
EG III (Cel 12A)	218	218	25	7.5	No	Reinikainen, 1994
EG IV (Cel 61A)	344	344	55	-	C	Karlsson et al., 2001
EG V (Cel 45A)	225	166	23	-	C	Reinikainen, 1994
BGL I (Cel 3A)	713	713	75	8.7	No	Reinikainen, 1994
BGL II (Cel 1A)	700	700	114	4.8	-	Foreman et al, 2003; Viikari et al, 2001

***** SDS-PAGE results. Abbreviations: CBH, cellobiohydrolase; EG, endoglucanase; BGL, β-D-glucosidases; CBD, cellulose binding domain; C, C-terminal; N, N-terminal.

Cellulases are currently sold to the textile industry for cotton softening and denim finishing and to detergent markets for color care, cleaning and anti-redeposition in washing powders. (Cherry & Fidantsef, 2003). Alkaline cellulase when it attacks cotton fiber relaxes the rigidity of the fiber and releases the stains within the interior of the fiber (Ohmiya et al., 1997). In the pulp and paper industry, cellulases are used together with hemicellulases to improve the drainage and runnability of the paper machines and to enhance the deinking of recycled fibres (Viikari et al, 2001; Cao & Tan, 2002).

Cellulases have replaced the use of volcanic lava stones in the treatment of denim in order to achieve the so-called "stone-washed" or abraded look appreciated by the consumers. The stones caused considerable damage to the machines and fibres, and nowadays the same effect can be obtained by the use of cellulases (Leisola et al., 2004).

In the future, the cellulase market is expected to increase dramatically if economical conversion of cellulosic biomaterial to ethanol can be demonstrated. The major barrier for this expansion is the current cost of cellulases in biomass saccharification (Cherry & Fidantsef, 2003).

1.3.3 Other enzymes

Besides xylanases and cellulases listed above (Table 2, 3), *T. reesei* is an efficient producer of many other enzymes also, which are listed in Table 4.

Table 4. Selected *T. reesei* enzymes with industrial potential.

Enzyme	Function	Application	Reference
β-mannanase	Degradation of mannan	Delignification of pulp	Ohmiya et al., 1997
α-L-arabinofuranosidase	Cleavage of side groups in xylan	Feed and baking	Roche et al., 1995
α-galactosidase	Cleavage of side groups in xylan	Digestion of guar gum; medicine	Golubev et al., 2004
Pectin methyl esterases	De-esterification and gelling of pectins	Clarification of cider	Haltmeier et al., 1983; Bhat, 2000
Acetylxylan esterases	Hydrolysis of acetyl side groups of xylan	Co-operation with xylanase	Hakulinen et al., 2000
Laccases	Oxidation of wide variety of compounds	Textile bleaching, biosensors, etc.	Kiiskinen et al., 2004

β-mannanase: Mannans and xylans are the main components of wood besides cellulose and lignin. For the complete hydrolysis of mannans the synergistic action of endo-1,4-β-mannanases, β-mannosidases, β-glucosidases, α-galactosidases and acetyl mannan esterases is required. Endo-1,4-β–mannanase, which hydrolyzes mannan yielding mannotriose and mannobiose, has been reported to be produced by *T. reesei* (Stålbrand et al., 1993).

α-L-arabinofuranosidase: D-xylose and L-arabinose are two most widespread pentose sugars in biosphere. Arabinan, arabinoxylan and some other arabinose-containing polysaccharides release arabinose when hydrolyzed by *T. reesei* α-L-arabinofuranosidase (Roche et al., 1995).

α-galactosidase catalyses cleavage of terminal α-galactose residues from α-*O*-galactosides including galactose-containing oligosaccharides and branched polysaccharides, such as galactomannans and galactoglucomannans. It may have an application in digestion of guar gum, which contains about 40 % galactoses with α-1,6-linkages on a β-mannosyl backbone. α-galactosidase can be used in modification of wood-derived materials because galactomannans and galactoglucomannans are the main groups of hemicelluloses in softwoods. It may have an application also in medicine for the treatment of Fabry disease (Zeilinger et al., 1993; Siika-aho et al., 1994; Shabalin et al., 2002; Golubev et al., 2004).

Pectinases are a group of enzymes (polygalacturonase, pectin lyase, pectate lyase, and pectin esterase) that break the glycosidic bonds of the long chains of galacturonic acid residues in pectic substances, which are the structural polysaccharides of plant cells. The pectinases have applications in fruit juice clarification and wine production. A potential utilization of pectinases is treatment of softwoods, which has been shown to improve the efficiency of preservative treatment by rendering the wood more permeable for chemical preservatives (Haltmeier et al., 1983; Gregorio et al., 2002).

Acetylxylan esterases represent a group of carbohydrate esterases with great potential in biotechnology and carbohydrate chemistry. They deacetylate partially acetylated 4-*O*-methyl-D-glucuronoxylan, the main hardwood hemicellulose, or its fragments generated upon the action of endo-1,4-β-xylanases (Hakulinen et al., 2000). Other important enzymes, such as laccases and proteases, are also secreted by *T. reesei* (Kiiskinen et al., 2004;

Eneyskaya et al., 1999). Laccases catalyse oxidation of a wide variety of compounds, and potentially apply in textile dye bleaching, pulp bleaching, effluent detoxification, biosensors and bioremediation (Kiiskinen et al., 2004). *T. reesei* protease digests the proteins in the medium under acidic conditions (pH below 2.7). At higher pH, the proteolytic reaction is limited. Glucose and cellobiose repress the proteolysis of cellobiohydrolase in a concentration-dependent manner (Eneyskaya et al., 1999).

T. lanuginosus is also an efficient producer of thermostable amylases (Arnesen et al., 1998; Puchart et al., 1999; Nguyen et al., 2002). Application of this amylase in the baking industry has been patented (Michelsen et al., 1996). However, *T. lanuginosus* produces only tiny amounts of other hemicellulases and cellulases (Singh et al., 2003).

1.4 CULTIVATION CONDITIONS

1.4.1 Carbon sources and inducers

In most studies on cellulase production by *Trichoderma*, cellulosic materials have been used as the substrate for fungus growth (Suto & Tomita, 2001). However, the high amount of solid material in these systems burdens the agitation and lowers the availability of oxygen in bioreactors, and absorbs some of the enzymes. The rate of enzyme synthesis depends on the hydrolysis of cultivation substrates (Oashima et al., 1990). Soluble substrates have some advantages compared to the cellulosic materials. The process conditions can be optimised and run as a fed-batch or a continuous culture system to maximize the productivity (Ju et al., 1999). A series of different carbohydrates have been studied for *T. reesei* QM9414 growth and xylanase induction (Table 5). In general, lactose has been used as a common carbon source and inducer of industrial enzyme production (especially cellulases) in *T. reesei* (Chaudhuri & Sahai, 1993, 1994; Morikawa et al., 1995; Olsson et al., 2003).

Table 5. Growth and expression levels of xylanase genes (*xyn*1, *xyn*2) in *T. reesei* QM9414.

Carbon source	Growth*	Induction¤	Reference
D-glucose	+ + +	-	Margolles-Clark et al.,1997
D-xylose	+ + +	-	Margolles-Clark et al.,1997
Mannose	+ + +	-	Margolles-Clark et al.,1997
D-galactose	+ +	-	Margolles-Clark et al.,1997
Xylobiose	+	+	Zeilinger et al., 1996
Sophorose	n.r.	+ + +	Zeilinger et al., 1996
Cellobiose	+	+	Margolles-Clark et al.,1997
Arabinose	n.r.	-	Zeilinger et al., 1996
Xylan	+ + +	+ +	Margolles-Clark et al.,1997
Cellulose	+ + +	+ +	Margolles-Clark et al.,1997

* Growth was estimated visually (+ + + best growth, + poor growth); ¤ induction was estimated by analysing mRNA expression. n.r., not reported.

Hydrolysates of many different bulk materials have been used for xylanase and cellulase production for decades. Wastepaper hydrolysate has a similar cellulose-inducing strength as cellulose and it induces a wide set of cellulases (Ju et al., 1999). A high xylanase/cellulase ratio of enzyme activities was found at neutral pH cultivation of *T. reesei* Rut C-30 on xylan- and cellulose-based media (Bailey et al., 1993a). Hemicelluloses were suitable substrates for the xylanase production with concomitant low production levels of cellulase activity (Gamerith et al., 1992). Notably, some filamentous fungi are known to produce higher xylanase activities when cultured on wood pulp than on pure xylan (Royer & Nakas, 1989).

Since xylan and cellulose are unable to enter the microbial cell, it has been suggested that low molecular weight degradation products of xylan and cellulose hydrolysis penetrate into the cells and induce the production of hydrolytic enzymes (Nikolaev et al., 1998; Haltrich et al., 1996). Both xylanases and cellulases are induced by monosaccharides and disaccharides. L-sorbose has been considered to be the only monosaccharide found so far to induce cellulase formation, and sophorose is regarded as the most efficient inducer of cellulases. Cellobiose is thought to be the inducer of these enzymes in natural conditions (Royer & Nakas, 1990; Nogawa et al., 2001).

There are only a few reports regarding the use of different soluble carbon sources for enzyme induction in *T. lanuginosus*. D-xylose seems to be an efficient inducer of xylanase activity in DSM 5826 (Table 6). Another report claims that D-xylose repressed xylanase production by *T. lanuginosus* RT 9 (Hoq et al., 1994).

Table 6. The xylanase and biomass production by *T. lanuginosus* DSM 5826 during growth on 15 g/l of different carbon sources (Purkarthofer & Steiner, 1995).

Carbon source	Xylanase (IU/ml)	Dry weight (mg/ml)
D-Glucose	0.21	5.92
D-Galactose	0.41	4.94
D-Mannose	0.29	5.32
D-Ribose	7.95	3.58
D-Arabinose	8.08	4.31
D-Xylose	73.50	4.55
D-Lyxose	51.00	3.89
L-Arabinose	0.83	3.83
D-Fructose	0.16	4.97
L-Sorbose	0.19	3.25
Cellobiose	0.15	3.96
Sucrose	0.66	3.85
Lactose	0.16	3.07
Maltose	0.46	4.99
Xylan	426	-
None	0.16	1.35

1.4.2 Nitrogen sources

Typical nitrogen sources in *T. reesei* cultivations are ammonium sulphate or ammonia water solution. Nitrate or urea are not suitable for *T. reesei* cultivations (Tangnu et al., 1981; Haltrich et al., 1996; Lieckfeldt et al., 2000), whereas trace peptone and yeast extract can stimulate an increase in enzyme production (Haapala et al., 1996; see also Pedersen & Nielsen, 2000). In the shake flask cultivations, the initial 30 g/l lactose and 5 g/l ammonium sulphate concentration are good choices. In batch and fed-batch fermentations, ammonia water solution has been used to adjust pH. The initial C:N ratio (w/w) should be close to 4:1 (approximately equal to the ratio of 4 grams lactose per 1 gram ammonium sulphate) (Ju & Afolabi, 1999).

Table 7. Common nitrogen sources for xylanase production by *T. reesei*.

Nitrogen source	Growth	Comments	Reference
Ammonium	+ + +	Decreases medium pH	Haapala et al., 1994
Urea	+	Increases medium pH	Haapala et al., 1994
Yeast extract	+ + + +	Stimulates enzyme production	Haapala et al., 1996;
Peptone	+ + + +	Stimulates enzyme production	Haapala et al., 1996;

The Maillard reaction may have a bad influence on the cultivation, when using plant hydrolysates as the carbon source. The preparation of plant hydrolysates to produce saccharides and proteins involves high temperatures, and the colour-forming Maillard reaction plays a significant role in this process. The Maillard reaction consumes nutrients such as amino acids and saccharides, and the colour products can be harmful for microbes and cell growth (Jing et al., 2000). High temperature, high substrate concentration and alkaline conditions greatly increase the intensity of the colour reaction (Ames, 1998).

A few studies have dealt with suitable nitrogen source for *T. lanuginosus*. It seems that yeast extract is a better nitrogen source and nutrient material than other common organic or inorganic nitrogen sources (Purkarthofer et al., 1993a).

1.4.3 Other nutrients and surfactants

Besides carbon and nitrogen sources, several other factors have also to be considered in designing the optimum cultivation conditions. The morphological and physiological changes of *T. reesei* influence enzymes production (Velkovska et al., 1997; McIntyre, 1998). It has been reported that only the second fungal stage of *T. reesei* can produce enzymes and the primary mycelium does not efficiently secrete enzymes (Velkovska et al., 1997). A summary of the metal ions and surfactants used for *T. reesei* cultivation are listed in Table 8.

Tween-80 is beneficial for the secretion of enzymes; its optimal concentration is close to 0.2 ml/l, while a higher concentration is harmful for the production of cellulases (Tangnu et al., 1981; Panda et al., 1987). A similar effect was observed in these studies also for other extracellular enzymes (Arnesen et al., 1998). The mechanism for the enhanced enzyme

production by Tween-80 may be related to the increased permeability of the cell membrane, allowing a more rapid secretion of the enzymes, which leads to greater enzyme synthesis (Arnesen et al., 1998; Eriksson et al., 2002). Another possible explanation is that Tween-80 has an influence on the level of glycosylation and thus, e.g., on protein stability (Kruszewska et al., 1990).

Table 8. Metal salt, organic nitrogen and Tween-80 concentrations in *T. reesei* cultivations.

	A	**B**	**C**	**D**	**E**
KH_2PO_4	15	2.0	3.8	2.0	2.0
$MgSO_4 \cdot 7H_2O$	1.23	0.3	0.6	0.3	0.3
$CaCl_2 \cdot 2H_2O$	0.8	0.3	0.8	0.3	0.3
$FeSO_4 \cdot 7H_2O$	0.0027	0.005	0.005	0.005	0.005
$MnSO_4 \cdot H_2O$	0.0016	0.0016	0.0016	0.0016	0.0016
$ZnSO_4 \cdot 7H_2O$	0.0014	0.0014	0.0014	0.0014	0.0014
$CoCl_2 \cdot 6H_2O$	0.0036	0.002	0.0037	0.002	0.002
Antifoam	-	-	0.1	0.1	-
Tween-80	0.3	0.2	0.2	0.2	0.1
Yeast extract	0.3	0.3	-	-	-
Peptone	0.75	0.75	-	2.0	1.0

A) Domingues et al., 1999, **B**) Leisola, 1979, **C**) Tholudur et al., 1999, **D**) Ju et al., 1999, **E**) Krishna et al., 2000. All units are g/l except Tween-80 unit is ml/l.

1.4.4 pH and temperature

pH is an important parameter in the production of enzymes by *T. reesei* (Denison, 2000). Earlier reports indicated that a rather high pH (7.0) is essential for good production of xylanases by *T. reesei* Rut C-30 on cellulose- and xylan-based growth media, although growth (broth viscosity) was evidently better at pH 4.0 than at pH 7.0. Meanwhile, good production of cellulases was found at low pH (4.0) (Bailey et al., 1993a). A high pH (7.0) was essential for high xylanase production by *Trichoderma longibrachiatum* in cellulose medium (Royer & Nakas, 1990). During the course of the fermentation, the nitrogen source can significantly influence the pH of the medium (Haapala et al., 1994). The pH of *T. reesei* culture broth decreased during the cultivation when ammonium salts were used as the nitrogen source, whereas the pH increased when urea was the nitrogen source.

The cultivation temperature does not only affect the growth rate of an organism, but it can also have a marked effect on the level of xylanase production. *T. reesei* Rut C-30 grew well at 17, 28 and 37 ^{o}C when cultivated on lactose substrate, but xylanase production was significantly increased at higher temperature, whereas cellulase production was reduced (Haltrich et al., 1996). An initial phase of cultivation of *T. reesei* Rut C-30 at 37 ^{o}C followed by a shift to 28 ^{o}C in the beginning of the enzyme production phase was advantageous for both the amount of xylanase activity obtained and the ratio of xylanase to cellulase. By applying the temperature shift during laboratory cultivation, xylanase activity could almost be doubled, whereas the xylanase/cellulase ratio was threefold higher in comparison to cultivation at a constant temperature of 28 ^{o}C (Haltrich et al., 1996; Smits et al., 1998).

1.4.5 Aeration and agitation

When bioreactors are used in the cultivation of filamentous fungi for industrial enzyme production, the agitation rate and aeration levels influence the fungal growth and secretion of enzymes. The shearing action of the impellers on the morphology and productivity of filamentous fungi also deserves attention (Ilias & Hoq, 1998; Gibbs et al., 2000). Too strong agitation and aeration have been shown to be harmful for the production of xylanase. In large-scale fermentations, the stirrer speed had an even more pronounced effect on the production of xylanase. Highest xylanase activities were obtained in 20,000-litre cultivations by *T. lanuginosus* when the stirrer was turned off after a certain cultivation time and then used only periodically (Haltrich et al., 1996; Reddy et al., 2002). Thus, conditions of low shear which are typical for an air-lift fermentor were approached by using this method.

The enzyme production by *T. reesei* QM 9414 was seriously affected by agitation (Lejeune et al., 1995). When using lactose as the substrate in a 15-litre fermentation, the optimal agitation rate was found to be 200 rpm. Low xylanase activities were obtained at 130 rpm, most probably due to oxygen or mass transfer limitations, while at 400 rpm almost no xylanase was produced. The latter result could be explained by a low production rate caused by the increased shear stress. When cellulose powder was used as the substrate, the effect of agitation rate was less pronounced than with lactose. With this substrate, the optimum stirrer speed was 300 rpm, and significant xylanase production occurred even with the highest agitation of 400 rpm. The particles of the insoluble substrate appeared to have a protective effect on the mycelium (Lejeune et al., 1995).

The effect of oxygen saturation has been studied for *T. reesei* Rut C-30 grown on 1 % cellulose or xylan. Enzyme and extracellular protein levels were not affected by oxygen levels of 20 % or above, but were severely reduced at 10 % oxygen saturation (Schafner & Toledo, 1992). On the contrary, low levels of dissolved oxygen or even oxygen limitation did not adversely influence xylanase production by *T. lanuginosus* (Purkarthofer et al., 1993b). Varying aeration rates used in laboratory fermentations of *T. lanuginosus* from 0.5 to 1.5 vvm (vvm: the income air volume per medium volume per minute) showed that slightly higher xylanase activities were obtained when aeration was increased from 0.5 to 1.0 vvm, whereas at the highest aeration rate of 1.5 vvm xylanase yields were significantly reduced (Hoq et al., 1994).

1.4.6 Fed-batch and continuous fermentation

Fed-batch operation involves a slow addition of highly concentrated nutrient media into the bioreactor with no effluent removal until the reactor is full. The aeration tank contains a large volume of highly active and dense organisms at the beginning of operation with slow feeding of concentrated nutrient solution, which is diluted inside the reactor. This operation can maintain low nutrient levels to minimize catabolite repression, or to extend the stationary phase by nutrient addition to obtain additional product. Fed-batch with feed-back control in the case of substrate inhibition is widely used in the industrial fermentations (Hendy et al., 1984; Bailey & Tähtiharju, 2003; Skolpap et al., 2004).

Continuous-flow stirred-tank reactor (CSTR) cultivations are usually operated as chemostats. It is usually preceded by growth of the fungus in batch culture to stationary phase. When

supply of fresh medium is initiated, the growth proceeds and material from the vessel is washed out, until the concentration of the medium is reduced to a level at which it limits specific growth rate (Papagianni, 2004). In an industrial production process, higher productivity and cell growth rate can be obtained by continuous culturing. Detailed *T. reesei* growth parameters are listed in the report by Chaudhuri and Sahai (1994).

1.4.7 Solid-state fermentation

Besides submerged fermentation, solid-state fermentation (SSF) is a popular method to produce xylanases and cellulases by fungi. SSF means that the microorganism grows on moist solid substrates in the absence of free-flowing water. Filamentous fungi grow typically in nature on solid substrates, such as wood, seeds, stems, roots and leaves of plants in symbiotic associations. Compared to the submerged fermentation, SSF possesses several advantages such as higher fermentation productivity, higher end-concentration of products, higher product stability, lower catabolic repression, cultivation of microorganisms specialized for water-insoluble substrates or mixed cultivation of various fungi, and lower demand of sterility due to the low water activity used in SSF (Hölker et al., 2004).

However, SSF is currently used only to a small extent for enzyme and secondary metabolite production because of severe process engineering problems. A scale-up of solid-state processes seems to be difficult due to the generally known problems of heat transfer, the fact that the media is not homogeneous, and difficulties with aeration. In *T. lanuginosus* cultivations, these problems are made worse by the shear sensitivity of the microbe (Purkarthofer et al., 1993b; Smits et al., 1996).

1.5 METHODS TO IMPROVE THERMOSTABILITY OF XYLANASE

There has accumulated during the years a large amount of information about factors related to thermostability of proteins (Lehmann & Wyss, 2001; van den Burg & Eijsink, 2002; Fágáin, 2003). The information has been gathered by structural comparison of mesophilic and thermophilic proteins, statistical comparison of amino acid composition between mesophilic and thermophilic proteins and mutagenesis studies of a large number of different proteins, including many industrial enzymes. To improve the protein thermostability, it is important to find the weak points in the protein structure, e.g. sites that are likely to unfold at elevated temperature. Computer simulations can be helpful in finding these sites. Sequence comparisons can be used to find features that are different in thermophilic enzymes when compared to mesophilic ones. This information is then used to plan site-directed mutations into a mesophilic protein. Random mutagenesis and directed evolution techniques do not require prior knowledge of the protein structure and can reveal stabilizing mutations that cannot be found by rational design (site-directed mutagenesis).

There are several strategies to plan thermostable mutations. Strategies to stabilize an enzyme can be to reduce the degrees of freedom in the main chain or to make unfolding more unfavourable (Shaw & Bott, 1996). Stabilizing mutations can increase the structural rigidity, e.g. by strengthening the attractive forces (hydrogen bonds, salt bridges, etc.). The approaches to stablize enzymes by salt bridges are not always successful (Shaw & Bott, 1996). Introduction of new disulphide bridges can have a very large stabilizing effect, although even they are not always successful. Replacement of lysine with arginine has been

shown to increase the thermostability of several proteins. Even a small structural modification can have a significant effect on the properties of the enzyme (Lee & Vasmatzis, 1997). A protein molecule can be stabilized also by adding stabilizing agents or particular ions and salts to the solution (Fágáin, 2003). This method is important in the production of commercial enzymes.

The thermostability of xylanases has been studied extensively because the first commercial xylanases were from mesophilic microbes, while the enzyme met harsh conditions in feed (high temperature) and pulp bleaching (high temperature and high pH) applications. Family 11 xylanases have been stabilized by introduction of disulphide bridges into the protein N-terminus and α-helix, extension of protein N-terminus and several single amino acid substitutions at N-terminal region, Ser/Thr surface, α-helix and other sites (Wakarchuk et al., 1994; Shibuya, 2000; Turunen et al., 2001, 2002; Sung, 2003; Fenel et al., 2004; Jänis et al., 2004). While the mesophilic family 11 xylanases are inactivated quickly above 50 ^{o}C, the engineered enzymes have at best 10-20 ^{o}C higher apparent temperature optimum (Georis et al., 2000; Sung, 2003; Fenel et al., 2004). The protein engineering of these enzymes has still not created the thermostability found in the *Dictyoglomus thermophilum* xylanase, which is the most stable known family 11 xylanase with the apparent temperature optimum (T_{opt}) at 85 ^{o}C (Morris et al., 1998). The thermostability of family 10 xylanases can be much higher than that of family 11 xylanases (Biely et al., 1997). The most thermostable known xylanases are active at temperatures above 100 ^{o}C (Table 9).

Table 9. A list of thermostable xylanases.

Xylanase	MW* (kDa)	Family	T_{opt} (^{o}C)	$T_{1/2}$ (min)	Reference
Thermomyces lanuginosus DSM5826	25.5	11	60-70	148 at 75 ^{o}C	Cesar & Mrsa, 1996; Singh et al, 2000
Nonomuraea flexuosa (*Actinomadura flexuosa*)	37	11	70-80	32 at 80 ^{o}C 273 at 80 ^{o}C (shortened form)	Leskinen et al., 2004; Hakulinen et al., 2003
Dictyoglomus thermophilum	33	11	85		Gibbs et al., 1995 Morris et al., 1998
Thermococcus zilligii	95		80	8 at 100 ^{o}C	Uhl & Daniel, 1999
Sulfolobus solfataricus	57		90	47 at 100 ^{o}C	Cannio et al., 2004
Thermotoga neapolitana	116	10	102	120 at 100 ^{o}C	Zverlov et al., 1996
Thermotoga sp. FjSS3-B.1		10	80	20 at 105 ^{o}C	Sunna et al., 1997; Simpson et al, 1991;
Pyrodictium abyssi			105-115		Sjöholm & Antranikian, 1997

* Molecular weight in SDS-PAGE.

2. AIMS OF THIS STUDY

Xylanase is used in various industrial applications. It is mainly produced by different fungi. However, more economical production systems and more stable enzymes are needed for industrial applications. Cheaper and better inducers of xylanase would therefore be advantageous. The problem with stability can in principle be overcome by finding more stable enzymes from nature or by stabilizing the existing enzymes to be suitable for industrial applications.

Trichoderma reesei and *Thermomyces lanuginosus* are two excellent xylanase producers. Therefore these organisms were chosen for this study. The specific goals of this thesis study were formulated during the research process and were as follows:

1. to study the regulation of xylanase production in *T. reesei*, especially the pH-dependent regulation of enzyme production;

2. to find better inducers and soluble carbon sources for xylanase production by *T. reesei*;

3. to study the temperature- and pH-dependent properties of *T. reesei* xylanase II mutants;

4. to study the growth properties of *T. lanuginosus* and characterize its xylanase.

3. MATERIALS AND METHODS

3.1 ORGANISMS AND CULTIVATION CONDITIONS

T. reesei Rut C-30 was obtained from VTT, Finland (www.vtt.fi). Dry powder spores were suspended in sterile 20 % (v/v) glycerol and the suspension inoculated on potato dextrose agar (PDA) slants (Difco Laboratories, USA). The PDA slants were incubated at 30 ºC for 7 days and then stored at 4 ºC. The formed spores were collected by washing the slant with 3 ml of sterile culture medium. The spore concentrate was pipetted into 250 ml shake flasks containing 100 ml culture medium and incubated on a rotary shaker (200 rpm) at 30 ºC. After 36 h growth the medium was used as the inoculum for bioreactor cultivations.

Batch cultivations were carried out in 2-litre glass-vessel bioreactors (Biostat MD system, B. Braun Biotech International, Germany). The cultivation parameters were as follows: temperature 28 ºC, agitation 400 rpm (tip speed 1.1 m/s, two Rushton type impellers), aeration 1 vvm and cultivation time 5 days. Foam was controlled by automatic addition of 10 % (v/v) silicone antifoaming agent (BDH Laboratories, UK). The pH was controlled by automatic addition of 12.5 % (v/v) ammonia water or 10 % (w/w) sulfuric acid. The working volume in the bioreactor was 1 litre.

The same Biostat MD system and above-mentioned conditions were used in the fed-batch cultivation. The initial working volume was 1 litre. The feeding was started at t = 48 h and maintained for 48 h. The pump rate was set at about 5.0 ml/h and the total input was 240 ml feeding solution into the bioreactor. In the fed-batch cultivation the initial mono- and disaccharide concentration was set to 20 g/l (10 g/l lactose monohydrate and 10 g/l plant hydrolysate sugars). The feeding solution comprised 100 g/l lactose monohydrate, 100 g/l plant hydrolysate sugars, 7.5 g/l peptone, 3 g/l yeast extract and trace metals. The trace metal concentrations were 10 times the concentrations in the culture medium.

T. reesei culture medium was: 5 g/l KH_2PO_4; 0.6 g/l $MgSO_4 \cdot 7H_2O$; 0.8 g/l $CaCl_2 \cdot 2H_2O$; 5.0 mg/l $FeSO_4 \cdot 7H_2O$; 1.6 mg/l $MnSO_4 \cdot H_2O$; 1.4 mg/l $ZnSO_4 \cdot H_2O$; 2.0 mg/l $CoCl_2 \cdot 6H_2O$; 0.2 ml/l Tween-80 (Fluka Chemie, Switzerland); 0.75 g/l Peptone (Difco Laboratories, USA); 0.3 g/l Yeast extract (Lab M, International Diagnostics Group, UK); and the carbon source was lactose monohydrate (the concentration is given in the text). If otherwise not indicated, the culture medium components were purchased from Sigma-Aldrich Chemie, Germany.

T. lanuginosus DSM 10635 (www.dsmz.de/strains/no010635.htm) (isolated from sludge in the Czech Republic) was purchased from the German type culture collection (DSMZ). The strain was grown on a potato dextrose agar slant at 50 ^{o}C for 5 days and stored at 4 ^{o}C. The growth conditions in shake flasks and bioreactor are described in article **V** (Xiong et al., **V**). The medium was 5 g/l KH_2PO_4; 0.3 ml/l Tween 80; 15 g/l yeast extract and 15 g/l various carbon sources as indicated in the text.

The generation of the mutants of *T. reesei* xylanase II by PCR method is described in article **IV** (Xiong et al., **IV**). *Escherichia coli* XL1-Blue (Strategene, USA) was used as the host strain for the plasmid growth and production of xylanase enzyme from the pALK143 vector (ROAL, Finland) as earlier described by Turunen et al. (2001). In the plate screening of the enzyme activity, the *E. coli* cells were grown on agar plates containing RBB-xylan (X-0502,

Sigma), in which the xylanase activity is seen as white halos around the positive colonies (Biely et al., 1985). For the production of xylanase by *E. coli*, the cultivation medium was 2 % (w/v) LB broth (Laboratorios Conda, Spain). Growth conditions were 30 ^{o}C and 200-250 rpm agitation in the shake flask. 1 mM isopropyl-thio-β-D-galactopyranoside (IPTG) was added to induce xylanase production (Turunen et al., 2001).

3.2 ACID HYDROLYSIS OF PLANT RAW MATERIALS

Oat husk (Suomen Viljava, Finland), spruce fiber (Metso, Finland) and sugar beet pulp (Suomen Sokeri, Finland) were hydrolyzed in 4 % (w/w) sulfuric acid at 100 °C for 2 hours (100 g solid in 1 litre acid solution). After hydrolysis the solutions were filtered with Whatman No.3 filter paper (Whatman International, UK) applying suction. The filtered solutions were neutralized with calcium carbonate and the precipitate removed by filtration. The sugar beet pulp was pre-treated before the acid hydrolysis with a protease solution at 60 °C and pH 8 for 2 hours and was then washed with excess water to remove the soluble material. After hydrolysis the solutions were concentrated by vacuum evaporation at 60 °C to ~30 % (w/w) of dry matter and re-filtered. The concentrated hydrolysates were autoclaved at 121 °C for 15 minutes immediately after concentration.

3.3 ANALYSIS OF SUBSTRATES AND FERMENTATION PRODUCTS

The concentration of monosaccharides was analyzed by high-performance liquid chromatography (HPLC). The following system was used: Waters 717 plus autosampler (Waters Corp., USA), Waters 510 pump, and Waters 410 refractive index detector. The components were separated in an Aminex HPX-87P column (Bio-Rad Laboratories, USA) at 70 °C with distilled water as the mobile phase. The elution rate was 0.6 ml/min and a deashing Micro-Guard pre-column (Bio-Rad Laboratories, USA) was used to remove the ions.

The oligosaccharide content of oat husk hydrolysate and sugar beet pulp hydrolysate was determined by gel permeation chromatography (GPC). The detailed method is described in article **III**. The Empower program version 4 (Waters) was used for calculations and Shodex standard P-82 (Showa Denko K.K., Japan) together with L-arabinose, cellobiose and raffinose (Sigma) were used as molecular weight standards.

The cell dry weight (cdw) was measured with pre-weighed filter papers (0.45 μm, diameter 50 mm, NC 45, Schleicher & Schuell, Germany). An aliquote of 3 ml of culture broth was pipetted onto the filter paper under suction. The cells were washed with 20 ml distilled water, dried in a microwave oven at full power (1000 W) for 7 minutes, cooled down and weighed.

The protein concentrations were determined by the method of Lowry et al. (1951). Bovine serum albumin (A-4503, Sigma) was the standard protein. The xylanase protein concentration of absorbance = 1 at 280 nm (1 cm cuvette) corresponded to the concentration of 0.7 g/l measured by Lowry. The standard protein (Bovine Albumin, Sigma, A-4503) of absorbance 1 at 280 nm (1 cm cuvette) corresponded to the weight concentration 1.66 grams

protein per litre. Pure *T. reesei* xylanase II protein with absorbance 1 at 280 nm corresponds to the protein concentration of 0.37 mg/ml (Turunen et al., 2001).

3.4 ENZYME PURIFICATION

T. lanuginosus xylanase was purified as follows. *T. lanuginosus* cells were separated from the medium by centrifugation. Ammonium sulfate was added to the supernatant to achieve 80 % saturation. The suspension was centrifuged and the precipitate was dissolved in 25 % saturated ammonium sulfate in 50 mM Tris-HCl (pH 7.5). The sample was applied to a Phenyl Sepharose column (Amershan Pharmacia Biotech, Sweden) pre-equilibrated with 25 % saturated ammonium sulfate in 50 mM Tris-HCl buffer (pH 7.5). The column was eluted with a linear gradient of 25 to 0 % saturated ammonium sulfate. The active fractions were pooled and concentrated by ultrafiltration (PM 10, Millipore) to approximately one tenth of the original volume and 20 mM ammonium acetate buffer (pH 6.0) was added to achieve the original volume. The concentration-dilution procedure was repeated once and the sample was applied to a DEAE Sepharose FF (Amershan Pharmacia Biotech) column pre-equilibrated with 20 mM ammonium acetate buffer, pH 6.0. The column was eluted with a linear NaCl gradient from 0 to 1 M NaCl (Nyyssölä et al., 2001). The fractions showing xylanase activity were pooled and concentrated by ultrafiltration (Centriplus 30, Amicon, USA). The sample was washed by adding 20 mM ammonium acetate buffer (pH 6.0) and concentrated again. The final sample was filtered by Ultrafree MC 30,000 NMWL (Millipore) and the xylanase was collected from the filtrate. The purified enzyme was stored at 4 ^{o}C in 20 mM ammonium acetate buffer, pH 6.0.

3.5 IEF AND SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using 12 % polyacrylamide gels according to standard protocol (Laemmli, 1970). Molecular weight was estimated using BIO-RAD low molecular weight standard. The denaturing SDS-PAGE was run at room temperature and 200 V. The isoelectric focusing (IEF) was performed using polyacrylamide gel (Ampholine PAG plate, Amersham Pharmacia Biotech, Sweden) with a pH range of 3.5-9.5. Samples were focused at 2500 Vh and the end voltage was 1500 V (~150 V/cm gel). Proteins were stained with Coomassie blue (Bio-Rad Laboratories, USA). The accurate molecular weight of the purified xylanase was determined by ESI FT-ICR mass spectrometry as described by Jänis et al. (2001).

3.6 ZYMOGRAM

Zymogram analysis after IEF was performed according to Biely et al. (1988). Remazol Brilliant Blue-Xylan (RBB-xylan) was used as the soluble substrate for detecting xylanase activity. The IEF gel was overlapped onto an RBB-xylan-agar gel. The gels were incubated at room temperature until the enzyme zones became clearly visible. The IEF gel was removed and the enzyme-degraded substrate zones on the RBB-xylan-agar gel were destained with a solution comprising two parts of 95 % (v/v) ethanol and one part of 0.05 M acetate buffer (pH 5.4).

The assumed xylanase III was extracted from IEF agarose. IEF was performed using agarose gel (Agarose IEF, Amersham Pharmacia Biotech) with a pH range of 3.5-9.5 using Ampholine preblended solution (Amersham Pharmacia Biotech). The IEF parameters were otherwise as described above. After running the agarose IEF, the gel region close to p*I* 9.1 was cut out of the gel and smashed in 0.05 M citrate-phosphate buffer (pH 5). The mixture was frozen and thawed twice to transfer the proteins into the buffer solution. A centrifugal filter device (Centriprep YM-3, Millipore, USA) was used to concentrate the sample solution to protein concentration of 2 g/l. This protein solution was used for the next SDS-PAGE zymogram analysis.

Zymogram analysis was also carried out after SDS-PAGE. The protein concentrate was run in a SDS-PAGE gel containing 0.1 % (w/v) xylan at 4 ºC and 100 V, with non-heated protein sample. SDS was washed out using 2.5 % (v/v) Triton X-100 solution (Sigma-Aldrich Chemie, Germany), and after this the gel was incubated in 0.05 M acetate buffer (pH 5.4) at 50 ºC for 20 min. Then the gel was soaked in 0.2 % (w/v) NaOH for 30 min. After removal of NaOH, 0.1 % (w/v) Congo-Red solution was added to stain xylan (20 min incubation). Finally, 1 M NaCl solution was used to remove unbound Congo-Red. Bio-Rad Coomassic blue was used to stain the protein standard lane (SDS-PAGE low range standard LS1610305, Bio-Rad Laboratories, USA).

3.7 ENZYME ACTIVITY ASSAYS

Xylanase activity was analyzed by measuring with the 3,5-dinitrosalicylic acid (DNS) method the reducing sugars released during a 10 min reaction (Bailey et al., 1992). The substrate was 1 % (w/v) xylan (X-0502, Sigma). The buffer was 0.05 M citrate-phosphate at pH 4-7 or 0.05 M Tris-HCl at pH 7-9 or 0.05 M Glycine-HCl at pH 9-10. The used pH and temperature values in each assay are mentioned in the text. The residual activities after inactivation at higher temperatures were measured at 50 ^{o}C, pH 5 (*T. reesei* XYN II) or 70 ^{o}C, pH 6 (*T. lanuginosus* XYN).

The cellulase activity was analyzed with filter paper according to the method of Ghose (1987). Whatman No.1 filter paper (~50 mg, Whatman International, UK) was incubated at 50 ºC for 1 h in 1 ml of 0.05 M Na-citrate buffer solution (pH 4.8) supplemented with 0.5 ml of enzyme solution. The liberated sugars were analyzed by the DNS method.

One unit (IU) of enzyme activity was defined as the amount of enzyme releasing 1 micromole reducing sugars in one minute reaction. D-xylose was the standard sugar for xylanase assay, and D-glucose was the standard sugar for cellulase assay.

3.8 E_A AND E_D OF ENZYME

The Arrhenius activation energy E_a and the deactivation energy E_d were approximately calculated with the Arrhenius Equation:

$$\mathbf{k = A * e^{-E/RT}}$$

Both the enzymatic reaction velocity constant k_a and the enzyme deactivation velocity constant k_d were calculated with this equation. k_a is obtained from the temperature-dependent enzyme activity curve (temperature optimum curve) and k_d is obtained from temperature-dependent enzyme inactivation profiles (Pauline, 1995). T is the absolute temperature.

3.9 HALF-LIFE IN THE PRESENCE OF SUBSTRATE

The half-life in the presence of the substrate was determined from the productivity curves (Xiong et al., **V**, Fig. 6A &B), in which the amount of reaction product was followed as a function of time. The inactivation of the enzyme was seen as decreased accumulation of the reaction product during the course of reaction. A series of parallel reactions with equal amounts of enzyme was performed with equal amounts of substrate, and then the reaction was terminated at time point t_1, t_2, t_3, t_4, t_5 and t_6 by DNS solution. The corresponding activity values (absorption at 540 nm) were A_1, A_2, A_3, A_4, A_5 and A_6. The time intervals were the same as the first reaction time, and thus, the reaction times were recorded as 0, 1t, 2t, 3t, 4t and 5t. The enzyme deactivation is estimated to decrease the activity exponentially with a function of time following the equation $V = V_o \times e^{-k_d t}$. The first interval phase reaction rate was assumed to be V_o and then the other values were calculated as shown in Table 10:

Table 10. Half-life calculation in the presence of substrate.

Interval phase	**t_1**	**t_2 -- t_1**	**t_3 -- t_2**	**t_4 -- t_3**	**t_5 -- t_4**	**t_6 -- t_5**
Activity	A_1	A_2-A_1	A_3-A_2	A_4- A_3	A_5-A_4	A_6-A_5
Time	0	1t	2t	3t	4t	5t
Natural Logarithm	LN (A_1 / A_1)	LN ((A_2-A_1) / A_1)	LN ((A_3-A_2) / A_1)	LN ((A_4- A_3) / A_1)	LN ((A_5-A_4) / A_1)	LN ((A_6-A_5) / A_1)

The slope of linear Time- Logarithm (X-Y) is the constant - k_d. Half-life can then be approximately calculated from the equation t_h = LN (2) / k_d.

4. RESULTS AND DISCUSSION

4.1 INFLUENCE OF pH ON XYLANASE PRODUCTION BY *T. REESEI*

Lactose was used as the main carbon source for the xylanase and cellulase production by *T. reesei* Rut C-30. The highest xylanase and cellulase activities were observed at pH 6.0 and pH 4.0, respectively (Fig. 1). The highest concentration of soluble protein was observed at pH 4.5. Compared with the cellulose- and xylan-based growth media, in which cellulase production was favoured at pH 4.0 and xylanase production was favoured at pH 7.0 (Bailey et al., 1993a), the lactose-based medium showed a lower pH for the maximal xylanase production (Xiong et al., **I**).

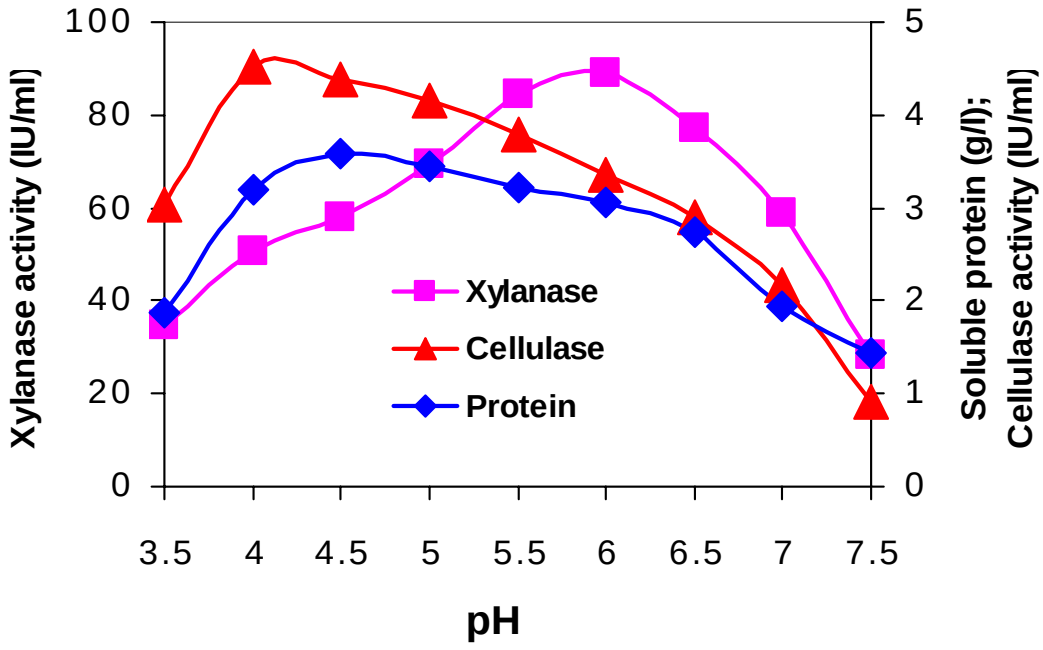

Fig. 1. Xylanase and cellulase activities (IU/ml) and soluble protein concentration (g/l) as a function of pH in batch cultivations of *T. reesei* Rut C-30. Culture conditions: 30 g/l lactose, 28 ^{o}C, 400 rpm and 5 days.

The apparent xylanase activity in the culture broth is formed by various xylanases and cellulases. By using the IEF and RBB–xylan zymogram analysis, it was possible to obtain information about the xylan degrading enzymes (Xiong et al., **I**, Fig. 2). The non-specific cellulases, such as endoglucanase I (EGI), which show xylanase activity, have p*I* value below 5.0 (Biely & Markovic, 1988; Bailey et al., 1993b). It could be seen in the zymogram analysis of IEF gels that xylanase activity corresponded to three *T. reesei* xylanases (Xiong et al., **I**, Fig. 2). The production of xylanases showed pH-dependence. Xylanase II (p*I* 9.0) was expressed at low (4.0) and high (6.0) pH, whereas xylanase I showed higher expression at pH 4.0 and xylanase III at pH 6.0.

The identity of both xylanases I and II was confirmed by comparing to purified enzymes, according to the active band in RBB-xylan zymogram and p*I* results in IEF gel (Xiong et al., **I**, Fig. 2). The cultivation of *T. reesei* at pH 6 produced a xylanase with a very high p*I* value

(higher than that of the pure xylanase II, p*I* 9.0). In the xylan-containing SDS-PAGE, the protein molecular mass weight was close to 32 kDa (Xiong et al., **I**, Fig. 2 & 3). Both p*I* and MW corresponded to the values of the *T. reesei* xylanase III (p*I* 9.1; 32 kDa) (Xu et al., 1998). Therefore, this indicated that the enzyme with p*I* higher than 9.0 was *T. reesei* xylanase III.

As a conclusion, *T. reesei* Rut C-30 reacts to the pH of the growth environment by modifying its enzyme production patterns. The pH-dependent activity profiles of the purified *T. reesei* xylanases and the pH-dependent production levels appear to be linked together. It means that, at a low pH, *T. reesei* produces xylanase I, which is most active at those pH values. At a high pH the fungus produces xylanase III, which is most active at those pH values. Although xylanase II is produced both at pH 4 and pH 6, a higher amount of xylanase II is produced at pH 6. The fungus apparently saves its energy by modifying its metabolism to produce the correct enzymes for the particular pH surroundings. The comparison of activity and production levels for different xylanases is shown in Table 11. Further research is needed to clarify what is the molecular basis for the influence of pH on the expression of XYN I, XYN II and XYN III and how the regulation at promoter level is involved.

Table 11. Summary of *T. reesei* xylanase activities and production levels by *T. reesei* Rut C-30 at pH 4 and 6. (+ + + best activity or production; + poor activity or production)

	pH 4	**pH 6**	**Reference**
XYN I activity*	**++**	**+**	Tenkanen et al., 1992
XYN I production	**++**	**+**	Xiong et al., **I**
XYN II activity*	**++**	**+++**	Tenkanen et al., 1992
XYN II production	**++**	**+++**	Xiong et al., **I**
XYN III activity*	**+**	**+++**	Xu et al., 1998
XYN III production	**+**	**+++**	Xiong et al., **I**

* Activity here means the pH-dependent activity. The optimum pH of XYN I, II, and III is 4.0, 5.5, and 6.0, respectively (Table 2).

4.2 XYLANASES INDUCTION BY L-ARABINOSE AND OTHER SUGARS

Eight aldopentoses were tested as the carbon source for xylanase production by *T. reesei* Rut C-30. The highest xylanase activity was achieved in cultivation with L-arabinose, and the obtained xylanase production was even higher than in the lactose cultivation under the conditions that were used in this study (Xiong et al., **II,** Table 1).

In the cultivation with mixture of D-glucose (15 g/l) and L-arabinose (5 g/l), the xylanase activity reached 96.6 IU/ml. The sole D-glucose (20 g/l) cultivation produced only 3.8 IU/ml, since D-glucose has a strong repression effect. With the mixture of lactose (15 g/l) and L-arabinose (5 g/l), the obtained xylanase activity was 121.7 IU/ml, whereas the sole lactose (20 g/l) cultivation produced 59.2 IU/ml in the same conditions (Xiong et al., **II**). Lactose is able to induce xylanase activity in *T. reesei* Rut C-30. However, 20 g/l of D-glucose caused strong repression of xylanase activity (Fig. 2).

Co-metabolism of L-arabinose with other sugars was also studied. The mixturing of L-arabinose with other sugars improved xylanase production to different extents when compared to the cultivation with the single sugars (Fig. 2). L-arabinose caused the maximum increase of xylanase induction for D-glucose cultivation. Thus, L-arabinose appeared to relieve the repression of xylanase production caused by D-glucose. L-arabinose showed limited effect on the xylanase induction when used in mixture with other sugars, except with D-glucose and lactose.

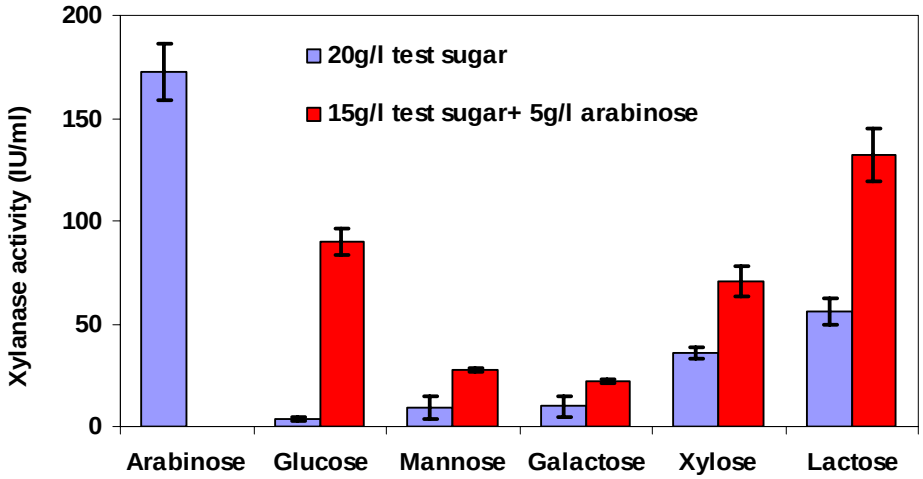

Fig. 2. Stimulation of xylanase production by L-arabinose in 4 days shake flask cultivation with different sugar mixtures.

T. reesei consumed lactose, D-glucose and L-arabinose at different rates in the cultivation with mixture of them. It consumed each type of sugar efficiently, but first lactose and D-glucose and after that L-arabinose (Fig. 3). When the lactose was cleaved to D-galactose and D-glucose during the early stages of cultivation, this increased the amount of D-glucose in the medium shifting the consumption curve of D-glucose to the right (Fig. 3). D-galactose formed from lactose was consumed quickly. D-galactose was a good carbon source but it induced only low amounts of xylanase (Fig. 2).

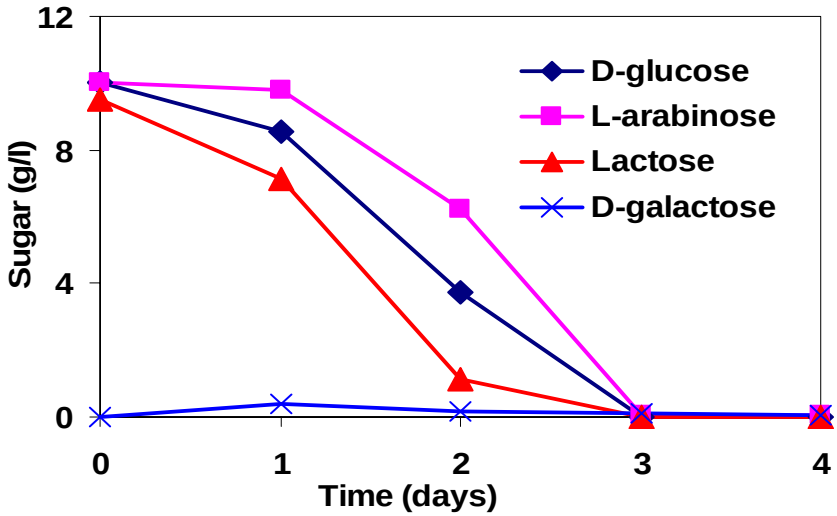

Fig. 3. Sugar consumption rates by *T. reesei* in cultivation with sugar mixture of D-glucose, L-arabinose and lactose. D-galactose is the hydrolysis product of lactose.

The positive effect of L-arabinose on xylanase production by *T. reesei* has not been reported before. L-arabinose is an abundant sugar in nature. Since L-arabinose is found in the arabinoglucurone side-chains of xylan in wood fibres and other hemicelluloses, it is possible, in principle, that the release of L-arabinose is a signal for the fungus to produce xylanase for xylan digestion. The efficient induction of xylanase by L-arabinose could be one reason why higher xylanase activities are achieved when some fungi are grown on wood pulp compared to growth on pure xylan (Royer & Nakas, 1990).

4.3 XYLANASE INDUCTION BY PLANT HYDROLYSATES

In order to find cheaper materials, L-arabinose-rich plant hydrolysates such as sugar beet pulp and oat husk hydrolysates were tested as the carbon sources and inducers of xylanase activity. Based on the sugar concentrations obtained by HPLC analysis of these hydrolysates (Xiong et al., **III,** Table 1), pure sugar models were tested first. The models consisted of pure monosaccharides with same ratio as in the corresponding hydrolysates (Xiong et al., **III,** Table 2). Surprisingly, the media made from the L-arabinose-rich plant hydrolysates produced higher xylanase activities than the corresponding media made from the mixture of pure sugars (Xiong et al., **III**). A fed-batch cultivation on the oat husk hydrolysate (30 g/l) and lactose (30 g/l) mixture is shown in Fig. 4.

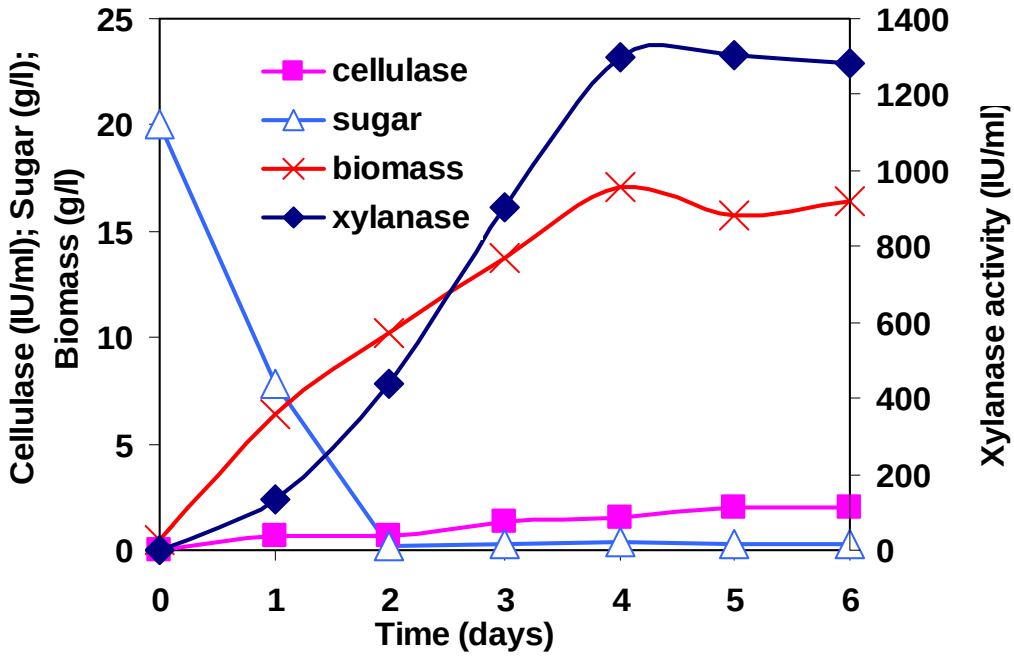

Fig. 4. *T. reesei* Rut C-30 growth and xylanase activity production in fed-batch cultivation with mixture of oat husk hydrolysate and lactose. The details are described in Materials and Methods 3.1. (Xiong et al., **III**).

The xylanase activity (1350 IU/ml) produced by the mixture of oat husk hydrolysate and lactose in fed-batch cultivation is amongst the highest xylanase activities achieved with *T. reesei* (Haltrich et al., 1996). The batch cultivation using solid substrates like beechwood xylan (30 g/l) and corn steep liquor (10 g/l) achieved about 1370 IU/ml xylanase activity (Bailey et al., 1993a). Meanwhile, the produced xylanase patterns (xylanases I, II and III) were similar regardless of the used carbon sources (Xiong et al., **III**, Fig. 7).

While xylanase III (srs.ebi.ac.uk/srsbin/cgi-bin/wgetz?-e+[UNIPROT-acc:Q9P973]+-vn+2) is produced by *T. reesei* strains Rut C-30 and PC-3-7, it is not produced by the strain QM9414 (Xu et al., 1998, 2000). It appears that the *xyn*3 gene of *T. reesei* QM9414 is in a dormant state, which may be caused by a mutation in *T. reesei* QM9414 that prevents the expression of *xyn*3 gene. QM9414 strain is missing also another xylanase-related property: there is no xylanase induction by arabinose in this strain (Zeilinger et al., 1996). In genealogy, the above three *T. reesei* strains are derived from the same ancestor strain *T.reesei* QM6a (Table 1). Hence, it is possible that the ancestor strain QM6a would show similar pH-dependent regulation and L-arabinose induction of xylanases as strain Rut C-30.

In addition, it was observed that *T. reesei* Rut C-30 can grow in entire plant hydrolysates solution with saturated calcium sulfate, which was formed by the neutralization with calcium carbonate (Xiong et al., **III**). The osmotic pressure caused by saturated calcium sulfate did not inhibit the *T. reesei* growth and enzyme secretion. This result is in agreement with results obtained by growing *T. reesei* in high concentration of mineral salts (NH_4NO_3, KH_2PO_4, $MgSO_4$, and KCl) (Haltrich et al., 1996).

Since the *T. reesei* cultivations with plant hydrolysates produced higher xylanase activities than the corresponding pure sugar models, it is possible that the oligosaccharides present in the hydrolysate are responsible for this effect. It could even be that the oligosaccharides themselves are more effective inducers of xylanase production in *T. reesei* than the monosaccharides (Xiong et al., **III,** Fig. 1).

4.4 THERMOSTABILITY OF *T. REESEI* XYLANASE II MUTANTS

Thermostability and broad pH range tolerance are desirable properties of xylanases when thinking the industrial usefulness of enzymes. There are several ways to improve these properties. In generally, many stabilizing amino acid substitutions and some of the factors determining the pH-dependent activity are known (Haki and Rakshit, 2003), and exploited also for xylanases (Turunen et al., 2004). Disulphide bridges have been used to stabilize many proteins, and if successful, they can have a remarkable stabilizing effect (Pace et al., 1990; van den Burg et al., 1998). Usually, finding of the stabilizing mutations for each particular enzyme is a process of trial-and-error. Disulphide bridges have been used to stabilize family 11 xylanases with a good success (Wakarchuk et al., 1994; Turunen et al., 2001; Fenel et al., 2004). In *T. reesei* XYN II, the bridges at position 2-28 (protein N-terminus) and 110-154 (α-helix) have increased the thermostability (Turunen et al., 2001; Fenel et al., 2004). Unlike the bridge at 110-154, bridge at 2-28 increased also the apparent temperature optimum. The *T. reesei* XYN II amino acid sequence (www.ebi.ac.uk/msd-srv/apps/Viewer/ViewerServlet?id=1xyp, for corresponding structure see 1 XYP in Protein Data Bank) is as follows:

```
  1 QTIQPGTGYN NGYFYSYWND GHGGVTYTNG PGGQFSVNWS NSGNFVGGKG

 51 WQPGTKNKVI NFSGSYNPNG NSYLSVYGWS RNPLIEYYIV ENFGTYNPST

101 GATKLGEVTS DGSVYDIYRT QRVNQPSIIG TATFYQYWSV RRNHRSSGSV

151 NTANHFNAWA QQGLTLGTMD YQIVAVEGYF SSGSASITVS
```

Different mutation combinations were engineered between two bridges at the N-terminus and two bridges at the α-helix. The bridges at the α-helix were 110-154 and 105-162 and they formed a crosslink between the α-helix and nearby β-strand. The bridges at the N-terminus were 2-28 and 7-16. The combinations were A) 2-28 and 110-154, B) 2-28 and 105-162, C) 7-16 and 110-154. The mutants contained also some other mutations (Xiong et al., **IV**, Table 1). To summarize, the result was that the combination of bridges 2-28 and 110-154 had an additive effect on the thermostability, whereas the combination of 7-16 and 110-154 had only a partly additive effect and the combination of 2-28 and 105-162 was not successful.

The combination of the disulphide bridges 2-28 and 110-154 created a superstable mutant (named DB1; Xiong et al., **IV**). The mutations in this mutant were: T2C, T28C, N11D, N38E, S110C, N154C, and Q162H (Xiong et al., **IV**). The presence of the disulphide bridges in this seven-fold DB1 mutant was verified for the purified enzyme by mass spectrometry. The DB1 mutant contained also an unintentional mutation Y27F, confirmed by sequencing and also mass spectroscopy of the protein (Jänis et al., 2004).

```
  1 QCIQPGTGYN DGYFYSYWND GHGGVTFCNG PGGQFSVEWS NSGNFVGGKG
    ↑_____________________________↑

 51 WQPGTKNKVI NFSGSYNPNG NSYLSVYGWS RNPLIEYYIV ENFGTYNPST

101 GATKLGEVTC DGSVYDIYRT QRVNQPSIIG TATFYQYWSV RRNHRSSGSV
        _______↑
        ↓
151 NTACHFNAWA QHGLTLGTMD YQIVAVEGYF SSGSASITVS
```

The DB1 mutant showed a very high thermostability and stability over a very wide pH range as shown in Fig. 5 and 6. Some enzymatic properties such as K_m, V_{max}, and pH_{opt} did not differ significantly between the mutant and the wild type xylanase, except that the apparent optimum temperature was increased from ~58 ^{o}C to ~71 ^{o}C (Xiong et al., **IV**, Table 3). The introduction of the bridge 110-154 to the bridge 2-28 mutant did not anymore increase the apparent optimum temperature from the level of the mutant with the 2-28 bridge. However, the thermostability increased 110-fold at 65 ^{o}C. Alltogether, the half-life of DB1 was at least 5000 times higher at 65 ^{o}C than that of the wild type enzyme. Since the kinetic values remained at the wild type level, it means that the extensive stabilization of the enzyme did not decrease the functional properties.

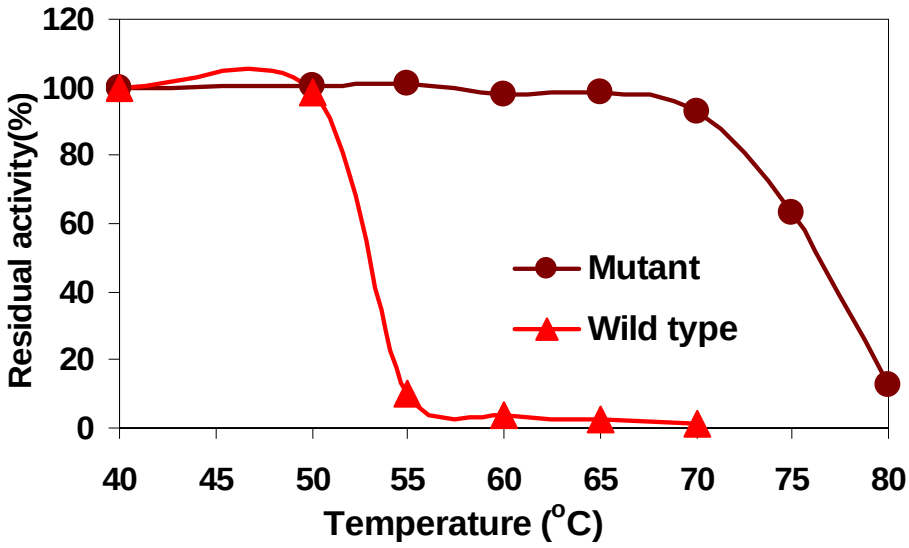

Fig. 5. Temperature-dependent inactivation of the thermostable DB1 mutant and the wild type *T. reesei* XYN II at pH 6. The enzymes were incubated for 10 min at each temperature and then the residual activity was measured.

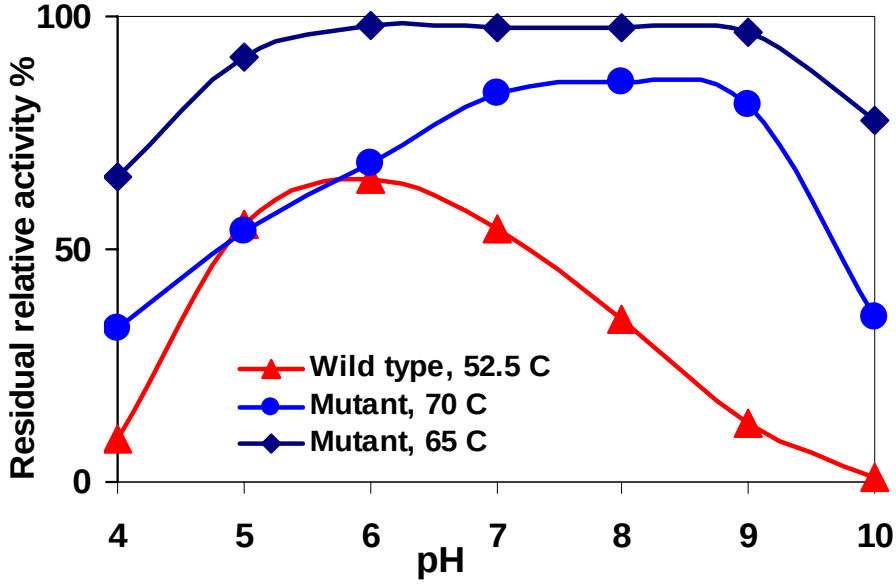

Fig. 6. pH-dependent inactivation of the thermostable DB1 mutant and the wild type *T. reesei* XYN II. After 30 min incubation at each pH, the residual activity was measured.

4.5 CHARACTERIZATION OF *T. LANUGINOSUS* XYLANASE

Since L-arabinose, lactose and oligosaccharides (Xiong et al., **II** and **III**) are typically good carbon sources and inducers of xylanase production in *T. reesei*, the cultivation of *T. lanuginosus* DSM 10635 was studied by using a similar medium as in the *T. reesei* cultivations. However, the results were fundamentally different. *T. lanuginosus* DSM 10635 cultivation with those carbohydrates produced a lower xylanase activity than with D-xylose (Xiong et al., **V,** Table 1). These cultivation results with DSM 10635 were similar to the results reported for *T. lanuginosus* DSM 5826 (Table 6). D-xylose was the best carbon source and inducer of xylanase production. L-arabinose and lactose were not efficient inducers of xylanase, and lactose even was not consumed by *T. lanuginosus* DSM 10635 (Xiong et al., **V**). These results showed that the molecular system controlling the xylanase induction is fundamentally different between *T. reesei* and *T. lanuginosus*.

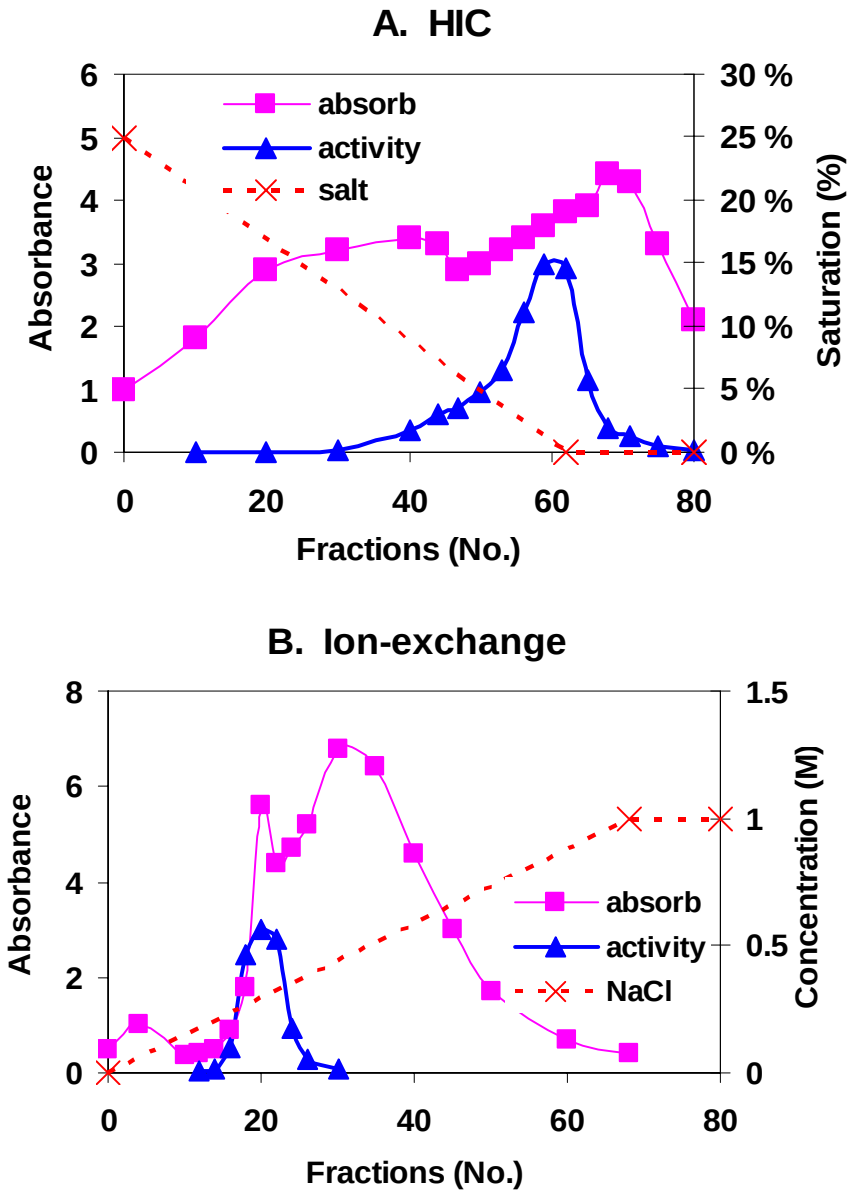

Fig. 7. *T. lanuginosus* xylanase purification by HIC and ion-exchange chromatography. The elution salt concentration is shown on the right; absorbance (at 280 nm) of the elution and xylanase activity (absorbance at 540 nm in DNS assay) are shown on the left.

T. lanuginosus DSM 10635 xylanase was purified to electrophoretic homogeneity and some of its enzymatic properties were characterized. The xylanase activity peak appeared at 2-10 % saturated ammonium sulphate in the HIC step and at approximately 0.3 M NaCl in ion-exchange chromatography step (Fig. 7A and 7B). A summary of *T. lanuginosus* xylanase purification steps is given in Table 12.

Table 12. The purification summary for *T. lanuginosus* DSM 10635 xylanase.

Steps	Protein (mg)	Activity (IU)	Specific activity (IU/mg)	Yield (%)
Broth	1350	15500	11.5	100
$(NH_4)_2SO_4$	294	12700	43.3	82
Phenyl Sepharose	26	8650	333	56
DEAE-Sepharose	3.3	5030	1520	32
Ultrafiltration	1.8	4340	2330	28

The molecular mass of the purified DSM 10635 xylanase was 21295.17 Da determined by mass spectrometry. This is very close to the theoretical molecular mass of 21294.96 Da deduced from the DSM 5826 xylanase sequence (www.ebi.ac.uk/msd-srv/msdlite/atlas/1yna_visualization.html), when the aminoterminal Gln is in a cyclic form as shown by the crystal structure (1YNA in Protein Data Bank; Schlacher et al., 1996; Gruber et al., 1998). In addition, the protein did not show molecular weight heterogeneity in the mass spectrometric analysis, indicating that there are no glycosylation variants of this protein. This is in agreement with the results obtained by lectin characterization of *T. lanuginosus* DSM 5826 xylanase (Cesar & Mrsa, 1996). Finally, the enzymatic properties are also similar between these two xylanases (Xiong et al., **V**).

T. lanuginosus DSM 5826 xylanase amino acid sequence (PDB code 1YNA; the protein structure is shown on the cover picture of this book):

```
  1 QTTPNSEGWH DGYYYSWWSD GGAQATYTNL EGGTYEISWG DGGNLVGGKG

 51 WNPGLNARAI HFEGVYQPNG NSYLAVYGWT RNPLVEYYIV ENFGTYDPSS

101 GATDLGTVEC DGSIYRLGKT TRVNAPSIDG TQTFDQYWSV RQDKRTSGTV
       _______↑
       ↓
151 QTGCHFDAWA RAGLNVNGDH YYQIVATEGY FSSGYARITV ADVG
```

When comparing the amino acid sequences of *T. lanuginosus* xylanase with *T. reesei* XYN-II, the similar structure was found for these two family 11 xylanases, specifically the α-helix (amino acids 153-162) and two β-sheets (Törrönen & Rouvinen, 1997; Singh et al, 2003). The disulfide bridge at position 110-154 is one of the reasons for the high thermostability of *T. lanuginosus* xylanase. Cleavage of the disulphide bridge resulted in a 25% loss of the β-sheet structure (Tatu et al., 1990). The same disulphide bridge in *T. reesei* xylanase II and *Bacillus circulans* xylanase increased considerably the thermostability (Wakarchuk et al., 1994; Turunen et al., 2001; Xiong et al., **IV**).

In conclusion, the properties of xylanases from different *T. lanuginosus* strains are highly similar, even though they have been isolated from different geographic locations (Xiong et al., **V**). The findings from mass spectrometry showed that DSM 10635 and DSM 5826 xylanases have identical or highly similar amino acid sequences. This conclusion is based also on the highly similar properties of xylanases of most of these strains (Singh et al., 2000a).

4.6 THERMOSTABILITY OF *T. LANUGINOSUS* XYLANASE

The thermostability of *T. lanuginosus* xylanase in the presence and absence of the substrate was studied in the article **V**. Typical to *T. lanuginosus* xylanase is that it loses its activity slowly during the elevation of temperature, when the experiment is done at neutral or slightly alkaline pH (Fig. 8). The same behavior has been reported for xylanases of other *T. lanuginosus* strains (Singh et al., 2000b). In line with this, the deactivation energy (86 kJ mol^{-1}; at pH 6.5) was smaller than the typical values (170-400 kJ mol^{-1}) of many enzymes (Pauline, 1995). However, at pH 5, the temperature-dependent inactivation curve is steep during the elevation of the temperature, and correspondingly, the deactivation energy is high (278 kJ mol^{-1}; Lischnig et al., 1993).

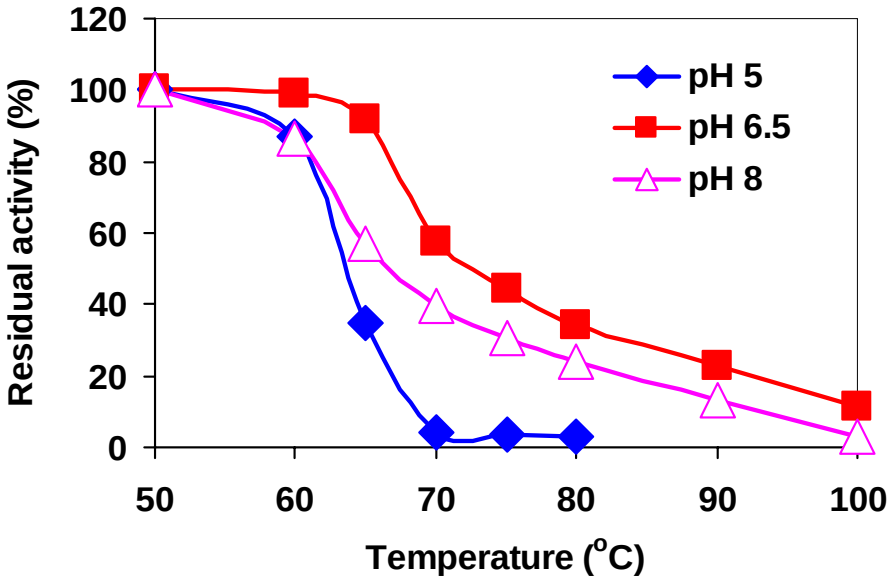

Fig. 8. Temperature-dependent inactivation profiles of *T. lanuginosus* xylanase. The enzyme was incubated for 30 min at each temperature and the residual activity was measured (Xiong et al., **V**).

During the incubation for 30 min at 70 °C, *T. lanuginosus* xylanase was inactivated very easily at pH 4-5 without presence of substrate, but still it was quite active at that pH (Xiong et al, **V**, Fig. 5). This apparent discrepancy called for further study into the stability of this enzyme in the presence of the substrate at low pH. It was found that the enzyme was much more stable in the presence of the substrate than when it was absent at acidic pH (pH 4.0-6.5), but not at neutral or alkaline pH (Table 13). The presence of the substrate increased the stability 7-fold at pH 4. The molecular mechanism for the protective effect of substrate on

xylanase at acidic pH is unclear. It is possible that the hydrogen bonds (the substrate forms in the active site) increase the stability of the enzyme at low pH. However, when the amount of OH^- ions is increased in the solution, the hydrogen bonds from the substrate are disrupted, and as a consequence, the substrate does not stabilize the enzyme in alkaline pH. Another possibility is that the substrate causes stabilizing structural changes in the enzyme in a pH-dependent manner. The effect of the substrate on the thermostability of xylanase has been shown earlier (Tenkanen et al., 1992; Tenkanen et al., 1995; Turunen et al., 2002), but this work is the first to find its pH-dependency. In studying the DB1 mutant of *T. reesei* XYN II it was observed that the protective effect of the substrate is low at higher temperatures (75 ^{o}C) (Xiong et al., **IV**).

Table 13. Half-life (min) of *T. lanuginosus* DSM 10635 xylanase in the absence or presence of 1 % birchwood xylan substrate (Xiong et al., **V**).

Incubation conditions	**Substrate absent**	**Substrate present**
65 ^{o}C, pH 4.0	~ 2	~ 14
65 ^{o}C, pH 5.0	~ 18	~ 71
70 ^{o}C, pH 5.0	~ 4	~ 13
70 ^{o}C, pH 6.5	~ 40	~ 60
70 ^{o}C, pH 9.0	~ 19	~ 12

5. CONCLUSIONS AND FUTURE PERSPECTIVES

This thesis presents results from studies of xylanase production by *T. reesei* and *T. lanuginosus,* as well as on stabilization of *T. reesei* xylanase II by disulphide bridges. However, some interesting topics could deserve further research:

- *T. reesei* produces higher xylanase activities and lower cellulase activities at pH 6; or produces lower xylanase activities and higher cellulase activities at pH 4 in the lactose-based medium. The fungus modifies the xylanase expression pattern to maximize the degradation of xylan at different pH conditions. The mechanism for the pH-dependent regulation of xylanase expression has still to be clarified.

- L-arabinose was one of the best inducers of *T. reesei* xylanases among the tested sugars. Under experimental conditions, the effect was even better than with lactose. The cultivation with a mixture of L-arabinose and lactose or D-glucose produced a higher xylanase activity than the sole lactose or D-glucose. Especially, L-arabinose relieves the repression of xylanase production by D-glucose. The molecular background for this regulation at promoter level requires further research.

- L-arabinose-rich plant hydrolysates are good carbon sources and inducers for xylanase production by *T. reesei*. It may be that oligosaccharides are more powerful inducers than monosaccharides. This possibility should be studied further, and there are questions such as which type of oligosaccharide is the best inducer, or whether the combination of different oligosaccharides gives better results than single ones.

- The thermostability of *T. reesei* xylanase II is significantly improved after introduction of two disulphide bridges into proper positions. This highly stable variant of xylanase is potentially very useful for industrial applications. However, the achieved stability level is still lower than the stability level of most stable family 11 xylanases. Engineering a mesophilic xylanase to be fully active at ~100 ^{o}C would be a great challenge.

- *T. lanuginosus* secretes a thermostable xylanase unaccompanied by cellulases. The produced cellulolytic/xylanolytic enzyme pattern is fundamentally different than that of *T. reesei*. An efficient inducer of xylanase for *T. lanuginosus* is D-xylose; the best inducers for *T. reesei,* such as L-arabinose or lactose, are not suitable for *T. lanuginosus.* The inactivation of *T. lanuginosus* xylanase proceeds slowly during the elevation of temperature at neutral or slightly alkaline pH. Substrate protection of this xylanase was influenced by pH. Industrial applications involving this xylanase, especially after modification of its properties by protein engineering, could bring interesting results, but this requires further research.

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