An investigation of the microbial hydrolysis of the lignin carbohydrate complex of grasses

By

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Declaration

I the undersigned hereby declare that the work contained in this thesis is my original work and has not previously in its entirety or in part been submitted at any university for a degree.

G. G. Stevens

Abstract

The microbial degradation of the lignin carbohydrate complex of plant material is only partially understood. Lignin carbohydrate complex was extracted from wheat straw and subsequently analysed. An adjustment to the standard protocol was required to increase the amount of lignin carbohydrate complex extracted from wheat straw. Characterization of the lignin carbohydrate complex after trifluoacetic acid hydrolysis was done by capillary electrophoresis. HPLC proved ineffective, as baseline separation could not be achieved. Characterization of the lignin carbohydrate complex revealed that a large portion (68 %) consisted of carbohydrate and lignin (20 %). Capillary electrophoresis of the trifluoroacetic acid hydrolysates of the lignin carbohydrate complex revealed that the carbohydrates consisted of mannose, xylose, arabinose, galactose, glucose and rhamnose. The major monosaccharide present in the lignin carbohydrate complex was mannose which made up 34 % of the total carbohydrate composition. Ferulic and *p*-coumaric acid were present in the lignin carbohydrate complex, but in concentrations less than 1 % of the lignin carbohydrate complex. The lignin carbohydrate complex of wheat straw probably had a heterogenous structure consisting of a variety of molecules, as molecular weight determination could not be accurately determined. An estimated molecular weight of 5.9 kDa was determined. Ten fungal strains (*Aspergillus niger, Aureobasidium pullulans*, *Bjerkandera adusta*, *Coriolus versicolor*, *Lenzitus betulina*, *Phanerochaete chrysosporium, Pycnoporus coccineus*, *Pycnoporus sanguineus 294, Pycnoporus sanguineus K5-2-3* and *Trichoderma reesei*) were evaluated for growth on the lignin carbohydrate complex. All strains except *B. adusta* showed growth after 5 days with *A. niger, A. pullulans, C. versicolor*, *P. chrysosoporium* and *T. reesei* showing the best growth on the lignin carbohydrate complex. The culture fluid revealed a number of proteins secreted by these organisms. The protein determination was confirmed by SDS-PAGE which revealed an array of proteins ranging from 8 kDa to 180 kDA. Prominent bands between 26 kDa and 80 kDa could be observed in the culture fluid of

A. niger, A. pullulans and *T. reesei*, but not in *C. versicolor*. Activity studies on the culture fluid of these four strains revealed activity for xylanase, xylosidase, arabinofuranosidase, ferulic acid esterase and laccase, with vast differences between the activities of the various fungi. The enzymes of these fungal strains were also evaluated for their ability to degrade xylan and sugar cane bagasse using capillary electrophoresis. It appeared that all the organisms produced enzymes to degrade birchwood xylan. However, the electropherograms revealed that the degradation patterns of birchwood xylan differed among these organisms over the same time interval, as xylotetraose, xylotriose, xylobiose and xylose were released in various concentrations. The electropherograms obtained from the enzyme hydrolysates of sugar cane bagasse, pointed to the substrate being inaccessible. Electropherograms of the culture fluid of *A. niger, A. pullulans, C. versicolor and T. reesei,* when incubated on the lignin carbohydrate complex indicated similar peaks to those obtained and identified in the trifluoroacetic acid hydrolysates. However, the electropherograms of the culture fluid of these organisms revealed additional smaller peaks which could not be identified. The electropherograms of the culture fluid of the various organisms also indicated a complete release of some sugars, using the trifluoacetic acid hydrolysate of the lignin carbohydrate complex as a control for the amount of sugars present. HPLC analyses revealed that after 72 h, no apparent degradation of the lignin carbohydrate complex took place as peak height and areas appeared to be similar. These peaks could however not be identified due to a lack of standards as well as baseline separation which could not be achieved.

Opsomming

Tans word die mikrobiese afbraak van die lignienkoolhidraatkompleks van plant materiaal slegs gedeeltelik verstaan. Lignienkoolhidraatkompleks was vanaf koringstrooi geïsoleer en gevolglik geanaliseer. Daar moes van die standaard prosedure vir die ekstraksie van lignienkoolhidraatkompleks afgewyk word ten einde beter lignienkoolhidraatkompleks opbrengs te lewer. Karakterisering van die lignienkoolhidraatkompleks na trifluoroasynsuurvertering was deur kapillêre elektroforese bepaal. Dit wou voorkom asof kapillêre elektroforese 'n beter opsie vir die analise van die verteerde monster van lignienkoolhidraatkompleks is, vergeleke met hoëdruk vloeistof chromatografie. Daar was gevind dat die lignienkoolhidraatkompleks uit 68 % koolhidraat en 20 % lignien bestaan. Kapillêre elektroforese het die teenwoordigheid van die volgende suikers bevestig naamlik, mannose, xilose, arabinose, glukose, galaktose en ramnose. Mannose was die dominerende suiker in die lignienkoolhidraatkompleks wat 34 % van die totale koolhidraat opbrengs uitgemaak het. Ferulien- en *p*-kumaarsuur kon ook identifiseer word, maar die twee sure het minder as 1 % van die totale inhoud van die lignienkoolhidraatkompleks uitgemaak. Vanuit resultate bekom wil dit voorkom dat die lignienkoolhidraatkompleks 'n heterogene molekuul is omdat die molekulêre gewig daarvan nie akkuraat bepaal kon word nie. 'n Geskatte molekulêre grootte van ongeveer 5.9 kDa was bepaal met verwysing na die hoogste piek wat in die chromatogram waargeneem was.

Tien fungus kulture was in die studie gebruik om hul vermoë te toets om op die lignien-koolhidraatkompleks te groei, naamlik *Aspergillus niger, Aureobasidium pullulans*, *Bjerkandera adusta*, *Coriolus versicolor*, *Lenzitus betulina*, *Phanerochaete chrysosporium, Pycnoporus coccineus*, *Pycnoporus sanguineus 294, Pycnoporus sanguineus K5-2-3* en *Trichoderma reesei*. *B. adusta* het nie groei na 5 dae getoon nie, en dit wou voorkom asof *A. niger, A. pullulans*, *C. versicolor*, *P. chrysosoporium* en *T. reesei* die beste kon groei op die substraat na 5 dae. Die kultuurvloeistof van die vier kulture het getoon dat proteïene deur

hierdie organisms uitgeskei was. Hierdie proteinbepaling was ook bevestig deur SDS-PAGE, wat bande tussen 8 kDa en 180 kDa gelewer het. Prominente bande tussen 26 kDa en 80 kDa kon waargeneem word in die kultuurvloeistof van *A. niger, A. pullulans*, en *T. reesei,* maar nie in die kultuurvloeistof van *C. versicolor* nie. Aktiwiteitstudies op die kultuur vloeistowwe het getoon dat daar aktiwiteit was vir die volgende ensieme, naamlik xilanase, xilosidase, arabinofuranosidase en feruliensuur esterase. Hierdie aktiwiteit het aansienlik verskil tussen die verskillende organismes.

Die ensieme van die vier organismes was ook gebruik om hul vermoë te toets om xilaan en suikerriet af te breek. Daar was gevind dat xilaanafbraak verskillend was vir die organisms oor dieselfde tydperk. Xilotetraose, xilotriose, xilobiose en xilose was in verskillende konsentrasies gevind vir die verskillende organismes. Die elektroferogramme van die kultuurvloeistof op suikerriet van die verskillende organismes het getoon dat die substraat nie toeganklik vir die ensieme was nie. Die elektroferogramme van die kultuurvloeistof op lignienkoolhidraatkompleks van die verskillende organismes het dieselfde pieke getoon soos geïdentifiseer in die elektroferogramme van die trifluoroasynsuur vertering. Die elektroferogramme met die ensiem vertering het egter addisionele pieke getoon wat nie sigbaar op die elektroferogramme van die trifluoroasynsuur vertering was nie. Hierdie pieke het min of meer dieselfde tyd ge-elueer as die monosakkariede. Kapillêre elektroforese het ook getoon dat die ensiematiese afbraak van die lignienkoolhidraatkompleks gelei het tot algehele vrystelling van sommige suikers, wanneer die trifluoroasynsuur vertering as maatstaaf dien vir die hoeveelheid suikers teenwoordig in die lignienkoolhidraatkompleks. Hoëdruk vloeistof chromatografie het getoon dat geen sigbare afbraak na 72 h van inkubasie met die ensieme op die lignienkoolhidraatkompleks plaasgevind het nie aangesien die piek hoogtes konstant gebly het. Hierdie pieke kon egter nie geïdentifiseer word nie as gevolg van lae resolusie van die pieke asook standaarde wat nie beskikbaar was nie.

This thesis is dedicated to my late father Mr. P. Stevens,

my mother Mrs. T. Stevens,

my wife, Valda

and children Keaton and Elcon.

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My sincere appreciation to the following:

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Introduction

Lignin carbohydrate complexes which are found in softwood, hardwood and grasses are highly complexed molecules, contributing to the rigidity of plant cell walls. The complexes consist of lignin which is attached to the carbohydrate moieties via various linkages (Jeffries 1994). The composition and quantities of lignin and carbohydrates vary between plant species as well as within the plant species itself. Due to this compositional variation, it follows naturally that the cross links between these polymers would also differ. Lignin is a complex phenolic polymer composed of phenylpropane monomeric units like *p*-coumaryl alcohol (*p*-hydroxyphenyl propanol), coniferyl alcohol (guaiacyl propanol), and sinapyl alcohol (syringyl propanol) which are interconnected by a variety of carbon-carbon bonds as well as ester and ether linkages (Jeffries 1994).

In softwood the carbohydrate moiety of lignin carbohydrate complexes consist of galactomannan, arabino-4-*O*-methylglucuronoxylan, and arabinogalactan linked to lignin at benzyl positions (Mukoyoshi *et al.* 1981). It was initially hypothesized that carbohydrate portions in the lignin carbohydrate complexes of hardwood and grasses are composed exclusively of 4-*O*-methylglucuronoxylan and arabino-4-*O*-methylglucuronoxylan, respectively (Azuma and Koshijima 1988). However, more recent results show that the lignin carbohydrate complex in grasses could also consist of arabinose, mannose, galactose, glucose and rhamnose. These sugars might be present as polymers or side chains in the lignin carbohydrate complexes of grasses (Luonteri *et al.* 1999, Tenkanen *et al.* 1997). Two major substances which appear in the lignin carbohydrate complexes of grasses are those of *p*-coumaric acid and ferulic acid. These substances could be esterified to lignin, or could also be ether-linked to lignin, depending on the type of grass (Azuma and Koshijima 1988). Degradation of lignin carbohydrate complexes by microorganisms appear to be not fully understood due to the complexity of the structure. It appears that microorganisms have developed effective ways of penetrating these

structures, and utilize them as energy source. However it was suggested that degradation cannot take place unless these lignin carbohydrate complexes are pretreated to some extent. This pretreatment can be achieved by the action of low molecular weight components and oxidants (Lequart *et al.* 2000). A number of enzymes of microbial origin involved in the degradation of lignin carbohydrate complexes have been identified and characterized such as esterases and polysaccharases (Jeffries 1990). These include ferulic acid esterase and *p*-coumaric acid esterase which attack hemicellulose side chains, and xylanases, mannanases and galactanases which react on the polymeric carbohydrate chains. Other accessory hemicellulases include α-L-arabinofuranosidase and α-methyl-glucuronidase (Jeffries 1990). Lignin degrading enzymes include lignin peroxidases, manganese peroxidases and laccases (Cullen 1997).

Investigations of these various enzymes have yielded information regarding the degradation of the lignin carbohydrate complexes which would be useful in applications such as biobleaching of pulp and paper and as a supplement to ruminant feeds. Hydroxycinnamyl esterases which cleave ester linkages between aromatic acids and lignin as well as between lignin and hemicelluloses can contribute to lignin solubilization in the pulp and paper industry in combination with other enzymes (Magnuson and Crawford 1992).

It is well known that plant cell walls provide an essential source of energy to ruminants. However a large portion of this energy source is excreted by the animals, due to the lignin which is poorly degraded by microbial enzymes present in mammals (van Soest 1994), as well as the complexity of the substrate (Jung *et al.* 1996). Thus an understanding of the limiting factors responsible for the degradation of lignin carbohydrate complexes would be useful to enhance the process.

Analyses of the degradation products formed after chemical and enzymatic digestion of these complexes is important in understanding the degradation process. Analytical techniques like

High Performance Liquid Chromatography (HPLC), Capillary Electrophoresis (CE) and Mass spectrometry are the most important tools available to conduct these analyses.

The objective of this study is:

- to isolate a lignin carbohydrate complex from wheat straw
- to characterize the chemical composition of the lignin carbohydrate complex of wheat straw
- to evaluate the ability of various fungi to grow on the lignin carbohydrate complex
- to evaluate the enzyme profiles
- to analyse the degradation process of the lignin carbohydrate complex by microbial enzymes using modern analytical techniques.

2. Literature review

2.1 Lignin carbohydrate complexes

2.1.1 Structure of lignin carbohydrate complexes

2.1.1.1 General structure

Lignin carbohydrate complexes appear in the cell walls of hardwoods (gymnosperms), softwoods (angiosperms) as well as in the cell walls of grasses (*Graminae*) (Azuma *and* Koshijima 1988). These complexes comprise of various macromolecules which are cross-linked to each other and are generally regarded to be highly intricate molecules. Due to the complexity of these structures and especially the way the different molecules are either ester- or ether-linked to each other, it is extremely difficult to hydrolyze these structures, chemically as well as enzymatically (Magnuson and Crawford 1992, Christov and Prior 1993). Despite the various modern chemical techniques available, the chemical structure of lignin carbohydrate complexes of a number of plants is only partially known. One of the major obstacles to elucidation of the chemical structure is the isolation of such material in a pure state (Atsushi *et al.* 1984). Lignin, which makes up a significant portion of the complex, can be described as a three-dimensional macromolecule with a molecular weight in the range of 100 kDa (dos Santos Abreu *et al.* 1998). It is composed of three principal building blocks: *p*-coumaryl alcohol (*p*-hydroxyphenyl propanol), coniferyl alcohol (guaiacyl propanol), and sinapyl alcohol (syringyl propanol) (Fig. 2.1). In softwoods, coniferyl alcohol is the principal constituent, while the lignin of hardwoods is composed of guaiacyl and syringyl units. Grass lignins contain guaiacyl-, syringyl-, and *p*-hydroxyphenyl-units. These phenylpropane monomeric units are interconnected by a variety of

carbon-carbon bonds as well as ether- and ester linkages. However lignin inter-monomer linkages are similar in softwood, hardwood, and grass lignins (Jeffries 1994).

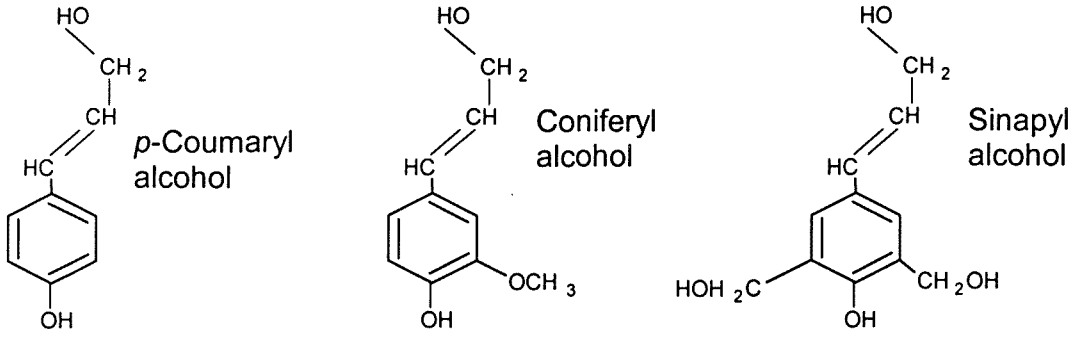


Fig. 2.1 Building blocks of lignin (Jeffries 1994).

The carbohydrates associated with the lignin carbohydrate complex varies from plant to plant and due to the nature of the cross-linkages between carbohydrates and lignin, the structures are difficult to hydrolyse. Recent developments in chromatography and nuclear magnetic resonance spectroscopy have shed new light on these complexes regarding their structures, different bonds, carbohydrate- and lignin content (Table 2.1) (Colquhoun *et al.* 1994).

2.1.1.2 Structure of lignin carbohydrate complexes in hardwood

In hardwood the carbohydrate portions of the lignin carbohydrate complexes are composed exclusively of 4-*O*-methylglucuronoxylan and is esterified to lignin (Fig. 2.2). However, many glucuronic acid groups may also be esterified within the xylan polymer. It was also found that mannose, galactose and glucose are *O*-6 ether-linked and xylose is *O*-2 or *O*-3 linked to the benzyl hydroxyl of the lignin carbohydrate complex (Jeffries 1990).

Table 2.1 Comparison of structures linking lignin to carbohydrate found in different grasses.

Type of grass	Structure linkage	Reference
Spinacia oleracea (Spinach)	4-*O*-(6-*O*-feruloyl-β-D-galactopyranosyl)-D-galactose and 3-*O*-(3-*O*-feruloyl-α-L-arabinofuranosyl)-L-arabinose	Fry 1982
Saccharum officinarum (Sugar cane bagasse)	*O*-[5-*O*-trans-feruloyl)-α-L-arabinofuranosyl]-(1-3)-*O*-β-D-xylopyranosyl-(1-4)-D-xylopyranose	Kato *et al.* 1983
Zea mays (Corn stalk)	*O*-[5-*O*-(trans-feruloyl)-α-arabinofuranosyl]-(1-3)-*O*-β-D-xylopyranosyl-(1-4)-D-xylopyranose	Kato and Nevins 1985
Hordeum vulgare (Barley straw)	*O*-[5-*O*-(trans-*p*-coumaroyl)-α-L-arabinofuranosyl]-(1-3)-*O*-β-D-xylopyranosyl-(1-4)-D-xylopyranose (PAXX) *O*-[5-*O*-(trans-feruloyl)-α-L-arabinofuranosyl]-(1-3)-*O*-β-D-xylopyranosyl-(1-4)-D-xylopyranose (FAXX)	Harvey *et al.* 1986
Triticum aestivum (Wheat straw)	2-*O*- [5-*O*-(trans-feruloyl)-β-L-arabinofuranosyl]-D-xylopyranose	Smith and Hartley 1983
Cynodon dactylon (Bermuda grass)	*O*-[5-*O*-(trans-*p*-coumaroyl)-α-L-arabinofuranosyl]-(1-3)-*O*-β-D-xylopyranosyl-(1-4)-D-xylopyranose (PAXX)	Borneman *et al.* 1990a
Beta vulgaris (Sugar beet)	*O*-[2-*O*-trans-feruloyl)-α-L-arabinofuranosyl]-(1-5)-L-arabinofuranose *O*-α-L-arabinofuranosyl]-(1-3)-*O*-[2-*O*-(trans-feruloyl)-α-L-arabinofuranosyl-(1-5)-L-arabinofuranose *O*-[6-*O*-(trans-feruloyl-β-D-galactopyranosyl]-(1-4-D-galactopyranose	Ralet *et al.* 1994
Phyllostachys pubescens (Bamboo shoot)	*O*-(4-*O*-trans-feruloyl)-α-D-xylopyranosyl-(1-6)-D-glucopyranose *O*-[5-*O*-(trans-p-coumaroyl)-α-L-arabinofuranosyl]-(1-3)-*O*-β-D-xylopyranosyl-(1-4)-D-xylopyranose (PAXX)	Ishii and Hiroi 1990

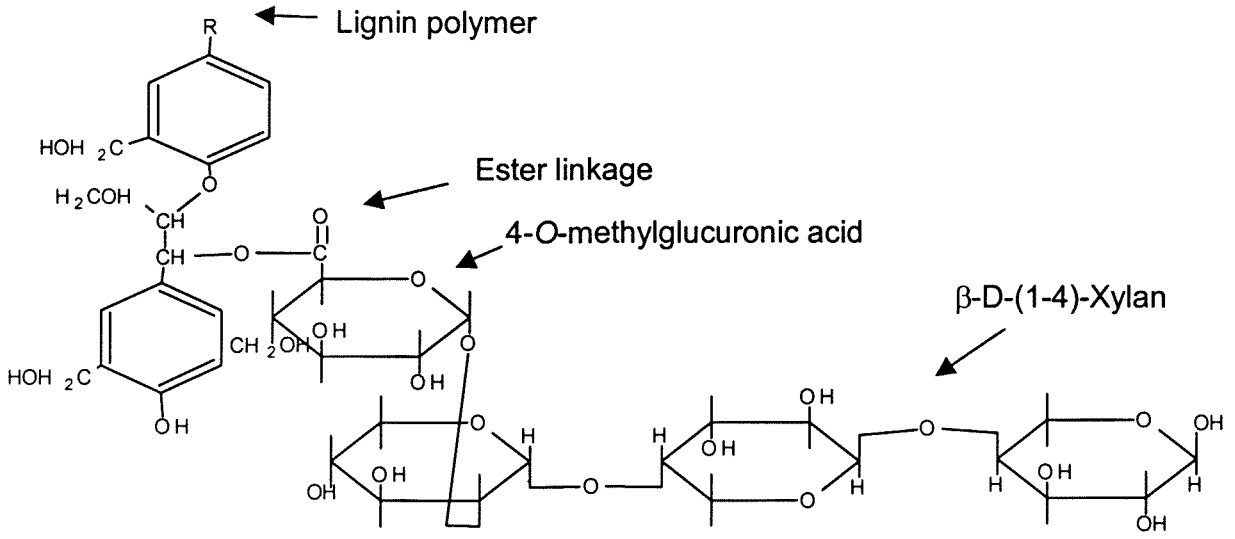


Fig. 2.2 Structure of lignin carbohydrate complexes in hardwood (Jeffries 1990).

2.1.1.3 Structure of lignin carbohydrate complexes in softwood

In softwood the lignin carbohydrate complexes are distinct in that their carbohydrate portions consist of either galactomannan, arabino-4-*O*-methylglucuronoxylan (Fig. 2.3), or arabino-galactan linked to lignin at benzyl positions, with either ether-or ester bonds. (Mukoyoshi *et al.* 1981).

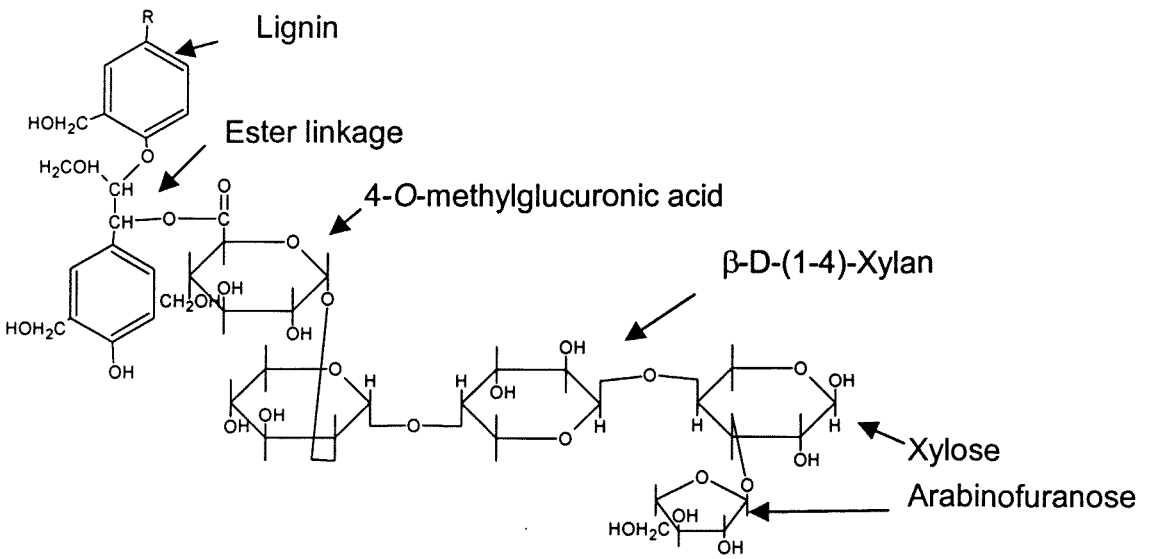


Fig. 2.3 Arabino-4-*O*-methylglucuronoxylan in softwood (adapted from Jeffries 1990).

2.1.1.4 Structure of lignin carbohydrate complexes in grasses

In grasses the carbohydrate portion of the lignin carbohydrate complex mainly consists of arabino-4-*O*-methylglucuronoxylan (Fig. 2.4) (Jeffries 1990). Compared to other plants the lignin carbohydrate complexes of grasses also contain an additional functional group such as phenolic hydroxyls of esterified phenolic acids which is linked to lignin.

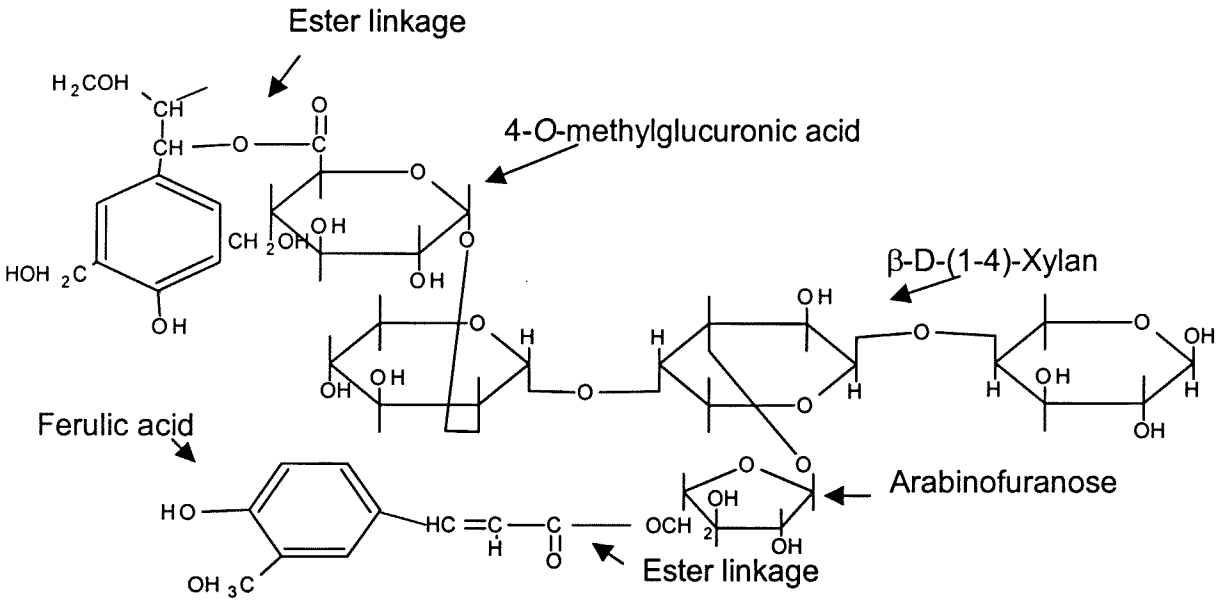


Fig. 2.4 Ferulic acid ester linkage to arabino-4-*O*-methylglucuronoxylan in grasses (adapted from Jeffries 1990).

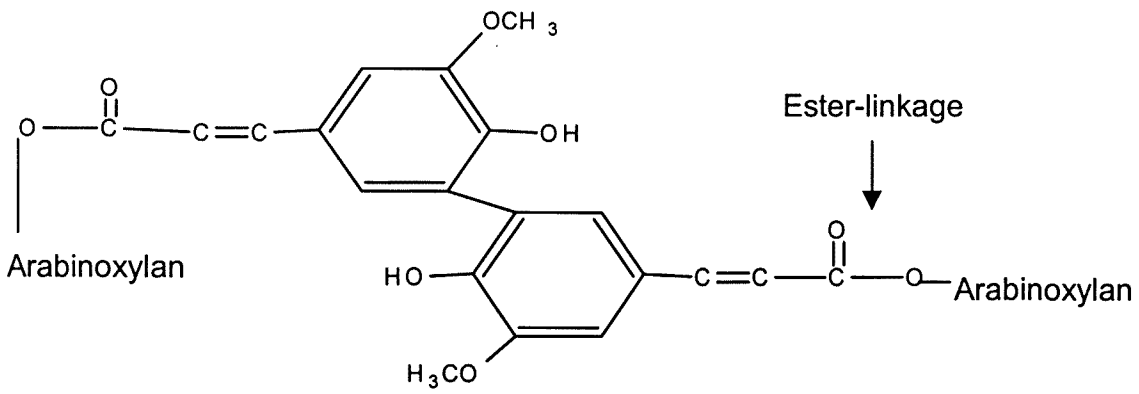


Fig. 2.5 Diferulic acid in grasses (Markwalder and Neukom 1976).

Ferulic- (4-hydroxy-3-methocinnamic) and *p*-coumaric (4-hydroxycinnamic) acids (Fig. 2.4 Table 2.1), are covalently bound to lignin in the cell walls of plants from several families, including those of the family *Gramineae*. From these studies it was concluded that grass arabinoxylans are esterified with ferulic acid (Harris and Hartley 1980, Hartley and Harris 1981). There are various ways in which the ferulic acid and *p*-coumaric acid could be linked to the sugar moiety in grasses (Table 2.1). The ferulic acid could be ester-linked at *O*-2 or *O*-5 of arabinofuranose or at *O*-6 of galactopyranose residues in the pectic side chains. They are also able to form dimers (Fig. 2.5) and cross-link cell wall polysaccharides and lignin (Colquhoun *et al.* 1994).

In some grasses, the feruloyl groups of the feruloylated arabinose oligosaccharides, are linked to *O*-2 of L-arabinofuranose-residues. Some of the structures which could be identified include feruloylated arabinose disaccharides which can be described as *O*-[2-*O*-(trans-feruloyl)-α-L-arabinofuranosyl]-(1 - 5)-L-arabinofuranose. The structure of the feruloylated trisaccharides can be described as *O*-α-L-arabinofuranosyl-(1-3)-*O*-[2-*O*-(trans-feruloyl)-α-L-arabinofuranosyl]-(1-5)-L-arabinofuranose (Table 2.1). In the case of feruloylated galactose disaccharides the structure can be described as *O*-[6-*O*-(trans-feruloyl)-β-D-galactopyranosyl]-(1-4)-D-galactopyranose (Colquhoun *et al.* 1994).

The above mentioned oligosaccharides (Table 2.1) could also be found in other members of the *Gramineae* family such as wheat (Smith and Hartley 1983), sugar cane bagasse (Kato *et al.* 1983), maize shoots (Kato and Nevins 1985), barley aleurone layers (Gubler *et al.* 1985), barley straw (Harvey *et al.* 1986) and bamboo shoots (Ishii and Hiroi 1990) which differ with regard to their linkage to the lignin structure and whether it occurs as a di- or a trisaccharide in the lignin carbohydrate complex (Fig. 2.6). It appears that the polysaccharides in the lignin carbohydrate complexes of grasses can consist of polymers of xylose, mannose, arabinose,

galactose, rhamnose and glucose. In grasses, phenolic acids like *p*-coumaric- and ferulic acid are relatively abundant and can be esterified or etherified to the carbohydrate polymers or lignin, and also link the carbohydrate polymers to lignin (Azuma and Koshijima 1988). It is hypothesized that the lignin carbohydrate complexes included in the cell wall structure are key elements combating degradation. Esterified phenolics in these complexes contribute to cell wall extensibility and growth, and decrease digestibility by ruminants. (Kroon *et al.* 1997).

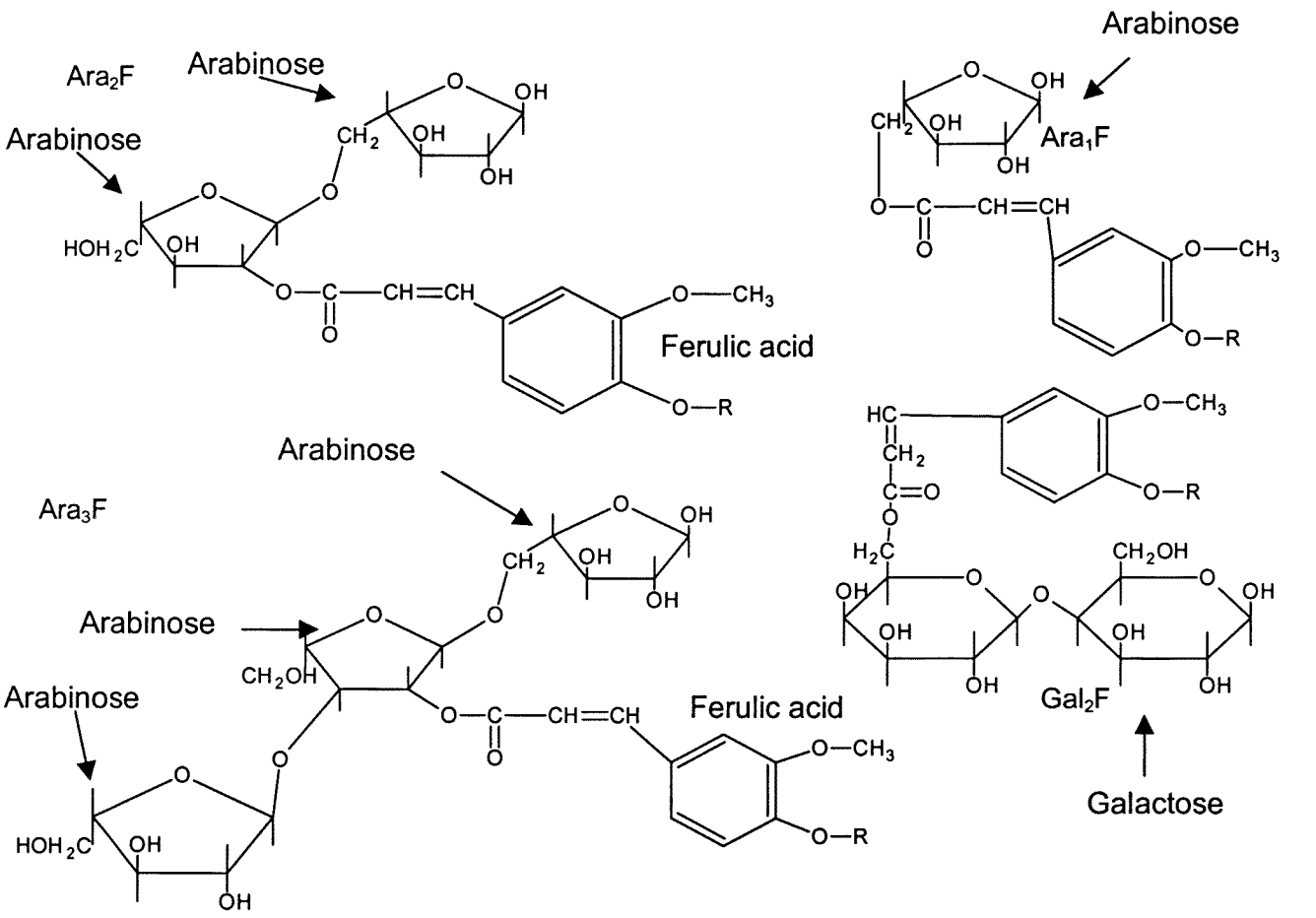
Based on what is known of current appendages and bonds in lignin carbohydrate complexes, a generalized comprehensive model with all the various linkages between lignin and carbohydrates was proposed (Fig. 2.7) (Bach *et al.* 1994).

2.1.2 Distribution of lignin carbohydrate complexes in various plants

Lignin forms complexes with carbohydrates (Figs. 2.1 - 2.7) and appear in the cell walls of vascular plants which include gymnosperms, angiosperms and *graminaceous* plants (Azuma and Koshijima 1988). As these plants have evolved separately, they also contain different lignin and hemicellulose constituents. Lignin and its association with carbohydrate complexes depends on plant variety and plant tissue (Cornu *et al.* 1994). Moreover, composition varies within a single plant (roots, stems, leaves), with age (heartwood versus sapwood), stage of growth (early wood versus late in annual rings) and with the conditions under which the plant grows (Jeffries 1994).

Futhermore, these complexes are usually associated with primary rather than with secondary cell walls (Engels and Schumans 1992) and maturing forages containing lignified cell walls which are more resistant to degradation compared to their younger counterparts. Table 2.1 gives a representative example of the different structures found in grasses and the way they are linked to their distinctive polymer backbone. With regard to the *Graminae* family, the carbohydrate composition, as well as the different bonds with which these carbohydrates are

linked to the lignin carbohydrate complex differ from species to species. Lignin is linked to other cell wall polymers during the process of primary cell wall formation and continues during cell wall aging (Yamamoto *et al.* 1989; Terashima 1993).



R = Lignin
Ara_1F = Arabinose attached to ferulic acid
Ara_2F = Two arabinose units attached to ferulic acid
Ara_3F = Three arabinose units attached to ferulic acid
Gal_2F = Two galactose units attached to ferulic acid

Fig. 2.6 Some of the structures of feruloylated arabinose mono-, di-, and trisaccharides (Ara_1F, Ara_2F, Ara_3F) and feruloylated galactose disaccharide (Gal_2F) in grasses (Colquhoun *et al.* 1994).

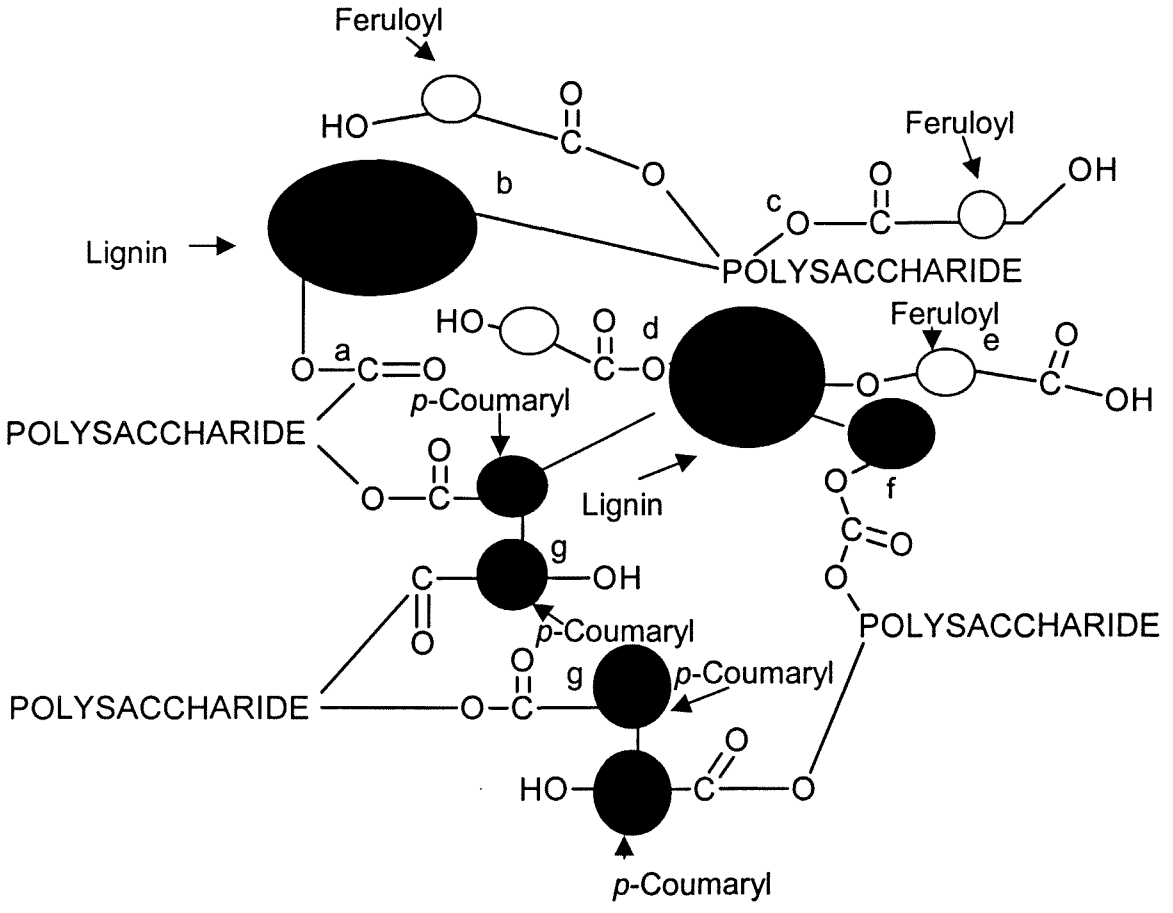


Fig. 2.7 Possible covalent crosslinks between polysaccharides and lignin in cell-walls. a = direct ester linkage; b = direct ether linkage; c = hydroxycinnamic acid esterified to polysaccharides; d = hydroxycinnamic acid esterified to lignin; e = hydroxycinnamic acid ester; f = ferulic acid ester-ether bridge; g = dehydroferulic acid diester-ether bridge (Bach *et al.* 1994).

2.1.3 Microbial degradation of lignin carbohydrate complexes

2.1.3.1 Enzymes degrading lignin carbohydrate complexes

A number of microbial hemicellulose polysaccharases and esterases including acetyl xylan esterase, ferulic acid esterase, and *p*-coumaric esterase attack hemicellulose side chains and appear to be critical in the early steps of hemicellulose utilization (Donelly and Crawford 1988). Enzymes removing the side chains of carbohydrate polymers like α-L-

arabinofuranosidase and α-methyl-glucuronidase can be regarded as accessory enzymes which many times act in synergism with other enzymes to destroy lignin carbohydrate complexes (Christov and Prior 1993, 1996).

2.1.3.2 Properties of the enzymes

A few of these enzymes as well as their function in the degradation process are briefly described. The enzymes which act on the carbohydrate portion of the lignin carbohydrate complex are classified in two groups, namely hydrolases and esterases. The hydrolases which are responsible for the hydrolysis of glycosidic bonds from polymeric substrates like xylan, mannan, galactan and arabinan, include the following enzymes, β-xylanases, β-mannanases, β-galactanases, arabinases, β-xylosidases, β-mannanases, α-L-arabinofuranosidases and α-glucuronidases. The endo 1,4-β-xylanases are responsible for the random hydrolysis of 1,4-β-xylopyranosyl linkages of the D-xylan backbone to yield shorter xylo-oligosaccharides (Fig. 2.8). These xylo-oligosaccharides are further cleaved by the action of β-1,4-xylosidases to xylose units. However these enzymes are more specific in their action and will only cleave a xylose unit from the non-reducing end of the xylo-oligosaccharides (Fig. 2.8). α-D-Glucuronidases which are also specific in their action, hydrolyse the bond between glucuronic acid and xylo-oligosaccharides and act in synergism with xylanases and xylosidases to hydrolyse glucuronoxylan (Fig. 2.8) (Christov and Prior 1993, 1996, Jeffries 1990). Arabinase act on the arabinose backbone and will cleave arabino-oligosaccharides to smaller arabino-oligosaccharides. α-L-Arabinofuranosidases are responsible for the hydrolysis of 1,3-glycosidic linkages of α-L- arabinofuranosyl units attached to the xylan or arabinan backbone (Christov and Prior 1993, 1996, Flipphi *et al.* 1994, Jeffries 1990).

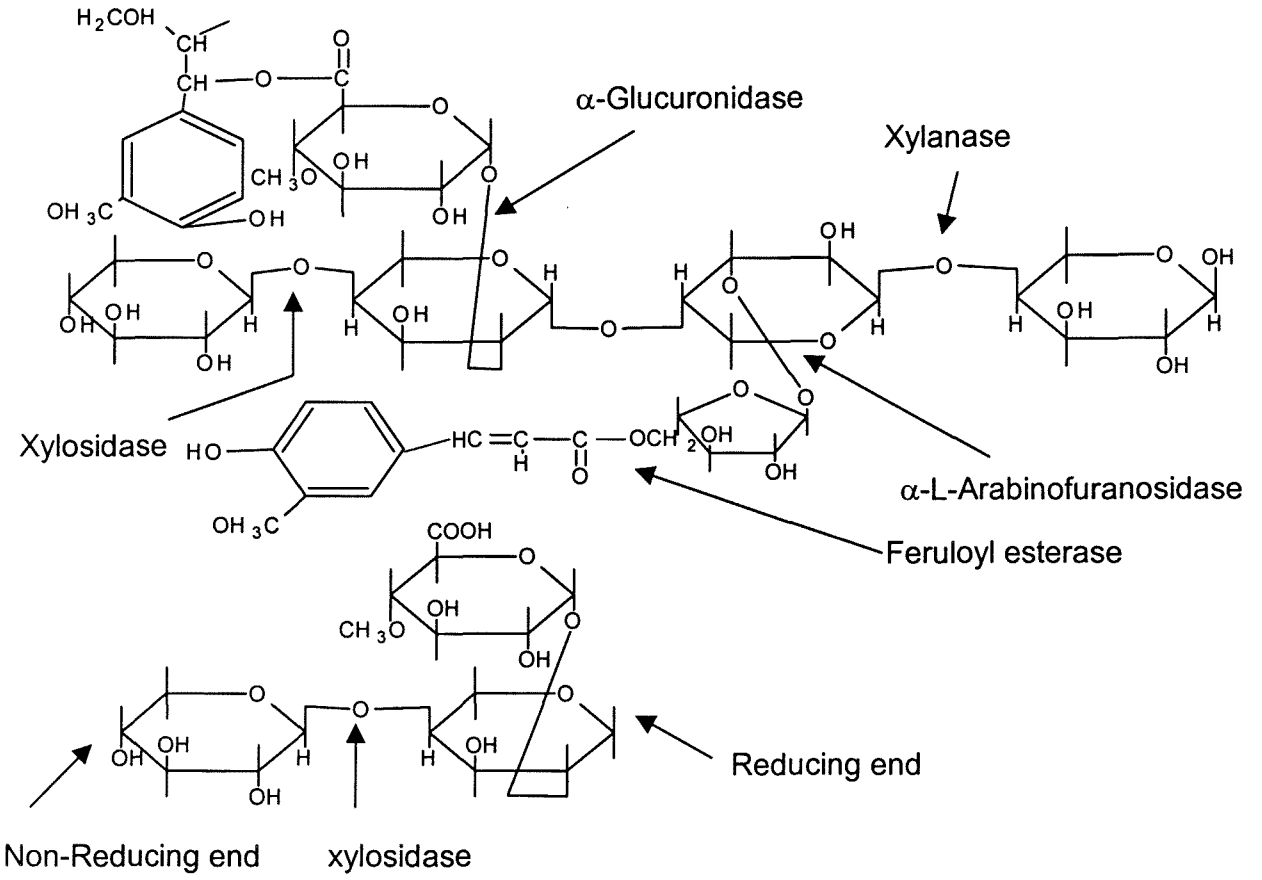


Fig. 2.8 Enzymatic sites on the xylan structure (adapted from Biely *et al.* 1997).

Endo-1,4-β-mannanases randomly cleaves β-D-1,4-mannopyranosyl linkages within polymeric chains of galactomannan, glucomannan and mannan. Complete degradation of these polymers requires the action of two additional enzymes, namely α-galactosidase and β-mannosidase. These enzymes break down the polymers to D-galactose and D-mannose, respectively (Ademark *et al.* 1998).

The second group of enzymes namely esterases are produced by some fungi and these enzymes are capable of releasing aromatic acids from hemicellulose. Ferulic- and *p*-coumaric acid esterases are responsible for the cleavage of ferulic- and *p*-coumaric acid from

hemicellulose respectively. In general it appears that ferulic acid esterases are active on plant cell wall feruloylated polysaccharides only in the presence of carbohydrases, although there are exceptions to this phenomena (Faulds and Williamson 1991). Carbohydrases which act on the oligosaccharide chain produce low molecular weight substrates which is an indication that the length of the oligosaccharide chain to which ferulic acid is attached plays an important role in the ferulic acid esterase activity (Faulds and Williamson 1991, Ferreira *et al.* 1993).

From recent results it appears that feruloyl esterases can be divided into two classes. The first class of feruloyl esterase cleaves ferulic acid from arabinose which can be attached to arabinose on the C-2 or C-5 position of arabinofuranose. Organisms like *Aureobasidium pullulans, Aspergillus niger, Penicillium funiculosum* and *Aspergillus oryzae* belong to a second class, all of which secrete feruloyl esterases that release ferulic acid from arabinose at the C-2 rather than the C-5 position (Rumbold *et al.* 2003a). In the study of feruloyl esterase from *Fusarium oxysporum* it appeared that this enzyme hydrolysed the C-2 substrate hundred fold more than the C-5 substrate (Topakas *et al.* 2003).

Little is understood about the lignin degrading enzymes, but four classes of enzymes have been identified which are secreted by *basidiomycetous* fungi namely, lignin peroxidases, manganese peroxidases, laccases and the hydrogen peroxide generating enzyme, glyoxal oxidase (Cullen 1997). Lignin peroxidase catalyzes a variety of oxidations which are all dependent on hydrogen peroxide. These include Cα-Cβ cleavage of the propyl side chains of lignin, hydroxylation of benzylic methylene groups, oxidation of benzyl alcohols to the corresponding aldehydes or ketones, phenol oxidation and aromatic ring cleavage of non-phenolic lignin model compouds. These enzymes are also very distinct, as they have a low pH optima (pH < 3) and high redox potential (Cullen 1997). Mangenese peroxidase, oxidize Mn^{2+} to Mn^{3+} and also uses hydrogen peroxide as oxidant. The activity of these enzymes are stimulated by organic acids which stabilizes the Mn^{3+}, which then produces diffusible oxidizing

chelates. Manganese peroxidase can oxidize lignin in an indirect mode without direct contact with lignin, as this enzyme is able to perform a one-electron oxidation of lignin. This is a very efficient way of degrading lignin, as lignin contributes to compactness of the cell wall and thus hardly accessible to enzymes. (Hilden *et al*. 2000).

Laccases are copper-containing enzymes which reduce molecular oxygen to water and also oxidize electron-rich substrates like phenolic compounds. Apparently non-phenolic compounds may also be oxidized but only in the presence of certain mediators. One of these mediators has been identified as 1-hydroxybenzotriazole (HOBT). Laccase/HOBT attack phenolic structures preferentially when it depolymerizes lignin in kraft pulp. It also degrades phenolic model compounds such as biphenyls, stilbenes and dephenylmethanes (Srebotnik and Hammel 2000). Glyoxal oxidase is secreted by some lignin degrading fungi and reacts on substrate with aldehyde-, α-hydroxycarbonyl-, and α-dicarbonyl compounds. An example of one of these compounds is glucoaldehyde, which is produced by the action of lignin peroxidases on lignin. A distinct characteristic of this enzyme is that it is reversibly inactivated, should the peroxidase/HOBT system be absent (Cullen 1997).

2.1.3.3 Production of the enzymes

Several species of fungi are known to produce enzymes that are capable of degrading lignocellulose. Most of these fungi produce hemicellulases and are only capable of removing side chains from lignin like carbohydrates and phenolic compounds, whereas others are capable of even destroying lignin (Cullen 1997).

Ferulic and *p*-coumaric acid esterases which appears to be restricted to certain fungi have been identified in the extracellular broths of *Streptomyces viridosporus* and *Streptomyces flavogriseus*, when grown on a native substrate like xylan (MacKenzie *et al.* 1987). In addition to

these esterases, these organisms also produce α-L-arabinofuranosidase and α-methylglucuronidase. Eight esterases were also reported from *S. viridosporus* to release *p*-coumaric and vanillic acid into the medium when concentrated. Usually *p*-coumaric acid is known to be esterified essentially only to the lignin and for this reason these organisms attack these substrates (Donelly and Crawford 1988).

Three ferulic acid esterases have been isolated from a commercial source of pectinase which have been produced by *A. niger*. They were designated FAE1, FAE11 and FAE111. FAE-1 with a molecular mass of 150 kDa, was specific for ferulic-, *p*-coumaric- and caffeic acids, whereas FAE-11 with a molecular mass of 29 kDa, had a preference for a methoxyl group at C3 of the phenolic ring and was specific for ferulic- and sinaptic acids. FAE111 showed similar substrate specificities to FAE11, but had much higher specific activities (Faulds and Williamson 1994).

Phenolic acid releasing enzymes have been reported in *Streptomyces olivochromogenes* (Faulds and Williamson 1991), *Penicillium pinophyllum* (Castanares *et al.* 1992), *Neocallimastix spp.* (Borneman *et al.* 1990b), *Schizophyllum commune* (Mackenzie *et al.* 1987), *Aspergillus spp.* (Tenkanen *et al.* 1991, Faulds and Williamson 1994), *Bacillus* and *Lactobacillus* spp. (Donaghy *et al.* 1998) and *Pseudomonas fluorescens* (Ferreira *et al.* 1993). From studies performed on a synthetic substrate such as methyl ferulate and on other feruloylated oligosaccharides from sugar-beet pulp and wheat-bran it appeared that the ferulic acid esterase from *P. fluorescens*, showed similar catalytic activities for methyl ferulate, methyl coumarate, methyl cinnamate, and methyl caffeate. However, studies performed on the ferulic acid esterases from the other organisms, as indicated above, revealed that they could be differentiated by their characteristic substrate specificities, where each enzyme recognizes specific substitution on the phenolic ring. Reports have also been made where ferulic and *p*-coumaric acid esterases in extracellular broths of *S. viridosporus* have not always been specific. These reports indicate that the esterases from this organism can hydrolyse ester

linkages of *p*-nitrophenyl butyrate, α-naphthyl acetate, α-naphthyl butyrate and lignocellulose. These esterases were produced when grown on mineral salts-yeast extract medium, and did not show any increase in activity when grown on lignocellulose-supplemented medium (Donelly and Crawford 1988).

High yields of feruloyl- and *p*-coumaroyl esterases were also reported for *Penicillium expansum, Penicillium brevicompactum* and *A. niger* in studies performed on synthetic substrates like *ONP*-butyrate, methyl ferulate and methyl coumarate. From these experiments it appears that optimum enzyme production by these fungi depend on whether solid- or liquid culture is used as well as the type of culture (Donaghy *et al.* 1998).

A variety of organisms are reported to solubilize grass lignin and these include *S. viridosporus, Streptomyces baddius* (Pometto and Crawford 1986), *Streptomyces cyanus, Thermomonospora mesophila* and *Actinomadura* sp. (Mason *et al.* 1988, Zimmerman and Broda 1989). In addition to these lignin-solubilizing enzymes they produce, other enzymatic activities from these organisms have been reported, including activities of endo-glucanase, xylanase and several esterases (Ramachandra *et al.* 1987).

2.2. Analytical techniques used for the determination of lignin carbohydrate complexes

In this study two techniques were used for the analyses of lignin carbohydrate complex related products, namely ion exchange chromatography and capillary electrophoresis. A brief introduction of the principles of these techniques is given below.

2.2.1 Chromatography

2.2.1.1 Ion exchange chromatography

Ion exchange chromatography is a type of chromatography whereby charge species can be separated according to their differences in charge and size. The stationary phase used in ion exchange chromatography usually consists of a macromolecular matrix from divinylbenzenestyrene copolymer, silica gel, cellulose, or dextran (Skoog *et al.*1998). Inorganic groups are then bonded to these surfaces. In this separation process, ions with opposite charge to the stationary phase will be retained to the column. The retention increases with increased charge of the analysed ion, decreasing solvated volume of the analysed ion and increasing similarity between the carbon chain of the analysed ion on the polymer matrix of the stationary phase. The first two effects are dependant on the charge and the dimensions of the ion and the third effect is the result of the non-specific interaction between the ion and the matrix. An aqueous solution of an electrolyte is used as a mobile phase in ion exchange chromatography (Skoog *et al.*1998). There are two types of ion exchangers, namely cationic exchangers and anionic exchangers. Cation exchangers, exchange positively charged ions from the sample with the ions of the electrolyte. Anion exchangers exchange negatively charged ions from the sample with the ions of the electrolyte. Emphasis is put on anion exchange chromatography as this was the technique used in this thesis (Skoog *et al.*1998; Anonymous 1994).

2.2.1.2 Anion exchange chromatography

A distinction is made between strong and weak anion exchangers. Strong anion exchangers contain a permanent cation (quatenary ammonium group) and weak anion exchangers are only charged with a certain pH range (primary-, secondary- and tertiary substituted amine groups). In this study, for the analyses of carbohydrates, a very high pH is required in order to obtain charged species of the analytes (pH 10-12). At this pH the weak anion exchange stationary phase would have lost most of its charge and little retention would be obtained. Therefore strong anion exchangers were used (Skoog *et al.*1998).

These consist of surface sulphonated polystyrenedivinyl benzene substrate and aminated porous beads of latex particles. These surfaces are then grafted with quaternary ammonium anion exchange functional groups. During the separation the negatively charged counter ions from the mobile phase (OH^-, CH_3COO^-) associate with the stationary phase. Sample ions compete with these counter ions for attachment to the exchanger and separation is achieved due to small differences in pKa values, charge, volume and length of carbon chain. The separation can be manipulated by changing parameters like pH, ionic strength, type of competing anions in the mobile phase and temperature (Skoog *et al.*1998, Anonymous 1994). The concentration of the NaOH or CH_3COONa in the mobile phase is inversely proportional to the retention times of the elutes. Usually the use of an aqueous solution of NaOH in different concentrations is sufficient for the analyses of mono- and disaccharides. For the separation of oligosaccharides, the addition of CH_3COONa to the mobile phase improves the quality of the chromatograms. It is not advisable to use the strongly binding CH_3COONa for the smaller sugars in the mobile phase, as they would not be able to compete for binding sites on the stationary phase and consequently elute without being separated. Generally the separation can be further improved by running the instrument with a mobile phase gradient. When running the

instrument at a gradient, the mobile phase changes during the analyses, whereas it remains the same during an isocratic run (Skoog *et al.*1998; Anonymous 1994).

2.2.1.3 Instrumentation

A typical high performance anion exchange chromatography (HPAEC) system (Fig. 2.9) consists of reservoir bottles (containing the solvents for the mobile phase), a valve injector, a column with guard column, a pump, a detector and a data system which contains the software to operate the HPLC system. The reservoir bottles are usually connected to a degasser before the solution goes to the pump.

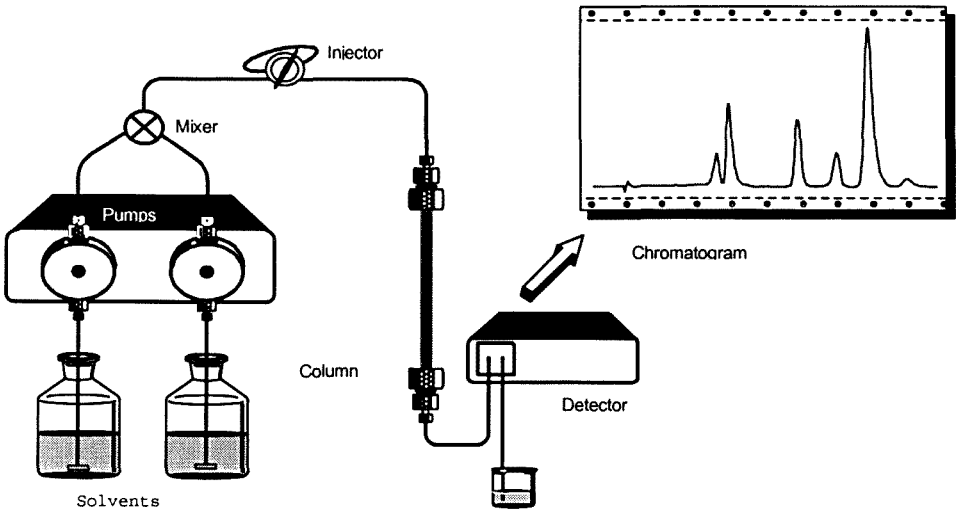


Fig. 2.9 A high performance liquid chromatography system (adapted from Skoog *et al.* 1998).

2.2.1.4 Anion exchange chromatography columns available for the analyses of carbohydrates

Several columns are available for the separation of saccharides as can be seen in Table 2.2. They differ in their composition which makes them suitable for various carbohydrates which differ in degree of substitution and polymerization.

Table 2.2 Different columns[a] in anion exchange chromatography for the analyses of sugars

Column	Resin composition	Function
PA1 (Dionex)	Pellicular. 10 μm polystyrene/divinyl benzene agglomerated with 580 nm MicroBead quaternary ammonium functionalised latex	Separation of monosaccharides
PA10 (Dionex)	Pellicular. 10 μm polystyrene/divinyl benzene agglomerated with 460 nm MicroBead difunctional quaternary ammonium ion	Separation of mono-to oligosaccahrides
PA100 (Dionex)	Pellicular. 8.5 μm ethylvinylbenzene/divinyl benzene agglomerated with 275 nm MicroBead quaternary ammonium functionalised latex	Separation of branched oligosaccharides
MA1 (Dionex)	Macroporous. 7.5 μm vinylbenzylchloride/divinyl benzene with alkyl quaternary ammonium group	Separation of sugar alcohols and glycols

[a] Obtainable from Astrochem in South Africa

2.2.1.5 Separation of carbohydrates using anion exchange chromatography

Carbohydrates are polar, hydrophilic, non-chromophoric and non-fluorophoric molecules and thus cannot be detected by absorbance detectors like UV-, VIS-, refractive index-, diode array and fluorescence detectors. In addition, carbohydrates have an enormous molecular diversity like oligo- and polysaccharides which can vary in degree of substitution and the way they are branched. In contrast to this, some monosaccharides have structural similarities and only vary with regard to their OH position. All these contribute to the difficulties that can be experienced during anion exchange analyses. Despite these problems it is possible to separate these molecules as they differ in their pKa values (12-14) of their hydroxyl groups. The α-and β-anomers can be separated due to the OH group at position 2 of an unsubstituted β-anomer which is believed to be more acidic compared to that of the α-anomer because of its proximity to the electron withdrawing acetal group (Torto *et al.* 1995). These properties make anion exchange possible with subsequent separation of the molecules.

2.2.1.6 Detection

One of the possibilities to detect carbohydrates is to exploit their ability to be oxidized. By oxidizing these compounds at a selected potential, it would leave the non-oxidizable ones undetected. Detection could be carried out by a variety of electrode material available like platinum, mercury, gold, silver, copper, nickel and lead. However it is important that the correct electrolyte is used for each electrode and that a constant composition of the electrolyte is maintained as it can limit the detection. High performance anion exchange chromatography with pulsed amperometric detection is a widely used chromatography technique for carbohydrate containing samples. With pulsed amperometric detection it is possible to sensitively detect carbohydrates, glycols and alditols and other alcohols at high pH following high performance anion exchange chromatography in one run (Hanko and Rohrer 2000). At high pH, carbohydrates are electrocatalytically oxidized at the surface of a gold electrode by the application of a positive potential. The current which is measured is directly proportional to the concentration of the analytes, thus providing a means to quantify the analytes through detection. Due to the production of oxidized products, the electrode surface needs to cleaned between measurements. This is accomplished by raising the electrode potential to a level which is sufficient to oxidatively clean the electrode surface. This results in the desorption of the carbohydrate oxidation products. In pulsed amperometric detection, carbohydrates are generally detected by sampling the electrode current at 16.7 ms at a certain constant potential over a defined time. After these two steps, a negative potential is applied to regenerate the electrode surface. This cyclic procedure forms the basis of pulsed amperometry (Hanko and Rohrer, 2000). The DIONEX system supports three types of detectors for pulsed amperometry namely: Pulsed Amperometric Detection-2 (PAD-2), Pulsed Electrochemical Detection (PED-2) and Electrochemical Detection (ED40). The ED40 detector is equipped with a combination pH-Ag/AgCl reference electrode. This combination electrode can be set up so that the pH or the

Ag/AgCl half of the electrode is used as the reference electrode. The working electrode is thus maintained by having a reference electrode included in the system. It is also important for high performance anion exchange chromatography with pulsed amperometric detection that the system remains metal free, as NaOH and CH_3COONa could react with the metal and thus have undesired separation (Anonymous, 1994).

2.2.2 Capillary Electrophoresis

2.2.2.1 Introduction

Capillary electrophoresis can be regarded as the separation of ions in an electric field, which will separate according to their charge to mass ratio. These ions will move at a characteristic velocity depending on their mobility as well as the specified voltage which is applied over the capillary.

2.2.2.2 Electrophoretic mobility

Separation is achieved due to the differences in electrophoretic mobility of the charged species in a constant electric field. These ions experience an electrostatic force (F), proportional to the electric field strength (E) and the charge (q) of the ion ($F = qE$). This electrostatic force results in an acceleration of the charged species towards the opposite charged electrode. Soon the ion will achieve a constant velocity because the functional force $F = 6\pi\eta\nu r$ (with η being the viscosity of the electrolyte, ν being the ion velocity and r being the ion radius) will equal the electrostatic force. Hence $qE = 6\pi\eta\nu r$ or $\nu = qE/6\pi\eta r$. The electromobility is defined as $\mu_{ep} = \nu_{ep}/E$, hence $\mu_{ep} = q/6\pi\eta r$ (Stokes law). The electrophoretic mobility is a characteristic property of a given species in a defined medium at defined temperature. This equation describes the way that separation by capillary electrophoresis is achieved, as it shows that the migration of

the charged species in an electric field depends on its charge, size, and shape as well as the viscous drag of the solvent (Paulus and Klockow-Beck 1999).

2.2.2.3 Electroosmotic flow (EOF)

Next to electrophoresis, the main transport mechanism in open tube capillary electrophoresis is electroosmosis. Capillary inner surfaces with uncoated fused silica are covered with silanol groups. These silanol groups can dissociate at a pH greater than 3 and generate an excess of negative charges at the capillary surface. In an electric field, cations from the buffer are attracted towards the negatively charged silanol groups and forms an ionic layer next to the capillary wall. When an electric field is applied over the capillary, the cations in the double layer next to the wall moves toward the cathode. In an aqueous solution, the cations are also surrounded by water molecules, resulting in a mass flow from the anode to the cathode. This flow is called the electroosmotic flow (EOF). The electroosmotic velocity (ν_{eo}) can be expressed as $\nu_{eo} = \mu_{eo} E$ and the electoosmotic mobility as $\mu_{eo} = \varepsilon\xi 4\pi\eta$, where ε= dielectric constant, ξ = zeta potential. The zeta potential could be described as a function of the surface charge density on the capillary wall. It is proportional to the double layer thickness of the wall and inversely proportional to the ionic strength of the buffer medium (Paulus and Klockow-Beck 1999).

The net mobility of analytes is the vectorial sum of both their electrophoretic mobility and the electroosmotic velocity. With a large electroosmotic flow the net mobility is mainly determined by the magnitude of the electroosmotic flow. At a pH greater than 5 the electroosmotic flow is the dominant flow factor and for fused silica capillaries it will flow from anode to cathode. Due to this, the on-line detector has to be placed at the cathodic side of the capillary irrespective of the charge of the sample that needs to be separated. Both negative and positive analytes are swept towards the detector (Paulus and Klockow-Beck 1999).

When the opposite scenario is encountered, at low pH, the silanol groups of fused silica are essentially not ionized, thus the zeta potential as well as the resulting electroosmotic flow at the capillary wall to the buffer interface is very low. The electroosmotic flow is then negligible compared to electrophoretic mobility of most charged species. Under these circumstances, the magnitude and the direction of the electrophoretic mobility of the analytes of interest determine their net mobility. Positive and negative analytes can thus not be separated in one single run. Negatively charged species can then only be detected by reversing the polarity of the electric field compared to the polarity of the conditions with large electroosmotic flow (Paulus and Klockow-Beck 1999).

2.2.2.4 Instrumentation

A typical capillary electrophoresis system consists of a separation capillary, a power supply, a detection system, a thermostaticing system and a data system as displayed in Fig. 2.10.

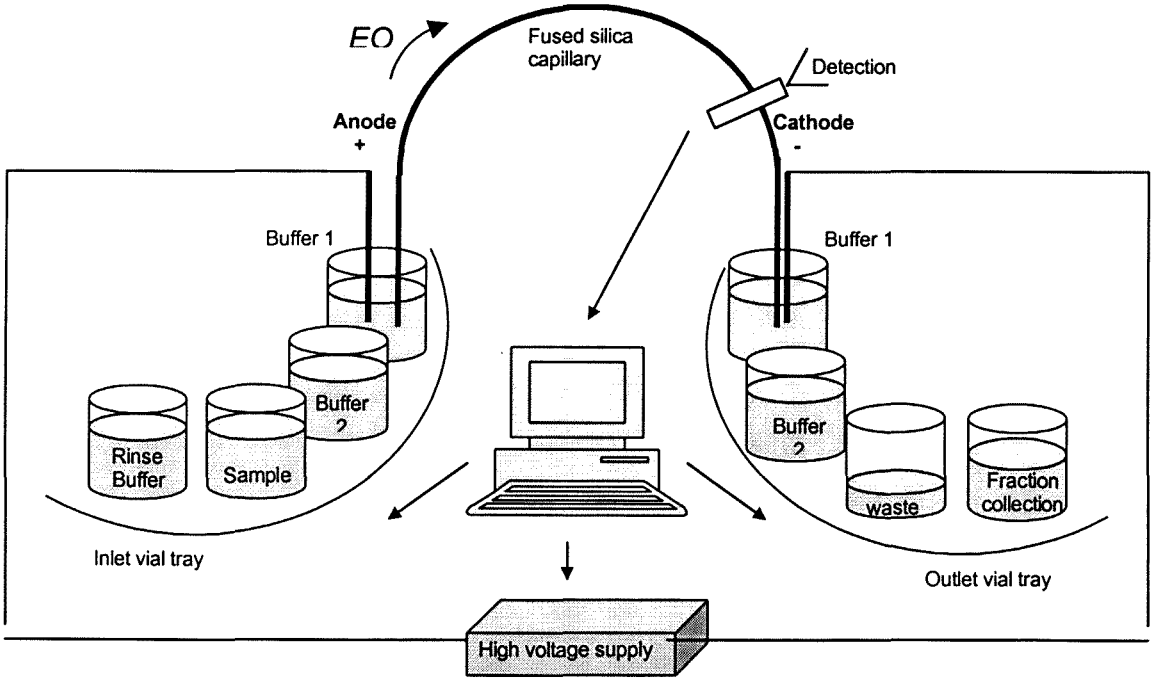


Fig. 2.10 Basic components of a capillary electrophoresis system.

Capillary electrophoresis is normally performed in fused silica capillaries. These capillaries are coated on the outside with a 10-20 µm thick film of polyamide, which makes them more flexible and thus easier to handle. These capillaries can have an internal diameter of up to 100 µm. In principle all the detection modes used in HPLC are also used in capillary electrophoresis. However mainly ultraviolet (UV) and laser induced fluorescence are used. Although UV detection is more popular and cheaper than laser induced fluorescence detection, laser induced fluorescence detection is more sensitive, as detection limits in the low nanomolar to picomolar range can be achieved (Paulus and Klockow-Beck 1999).

2.2.2.5 Modes

Capillary electrophoresis can be operated in various modes like Capillary Zone Electrophoresis (CZE), Micellar Electrokinetic Capillary Chromatography (MECC), Capillary Gel Electrophoresis, Isotachophoresis (ITP), Capillary Isoelectric Focusing (CIEF) and Capillary Electro Chromatography (CEC), (Table 2.3) (Paulus and Klockow-Beck 1999).

Table 2.3 Modes of Capillary Electrophoresis

Mode	Separation Principle
Capillary Zone Electrophoresis	Electrophoretic mobility
Micellar Electrokinetic Capillary Chromatography	Partition between micellar and buffer phase
Capillary Gel electrophoresis	Size
Isotachophoresis	Electrophoretic mobility
Capillary Isoelectric Focusing	Isoelectric point
Capillary Electro Chromatography	Chromatographic interaction

CZE was the only mode used during this thesis and will be discussed in more detail. CZE is together with MECC the most popular and widely used capillary electrophoresis mode, where separation is achieved using the differences in charge and size of the solutes in an electrical field (Jorgenson and Lukacs 1981). Both positive and negative solutes are separated in these modes, where the positive solutes elute in front of the electroosmotic flow and the negative ones are retarded with regard to the electroosmotic flow (Paulus and Klockow-Beck 1999).

2.2.2.6 Capillary electrophoresis for the separation of carbohydrates

Carbohydrates are very diverse molecules which could vary from mono- to oligo- to polysaccharides. The diversity of the structures, makes the separation of these molecules very difficult. In addition the α- and β- anomerity of the glycosidic bonds requires extreme selectivity. The polar and non volatile nature of carbohydrates and the absence of chromophoric and fluorophoric functional groups, also contribute to the difficulty in separating these molecules (Paulus and Klockow-Beck 1999). Furthermore neutral carbohydrates have no charge except under strong alkaline conditions. Consequently neutral carbohydrates have no electrophoretic mobility in acidic, neutral or mild alkaline conditions (Ristolainen 1999).

The situation regarding carbohydrate analyses changed with the introduction of capillary electrophoresis. Capillary electrophoresis is compatible with minute amounts of sample, could be detected online, diversity of neutral carbohydrates could be separated after pre-column derivitization and quantitation could be done with a high degree of reproducibility. Thus, it appears that capillary electrophoresis could be a very powerful technique regarding separation which could not be achieved in the past, regarding carbohydrate research (Paulus and Klockow-Beck 1999).

2.2.2.7 Capillary electrophoresis for the analyses of carbohydrates

Carbohydrates are traditionally not easy to analyse by capillary electrophoresis because they lack a charge under normal pH conditions and because they do not contain chromophoric groups. As is the case in anion exchange chromatography, a charge can be induced on the sugars by working at high pH. The carbohydrates will, however, never be fully deprotonated leading to sometimes difficulties obtained to achieve baseline separation between the analytes. The detection by UV absorbance can be achieved by using indirect UV detection. In this mode, a strongly UV absorbing buffer is used as a background analyte. This leads to a negative signal in the electropherograms when the UV-transparent carbohydrates are passing the detector. A drawback of indirect UV-detection is, however, generally its limited sensitivity. A good alternative for the above mentioned solutions is found in the pre-column derivitization of the carbohydrates.

Several derivitizing reagents are available, like 2-aminopyridine (2-AP), 4-aminobenzoic butyl ester (4-ABBE), 6-aminoquinoline, 1-aminopyrene-3,6,8-trisulphonate (APTS) and 8-aminonaphthalene-1,3,6-trisulphonic acid (ANTS) and 4-Aminobenzoic acid ethyl ester (ABEE). The carbohydrates are derivitized with these amino-compounds in the presence of a reducing agent by a procedure called reductive amination (Fig. 2.11) (Lamari *et al.* 2003).

As neutral carbohydrates have no charge (except strong alkaline conditions) and thus have no electrophoretic mobility in neither acid-, neutral- or mild alkaline solutions, a charge should be introduced into them. In reductive amination, the amino groups of the derivitizing reagent attacks the carbon of the carbonyl groups of the reducing sugar and should be performed in mild acid conditions to promote sugar ring opening. This process yields a Schiff base which is acid labile and is then reduced with sodium cyanoborohydrate to a stable secondary amine (Fig. 2.11) (Honda S. 1996).

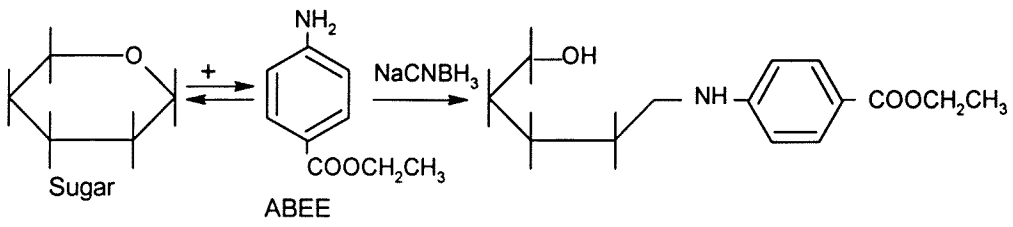


Fig. 2.11 Conversion of a reducing sugar to glycamine derivative by reductive amination.

Due to the equilibrium nature of the first step, it is important that the reaction be performed in such a way that promotes maximum labeled sugars. In order to obtain a high degree of purity it is important that the derivitizing reagent should be used in excess as any impurities carrying amino groups will also react. Caution should also be taken with regard to excess amount of derivitizing reagents as it could influence some separation mechanism (Lamari *et al.* 2003).

3. Methods

3.1 Microorganisms

The microorganisms used in this study to screen for enzyme production to degrade the lignin carbohydrate complex were all fungi (Table 3.1). The cultures were maintained by cultivation on yeast extract-malt agar and stored at 4°C.

Table 3.1 Fungi used to screen for growth on the lignin carbohydrate complex substrate

Organisms	Strain number	Source
Aspergillus niger	ATCC 90196	ATCC
Aureobasidium pullulans	NRRL Y2311-1	C. Kurtzman
Bjerkandera adusta	169	F. Wolfaardt, Sappi Ltd (UOFS)
Coriolus versicolor	S12-1-1	F. Wolfaardt, Sappi Ltd (UOFS)
Lenzites betulina	382	F. Wolfaardt, Sappi Ltd (UOFS)
Phanerochaete chrysosporium	ME-446	M Hammel, USDA (Forest Products)
Pycnoporus coccineus	S12-1-1	A. Kapich
Pycnoporus sanguineus	294	F. Wolfaardt, Sappi Ltd (UOFS)
Pycnoporus sanguineus	K5-2-3	A. Kapich
Trichoderma reesei	Rut C30	D. Eveleigh Rutgers University (USA)

3.2 Cultivation of microorganisms

The organisms were cultivated on Yeast Nitrogen Base (YNB) (DIFCO, USA) without amino-acids and ammonium sulphate. However, the YNB contained *p*-aminobenzoic acid, niacin, pyridoxine hydrochloride, thiamine hydrochloride, riboflavin, folic acid and biotin which serves as nitrogen source. Stock solutions of YNB (3.4% w/v; 2 x strength) and lignin carbohydrate complex (4 % w/v) were separately prepared and sterilised by autoclaving. The cultivation medium was prepared by pipetting 4 ml each of the stock solutions of lignin carbohydrate complex and YNB into sterile 10 ml test tubes to yield a 2 % lignin carbohydrate complex and a 1 x YNB solution, respectively. The test tubes were covered with sterile wired metal caps to ensure adequate aeration.

The test tubes were inoculated with mycelium of the different organisms and incubated at 30°C on a rotating wheel at 40 rpm for 4 days. Samples of 1 ml were taken every 24 h under sterile conditions and the biomass was separated from the culture fluid by centrifugation (8000 rpm for 10 min). Some selected fungi were cultivated in the 100 ml lignin carbohydrate complex (2 %)-YNB in 300 ml Erlenmeyer flasks for 5 days at 30°C on an orbital shaker (1000 rpm). The cells were separated from the culture fluid by centrifugation at 8000 rpm for 10 min. The supernatant of these samples were concentrated 8-fold through a 3000 kDa regenerated cellulose membrane (Millipore) under nitrogen using an Amicon stirred ultrafiltration cell apparatus (Model 8200) (Millipore, MA, USA). Sodium azide (0.02 %) was added to the supernatant to prevent bacterial growth.

3.3 Extraction of lignin carbohydrate complex

A slight modification to the method used for the extraction of lignin carbohydrate complexes as described by Azuma and Koshijima (1988) was followed. One kilogram of wheat straw was hammer-milled to 24-80 mesh at room temperature. The finely divided wheat straw was then extracted with 2.5 L of a 80 % aqueous 1,4-dioxane (AR Grade, Riedel-deHaën) for 48 h. The aqueous 1,4 dioxane with lignin carbohydrate complex was removed from the wheat straw by squeezing the residue through a 150 μm mesh sieve. The residue was re-extracted with the same amount of solvent. The filtrates of the lignin carbohydrate complex in aqueous 1,4-dioxane were concentrated at 38°C on a rotary evaporator (Rotavap) and then dialyzed against distilled water. The precipitate was washed three times with distilled water and the supernatants were added to the lignin carbohydrate complex filtrate in order to achieve maximum yield. The combined aqueous solution was concentrated to a small volume at 38°C with the rotary

evaporator and lyophilized (Azuma and Koshijima 1988). No further purification methods were followed after this step.

3.4 Charaterization of isolated lignin carbohydrate complex

3.4.1 Chemical hydrolysis

Trifluoroacetic acid (TFA) was used to hydrolyse the lignin carbohydrate complex by adding 1 ml of 2 N TFA to 10 mg of lignin carbohydrate complex sample in a 10 ml test tube and heated in boiling water for 4 h. Further hydrolysis of the sample was preformed with 5 N TFA for 3 h. The hydrolysates were dried under nitrogen gas and dissolved in 1 ml sterile water.

3.4.2 Monosaccharide composition

The monosaccharide composition was determined using capillary electrophoresis and high performance liquid chromatography. Analytes in the samples were identified by comparing the retention times of the sample with that of the known quantities of the standards. The calibration curves for the standards were constructed by plotting peak area *vs* amount injected.

3.4.3 Carbohydrate content

Carbohydrate content was determined by the phenol-sulphuric acid method with slight modification (Dubois *et al.* 1956). Briefly, 0.1 ml of phenol (5 %)-water solution was added to 0.1 ml of lignin carbohydrate complex hydrolysate. Concentrated H_2SO_4 (1 ml) of analytical grade was pipetted directly into the phenol/hydrolysate solution and allowed to cool. The absorbance of the samples was measured spectrophotometrically (490 nm, Beckman Coulter, DU 630 series spectrophotometer). The spectrophotometer was zeroed with a solution of 0.1 ml of 5 % phenol-water and 1 ml H_2SO_4.

3.4.4 Lignin content

The lignin content of the lignin carbohydrate complex was determined by using the acetyl-bromide method (Yifang *et al.* 1998). The lignin carbohydrate complex (10 mg) was dissolved in 5.0 ml 1,4-dioxane and 0.5 ml of 25 % acetyl bromide in glacial acetic acid was added. A blank without lignin carbohydrate complex was included to correct for reagent background absorbance. The samples were heated at 50°C in a water bath for 30 min, cooled to room temperature and 2.5 ml acetic acid, 1.5 ml 0.3 M NaOH, and 0.5 ml 0.5 M hydroxylamine hydrochloride solution were added. The samples were shaken and glacial acetic acid was added to a final volume of 10 ml. The lignin content was measured spectrophotometrically (280 nm). The lignin content was calculated using the following formula (Yifang *et al.* 1998):

$$\text{Lignin content (\%)} = [\, A \times 100]/[\, 20.2\ l/(g.cm) \times \text{Sample concentration}(g.l^{-1})]$$

3.4.5 Molecular weight determination

The molecular weight of the lignin carbohydrate complex was determined by an ÄKTA purifier (Amersham Pharmacia Biotech, Sweden) with a HR 10/30 column with an internal diameter of 10 mm. The total bed volume was approximately 24 ml, consisted of a matrix of dextran covalently cross-linked to porous agarose beads (Superdex 75). The mobile phase consisted of 50 mM Tris-HCl and 100 mM NaCl at pH 7.5 and was maintained at a constant flow rate of 0.4 ml/min. The concentration of the lignin carbohydrate complex in the various fractions was quantified at 280 nm. Dextran standards (Fluka, Switzerland) were used to calibrate the column by quantifying the carbohydrate content of each fraction by the phenol-sulphuric acid method (see section 3.4.3).

3.5 Enzyme activity determination

3.5.1 Xylanase activity

The activity of β-xylanase was determined by the method of Bailey *et al.* (1992). The activity was determined by following the release of reducing sugars from a 1 % birchwood xylan (Roth, Karlsruhe, Germany) solution at 50°C for 5 min. The reaction was terminated after 5 min by the addition of dinitrosalicylic acid (DNS) reagent. A 5 min boiling step was also included in order for the colour reaction to take place. The release of reducing sugars were spectrophotometrically determined at 540 nm. One unit of activity is defined as the amount of enzyme that released 1 μmol of xylose per min.

3.5.2 α-L-Arabinofuranosidase assay

Activity of α-L-arabinofuranosidase was determined by measuring the release of *p*-nitrophenol from *p*-nitrophenol-β-D-arabinofuranoside (5 mM in 0.1 M sodium acetate buffer, pH 5.0) substrate at 37°C for 10 min (Biely 2003). The reaction was terminated by addition of saturated sodium borate solution. The release of *p*-nitrophenol was quantified spectrophotometrically at 410 nm. One unit of activity is defined as the amount of enzyme releasing 1 μmol of *p*-nitrophenol in 1 min.

3.5.3 β-Xylosidase assay

Activity of β-xylosidase was determined by measuring the release of *p*-nitrophenol from *p*-nitrophenyl-β-D-xylopyranoside (5 mM in 0.1 M sodium acetate buffer, pH 5.0) substrate at 37°C for 10 min (Biely 2003). Termination of the reaction took place by addition of saturated sodium borate solution. The release of *p*-nitrophenol was quantified spectrophotometrically at

410 nm. One unit of activity is defined as the amount of enzyme releasing 1 μmol of *p*-nitrophenol in 1 min.

3.5.4 Ferulic acid esterase activity

The coupled enzyme assay method of Biely *et al.* (2003) was used. A stock solution of *p*-nitrophenyl 5-*O*-*trans*-feruloyl-α-L-arabinofuranoside (NPh-Ara*f*) (50 mM) was dissolved in dimethylsulphoxide (DMSO) solution. In preparation for the assay, 12 μl of DMSO and 3 μl of Tween 20 was added to 1 ml of stock solution and diluted further with 0.1 M sodium phosphate buffer (pH 5.5) to yield a final concentration 0.6 mM. The assay consisted of the following: 440 μl sodium phosphate buffer (0.6 mM), 5 μl substrate (NPh-Ara*f*), 5 μl arabinofuranosidase (Megazyme, Ireland) and 50 μl of sample. The reaction was performed in 1.5 ml Eppendorf tubes at 30°C for 10 min. The reaction was terminated with 180 μl saturated sodium borate and the *p*-nitrophenol released was quantified spectrophotometrically at 420 nm. One unit of feruloyl esterase is defined as the amount of enzyme that releases 1 μmol of *p*-nitrophenol in 1 min.

3.6 Analytical techniques

3.6.1 High Performance Liquid Chromatography (HPLC)

Analytical grade standards of xylose, glucose, mannose, rhamnose, galactose, arabinose, glucuronic acid and methyl glucuronic acid (Sigma-Aldrich) in concentrations of 25-, 50-, 100-, 250-, 500- and 1000 μg/ml were used to calibrate the HPLC. The dilution series was performed in sterile conditions using MilliQ water (18 mOhm) and were filtered through 0.22 μm nitrocellulose disposable syringe filters (Millipore, Bedford, MA) prior to injection. Quantitative analyses of the monosaccharides was carried out using a HPLC system (Dionex, Sunnyvale, CA) with a CarboPac PA10- and MA1-column for carbohydrate analyses and a pulsed

amperometric detector (PAD). The ED40 waveform was +0.05 V for 0.00 s to 0.40 s, then +0.75 V from 0.41 s to 0.60 s followed by 0.15 V from 0.61 s to 1.00 s. Integration occurred between 0.20 s and 0.40 s.

The eluents used to separate the sugars were 250 mM NaOH (Fluka Chemie, Buchs, Switzerland) and de-ionised water. All mobile phases were degassed prior to use. The following gradient programmes were used at a flow rate of 1.5 ml/min: (a) PA10 column, initially 15 % NaOH and 85 % de-ionised water; after 11 min 60 % NaOH and 40 % de-ionised water; after 21 min, 100 % NaOH and 0 % de-ionised water; from 40 until 45 min, 15 % NaOH and 85 % de-ionised water; (b) MA1 column, isocratic separation with 60 % NaOH and 40 % degassed de-ionised water.

Qualitative analyses of the lignin carbohydrate complex and the degradation thereof was performed on the HPLC using a PA 100- column (Dionex, Sunnyvale, CA). The analyses were performed isocratically with the following mobile phases; 50 % 500 mM NaOH, 10 % 250 mM CH_2COONa and 40 % de-ionised water. The ED40 waveform used in the analyses of the monosaccharides, remained unchanged for the analyses on the PA-100 column. The column was flushed after each analyses with 100 % 500 mM NaOH.

3.6.2 Reversed Phase High Performance Liquid Chromatography (RPHPLC)

The phenolic acid content (*trans*-*p*-coumaric and *trans*-ferulic acid) was determined by reversed phase high performance liquid chromatography (RPHPLC) (Waters, Milford, MA, USA). Separation was achieved at 25°C with a Phenomenesc Luna C18 column with the following dimensions: 250 cm (length) x 4.6 mm (internal diameter) packed with 5 μm silica particles. For the mobile phase the following eluents were used: (A) 2 % acetic acid or (B) 0.5 % acetic acid in 50 % ($^{v}/_{v}$) acetonitrile (Merck). Separation was achieved using a gradient

with the following conditions: 90 % (A), 10 % (B) to 25 min, 45 % (A), 55 % (B) to 30 min. The phenolic acids were measured at 320 nm.

3.6.3 Capillary electrophoresis

The same monosaccharide standards and concentrations described above were also used for capillary electrophoresis analyses. Detection of monosaccharide was achieved by derivitization of the standards and samples by reductive amination (Dahlman *et al.* 2000) of saccharides using ethyl *p*-aminobenzoate (Sigma-Aldrich) as derivitizing agent. The stock solution was prepared by dissolving ethyl *p*-aminobenzoate (100 mg/ml) and acetic acid (Merck, AR; 100 mg/ml) in methanol (Merck, AR). Prior to use, 10 mg sodium cyanoborohydrate (Sigma-Aldrich) was added to 1 ml of this stock solution to obtain the ethyl *p*-aminobenzoate reagent solution. This solution (240 µl) was mixed with the monosacharides (200 µl) in Eppendorf tubes, sealed and held in a water bath at 80°C for 1 h. Thereafter, the mixture was diluted with alkaline sodium borate buffer (400 mM, pH 10.5) and vortexed vigorously resulting in the precipitation of the excess ethyl *p*-aminobenzoate. The mixture was filtered through a 0.45 µm filter (Millipore) and recovered in a capillary electrophoresis vial.

Capillary electrophoresis analyses were performed on a HP^{3D}capillary electrophoresis system (Agilent Technologies, Waldbronn, Germany) equipped with a diode array detector (DAD). Separation was achieved using a bare fused silica capillary with the following dimensions: 48.5 cm total length, 40 cm effective length and 50 µm internal diameter (Composite Metal Services, Ltd., Worcester, UK), The separations were performed at +15 kV at 25°C. The samples were injected for 5 sec at 0.5 psi. The running buffer was initially 400 mM sodium borate adjusted to pH 10.5 with NaOH. To optimise separation, the borate buffer was increased to 440 mM (pH 11.5). The derivitized sugars were detected at a wavelength of 306 nm. The capillary column

was flushed for 10 min between cycles with the borate buffer. Data analyses was achieved using Chemstation software (Agilent).

3.6.4 Thin Layer Chromatography

Qualitative analyses of the digested trifluoroacetic acid products was performed using thin layer chromatography on Silica gel 60 plates (Merck). Two microlitres of each sample were spotted onto the Silica gel plates together with standards (1 mg/ml) of xylose, glucose, mannose, rhamnose, galactose, arabinose, glucuronic acid and methyl glucuronic acid (Sigma –Aldrich). Samples and standards were separated over 24 h with a mobile phase consisting of butanol, acetic acid (Sigma-Aldrich) and distilled water (2:1:1) and visualized by spraying the silica gel plates with a solution containing 90 % ethanol, 10 % sulphuric acid and 0.2 % orcinol. After drying by evaporation, the silica gel plate was placed in a preheated oven at 120°C for 2 min to develop the colour of the spots.

3.6.5 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed using 12 % polyacrylamide gels and running the gels for 75 min at 150 V and ~70 Amps. Gels were stained by the standard silver stain method of Blum *et al.* (1987) except that a 1 h incubation period in the fixing solution was used. The various protein bands were identified by comparing their relative positions on the gel to that of standard marker proteins (Full range rainbow marker, RPN 800; Amersham Biosciences, Vienna, Austria).

3.6.6 Protein determination

The total amount of protein in the supernatant of the cultures was determined by the method of Bradford (1976), using a stock solution of bovine serum albumin (BSA) (12.5 μg/ml). A dilution

series of 0-, 1.25-, 2.5-, 5-, 7.5- and 10 μg BSA was prepared by diluting the stock solution BSA to 800 μl, respectively. This dilution was linear over this concentration range, and used as standard curve. Coomassie brilliant blue stain (Cat. Number 500-0006, Bio-Rad Laboratories, München, Germany) (200 μl) was added to yield a final volume of 1 ml in order to visualize the proteins. The protein concentration was quantified spectrophotometrically at 595 nm.

3.7 Enzymatic degradation of substrate

Enzymatic degradation on the lignin carbohydrate complex was performed in a water bath at 40°C in 10 ml reaction glass tubes. The lignin carbohydrate complex (20 mg) was dissolved in 5 ml of 50 mM sodium acetate buffer (pH 5). The crude concentrated enzymes were added to the substrate every 24 h for 4 days. The controls were treated similarly except that no enzymes were added. Analyses were performed on a HPLC system (Dionex) using a PA-100 column.

4. Results

4.1 Extraction and characterization of the lignin carbohydrate complex

4.1.1 Extraction of the lignin carbohydrate complex

As indicated in section 3.3, the lignin carbohydrate complex in wheat straw was isolated using a modification of the standard protocol (Azuma and Koshijima 1988). Originally the lignin carbohydrate complex was extracted from 1 kg wheat straw, which resulted in a yield of 0.01 g. According to the protocol, the procedure should yield greater than 10 g of water soluble lignin carbohydrate complex. However, it appeared that the extracted lignin carbohydrate complex was not completely soluble in water, as a precipitate always remained after trying to solubilize the lignin carbohydrate complex. Filtering the lignin carbohydrate complex for sterility purposes via the standard protocol always resulted in the clogging of the 0.45 μm (pore size) membrane due to the apparent insolubility of the lignin carbohydrate complex. After numerous unsuccessful attempts to obtain an improved yield it was decided to eliminate the final extraction step from the protocol. As a result, the yield increased to almost 50 g lignin carbohydrate complex from 1 kg wheat straw. Chemical composition analyses indicated that the lignin carbohydrate complex isolated by the standard and the modified protocols had the same composition (data not shown).

4.1.2 Characterization of the lignin carbohydrate complex

4.1.2.1 HPLC analyses

The monosaccharide content of the lignin carbohydrate complex could not be quantified, as baseline separation could not be achieved on either the PA10 or MA1column (data not shown). The relative standard deviation for these analyses was greater than 10 %. Only galactose and glucose could be separated from the trifluoroacetic acid hydrolysed lignin carbohydrate complex sample. All the other sugars co-eluted as peaks with very broad bases (data not shown). Attempts to achieve better separation on the MA1 column also

failed, although the MA1 column appears to separate the lignin carbohydrate complex sample better than the PA10 column (Fig. 4.1). Peak 1 appears to be very prominent in the three repeated chromatograms obtained, from the trifluoroacetic acid hydrolysis of the lignin carbohydrate complex sample. This peak could not be identified due to a lack of proper standards.

From literature supplied by the equipment manufacturer (Anonymous 1994), the PA1 column could have been the better option to separate all these sugars.

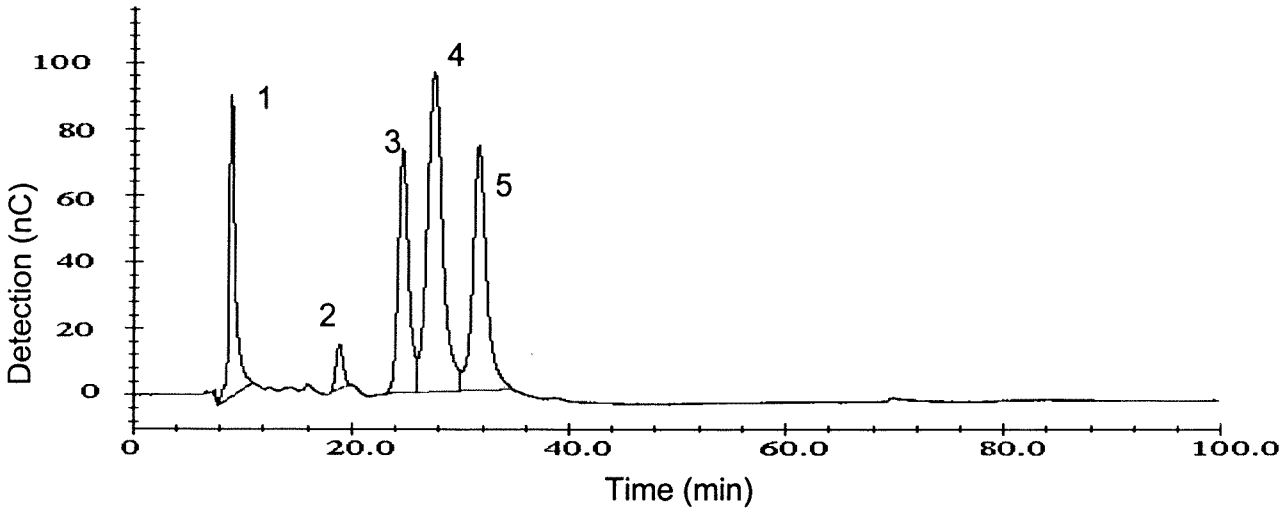


Fig. 4.1 HPLC chromatogram of trifluoroacetic acid hydrolysate of the lignin carbohydrate complex using the MA1 column (Dionex). 1, not identified; 2, rhamnose; 3, arabinose; 4, glucose; 5, xylose.

However, the results would probably be difficult to interpret should separation of the sugars be achieved using the PA1 column, as the impurities in the lignin carbohydrate complex interfere with separation causing changes in retention times. Any impure substance in the sample which possesses ionic groups at the pH where the samples are analysed, will also compete for binding sites on the stationary phase. If these impurities can be oxidized, they will probably be detected by pulsed amperometric detection, and their detection can overlap with the peaks of interest.

Whereas others have determined glucuronic acid by anion exchange chromatography (Anonymous 1994), the compound could not be separated or detected in these experiments. Attempts to achieve separation of glucuronic acid, by using different

columns, running conditions, mobile phase concentrations as well as changing the detector (ED40) waveform did not yield a detectable response. Thus, none of the sugars could be quantified using the available columns (PA10, MA1) on the Dionex HPLC.

4.1.2.2 Capillary Electrophoresis analyses

Capillary electrophoresis was more successful in separating the component sugars of the lignin carbohydrate complex than HPLC. A calibration curve was constructed for each sugar by plotting the peak area against the amount injected. A correlation coefficient of at least 0.99 and a relative standard deviation of less than 3 % of three consecutive injections was obtained for the analyses of all the sugars (Table 4.1). The lowest level of sensitivity where the sugars could be detected was at a concentration of 0.025 mg/ml for each individual sugar (Fig. 4.2). The first peak detected in the electropherograms is that of the derivatizing agent ethyl *p*-aminobenzoate. This agent is neutral under these circumstances and it elutes first as it migrates to the cathode with the electroosmotic flow. All the ethyl *p*-aminobenzoate complexed sugars which are negatively charged under the conditions employed would migrate after the ethyl *p*-aminobenzoate. The sugars would then migrate according to their charge to mass ratio. In the lignin carbohydrate complex hydrolysed sample, this property would imply that the larger molecules like xylose, glucose and mannose polymers would elute first after the ethyl *p*-aminobenzoate peak and would then be followed by the smaller molecules such as monosaccharides. The use of 440 mM borate buffer (pH 11.5) contributed to the effective separation of the sugars especially mannose, glucose and arabinose. Initially a concentration of 400 mM borate buffer (pH 10.5) was used. This resulted in the co-elution of glucose and arabinose and this peak was very close to that of mannose. After using 440 mM buffer (pH 11.5), the sugars were clearly separated from each other. The reproducibility of the results were further enhanced by flushing between cycles with the same borate buffer instead of 0.1M NaOH. The retention times of the sugars varied considerably when the column was flushed with 0.1M

NaOH, but was more constant when the capillary was flushed with the borate buffer for 10 minutes between cycles.

Table 4.1 Statistical analyses of monosaccharide and glucuronic acid determination by capillary electrophoresis.

Compound	RSD(%)[a]	Regression equation	Correlation coefficient (r^2)
Mannose	1.1	$y = 48.465x - 0.0742$	0.9981
Xylose	1.2	$y = 63.931x - 0.1977$	0.9968
Arabinose	1.1	$y = 69.408x - 0.6807$	0.9972
Galactose	2	$y = 50.4x - 0.2482$	0.9967
Rhamnose	0.5	$y = 45.121x - 0.631$	0.9982
Glucose	1.1	$y = 44.199x - 0.075$	0.9965
Glucuronic acid	3	$y = 25.371x - 1.125$	0.9922

[a] Relative standard deviation of triplicate independent determinations

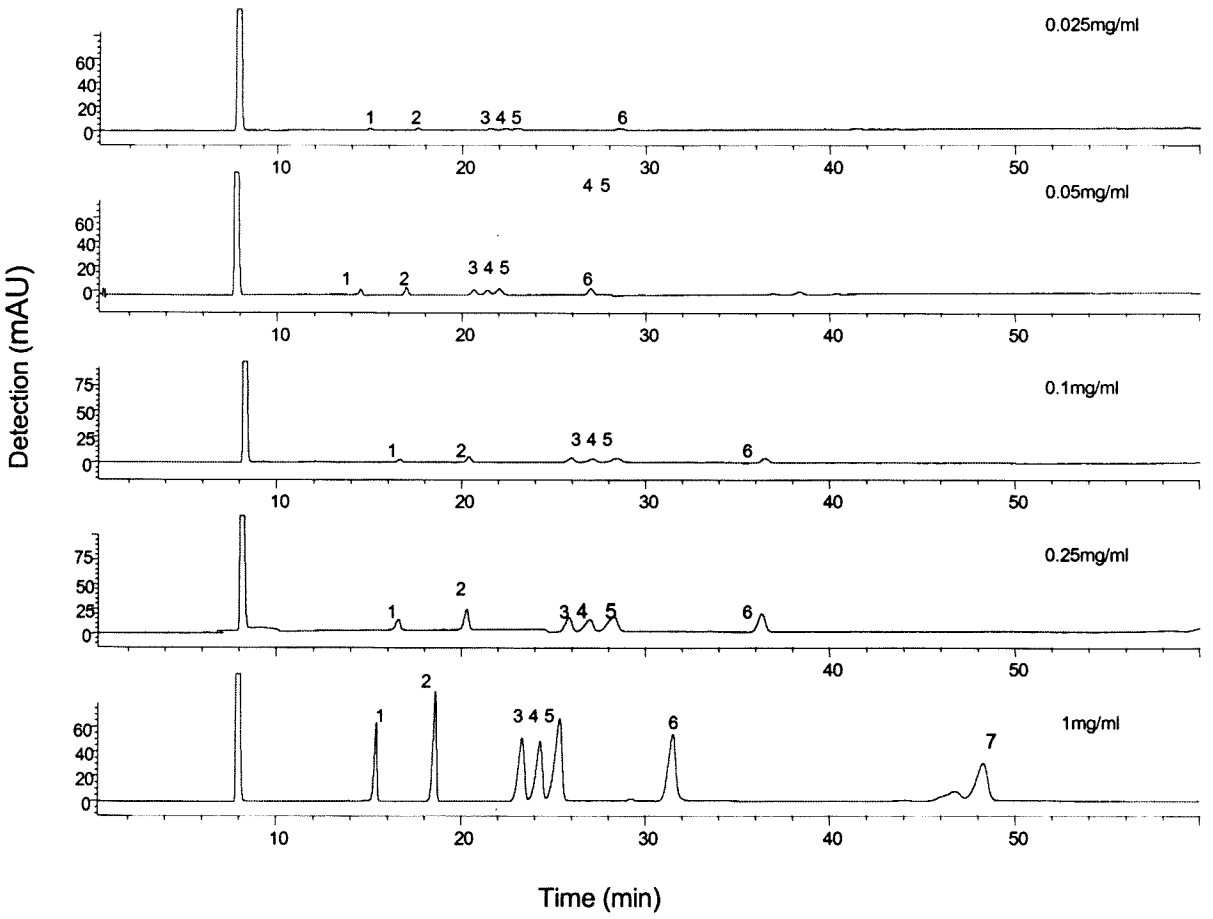


Fig. 4.2 Electropherograms of different concentrations of individual sugars ranging from 0.025 mg/ml to 1 mg/ml determined by capillary electrophoresis. 1, rhamnose; 2, xylose; 3, mannose; 4, glucose; 5, arabinose; 6, galactose; 7, glucuronic acid.

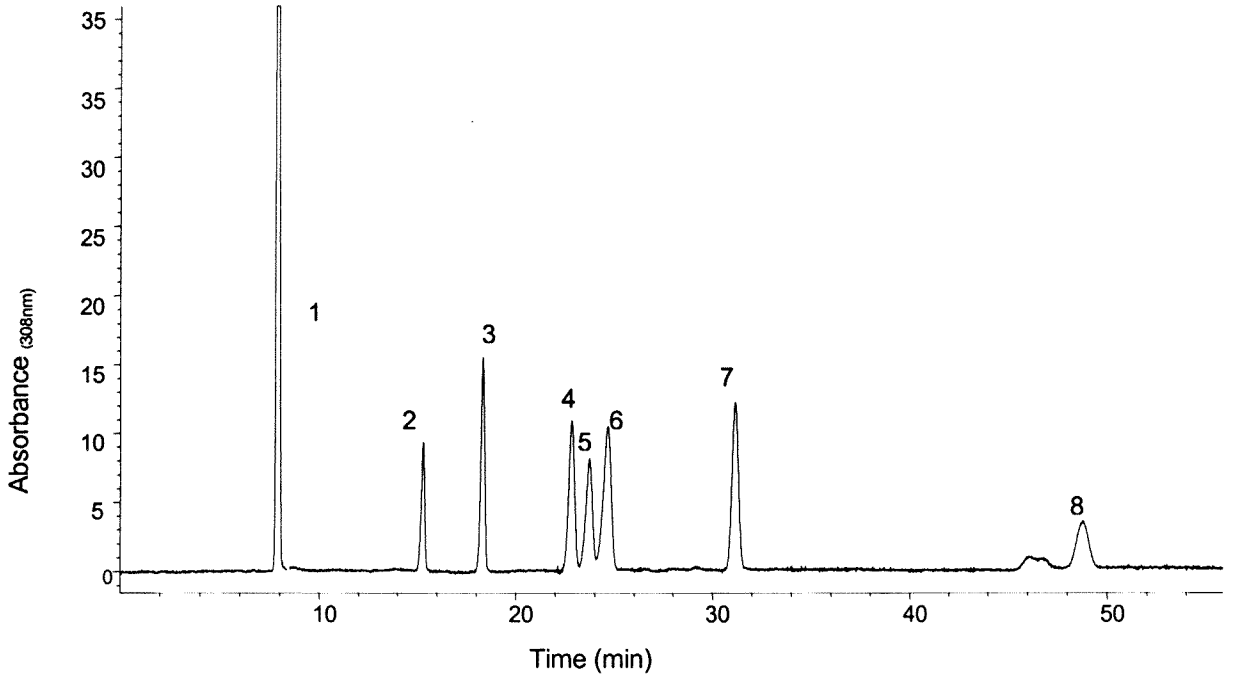


Fig. 4.3 Electropherogram of the monosaccharides and glucuronic acid standard (1 mg/ml per standard). 1, ethyl *p*-aminobenzoate; 2, rhamnose; 3, xylose; 4, mannose; 5, glucose; 6, arabinose; 7, galactose; 8, glucuronic acid.

A difficulty which could not be resolved was the separation of glucuronic acid. Despite the use of analytical grade glucuronic acid as a standard, the diode array detector always detected two peaks (Fig. 4.3). The possibility of the glucuronic acid being contaminated is unlikely as glucuronic acid samples from different suppliers yielded similar pairs of peaks. Introducing a higher buffer concentration (450 mM, pH 11.5) as eluent did not improve the resolution. Quantification of glucuronic acid was achieved by using the data obtained from the larger of the two peaks.

Capillary electrophoresis analyses of the trifluoroacetic acid hydrolysed lignin carbohydrate complex yielded electropherograms with effective separation of the compounds. The peaks were identified by comparing their retention times to the corresponding sugar standards. All the sugars which have been reported by Azuma and Koshijima (1988) to be present in hydrolysates of grasses (rice, bamboo, barley, sugar beet, spinach, corn stalk, Bermuda grass) could be separated and quantified in the samples analysed in this study. The elution profile of the standards in Fig. 4.3 differs from electropherograms previously

reported (Dahlman *et al.* 2000), as glucose eluted after mannose in the study reported here. In an attempt to confirm this elution profile, a number of samples with different concentrations of these two sugars were analysed. In all instances, glucose eluted after mannose.

Fig. 4.4 displays an electropherogram of hydrolysed lignin carbohydrate complex from wheat straw. Peak 2 might be polymerized carbohydrates like xylan, mannan, arabinan or galactan which were not completely hydrolysed, but this requires confirmation. The presence of relatively large quantities of xylose, mannose, galactose and arabinose which forms the backbone of xylan, mannan, galactan and arabinan respectively, is an indication that these polymers could be present in the sample. By calculating the peak areas of the sample and substituting them in the equations obtained from the standards, it was possible to calculate their concentrations in the lignin carbohydrate complex sample (Table 4.1, 4.2).

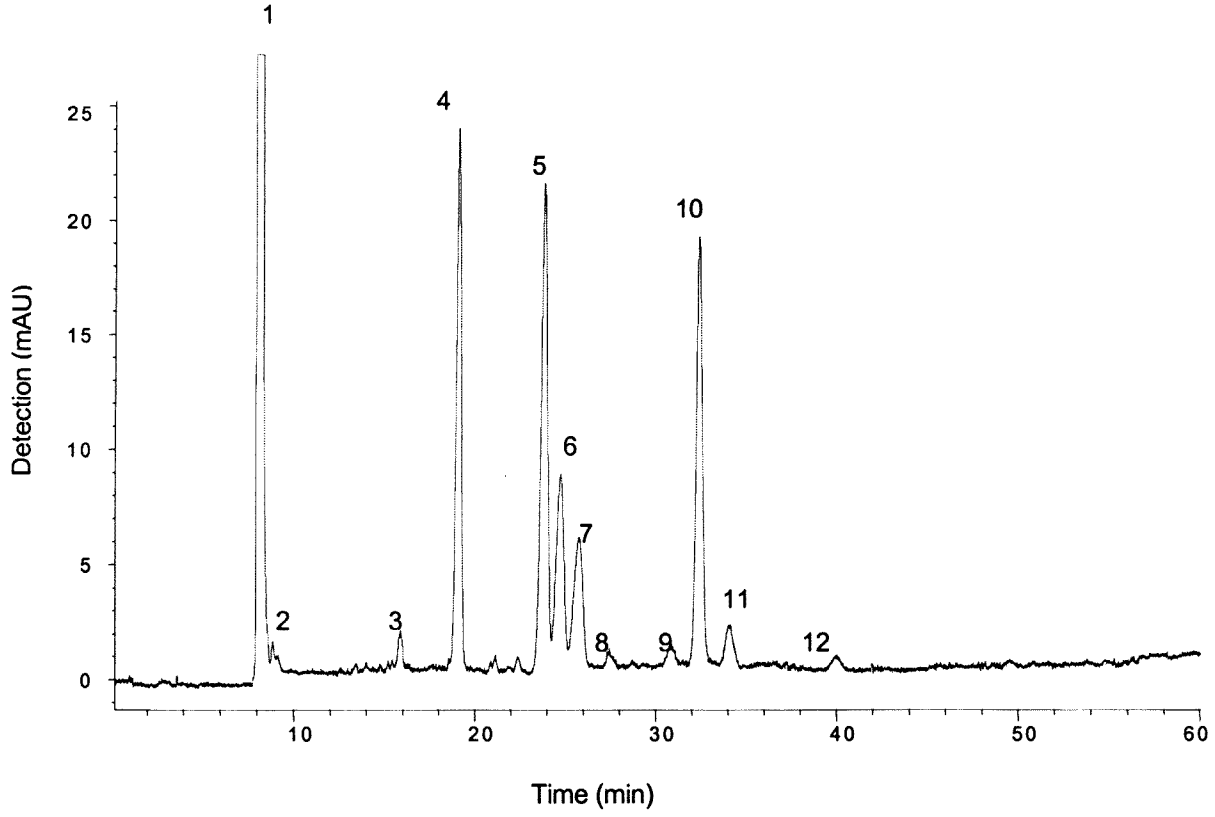


Fig. 4.4 Electropherogram of trifluoroacetic acid hydrolysed lignin carbohydrate complex from wheat straw (10 mg/ml). 1, ethyl *p*-aminobenzoate; 2, polysaccharides not hydrolysed; 3, rhamnose; 4, xylose; 5, mannose; 6, glucose; 7, arabinose; 8, not identified; 9, not identified; 10, galactose; 11, not identified; 12, glucuronic acid.

These data show that mannose was the main sugar present in the lignin carbohydrate complex with a concentration of 0.16 g/g lignin carbohydrate complex (Table 4.2), followed by xylose, glucose, arabinose, galactose and rhamnose. Due to a lack of standards some of the peaks in the electropherogram could not be identified (Fig. 4.4). The "ghost" peak which almost co-elutes with glucuronic acid in the standards did however not appear in the trifluoroacetic acid hydrolysed sample. It is also very noticeable that all the peaks shifted by almost 5 minutes later in the trifluoroacetic acid hydrolysed sample compared to those in the standards. However the repeated runs of the lignin carbohydrate complex sample yielded the same result, with very small shifts in peaks (less than 1 min shifts). Impurities could possibly cause changes in the retention times of the sugars, as observed with HPLC analyses.

Table 4.2 Concentration of carbohydrates (mean of triplicate determinations ± standard deviation) in lignin carbohydrate complex obtained from capillary electrophoresis analyses.

Carbohydrate	Concentration (g/g LCC)
Rhamnose	0.04 ± 0.02
Xylose	0.13 ± 0.03
Glucose	0.11 ± 0.03
Mannose	0.16 ± 0.04
Arabinose	0.085 ± 0.05
Galactose	0.075 ± 0.05
Glucuronic acid	0.015 ± 0.01

Comparing these results to previously reported work on rice straw (Azuma and Koshijima 1988), it appears that the concentrations of the released sugars from the lignin carbohydrate complex of wheat straw are much higher, with the exception of xylose and arabinose (Table 4.3). Mannose and xylose were the most abundant sugars present in lignin carbohydrate complexes of wheat straw and rice straw, respectively. It appears that the sugar composition of rice straw consists mainly of xylose and arabinose (Azuma and

Koshijima 1988). The remaining sugars in rice straw like galactose, rhamnose, mannose and glucose appear to be 10-fold and 80-fold less than xylose and arabinose respectively (Azuma and Koshijima 1988). Rhamnose which was not determined in rice straw, could be quantified in wheat straw. In contrast to these results, the composition of the lignin carbohydrate complex of rye grass, oats and barley straw (Wallace *et al.* 1995, Lam *et al.* 2001) appear to have very high amounts of glucose. The glucose amount in these samples is more than twice the amount of xylose and 10 fold higher than the remaining sugars. Rhamnose could also be determined in these samples.

4.1.2.3 Carbohydrate content

The total carbohydrate content of the lignin carbohydrate complex was 68.13 ± 4.0 %. The total monosaccharide content, recovered from the total carbohydrate (Table 4.3) of the hydrolysed lignin carbohydrate complex was 90.3 %. The remaining carbohydrate not detected could be unhydrolysed carbohydrate of the lignin carbohydrate complex or unidentified carbohydrate.

4.1.2.4 Lignin content

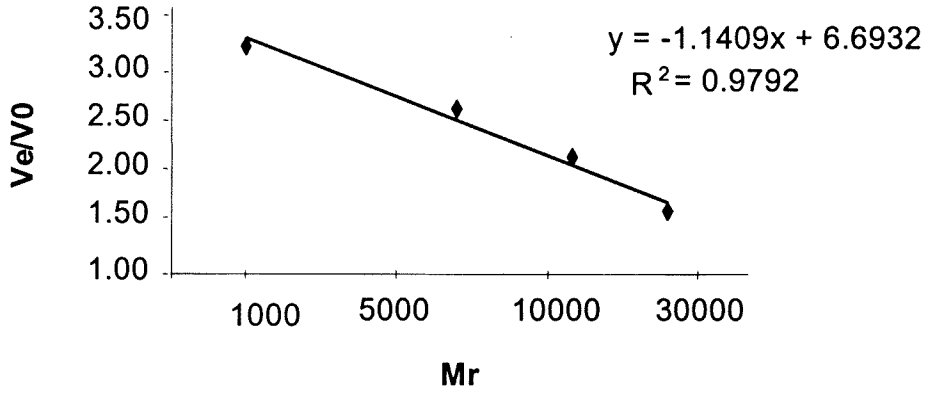
The lignin content of the lignin carbohydrate complex was found to be 20.5 ± 5 % (Table 4.3). The lignin content of the lignin carbohydrate complex of wheat straw was similar to levels found in rice straw and oats but is much higher than the concentration found in barley straw and rye straw (Table 4.3).

4.1.2.5 Molecular weight determination

A Superdex 75 gel permeation column was calibrated with dextran standards and yielded a linear relationship between 1 000 and 25 000 Da (Fig. 4.5). The lignin carbohydrate complex yielded a very broad peak with most of the material occurring between 25 000

and 2 000 Da and peak at 5 900 Da (Fig 4.6). Furthermore the sample appeared to contain significant material with a molecular weight less than 2 000 Da. These results point to the lignin carbohydrate complex being a heterogenous molecule.

Fig. 4.5 Calibration of a Superdex 75 gel permeation column with dextran standards of 1 000, 5 000, 12 000 and 25 000 molecular weight.



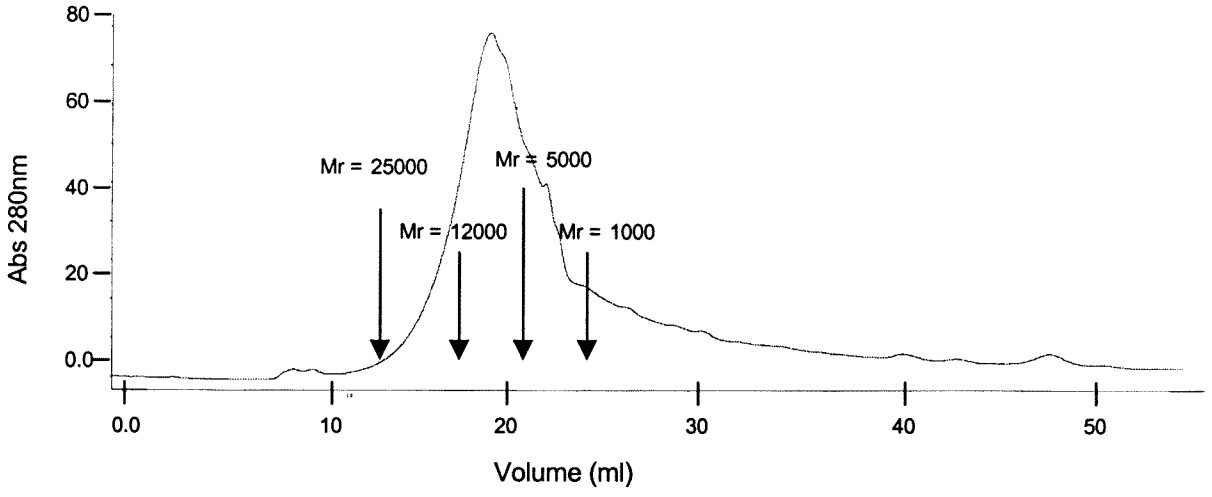


Fig. 4.6 Determination of the molecular weight of lignin carbohydrate complex from wheat straw using an Superdex 75 gel permeation column.

4.1.2.6 Phenolic acid determination in the lignin carbohydrate complex

The phenolic acid content of the lignin carbohydrate complex was determined by reversed phase high performance liquid chromatography and the results are displayed in Table 4.3. Similar concentrations of ferulic and *p*-coumaric acids were found. The concentration of *p* -

coumaric acid in wheat straw is relatively low compared to the values found in rice straw and barley straw, but are similar to values obtained in the lignin carbohydrate complexes of oats and rye grass. The concentration of ferulic acid in wheat straw was similar to values found in rice straw, oats and barley straw, but much lower compared to rye grass (Azuma *et al.*1988; Wallace *et al.* 1995; Lam *et al.* 2001).

Table 4.3 Composition of lignin carbohydrate complexes from different grasses.

Components	Wheat straw[a]	Rice straw[b]	Oats[c]	Barley straw[d]	Rye grass[d]
Total carbohydrate (% $^{w}/_{w\ LCC}$)	68.13 ± 4.0[e]	63.9	ND	ND	ND
Neutral sugar composition (% $^{w}/_{w\ carbohydrate}$)					
Arabinose	12.5 ± 1.21[f]	13.0	1.2	2.81	5.7
Xylose	19.1 ± 0.5[f]	80.1	24.6	20.0	20.9
Glucose	16.2 ± 0.12[f]	2.85	50.9	43.1	45.5
Mannose	23.5 ± 0.9[f]	0.43	0.2	0.47	0.1
Rhamnose	5.9 ± 1.01[f]	ND	1.4	0.07	0.2
Galactose	11.0 ± 0.5[f]	2.3	0.4	1.05	1.6
Uronic acid (% $^{w}/_{w\ carbohydrate}$)					
Glucuronic acid	2.2 ± 0.1[f]	ND	ND	ND	ND
Lignin content (% $^{w}/_{w\ LCC}$)	20.5 ± 5[g]	27.7	18.4	10.1	6.2
Phenolic acid content (% $^{w}/_{w\ LCC}$)					
trans-p-Coumaric acid	0.3 ± 0.1[g]	4.0	0.4	1.33	0.7
trans-Ferulic acid	0.4 ± 0.1[g]	0.8	0.4	0.45	1.1
Apparent molecular weight (Da)	5 900[h]	ND	ND	ND	ND

[a] This study
[b] Azuma and Koshijima, 1988
[c] Wallace *et al.* 1995
[d] Lam *et al.* 2001
[e] Mean of 5 determinations ± standard deviation
[f] Mean of 3 determinations ± standard deviation
[g] Mean of 2 determinations
[h] Estimated after 2 determinations
ND, not determined

4.2 Growth of fungi on the lignin carbohydrate complex

Ten fungal strains were evaluated for their ability to grow on the lignin carbohydrate complex. *B. adusta* showed little growth on the lignin carbohydrate complex after a 5 day incubation period at 30°C (Table 4.4) and was not investigated further. *A. niger, A. pullulans*, *C. versicolor*, *P. chrysosporium* and *T. reesei* showed substantial growth on the lignin carbohydrate complex after 5 days and appeared to be the strains with the best ability to degrade the substrate.

Table 4.4 Qualitative growth of organisms on lignin carbohydrate complex estimated by visual evaluation.

Days	Fungi									
	AN	AP	BA	CV	LB	PC	Pcoc	PS1	PS2	TR
1	+++	+	-	+	-	+	++	++	+	++
2	++++	++	-	++	+	++	++	+++	++	++++
3	++++	++++	-	+++	++	++	++	+++	++	++++
4	++++	++++	-	++++	++	++++	++	+++	++	++++
5	++++	++++	-	++++	++	++++	++	+++	++	++++

-, no growth
+, minimal growth
++, growth
+++, good growth
++++, very good growth

AN, *A. niger*
AP, *A. pullulans*
BA, *B. adusta*
CV, *C. versicolor*
LB, *L. betulina*
PC, *P. chrysosporium*
PCoc, *P. coccineus*
PS1, *P. sanguineus 294*
PS2, *P. sanguineus K5-2-3*
TR, *T. reesei*

4.3 Production of protein by fungi

The culture fluid of the various fungi were evaluated, for the concentration of protein secreted and the enzymes possibly involved in the lignin carbohydrate complex degradation. SDS-PAGE analyses on these culture fluids was conducted to confirm the presence of the various proteins.

Table 4.5 Protein content (mean of triplicate determinations ± standard deviation) in the culture fluids of fungi cultivated on lignin carbohydrate complex and wheat straw for 5 days.

Organisms	LCC [ac]	LCC [ad]	WS [bc]	WS [bd]
A. niger	7.4 ± 0.2	74.3 ± 0.7	4.3 ± 1.2	42.9 ± 0.1
A. pullulans	10.8 ± 0.3	108.0 ± 0.2	3.6 ± 0.5	36.0 ± 1.2
C. versicolor	9.0 ± 0.1	89.9 ± 0.1	3.8 ± 0.3	37.8 ± 0.7
L. betulina	10.6 ± 0.1	106.1 ± 0.2	3.7 ± 1.4	37.0 ± 0.1
P. chrysosporium	8.5 ± 0.6	84.7 ± 0.5	3.9 ± 0.4	38.7 ± 0.2
P. coccineus	9.1 ± 0.1	90.9 ± 0.2	3.2 ± 0.2	32.4 ± 1.1
P. sanguineus 294	8.9 ± 0.2	89 ± 1.0	4.3 ± 0.1	43.3 ± 0.3
P. sanguineus K5-2-3	8.0 ± 0.2	79.8 ± 0.1	6.8 ± 0.5	67.7 ± 1.4
T. reesei	9.9 ± 0.1	98.8 ± 0.3	3.9 ± 0.5	39.2 ± 0.2

[a] LCC = lignin carbohydrate complex
[b] WS = wheat straw
[c] Expressed as μg protein/g LCC or wheat straw
[d] Expressed as μg protein/ml LCC or wheat straw

All the organisms showed substantial amounts of protein secreted in the respective growth media (Table 4.5). In the medium containing the lignin carbohydrate complex, *A. pullulans* and *L. betulina* secreted the highest amount of protein. Protein secretion in the wheat straw medium was much lower than the concentration found in the lignin carbohydrate complex medium. *P. sanguineus K5-2-3* had the highest amount of protein secreted (67.7 μg/ml)

when grown on wheat straw. The protein content of the other organisms grown on wheat straw had almost similar values ranging from 32.4 μg/ml to 43.3 μg/ml.

4.4 Evaluation of extracellular enzymes

The activity of the enzymes from the various organisms grown on lignin carbohydrate complex were much higher compared to those of the wheat straw culture fluid (Tables 4.6-4.9). The xylanase activity, of the culture fluid of lignin carbohydrate complex was higher for *A. niger* compared to the culture fluid of the other organisms, irrespective the way it is expressed. Xylanase activities fell in a range between 9 and 30 U/ml.

The highest xylosidase activity expressed as units per milliliter lignin carbohydrate complex was found in the lignin carbohydrate complex culture fluid of *P. chrysosprorium*, followed by almost similar values for *A. pullulans* and *A. niger*. However, when the values are expressed as units per milligram protein it appears that *A. pullulans* had a higher activity. The xylanase and xylosidase activity appears to be higher in the culture fluid of lignin carbohydrate complex (Table 4.6) compared to activities found in the culture fluid of organisms grown on wheat straw (Table 4.8).

The arabinofuranosidase activity was also relatively high, when strains were cultivated on lignin carbohydrate complex and this might indicate that the relatively high arabinose content might induce this enzyme. *P. sanguineus 294* had the highest activity for arabinofuranosidase irrespective of the growth medium or the way it is expressed.

The ferulic acid esterase activity was also relatively high in both sets of culture fluids (lignin carbohydrate complex and wheat straw) (Tables 4.6 - 4.9). *C. versicolor* appeared to have the highest ferulic acid esterase activity when expressed as units per millilitre lignin carbohydrate complex culture fluid. However, when expressed as units per milligram protein, *P. sanguineus K5-2-3* appeared to have a higher value. Compared to the lignin carbohydrate complex culture fluid, it appears that *P. coccineus* had the highest ferulic acid

esterase activity in the wheat straw culture fluid when expressed as units per millilitre and per milligram protein (Tables 4.8, 4.9).

Table 4.6. Enzyme activities (U/ml) of the culture fluid of various organisms grown on lignin carbohydrate complex for 5 days.

Organism	Xylanase [a]	Xylosidase [b]	Araf [bc]	FeA [bd]
A. niger	29.79 ± 2.35	2.53 ± 0.21	3.04 ± 0.12	0.44 ± 0.01
A. pullulans	14.33 ± 3.11	2.70 ± 0.81	2.16 ± 0.22	0.54 ± 0.33
C. versicolor	20.14 ± 1.98	1.95 ± 0.02	1.16 ± 1.11	0.94 ± 0.01
L. betulina	9.03 ± 1.67	1.32 ± 0.12	1.13 ± 0.13	0.9 ± 0.10
P.chrysosporium	19.43 ± 2.85	2.85 ± 0.12	1.20 ± 1.10	0.5 ± 1.20
P. coccineus	16.31 ± 6.38	0.12 ± 0.11	1.76 ± 0.13	0.78 ± 0.01
P. sanguineus 294	16.87 ± 1.97	1.08 ± 0.21	8.50 ± 2.50	0.12 ± 0.11
P. sanguineus K5-2-3	8.88 ± 0.76	0.98 ± 0.10	5.48 ± 2.22	0.86 ± 0.25
T. reesei	19.43 ± 2.00	2.20 ± 0.95	3.01 ± 2.41	0.22 ± 0.02

[a] mean of 6 determinations ± standard deviation
[b] mean of 2 determinations ± range
[c] arabinofuranosidase
[d] ferulic acid esterase

Table 4.7 Enzyme activities (U/mg protein) of the culture fluid of various organisms grown on lignin carbohydrate complex for 5 days.

Organism	Xylanase [a]	Xylosidase [b]	Araf [bc]	FeA [bd]
A. niger	400.94 ± 10.54	34.05 ± 1.01	40.91 ± 0.12	5.92 ± 0.01
A. pullulans	132.68 ± 2.36	25.00 ± 0.98	20.0 ± 0.22	5.00 ± 0.33
C. versicolor	224.03 ± 5.89	21.69 ± 1.00	19.90 ± 1.11	10.4 ± 0.01
L. betulina	85.11 ± 2.31	12.44 ± 0.75	10.65 ± 0.13	8.48 ± 0.10
P. chrysosporium	229.40 ± 5.23	33.65 ± 2.14	14.17 ± 1.10	5.90 ± 1.20
P. coccineus	179.42 ± 2.14	1.32 ± 0.12	19.36 ± 0.13	8.58 ± 0.01
P. sanguineus 294	189.55 ± 2.11	12.13 ± 1.00	95.51 ± 2.50	1.34 ± 0.11
P. sanguineus K5-2-3	111.28 ± 3.89	12.28 ± 1.03	68.67 ± 2.22	10.7 ± 0.25
T. reesei	196.66 ± 7.66	22.27 ± 2.00	30.0 ± 2.41	2.22 ± 0.02

[a] mean of 6 determinations ± standard deviation
[b] mean of 2 determinations ± range
[c] arabinofuranosidase
[d] ferulic acid esterase

Figs. 4.7 and 4.8 gives an indication of the various proteins secreted by the selected fungi. It appears that these fungi secreted different types of protein between 8.4 kDA – 182.9 kDA. Similar patterns were obtained from the culture fluid of lignin carbohydrate complex and wheat straw. Relatively high amounts of protein from the culture fluid of *C. versicolor* and *L. betulina* grown on lignin carbohydrate complex was loaded on the gel (Fig. 4.7). Despite

the amounts loaded, the protein sectretion levels of these two organisms were much lower compared to those of the remaining organisms. Intense protein bands were observed between 37.4 kDa and 113 kDa. Similar amounts of protein was loaded on the gels of the culture fluid of the organisms grown on wheat straw, with the exception of *P. sanguineus K5-2-3* which had a much higher protein content loaded (Fig. 4.8). The secretion levels of *C. versicolor* and *L. betulina* were once again lower compared to the other organisms. Furthermore these two organisms did not secrete all of the proteins (~bands 182.9, 113, 20.5 and 8.4 kDa) compared to the other organisms (Figs. 4.7, 4.8).

Table 4.8 Enzyme activities (U/ml) of the culture fluid of various organisms grown on wheat straw for 5 days.

Organism	Xylanase[a]	Xylosidase[b]	Araf[bc]	FeA[bd]
A. niger	23.8 ± 1.96	1.5 ± 1.01	0.98 ± 1.10	1.75 ± 1.00
A. pullulans	14.2 ± 2.30	0.2 ± 0.80	1.80 ± 0.12	4.23 ± 0.13
C. versicolor	3.3 ± 0.19	0.1± 0.13	0.10 ± 1.00	0.25 ± 0.28
L. betulina	2.9 ± 0.95	0.11 ± 0.10	0.10 ± 0.58	3.16 ± 1.21
P. chrysosporium	4.1± 0.12	0.2 ± 0.11	0.19 ± 0.23	3.45 ± 0.59
P. coccineus	3.6 ± 1.00	0.1± 0.23	0.25 ± 0.17	4.62 ± 1.00
P. sanguineus 294	3.1 ± 0.85	0.2 ± 0.52	2.73 ± 1.29	4.20 ± 0.98
P. sanguineus K5-2-3	3.3 ± 0.69	0.2 ± 0.14	2.20 ± 2.22	4.49 ± 1.25
T. reesei	15.9 ± 1.21	0.7 ± 0.21	0.94 ± 0.18	2.71 ± 0.86

[a] mean of 6 determinations ± standard deviation
[b] mean of 2 determinations ± range
[c] arabinofuranosidase
[d] ferulic acid esterase

Table. 4.9 Enzyme activities (U/mg protein) of the culture fluid of various organisms grown on wheat straw for 5 days.

Organism	Xylanase[a]	Xylosidase[b]	Araf[bc]	FeA[bd]
A. niger	554.7± 6.59	34.9 ± 1.22	22.8 ± 1.10	40.7 ± 1.89
A. pullulans	394.4 ± 2.58	5.55 ± 0.98	50.0 ± 2.11	117.5 ± 2.33
C. versicolor	87.3 ± 3.69	2.64 ± 0.19	2.64 ± 1.00	6.61 ± 1.00
L. betulina	78.3 ± 1.98	2.97 ± 0.23	2.70 ± 0.58	85.4 ± 3.21
P.chrysosporium	105.9 ± 3.11	5.16 ± 0.68	4.90 ± 0.97	89.1 ± 1.59
P. coccineus	111.1 ± 8.16	3.08 ± 0.84	7.71 ± 0.69	141.9 ± 3.00
P. sanguineus 294	71.5 ± 2.33	4.61 ± 0.87	63.0 ± 1.29	96.9 ± 1.98
P. sanguineus K5-2-3	48.7 ± 1.89	2.95 ± 0.74	32.4 ± 2.22	66.3 ± 2.25
T. reesei	405.6 ± 8.57	17.8 ± 1.02	23.9 ± 1.11	69.1 ± 1.82

[a] mean of 6 determinations ± standard deviation
[b] mean of 2 determinations ± range
[c] arabinofuranosidase
[d] ferulic acid esterase

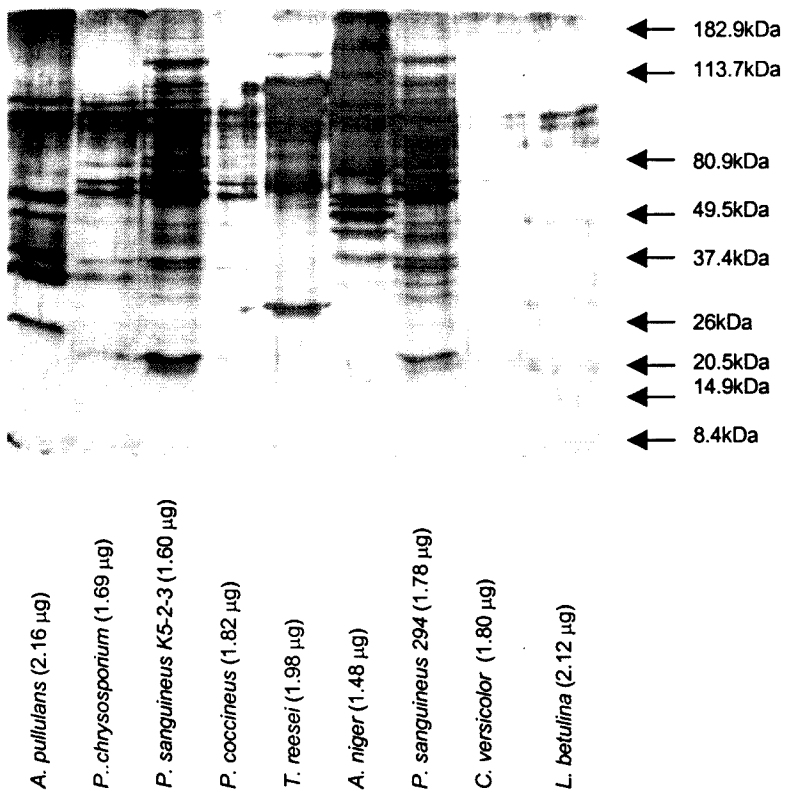


Fig. 4.7 SDS-PAGE of proteins (µg protein loaded indicated in brackets) secreted by fungi grown on lignin carbohydrate complex for 5 days.

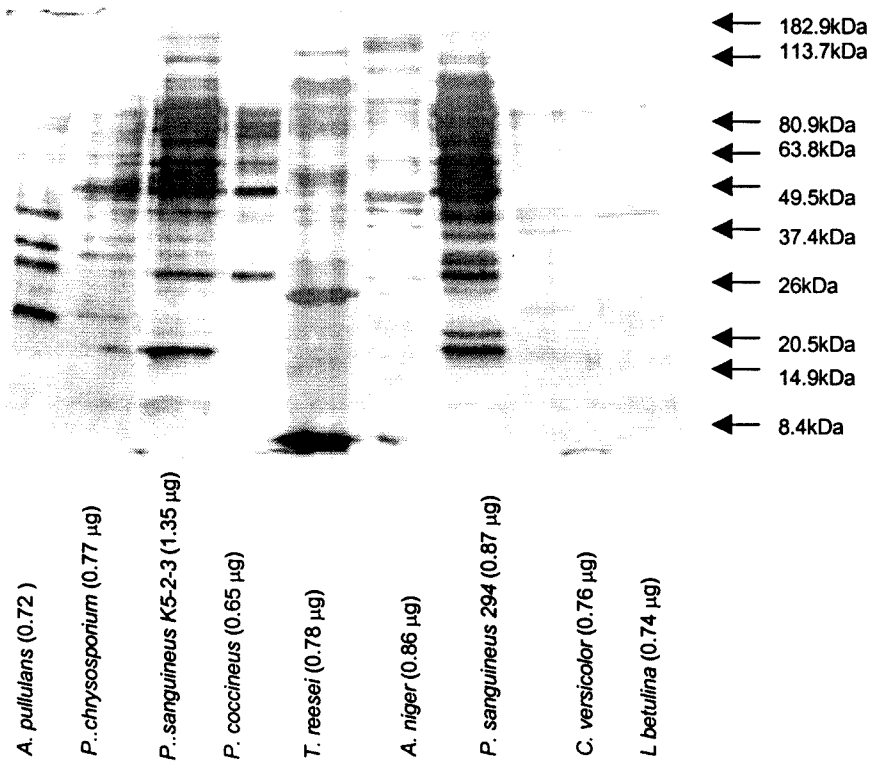


Fig. 4.8 SDS-PAGE of proteins (µg protein loaded indicated in brackets) secreted by fungi grown on wheat straw for 5 days.

4.5 Production of hemicellulolytic enzymes by fungi grown on various substrates

Table 4.10 Xylanase activity (U/ml) in culture fluid of different organisms grown on 1 g/L of various substrates for 5 days.

Organism	Xylose	Birchwood xylan	Glucose	Sugarcane bagasse
A. niger	130.7± 1.30[a]	179.2 ± 3.74[a]	0	70.6 ± 3.25[a]
A. pullulans	206.7 ± 2.10[a]	214.2± 2.25[a]	0	117.2 ± 4.17[a]
C. versicolor	64.5± 5.16[a]	72.0 ± 2.23[a]	0	0
T. reesei	69.3 ± 3.10[a]	27.0 ± 3.25[a]	0	18.0 ± 3.58[a]

[a] mean of triplicate determinations ± standard deviation.

Table 4.11 Xylosidase activity (U/ml) in culture fluid of different organisms grown on 1 g/L of various substrates for 5 days.

Organism	Xylose[a]	Birchwood xylan[a]	Glucose[a]	Sugarcane bagasse[a]
A. niger	0.40 ± 0.33[a]	1.02 ± 3.65[a]	0	0.82 ± 0.13[a]
A. pullulans	1.02 ± 1.20[a]	1.17 ± 2.35[a]	0	0.10 ± 0.10[a]
C. versicolor	0.25 ± 2.33[a]	0.26 ± 2.01[a]	0	0.07 ± 0.01[a]
T. reesei	5.97 ± 1.11[a]	7.90 ± 0.01[a]	0	0.16 ± 0.12[a]

[a] mean of triplicate determinations ± standard deviation.

Table 4.12 Arabinofuranosidase activity (U/ml) in culture fluid of different organisms grown on 1 g/L of various substrates for 5 days.

Organism	Xylose	Birchwood xylan	Glucose	Sugarcane bagasse
A. niger	0	2.93 ± 0.12[a]	0	0.16 ± 0.11[a]
A. pullulans	0	1.21 ± 0.18[a]	0	1.08 ± 0.67[a]
C. versicolor	0	1.12 ± 0.10[a]	0	0.12 ± 0.11[a]
T. reesei	0	1.44 ± 0.10[a]	0	1.44 ± 0.69[a]

[a] mean of triplicate determinations ± standard deviation.

Table 4.13 Protein concentration (µg/ml) of culture fluid of selected fungi grown on different substrates for 5 days.

Organism	Xylose	Sugarcane bagasse	Birchwood xylan	Glucose
A. niger	86.7 ± 2.35[a]	68.4 ± 1.69[a]	139.2 ± 0.69[a]	18.8 ± 0.26[a]
A. pullulans	138.4 ± 2.52[a]	90.0 ± 1.05[a]	219.2 ± 3.25[a]	20.0 ± 1.35[a]
C. versicolor	6.0 ± 1.29[a]	85.2 ± 3.56[a]	8.7 ± 2.31[a]	11.2 ± 1.00[a]
T. reesei	112.0 ± 2.36[a]	85.1 ± 1.00[a]	166.7 ± 1.00[a]	22.6 ± 1.25[a]

[a] mean of triplicate determinations ± standard deviation.

Four strains showing high level of protein secretion and a range of enzyme activities were selected for further study by cultivation on various substrates. *C. versicolor* was also included, as the organism has a well known ability to degrade lignin (Kondo and Imamura 1989). The culture fluids of the four strains were investigated for hemicellulolytic enzyme

activities, protein secretion profiles (SDS-PAGE) and ability to hydrolyse various substrates (capillary electrophoresis).

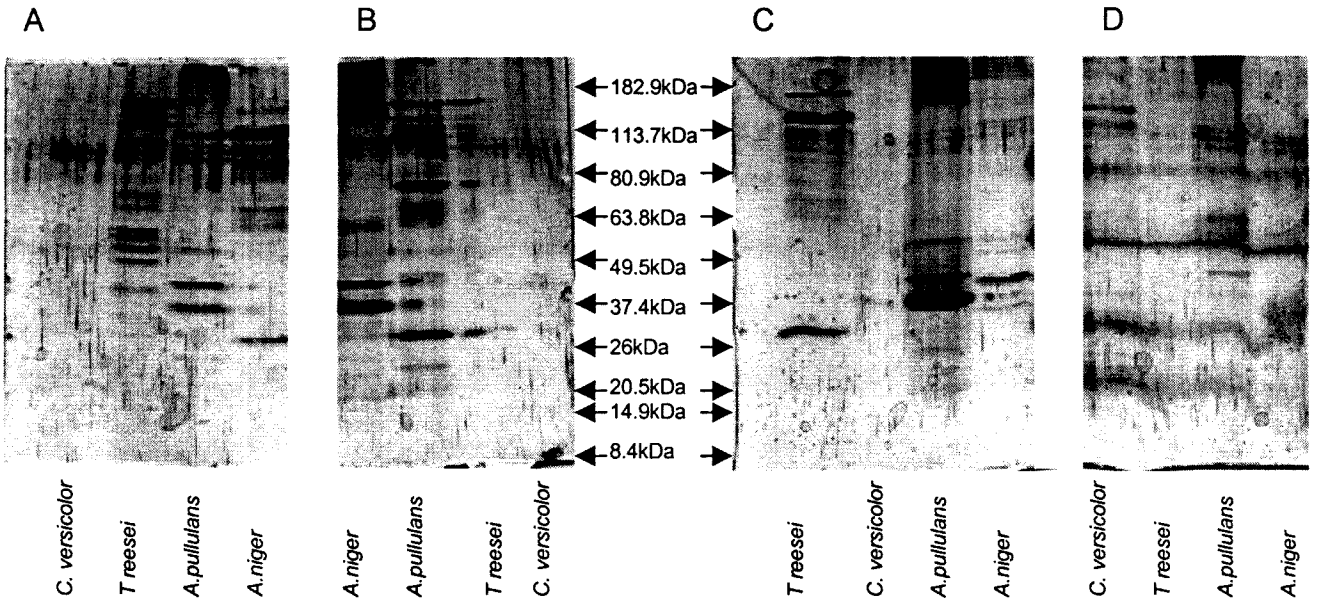


Fig 4.9 Secretion protein profiles of the culture fluid of fungi grown on 1g/L xylose (A), sugar cane bagasse (B), birchwood xylan (C) and glucose (D).

4.5.1 Enzyme activities

The four fungal strains produced xylanase (Table 4. 10) and xylosidase (Table 4.11) when the organisms were grown on birchwood xylan and xylose. No xylanase or xylosidase activity was observed when grown on glucose. When grown on sugar cane bagasse, lower levels of xylanase and xylosidase activity were observed and with *C. versicolor* no xylanase activity was found. Arabinofuranosidase activity was produced by the four fungi when grown on xylan and sugar cane bagasse but not xylose or glucose (Table 4.12). This suggests that either arabinose in the substrate or more complex hemicellulose structures are required to induce this enzyme. *A. pullulans* was found to produce the highest xylanase activities where as *T. reesei* produced the highest xylosidase levels.

4.5.2 Protein levels

Proteins were secreted by the four fungi when grown on birchwood xylan, xylose, sugar cane bagasse and even glucose (Table 4.13). With the exception of *C. versicolor*, the

highest protein levels were found in the culture fluids of fungi grown on birchwood xylan and lower levels were found in xylose and sugar cane bagasse grown cultures. *C. versicolor* secreted protein poorly when grown on birchwood xylan, xylose and glucose. Higher levels were found in a sugar cane bagasse grown culture suggesting that a complete lignocellulose substrate might be necessary for induction and secretion of protein involved in breakdown. On glucose lower protein levels were secreted.

4.5.3 Protein profiles

SDS-PAGE analyses revealed that all fungi except *C. versicolor* secreted a range of protein bands between 20 and 182 kDa into the culture fluid when grown on xylose, birchwood xylan and sugar cane bagasse. Surprisingly, some proteins were also observed in culture fluids of fungi grown on glucose. Prominent protein bands were often observed in the range of 30 to 40 kDa typical of family 11 xylanase (Biely *et al.* 1997). A series of bands were commonly found in the 80 to 180 kDa range and some of these may represent the accessory enzymes of hemicellulose degradation (Fig. 4.9) (Rumbold *et al.* 2003a & b; Ralet *et al.* 1994; Tenkanen and Siika-aho 2000).

4.6 Evaluation of the enzymes of the four selected strains grown on lignin carbohydrate complex

4.6.1 Evaluation of enzyme activities

The xylanase activity of *T. reesei* was the highest for the four organisms, irrespective the way it is expressed and was three fold higher than *A. pullulans* which also secreted relatively high amounts of xylanase (Tables 4.14, 4.15). Despite the high xylanase activity obtained for *T. reesei*, the xylosidase activity for the same organism was low. For the xylosidase activity *A. pullulans* had the highest activity which was double that of *T. reesei*. *A. pullulans* also had the highest activity for the arabinofuranosidase assay, as well as for the ferulic acid esterase activity. *T. reesei* had the highest laccase activity (Table 4.14, 4.15). Laccases oxidizes

substrates like phenolic compounds and play important role in ligninolysis by fungi. (Srebotnik and Hammel 2000). These laccase activity results could imply that *T. reesei* could probably degrade the lignin portion of the lignin carbohydrate complex more effectively than the other organisms. *A. pullulans* does not seem to be a good producer of laccases, as it had the lowest specific activity.

Table 4.14 Enzyme activity (U/ml protein) produced by the selected fungi of a concentrated culture fluid grown on lignin carbohydrate complex for 5 days.

Organism	Activity (U/ml)				
	Xylanase[a]	Xylosidase[b]	Arafsidase[bc]	FeA [bd]	Laccase[b]
A.niger	306.5 ± 1.21	7.3 ± 1.00	1.4 ± 0.12	0.87 ± 0.15	2.5
A.pullulans	569.2 ± 3.23	16.9 ± 2.13	2.5± 0.89	1.06 ± 0.2	2.9
C.versicolor	234.7 ± 1.59	10.4 ± 1.51	1.1 ± 0.96	0.75 ± 0.18	3.8
T. reesei	1961.0 ± 6.32	8.2 ± 0.94	1.8 ± 0.78	0.61± 0.12	3.8

[a] mean of 6 determinations ± standard deviation
[b] mean of 2 determinations ± range
[c] arabinofuranosidase
[d] ferulic acid esterase

Table 4.15 Specific activity (U/mg protein) produced by the selected fungi of a concentrated culture fluid grown on lignin carbohydrate complex for 5 days.

Organism		Activity (U/mg)				
	Protein (μg/ml)	Xylanase	Xylosidase	Arafsidase[a]	FeA[b]	Laccase
A. niger	1500 ± 1.11	204.3	4.86	0.93	0.58	1.67
A. pullulans	7700 ± 10.12	73.9	2.19	0.32	0.14	0.38
C. versicolor	2200 ± 8.99	106.7	4.72	0.50	0.34	1.72
T. reesei	1900 ± 8.64	1032	4.31	0.95	0.32	2.00

[a] arabinofuranosidase, [b] ferulic acid esterase

4.6.2 Protein profiles

The culture fluid of the various fungi grown on lignin carbohydrate complex were concentrated and diluted to various degrees in order to improve the resolution of the SDS-PAGE assay (Fig. 4.10). The profiles of concentrated culture fluid yielded smears with poor resolution of the bands, whereas the 2-fold diluted sample revealed faint bands. The undiluted sample showed the best resolution overall. *T. reesei* appeared to produce the greatest number of protein bands followed by *A. niger* and *A. pullulans*, whereas *C. versicolor* produced relatively few proteins when grown on lignin carbohydrate complex. *A. niger* culture fluid contained prominent bands at ~8 kDa, ~38 kDa and ~50 kDa and faint

bands at higher molecular weight. *A. pullulans* revealed a prominent band at ~ 23 kDa and faint bands with higher molecular weight. The culture fluid of *T. reesei* contained a variety of bands with the intense bands at ~26 kDa, ~37.4 kDa.

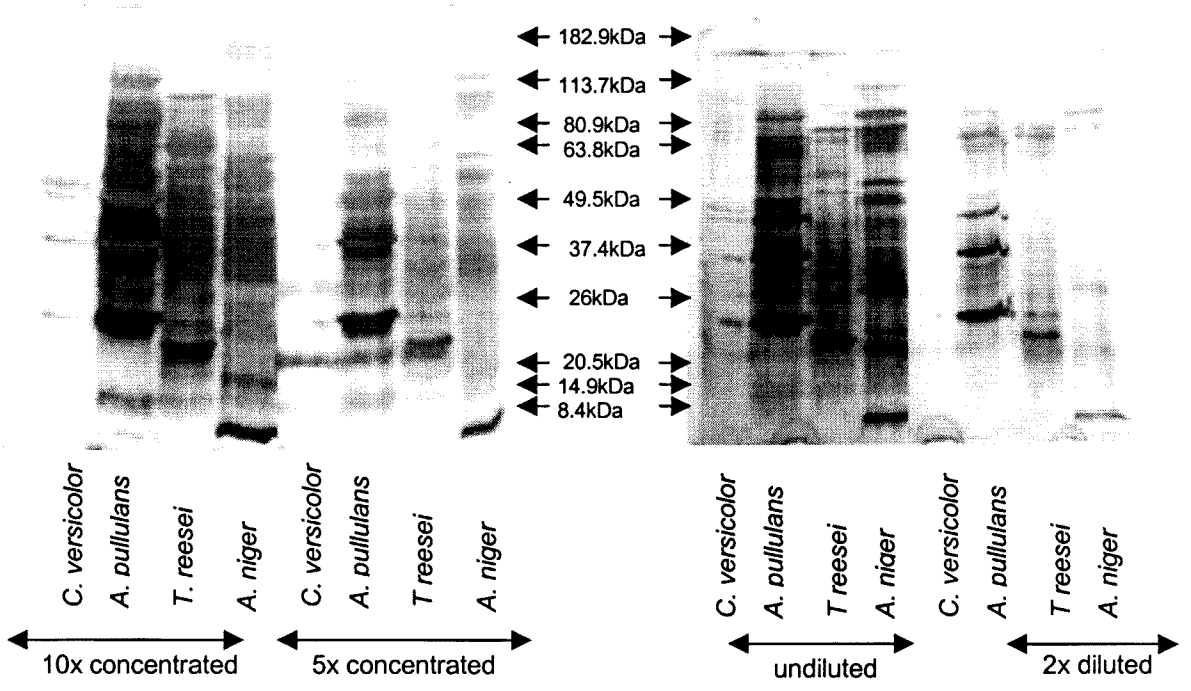


Fig. 4.10 SDS-PAGE gels of the culture fluid of selected fungi used in different concentrations. Original protein content of undiluted samples: *A. niger* (1500 μg/ml); *A. pullulans* (7700 μg/ml); *C. versicolor* (2200 μg/ml); *T. reesei* (1900 μg/ml)

4.6.3 Evaluation of substrate degradation by the culture fluid of lignin carbohydrate complex grown fungi

4.6.3.1 Birchwood xylan degradation

Capillary electrophoresis was performed on the culture fluid of the various organisms, to evaluate it for the presence of released sugars from birchwood xylan and sugar cane bagasse. The electropherograms revealed that the four fungal strains could degrade birchwood xylan to various monosaccharides. The electropherograms differed with regard to the various types of monosaccharides and their individual amounts (data not quantified). A large portion of apparent unhydrolysed birchwood xylan is visible in the electropherograms of *C. versicolor* (Fig. 4. 13) and *T. reesei* (Fig. 4.14). However this birchwood xylan peak was not visible in the electropherograms of *A. niger* (Fig. 4.11) and *A. pullulans* (Fig. 4.12).

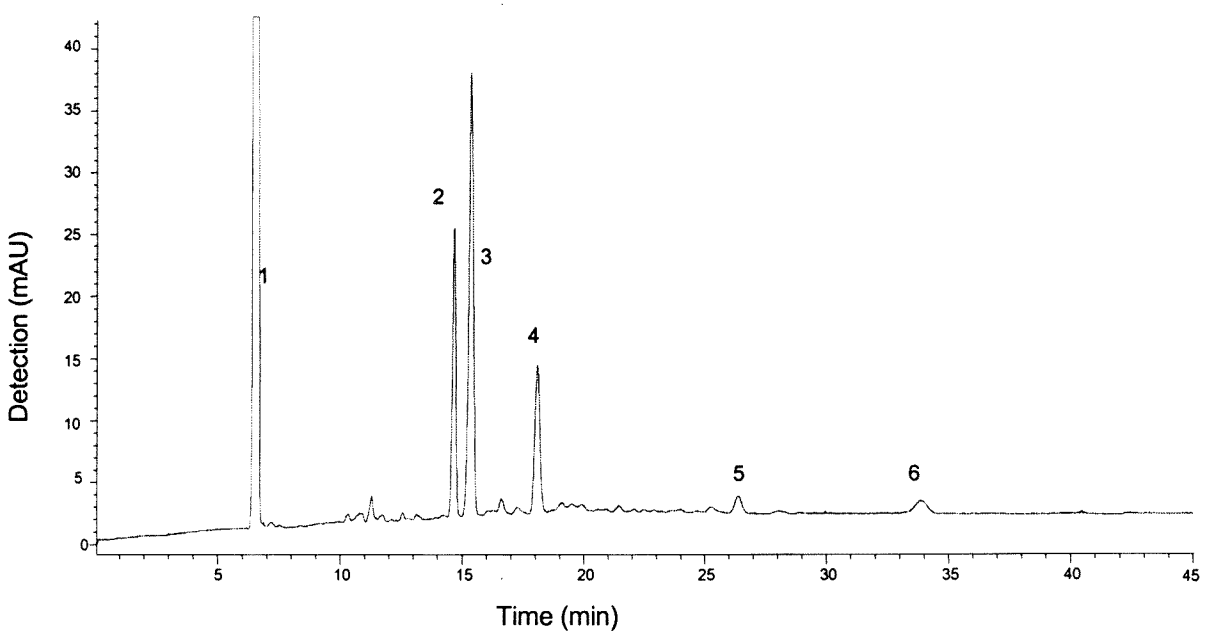


Fig. 4.11 Electropherogram of the culture fluid of *A. niger* grown on lignin carbohydrate complex and incubated on birchwood xylan (10 mg/ml) for 5 days. 1,ethyl *p*-aminobenzoate; 2, xylotetraose; 3, xylotriose; 4, xylose; 5, not identified; 6, glucuronic acid.

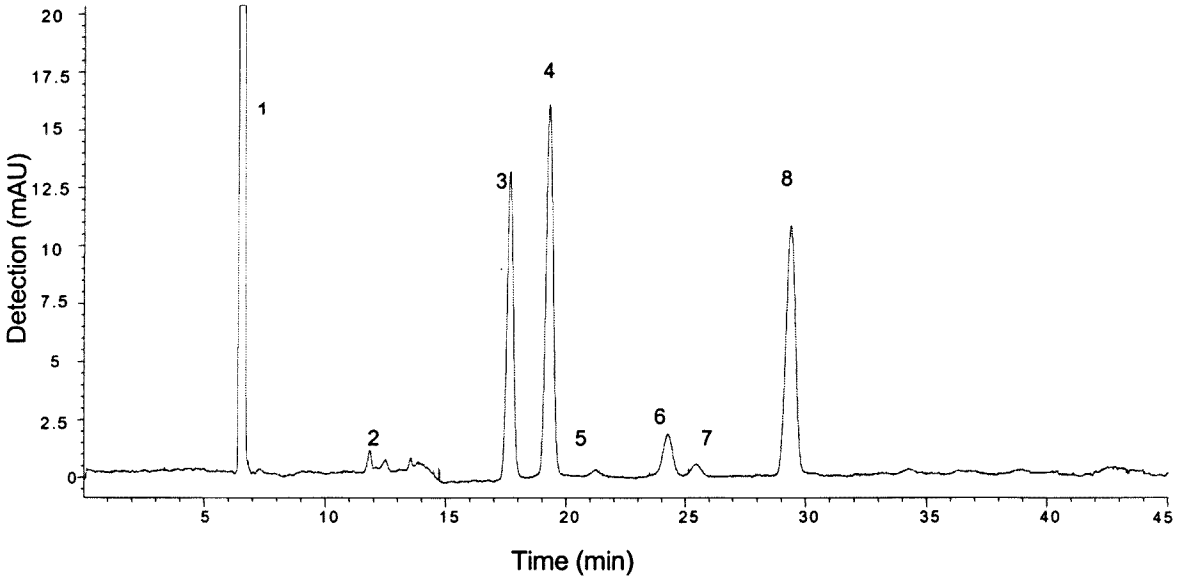


Fig. 4.12 Electropherogram of the culture fluid of *A. pullulans* grown on lignin carbohydrate complex and incubated on birchwood xylan (10 mg/ml) for 5 days. 1, ethyl *p*-aminobenzoate; 2, not identified; 3, xylobiose; 4, xylose; 5,6,7, not identified; 8, arabinose.

Birchwood xylan which is a polymer elutes closest after the ethyl *p*-aminobenzoate, as separation is achieved according to the charge to mass ratio. The main breakdown products

were xylose and xylooligomers up to xylotetraose. Oligomers greater than C4 could not be identified. Apart from xylose, arabinose and glucuronic acid could also be identified.

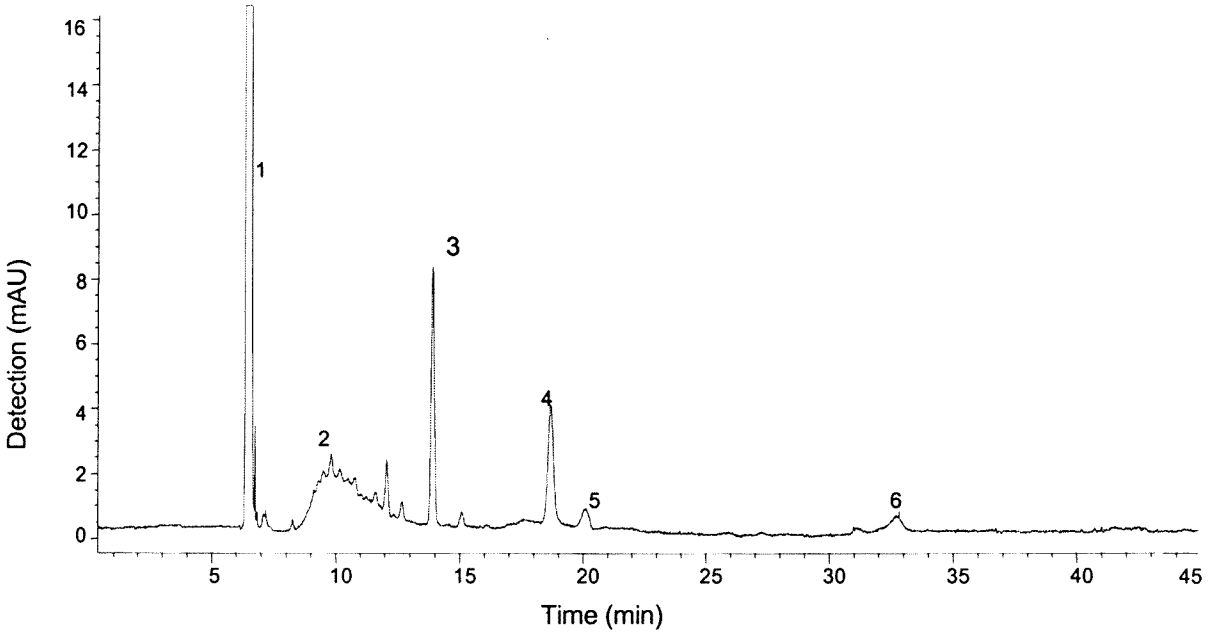


Fig. 4.13 Electropherogram of culture fluid of *C. versicolor* grown on lignin carbohydrate complex and incubated on birchwood xylan (10 mg/ml) after 5 days. 1, ethyl *p*-aminobenzoate; 2, xylan; 3, xylotetraose; 4, xylose; 5, not identified; 6, glucuronic acid.

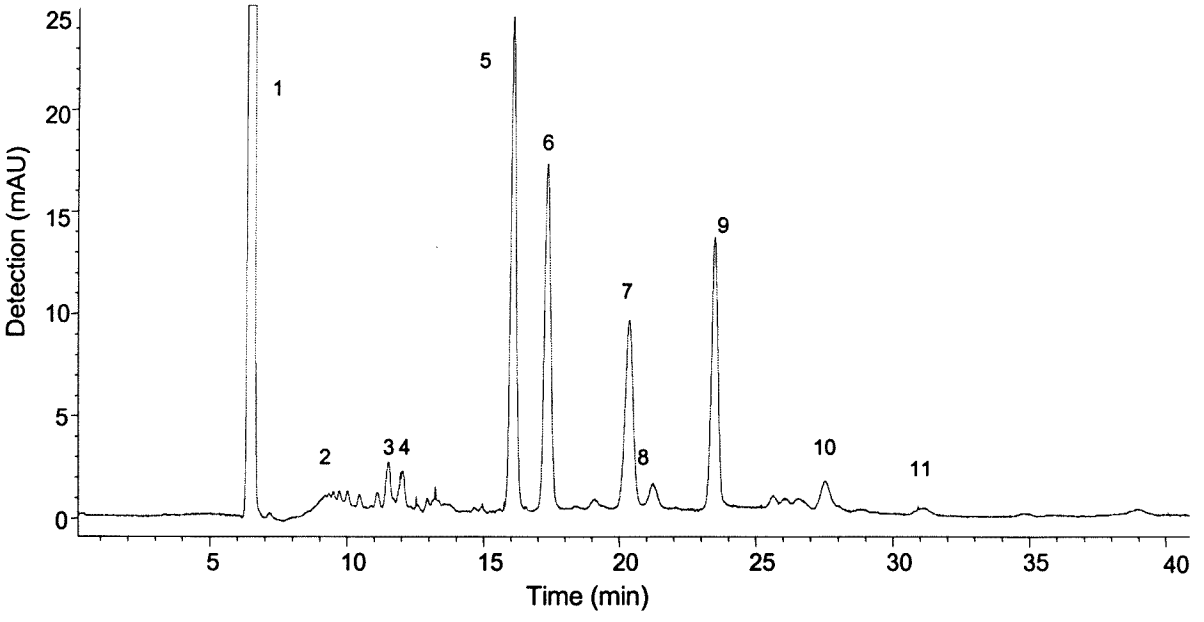


Fig. 4.14 Electropherogram of culture fluid of *T. reesei* grown on lignin carbohydrate complex and incubated on birchwood xylan (10 mg/ml) for 5 days. 1, ethyl *p*-aminobenzoate; 2, xylan; 3,4, not identified; 5, xylotriose; 6, xylobiose; 7, xylose; 8, not identified; 9, arabinose; 10, not identified; 11, glucuronic acid.

Arabinose could only be identified in the electropherograms of *A. pullulans* and *T. reesei*. Glucuronic acid was identified in all the electropherograms with the exception of *A. pullulans*.

4.6.3.2 Sugar cane bagasse degradation

No identifiable peaks of the degradation of sugar cane bagasse by culture fluids of the lignin carbohydrate complex could be observed after 1 h, 5 h and 5 days (Fig. 4.15). This suggest that growth on lignin carbohydrate complex failed to induce the enzymes required to degrade sugar cane bagasse. However this should be evaluated further under controlled conditions with culture fluids from other growth conditions.

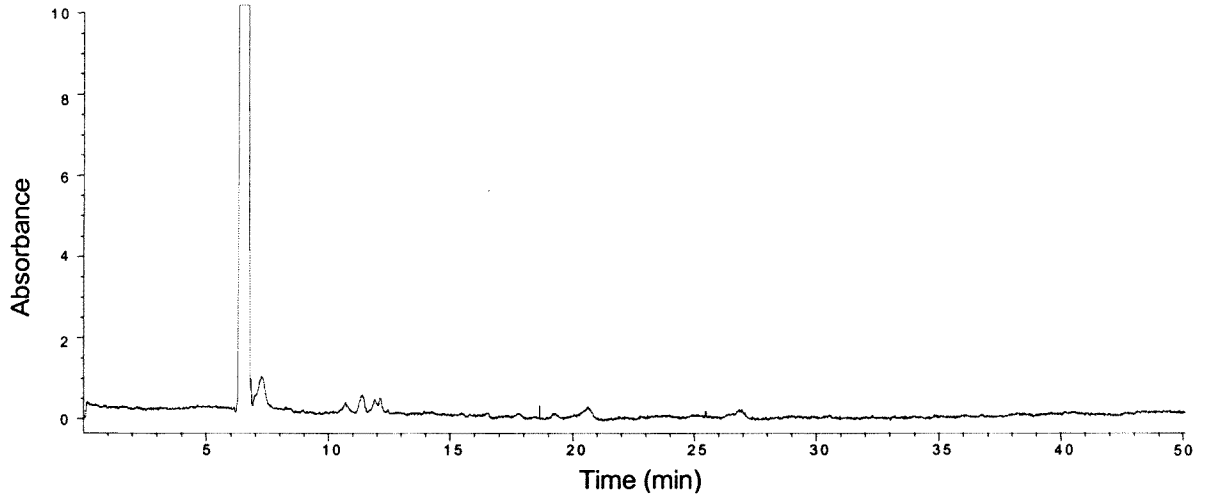


Fig. 4.15 Electropherogram of the culture fluid of *A. pullulans* grown on lignin carbohydrate complex and incubated on sugar cane bagasse (10 mg/ml) for 1 hour (peaks not identified).

4.6.3.3 Lignin carbohydrate complex degradation

Capillary electrophoresis was done on the concentrated culture fluid of the various organisms grown on lignin carbohydrate complex, to evaluate the ability of the enzymes to hydrolyse the lignin carbohydrate complex over a 5 day incubation period. Similar peaks of the release of saccharides from the lignin carbohydrate complex of the various organisms were obtained. The first few peaks in the electropherograms which did not separate properly, possibly constitutes the undigested carbohydrate polysaccharides cleaved from

lignin in the lignin carbohydrate complex. Depending on the degree of polymerization and thus the charge to mass ratio, the larger molecules would elute closer to the ethyl *p*-aminobenzoate peak (Figs. 4.16 - 4.19). These peaks differed between the electropherograms of the culture fluid of the different organisms and was not visible in the electropherograms of *A. pullulans*.

The first monosaccharide peak to elute after the polysaccharide peaks was that of rhamnose; identification of the peaks was done by spiking the sample. The next peak, xylose was one of the highest peaks in all the electropherograms. The presence of this relative high amount of xylose could be an indication that the xylan polymers in the lignin carbohydrate complex had been digested exhaustively. The two peaks which eluted after the xylose peak (Figs. 4.16 – 4.19), could not be identified due to a lack of standards. The next peak, mannose was also the most abundant sugar to be released through the enzymatic action of the various organisms.

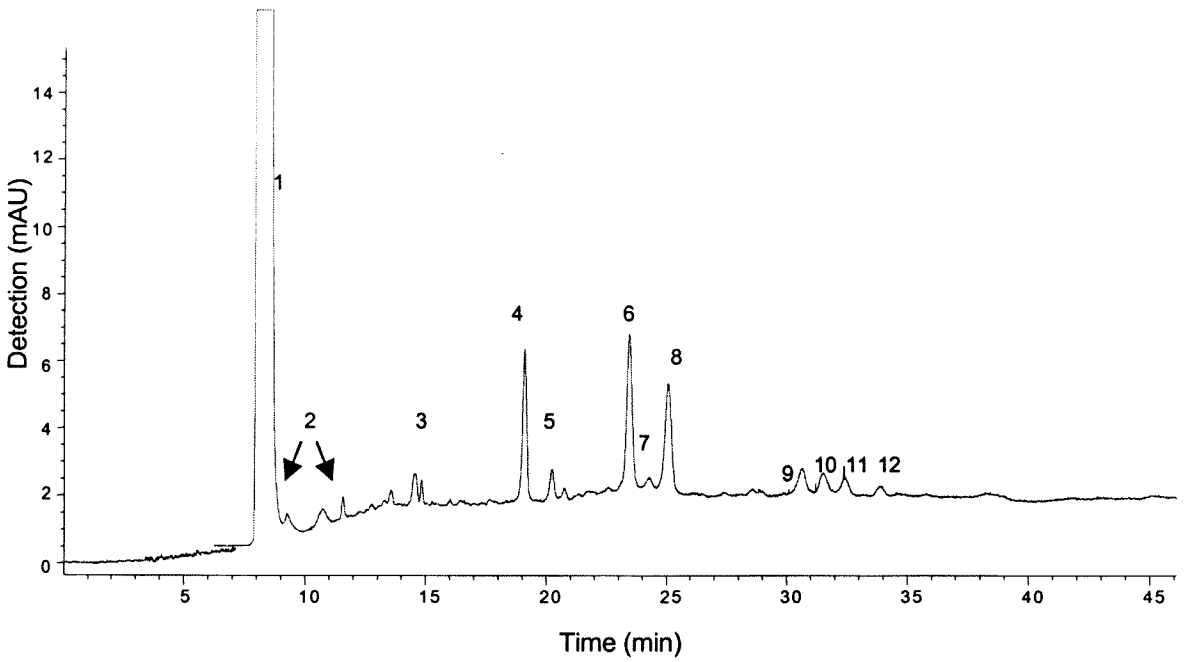


Fig. 4.16 Enzymatic digestion of lignin carbohydrate complex (20 mg/ml) by enzymes in culture fluid of *A. niger* after 5 days. 1 ethyl *p*-aminobenzoate; 2, polymers; 3, rhamnose; 4, xylose; 5, not identified; 6, mannose; 7, glucose; 8, arabinose; 9, galactose; 9,10,11,12, not identified.

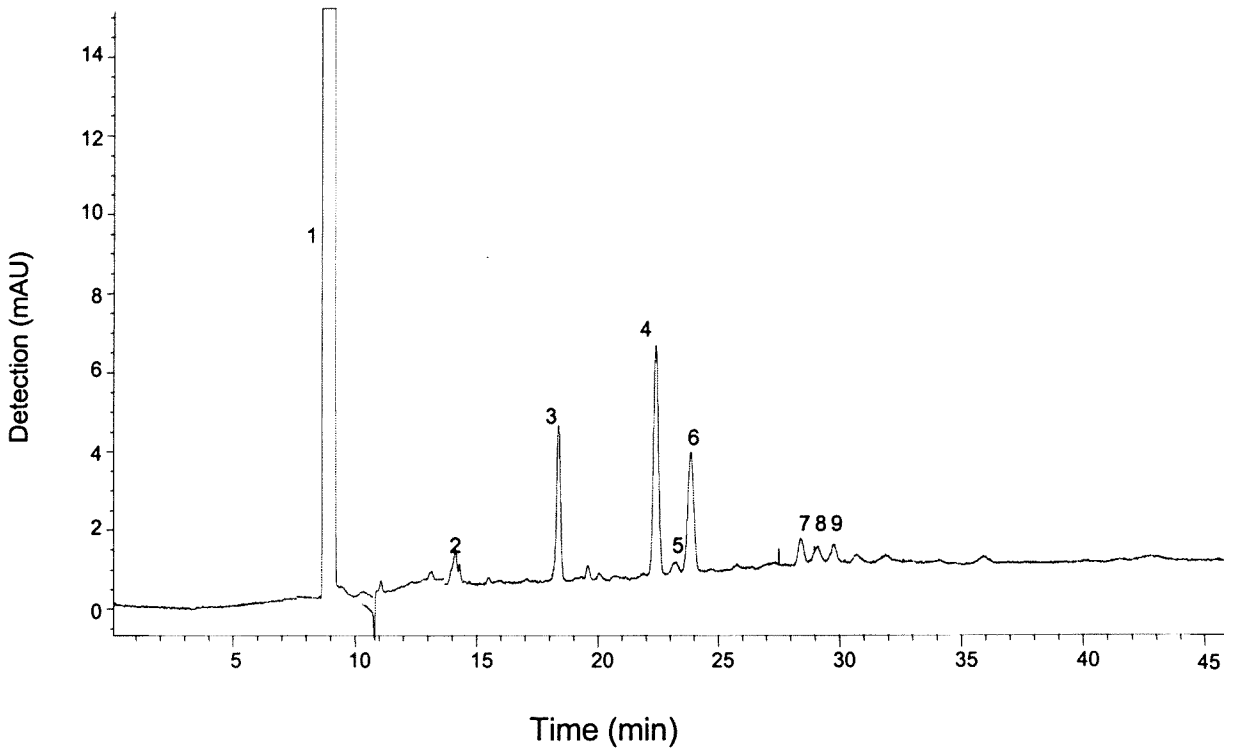


Fig. 4.17 Enzymatic digestion of lignin carbohydrate complex (20 mg/ml) by enzymes in culture fluid of *A. pullulans* after 5 days. 1, ethyl *p*-aminobenzoate; 2, rhamnose; 3, xylose; 4, mannose; 5, glucose; 6, arabinose; 7, galactose; 8,9, not identified.

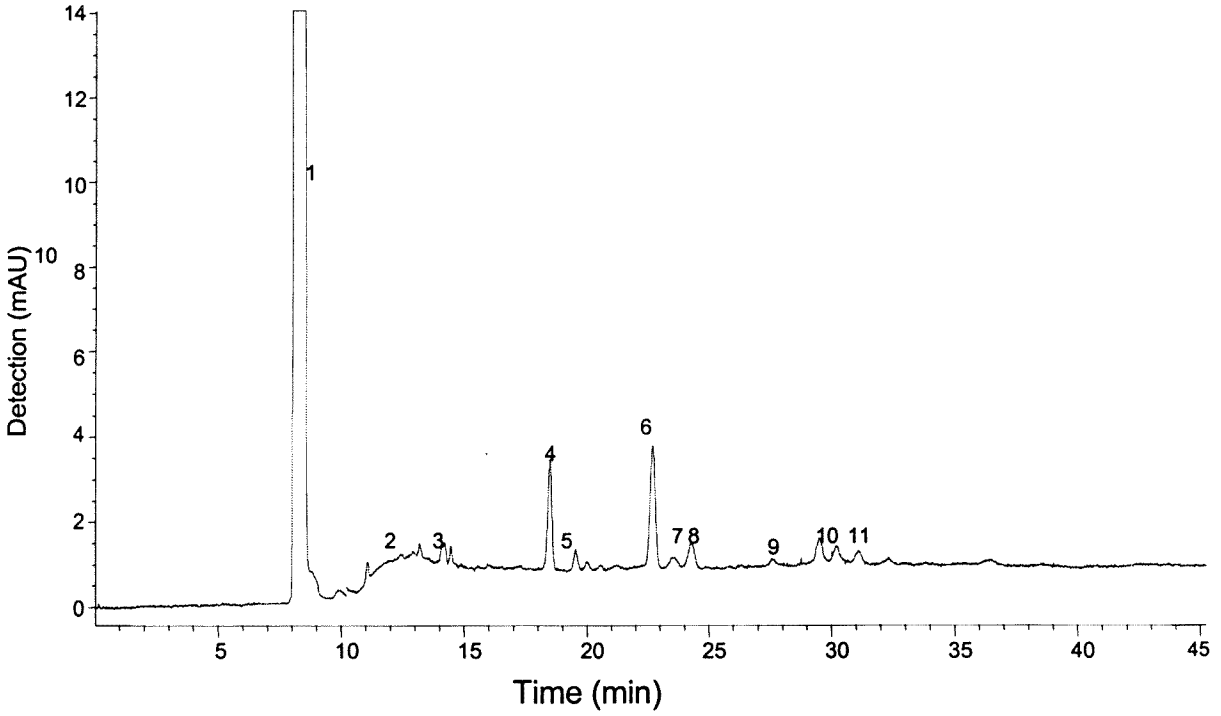


Fig. 4.18 Enzymatic digestion of lignin carbohydrate complex (20 mg/ml) by enzymes in culture fluid of *C. versicolor* after 5 days. 1, ethyl *p*-aminobenzoate; 2, polymers; 3, rhamnose; 4, xylose; 5, not identified; 6, mannose; 7, glucose; 8, arabinose; 9, galactose; 10,11, not identified.

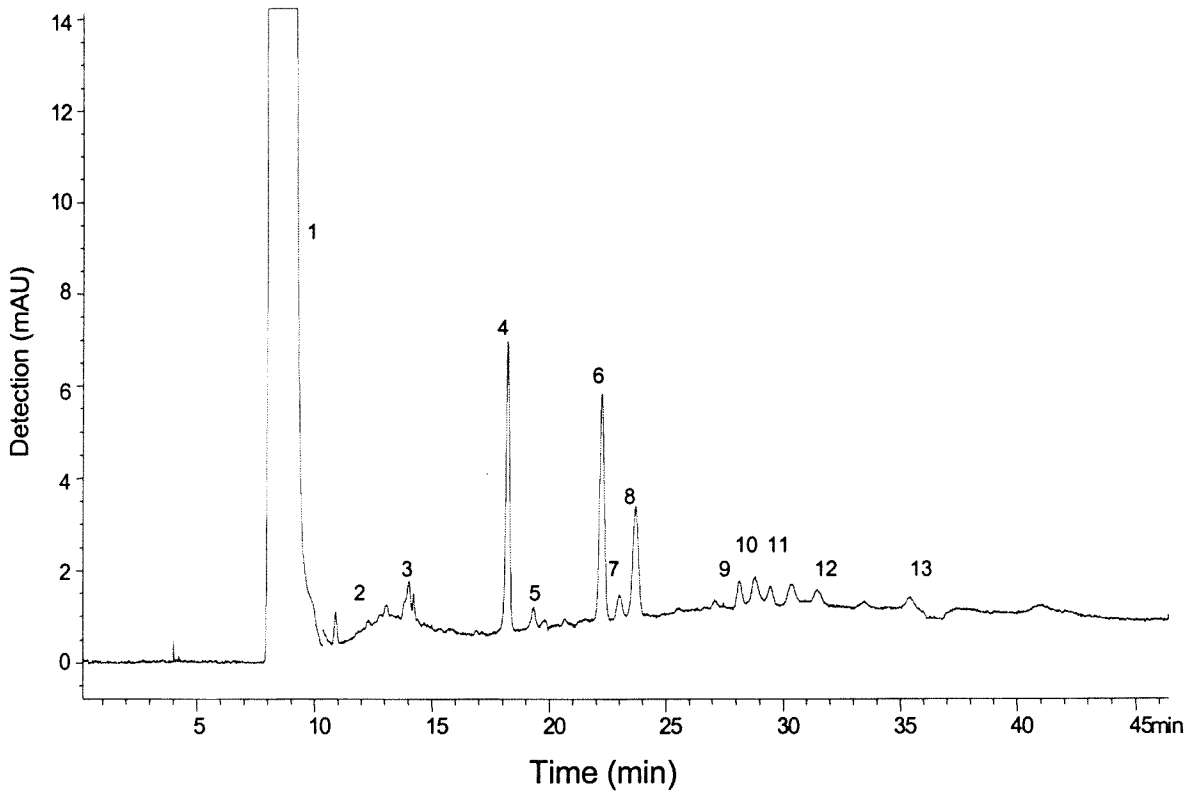


Fig. 4.19 Enzymatic digestion of lignin carbohydrate complex (20 mg/ml) by enzymes of *T. reesei* after 5 days. 1, ethyl *p*-aminobenzoate; 2, polymers; 3, rhamnose; 4, xylose; 5, not identified; 6, mannose; 7, glucose; 8, arabinose; 9, galactose; 10,11,12,13, not identified.

The released mannose could be a result of the mannan present in the lignin carbohydrate complex which has been digested through the action of mannanases and β-mannosidases. The next peak is that of glucose which has been released in relatively low amounts in all the electropherograms.

The peak representing arabinose, shows relatively high amounts of this sugar released. This is surprising as it was presumed that this sugar would be in relatively low amounts, as arabinose is an appendage to the xylan polymer (Fig. 2.4 of section 2). However the trifluoroacetic acid hydrolyses also yielded high amounts of arabinose. There were reports that arabinose could also be present in wheat straw as an arabinan polymer (Saha and Bothast 1998). The relatively high amount of arabinose could be a result of this polymer (should it be present in the lignin carbohydrate complex) being cleaved as well as the individual arabinose units cleaved as appendages from the xylan backbone.

The peak representing galactose, eluted with a number of other peaks with almost the same retention time. Galactose could thus not be identified by comparing the retention times, but needed to be spiked, as was the case with rhamnose. The peaks which eluted in close proximity of galactose were the highest in the electropherogram of *T. reesei*, but could not be identified due to a lack of standards.

From all the electropherograms it appears that the culture fluid from the various organisms contain the same degraded products which have been cleaved from the lignin carbohydrate complex by the various enzymes. This could be an indication that similar enzymes have been secreted by all the organisms to degrade the lignin carbohydrate complex. This coincides with the results obtained from the SDS-PAGE, which had similar bands for these organisms. No glucuronic acid could be detected by the diode array detector in any of the samples. This could indicate that low amounts of glucuronic acid were released, or that the enzyme was not secreted or if it was secreted, it probably belongs to a family which is very specific regarding its degradation. Should the latter be true, this could imply that some structure(s) on the polymer to which glucuronic acid is attached prevents the action of this enzyme. From the electropherograms it appears that the lignin carbohydrate complex has been effectively degraded by the enzymes produced by the different fungi.

Table 4.16 Amounts (mg/g LCC) of monosaccharides released by concentrated enzymes of various organisms. Values in brackets expressed as percentage of the monosaccharides released from the trifluoroacetic acid digestion of lignin carbohydrate complex.

Organism	Rham[a](0.04)[g]	Xyl[b](0.13)[g]	Man[c](0.16)[g]	Glu[d](0.11)[g]	Ara[e] (0.085)[g]	Gal[f] (0.075)[g]
A. niger	1.5 (19%)	25.9 (100%)	33.5 (100%)	2.3 (11%)	8.5 (50%)	3.5(23%)
A. pullulans	3.8 (48%)	26.0 (100%)	9.2 (29%)	15 (7%)	16.7 (100%)	15(100%)
C. versicolor	2.7 (34%)	25.7 (100%)	34.0(100%)	56 (25%)	18.1(100%)	14.8(100%)
T. reesei	2.9 (36%)	27.0 (100%)	33.9 (100%)	48 (22%)	18.04(100%	14.9(100%)

a, Rhamnose; b, Xylose; c, Mannose; d, Glucose; e, Arabinose; f , Galactose; g, concentration (g/g LCC) on sugars (see Table 4.2)

From Table 4.16 it appears that *A. niger* had a complete release of xylose and mannose from the lignin carbohydrate complex. The release of rhamnose, glucose, arabinose and galactose appears to be much less, which could indicate that these monosaccharides were inaccessible for the enzyme and thus not liberated. *A. pullulans* had a complete release of

xylose, arabinose and galactose, but failed to have a complete release of rhamnose, mannose and glucose, which were possibly inaccessible to the enzymes. *C. versicolor* and *T. reesei* achieved complete release of xylose, mannose, arabinose and galactose. These organisms failed to accomplish a complete release of rhamnose and glucose.

4.6.3.4 HPLC analyses of the degradation of the lignin carbohydrate complex

Degradation of the lignin carbohydrate complex by enzymes of concentrated culture fluid of *A. pullulans*, *A. niger*, *T. reesei* and *C. versicolor* grown on lignin carbohydrate complex was also followed using HPLC analyses. From these analyses an attempt was made to evaluate the degradation of the lignin carbohydrate complex by the culture fluid of these organisms. *C. versicolor* culture fluid was not evaluated further as the chromatogram of the treated and control samples were similar, suggesting that the enzymes of the preparation were inactive (data not shown).

The control sample of the lignin carbohydrate complex revealed a signal eluting between 1.5 and 10 minutes which could be attributed to the complexity of the lignin carbohydrate complex. The chromatogram reveals a few large peaks between 1 and 2 minutes followed by a number of smaller peaks. It is assumed that the larger peaks represent the lignin carbohydrate complex, and the smaller peaks are those of oligosaccharides present in the sample. Generally with the PA-100 column, the smaller oligosaccharides would elute before the larger oligosaccharides (Anonymous 1994). However, the lignin carbohydrate complex possibly did not bind to the column and would elute in the void volume of the column (Fig. 4.20).

The peak areas from these chromatograms could however not be quantified, as baseline separation could not be achieved for the either the larger or the smaller peaks. Furthermore suitable standards could not be obtained to identify these peaks. Figure 4.20 B indicates a substantially degraded lignin carbohydrate complex by the enzymes of *T. reesei* after an incubation period of 12 hours, compared to the peak height of the control (Fig. 4.20 A).

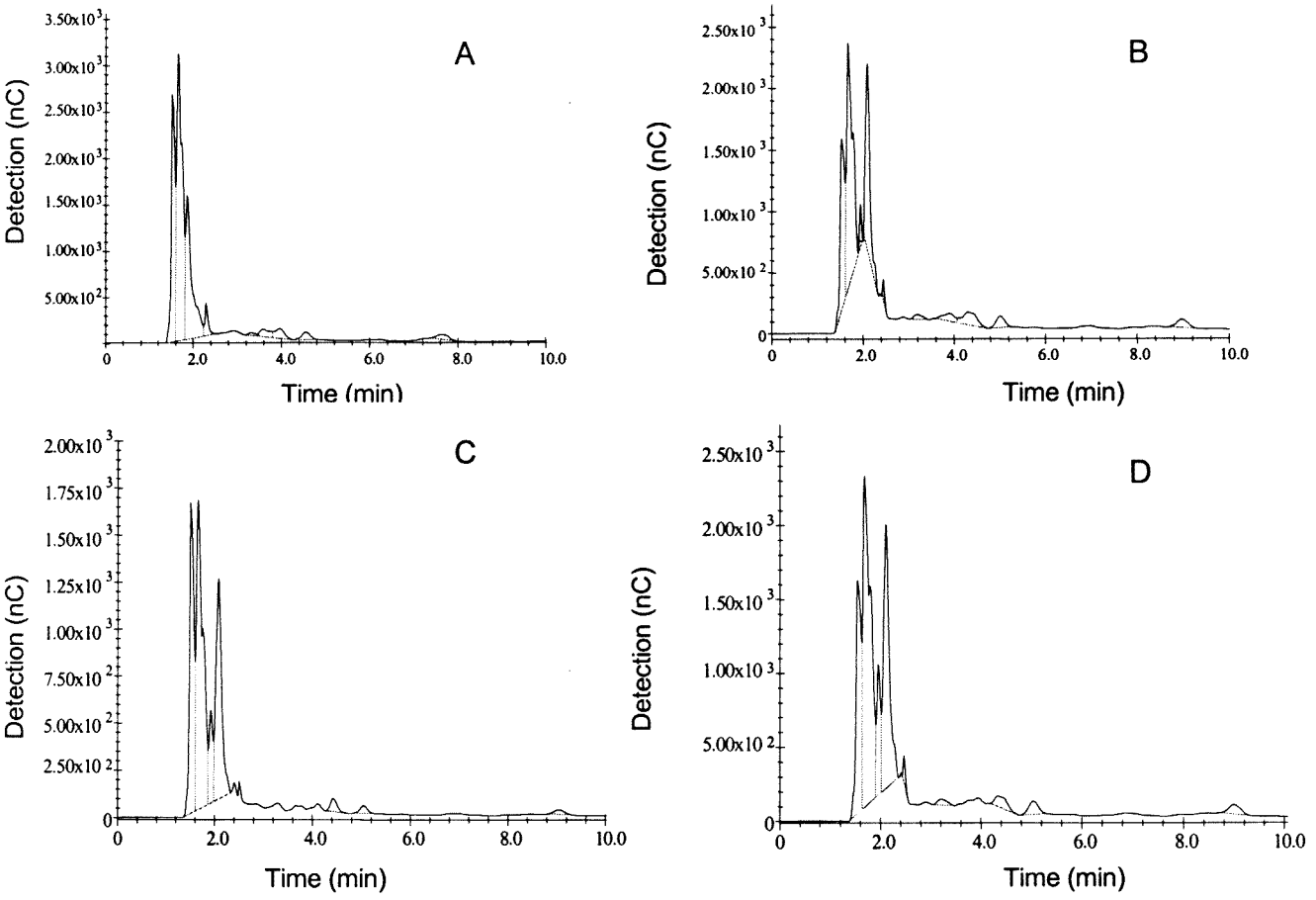


Fig. 4.20 HPLC chromatogram of the intact lignin carbohydrate complex (A), 12 h treatment with culture fluid of *A. pullulans* (B), 12 h treatment with culture fluid of *T. reesei* (C), 12 h treatment with culture fluid of *A. niger* (D).

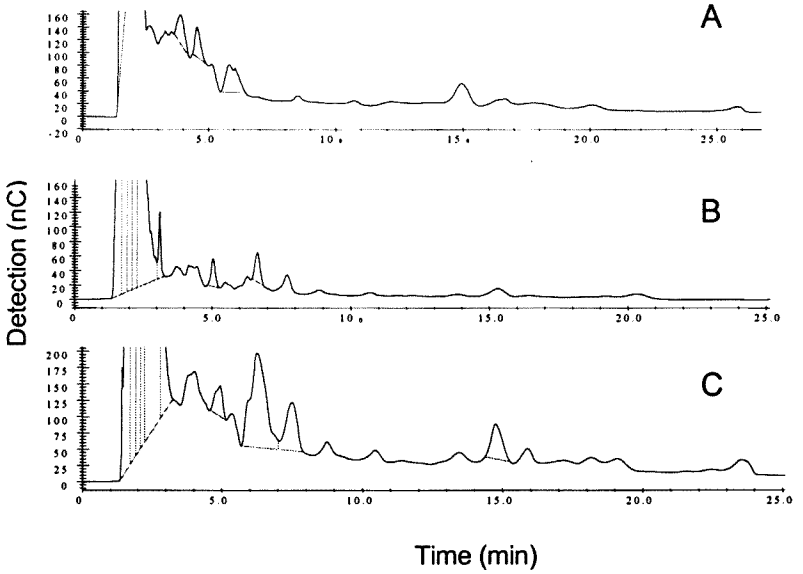


Fig 4.21 Comparison of the various HPLC chromatograms of liberated saccharides (12 h) from the lignin carbohydrate complex treated by the culture fluid of *A. niger* (A), *A. pullulans* (B) and *T. reesei* (C).

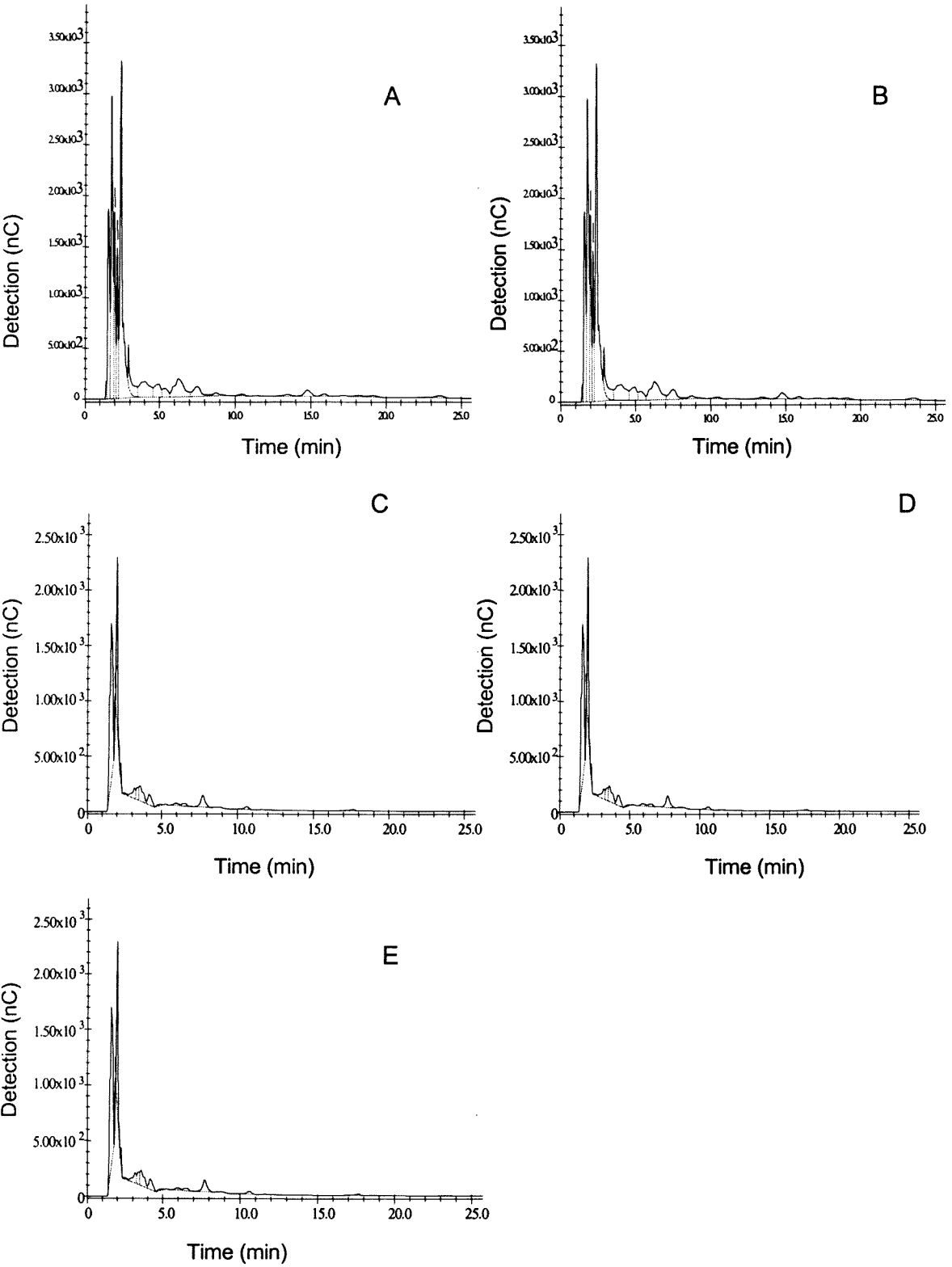


Fig. 4.22 Chromatograms of the different time intervals of the degradation of the lignin carbohydrate complex by the culture fluid of *T. reesei*, A, 1 h; B, 12 h; C, 36 h; D, 72 h; E, 96 h.

Peaks in the control sample (Fig 4.20 A) with retention times between 1 and 2 minutes show a significant decline in peak height when the lignin carbohydrate complex is treated with culture fluid for 12 h (Figs. 4.20 B-C). As treatment proceeded, the peaks between 1 and 2 minutes decreased in height whereas the peaks between 2 and 25 minutes representing oligosaccharides increased in height and number (data not shown). A comparison between the chromatograms of the released smaller saccharides of these three organisms is given in Fig. 4.21. From these chromatograms it appears that *T. reesei* culture fluid liberated the highest amount of the smaller saccharides as these peaks have greater height compared to the peak heights in chromatograms of *A. pullulans* and *A. niger* treated samples. Lack of suitable standards precluded identification of the peaks. After 72 h treatment, no change in the pattern of the chromatograms were observed which suggests that portions of the lignin carbohydrate complex was resistant to further degradation (Fig. 4.22).

5. Discussion

5.1 Extraction of lignin carbohydrate complex from wheat straw

The extraction of the lignin carbohydrate from wheat straw proved to be difficult according to the method of Azuma *et al.* (1988), as solubility in water and yield was low. Wallace *et al.* (1995) and Lam *et al.* (2001) following a similar DMSO/methanol extraction after dioxane extraction and found that the lignin carbohydrate complex consisted of methanol soluble and methanol insoluble fractions. These results suggest that the lignin carbohydrate complex appears to be heterogeneous. Another approach to the extraction of the lignin carbohydrate complex is by using specific enzymatic digestion of the plant material. Fry (1983) reported on the extraction of lignin carbohydrate complex by driselase, which consists of a mixture of fungal exo-and endo-hydrolases but lacking feruloyl-esterase. It was found that this approach yielded more than 60 % of the ferulic acid in the form of two feruloyl disaccharides, namely 3-*O*-(3-*O*-feruloyl-α-L-arabinopyranosyl)-L-arabinose and 4-*O*-(6-*O*-feruloyl-β-D-galactopyranolsyl)-D-galactose. Luonteri *et al.* (1999) and Borneman *et al.* (1990b) reported on the isolation of *O*-[5-O-(trans-feruloyl)-α-L-arabinofuranosyl]-(1-3)-*O*-β-D-xylopyranosyl-(1-4)-D-xylopyranose (FAXX) and *O*-[5-O-(trans-*p*-coumaroyl)-α-L-arabinofuranosyl]-(1-3)-*O*-β-D-xylopyranosyl-(1-4)-D-xylopyranose (PAXX) respectively. These substrates are useful to conduct specific assays like the determination of esterase activity, but substrates like these do not allow for a more comprehensive study of the entire lignin carbohydrate complex.

5.2 Characterization of lignin carbohydrate complex and breakdown products

5.2.1 Sugar composition of the lignin carbohydrate complex

The trifluroacetic acid digestion of the lignin carbohydrate complex using capillary electrophoresis to reveal the hydrolysed products, showed that the complex consisted of six sugars, which are mannose, xylose, arabinose, rhamnose, glucose and galactose. Fucose

which was reported to be present in barley straw and ryegrass (Wallace *et al.* 1995), could not be quantified in this study using capillary electrophoresis and HPLC. However there were a number of peaks in the electropherograms which could not be identified, and fucose standard was not available. These peaks eluted within the retention times of the other monosaccharides, suggesting that those unidentified peaks could also be monosaccharides. Mannose was the most abundant sugar in the lignin carbohydrate complex of wheat straw and the amount was much higher compared to results obtained in rice straw (Azuma and Koshijima 1988). This could suggest that the polymer of mannose (mannan) has a much higher degree of polymerization in the lignin carbohydrate complex of wheat straw compared to the lignin carbohydrate complex in rice straw (Azuma and Koshijima 1988). Except for xylose and arabinose, all the other sugars appeared to be in higher concentration than that of the lignin carbohydrate complex in rice straw. It appears that the lignin carbohydrate complex in rice straw has large quantities of xylose, suggesting a very high degree of polymerization of this monosaccharide. Rhamnose, which had the lowest concentration in the lignin carbohydrate complex of wheat straw could not be quantified in the lignin carbohydrate complex of rice straw. This sugar also had a much higher concentration in the lignin carbohydrate complex of wheat straw compared to the lignin carbohydrate complexes in oats, barley straw and rye grass (Lam *et al.* 2001, Wallace *et al.* 1995). Jeffries (1990) suggested that rhamnose might be involved in lignin carbohydrate bonds in the lignin carbohydrate complexes of grasses. High amounts of glucose have been obtained in the lignin carbohydrate complex of oats, barley straw and rye grass. These amounts were almost three fold higher than the amount for glucose obtained in this study (Azuma and Koshijima 1988, Lam *et al.* 2001, Wallace *et al.* 1995). This points to the cellulose, from which the glucose was released in the lignin carbohydrate complex of wheat straw to be present in very low amounts or that it could have been destroyed during the trifluoroacetic acid hydrolysis. Glucuronic acid found in this study could not be detected or was absent in

the lignin carbohydrate complexes of rice straw, barley straw, oats or rye grass (Wallace *et al.* 1995, Lam *et al.* 2001, Azuma and Koshijima 1988). Similar values for the hydroxycinnamic acids, ferulic- and *p*-coumaric acids were obtained in all the abovementioned grasses, with the exception of rice straw where a relatively high *p*-coumaric acid concentration was found (Azuma and Koshijima 1988).

5.2.2 Lignin determination in the lignin carbohydrate complex

The lignin content of plant cell walls appears to vary widely between 2 and 80 % lignin when the acetyl bromide analyses is used but is also dependent upon the solvent concentration (Fukushima and Hatfield 2001). For example Lam *et al.* (2001) reported a lignin content of 73.2 % in oats using 90 % dioxane as extraction solvent, compared to 44.6 % in a 50 % dioxane solution. On the other hand, Wallace *et al.* (1995) reported a lignin content of 6.18 % in grass using the same method. The reasons for the variation between grasses could be the syringyl/guaiacyl ratio, how these molecules are polymerized to form the lignin structure and the age of the plant (Wallace *et al.* 1995). The lignin content in this study which was determined to be 20 % of the lignin carbohydrate complex of wheat straw falls within the range of the results obtained in the samples of rye grass, barley straw, oats and rice straw (Wallace *et al.* 1995, Lam *et al.* 2001). Similar results were obtained when lignin was determined in wheat straw (32.6 %), without the lignin carbohydrate complex being extracted (Durot *et al.* 2002). The individual sugar concentration appears to be lower in wheat straw compared to the individual sugar concentration in the extracted lignin carbohydrate complex. However all the individual sugars which are found in the extracted lignin carbohydrate complex could also be found in wheat straw.

The molecular weight analyses yielded a peak with a range between ~1 000 and ~15 000 kDa. An apparent molecular weight of 5 900 kDa was obtained as a result of the highest peak obtained in the chromatogram. The wide range in molecular weights that was obtained

in this study could be attributed to the fact that in *Graminaceous* plants, the lignin does not form a single macromolecular complex throughout the cell wall, but exists as different small units attached to the carbohydrate in various ways (Wallace *et al.* 1995).

5.2.3 Analytical techniques for the analyses of the lignin carbohydrate complex

High performance anion exchange chromatography with pulsed amperometric detection has previously been shown to separate carbohydrates in their enolate form using NaOH at pH 10-14 (Torto *et al.* 1995). Another advantage of high performance anion exchange chromatography with pulsed amperometric detection is the fact that the carbohydrates do not need to be derivitized. However there are drawbacks to this system, namely limited choice of buffer conditions and an only moderate separation efficiency (Paulus and Klockow-Beck 1999). The PA10 and MA1 columns of Dionex have different abilities to separate carbohydrates, and the appropriate column choice would be dictated by the analytes present, the analytes of interest, analyte concentrations and the desired analyses times (Hanko and Rohrer 2000). These criteria, also has a remarkable influence on the retention times of the various analytes, which could differ from standards injected. It is thus important that peak identification be verified with methods like mass spectrometry or even by spiking the sample. As indicated, the lignin carbohydrate complex which was degraded enzymatically and chemically could not be separated properly by using the MA1 or PA10 columns. Very low amounts of NaOH (2 mM) was used to achieve separation. However lower NaOH concentrations limits the detector's response, as well as a much lower resolution between peaks. Clean-up steps like microdialysis sampling, prior to injection into the HPLC could remove most of the impurities which limits the separation efficiency of the HPLC and thus, better separation achieved (Torto *et al.* 1995).

Capillary electrophoresis of carbohydrates appeared to be more sensitive in separating carbohydrates under the conditions which these experiments were done, as the results were

very reproducible. However, there are many variables of the system that needs to be optimised in order to achieve effective separation using capillary electrophoresis. Capillary electrophoresis, like any other separation technique, unfortunately does not allow for the simultaneous separation of complex compounds consisting of a variety of molecules with diverse properties like lignin carbohydrate complex. Reports were made in the identification of different degrees of polymerization of macromolecules which can be achieved by capillary electrophoresis after digestion. (Peng *et al.* 2000). Lignin, ferulic- and *p*-coumaric acid might be separated by capillary electrophoresis, but at a lower buffer pH and concentration, compared to the buffer used for carbohydrate separation. Fortunately these compounds can be separated without pre-column derivitization, as they do possess chromophoric groups. These analyses could not be performed as the option of capillary electrophoresis with laser induced fluorensece was not available. The process of reductive amination of carbohydrates by ethyl *p*-aminobenzoate prior to injection appears to be very successful in their application to separate the carbohydrates as a derivitized borate complex. It is however important that an excess amount of ethyl *p*-aminobenzoate reagent be applied to the saccharides. This excess amount of ethyl *p*-aminobenzoate should however be removed by filtration, prior to injection, as it could interfere with the separation process (Dahlman *et al.* 2000, Honda 1996, Paulus and Klockow-Beck 1999).

In addition to the successful application of ethyl *p*-aminobenzoate, it is also important that the elution period should not exceed 60 min with the same buffer, as the buffer becomes ionized during the separation period. Drifting of peaks were frequently experienced as a result of the depletion of the borate buffer where elution periods were extended to 80 min including the flushing between cycles with 0.1 M NaOH. Peak shifting was also minimized by flushing between cycles with the same borate buffer as used for separation. An initial borate buffer concentration of 400 mM was used for the analyses of the hydrolysed lignin carbohydrate complex. The separation obtained at 400 mM appeared to have poor

resolution amongst the different peaks, and it was decided to increase the borate buffer concentration to 440 mM. A borate buffer concentration of 440 mM has its disadvantage, as heat generation takes place (exceeding 25°C), which can damage the system. A lower voltage (15 kV) had to be applied to compensate for the higher borate buffer concentration, and this resulted in longer retention times for the different carbohydrates. From literature it appears that this problem could be overcome by using an internal cooling system (Dahlman *et al.* 2000). Heat generation (exceeding 25°C) also results in poor separation, migration profiles, electrophoretic mobilities and resolution between adjacent peaks (Dahlman *et al.* 2000). This might be the reason why glucose eluted before arabinose in this study, and not visa versa as indicated in literature (Dahlman *et al.* 2000). It was initially thought that the impurities in the sample could be responsible for this observation, however the mixture of pure standards also had the same elution order. Despite the various problems which were experienced with the analyses, capillary electrophoresis equipped with a diode array detector could effectively separate all the carbohydrates which were present in the sample, whether obtained enzymatically or chemically. From these large amounts of monosaccharides present in the sample, it might be that these monosaccharides were cleaved from polymers like mannan, xylan and arabinan which could have been present in the lignin carbohydrate complex.

5.3 Growth of fungi on lignin carbohydrate complex

Jeffries (1994) reported the ability of *A. pullulans, A. niger, T. reesei, C. versicolor* and *P. chrysosporium* to degrade lignin carbohydrate complexes. These organisms appeared to produce some of the enzymes to degrade lignin carbohydrate complex. Reports were also made for *P. sanguineus* strains to degrade lignin associated plant material (Tekere *et al.* 2001). In another study, *P. coccineus* appeared to have the ability to degrade wood material to a certain extent (Watanabe *et al.* 2003). Nakamura *et al.* (1999) also reported on

B. adusta which produced lignin degrading enzymes when grown on wood material. These organisms were subsequently used in this study, since they appeared to have the ability to degrade lignin associated plant material. From the preliminary growth studies of the different fungi performed on the lignin carbohydrate complex, all the strains with the exception of *B. adusta* could utilize the lignin carbohydrate complex as carbon source. It was discovered after these growth studies that *B. adusta* could have lost the ability to grow, as the carbon source from which this organism was cultivated could have been depleted. Effective growth of *A. niger, P. sanguineus 294* and *T. reesei* on the lignin carbohydrate complex of wheat straw was already observed after 24 h. However *P. sanguineus 294* did not show an increase in growth after 24 h. *A. niger, A. pullulans, C. versicolor, P. chrysosporium* and *T. reesei* showed a substantial amount of growth after 5 days, suggesting that these organisms could probably utilize the lignin carbohydrate complex as carbon source under limiting nitrogen conditions. Thus, these organisms are probably capable of producing enzymes which are able to partially degrade the lignin carbohydrate complex.

5.3.1 Enzymes involved in the degradation of the lignin carbohydrate complex

It was reported that a variety of enzymes are involved in the degradation of lignin carbohydrate complexes, like cellulases, xylanases, mannanases, peroxidases (Jeffries 1990). In addition to these accessory enzymes like acetyl xylan esterase, ferulic and *p*-coumaric acid esterase, α-L-arabinofuranosidase and α-4-*O*-methyl glucuronidases are also critical in the early stages of hemicellulose utilization (Jeffries 1990). The activity studies on the culture fluid of the various organisms revealed activity for xylanase, xylosidase, arabinofuranosidase and ferulic acid esterase. From these results, it could be that *A. niger, A. pullulans, C versicolor* and *T. reesei* produced these enzymes as there was a substantial amount of enzyme activity found in the culture fluid for these organisms. The xylanase activity of all the organisms were much higher when grown on birchwood xylan, compared to

the xylanase activity of the lignin carbohydrate complex. Leathers (2003) reported a xylanase activity of 294 U/ml (when grown on arabinoxylan) for *A. pullulans*, which is slightly higher than the results obtained in this study. Xylanase activity was also reported for the enzymes of *A. pullulans* (Christov and Prior 1996). The xylanase activity was reported to be 108 U/ml which is much lower than the xylanase activity for this study on the lignin carbohydrate complex of wheat straw. Xylanase activity was also found in culture fluids of *T. reesei* and *A. niger* when grown on wheat straw (Thygesen *et al.* 2003), where the activity was found to be 81 and 115 U/ml, respectively. These amounts are much lower than the results obtained in this study (400 and 196 U/ml). *C. versicolor* also expresses a xylanase enzymes, which was reported in a glucose culture (10 U/ml) (El-Nassar *et al.* 1996). The xylanase activity of the fungi grown on the lignin carbohydrate complex was higher compared to the xylanase activity of the fungi when grown on wheat straw. Comparing these results with regard to the various substrates it might be that xylanase is secreted by the organisms in various amounts depending on substrate accessibility.

The xylosidase activity obtained in this study was hundred fold higher compared to results obtained in previous studies where a value of 0.001 U/ml (grown on oat spelt arabinoxylan) was obtained, for *A. pullulans* when grown on birchwood xylan (Leathers 2003). The xylosidase activity was also much higher in the culture fluid of *A. pullulans* (2.70 U/ml) when grown on lignin carbohydrate complex compared to the results obtained from Leathers (2003), which was 0.001 U/ml grown on oat spelt arabinoxylan. Xylosidase activity was also reported for *T. reesei* and *A. niger* when grown on wheat straw (Thygesen *et al.* 2003). The xylosidase appeared to be 0.063 U/ml and 7.5 U/ml for *A. niger* and *T. reesei* respectively. The highest xylosidase activity in this study was achieved in the culture fluid of *A. niger* (34 U/ml). The much higher xylosidase activity found in this study may point to the lignin carbohydrate complex acting as an efficient inducer of the enzymes. This could imply that a number of fragments of xylan could have been accessible to induce xylosidase activity.

The highest arabinofuranosidase activity in this study was achieved in the culture fluid of *A. niger* (3.04 U/ml). It was found that *T. reesei* also secretes an arabinofuranosidase (Olsson *et al.* 2003). The arabinofuranosidase activity (0.006 U/ml) for this organism grown on sugar beet pulp was much lower than the results obtained in this study for the same organism grown on lignin carbohydrate complex (3.01 U/ml). Similar to xylanase and xylosidase, it appears that induction of this enzyme is substrate dependant. The highest ferulic acid esterase activity (1.06 U/ml) in this study was found in *A. pullulans*. Rumbold *et al.* (2003b) also reported ferulic acid esterase activity for this organism, and secretion of this enzyme also appears to be substrate dependant

SDS-PAGE results indicated an array of enzymes produced by all these organisms ranging from 8 kDa – 200 kDa. From SDS-PAGE gels it might be that secretion of the various enzymes in the different fungi are substrate dependent, as different secretion levels are obtained from SDS-PAGE on various substrates. All the fungi used in the study only secreted a few enzymes when grown on substrates like glucose and xylose. These enzymes which are shown by SDS-PAGE analyses, were almost similar amongst *A. niger, A. pullulans, C. versicolor* and *T. reesei. T. reesei* and *A. pullulans* might have secreted a high amount of the same enzyme with an apparent molecular weight of ~30 kDa (Biely *et al.* 1997) *A. niger* secreted a large amount of enzyme corresponding to the 8.4 kDa band. Faulds and Williamson (1994) reported on ferulic acid esterase for two *A. niger* strains with a molecular weight of 29.7 and 145 kDa respectively. Kuroyama *et al.* (2001) reported a β-glucuronidase from *A. niger* with an apparent molecular weight of ~70 kDa. An α-L-arabinofuranosidase of *T. reesei* was by purified by Poutanen (1988) and had an apparent molecular weight of 53 kDa. These reports indicate the presence of accessory enzymes present in these organisms to cleave the side chains from the polymers as found in the lignocellulotic material. SDS-PAGE analyses in this study indicates bands which could not be identified but corresponds to those found in literature.

5.4 Enzymatic degradation of lignin carbohydrate complex

From the results obtained by hydrolysis by trifluroacetic acid, between 60- and 70 % consists of carbohydrate. The HPLC chromatograms revealed that despite the high concentration of enzymes secreted by the different organisms, a portion of the lignin carbohydrate complex still remained intact. No apparent degradation of the lignin carbohydrate complex took place after 72 h, as the peak heights and peak areas remained the same after 72 h. The peaks indicating the apparent liberated monosaccharides also did not increase after 72 h. This could indicate that the lignin carbohydrate complex became inaccessible for the enzymes of *T. reesei, A. pullulans* and *A. niger*, and that no carbohydrate could be liberated from the lignin carbohydrate complex.

In literature it is quite often shown that synergy plays an important role in the degradation of complex structures. Synergy refers to enzyme cooperativity in order to hydrolyse the substrate. These reports indicate that synergy depends on the various substrates, the different compounds in the substrate, the different bonds in the substrate and the various side chains which occur in the substrate (Christov and Prior 1993, Ademark *et al.* 1998). It is thus not surprising that the results obtained in this study, regarding the liberation of molecules from the lignin carbohydrate complex differs substantially compared to results obtained from studies on similar grasses like, rice straw, corn straw, sugar cane and other grasses (Azuma and Koshijima 1988, Lam *et al.* 2001, Wallace *et al.* 1995). A higher glucose content was observed in barley straw, rye grass and oats. These values were almost thrice the amount obtained in the lignin carbohydrate complex of wheat straw and almost fifty times higher than the glucose content of rice straw. However, higher amounts of arabinose, mannose, galactose and rhamnose were obtained in the lignin carbohydrate complex of wheat straw compared to the lignin carbohydrate complex in the other grasses. Capillary electrophoresis also revealed different degradation profiles on the degradation of xylan by the various organisms for the same time interval. Xylotetraose which was detected

in the culture fluid of *A. niger* and *C. versicolor* could not be detected in the culture fluids of *A. pullulans* and *T. reesei*. This could indicate that xylanase (which breaks down xylan to smaller fragments) acted in synergy with xylosidase to release xylose. However this synergistic effect apparently differs between *A. niger, C. versicolor, A. pullulans and T. reesei* over the same time interval.

Capillary electrophoresis of the enzymatic hydrolysis of the lignin carbohydrate complex also revealed that some of the released sugars had a 100 % hydrolysis rate when the trifluoroacetic acid hydrolysis is used as control. *C. versicolor* and *T. reesei* had a complete release of mannose, arabinose and galactose. *A. pullulans* completely released arabinose and galactose, whereas *A. niger* only had a complete release of mannose. These results might indicate that despite similar enzymes of the various organisms being released, they act in different ways to degrade the substrate or that inaccessibility of the substrate might play a role in the degradation thereof.

5.5 Future work in the analyses of lignin carbohydrate complex

There are many aspects in this study that need to be addressed, as inconclusive results were obtained. Optimization of the isolation of the lignin carbohydrate complex from wheat straw could be useful to characterize the complete lignin carbohydrate complex and not only portions of it. The isolated lignin carbohydrate complex needs to be hydrolysed in a mild way, without destroying any of the components in order to characterize it. Characterization of this isolated material of the lignin carbohydrate complex could yield additional information substantiating previous reports. The molecular mass of the lignin carbohydrate complex could probably be detected with more advanced equipment which could simultaneously detect the lignin as well as the carbohydrate of the entire complex.

Capillary electrophoresis should be optimized regarding the separation of the phenolic compounds present in the sample. It would be desirable if the degradation of the lignin

carbohydrate complex by the various enzymes could be followed over a time period using capillary electrophoresis and view the degradation profile of the degraded substrate. This could shed light on the different degrees of polymerization obtained after certain time intervals. Quantitative analyses should also be performed on the lignin carbohydrate complex which might be inaccessible to the enzymes after degradation ceases.

SDS-PAGE gels revealed the presence of a variety of proteins. These proteins need to be purified, identified and characterized to give an indication of the enzymes involved in the degradation of lignin carbohydrate complex.

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"I have discovered the secret that after climbing a great hill, one only finds that there are many more hills to climb. I have taken a moment here to rest, to steal a view of the glorious vista that surrounds me, to look back on the distance I have come. But I can rest only for a moment, for with freedom come responsibilities and I dare not linger for my long walk is not yet ended."

Nelson R. Mandela