

**Cloning of a novel esterase gene from
Bacillus pumilus and its characterisation in
*Escherichia coli***

BY

ANTON PIETERSE



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degree of Masters of Science at the University of Stellenbosch**

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Supervisor: Prof. W.H. van Zyl

DECLARATION

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

A. Pieterse

SUMMARY

Esterases play a variety of roles in nearly every aspect of life ranging from cellular metabolism, signal transduction to defence mechanisms in plants. One aspect of esterases that recently is receiving more attention is the role esterases play in the degradation of plant material. With fossil fuels (coal and oil) estimated to run out in the next 20 to 30 years, renewable sources such as plant biomass are becoming increasingly important. Plant biomass contains hemicellulosic and cellulosic materials that need to be degraded to their different constituents before they can be optimally used for the production of commodities.

Although the enzymes needed to hydrolyse the xylan backbone (xylanases and β -xylosidases) are important, enzymes that remove side chains from the polymer are equally important. They facilitate hydrolysis by xylanases and β -xylosidases and will improve the availability of monomeric sugars for utilisation when used in conjunction with other xylanolytic enzymes. Many of these side-chains are esters and they need to be removed through the action of esterases, either directly from the xylan backbone or from shorter xylo-oligomers.

An existing genomic DNA library of *Bacillus pumilus* in *Escherichia coli* was screened for the presence of an acetyl esterase encoding gene. Positive clones were identified by the formation of clearing zones on plates containing glucose pentaacetate. Plasmid DNA was isolated from a positive *E. coli* clone. The DNA insert was sequenced and found to contain two open reading frames, one of which encoded a novel esterase (*estA*). Using different primers the gene was amplified by

polymerase chain reaction and inserted into an inducible expression vector (pKK223-3) for expression in *E. coli*. The plasmid was introduced into *E. coli* and the esterase activity determined, using the chromogenic substrate α -naphthyl acetate. Activity levels decreased shortly after induction with IPTG and therefore plasmid pAP4 was used for enzymatic assays. Cultures containing plasmid pAP4 produced extracellular activity of 2.5 nkatal/ml. The pH and temperature optima as well as temperature stability of the enzyme was determined. The enzyme exhibited optimal activity at pH 6 and 60°C and was stable at 60°C after 2 h. Enzyme assays on different substrates yielded activity on methylumbelliferyl butyrate and methylumbelliferyl acetate in addition to the glucose pentaacetate and α -naphthyl acetate. The *estA* gene was cloned into a yeast expression vector between the *PGK* promoter and terminator sequences for expression of the gene in *Saccharomyces cerevisiae*. The *estA* open reading frame was also fused to the MF α 1 secretion signal for secretion of the protein from *S. cerevisiae*. The expression vector was successfully transformed into *S. cerevisiae*, but no extracellular activity was detected. Only low intracellular activity of 0.260 nkatal/ml was detected in *S. cerevisiae*.

OPSOMMING

Esterases speel 'n verskeidenheid van rolle in feitlik elke aspek van lewe, van sel metabolisme, sein transduksie tot verdedigingsmeganismes in plante. Een aspek van esterases wat al hoe meer aandag geniet, is die rol wat esterases in die afbraak van plant en plantmateriaal speel. Met olie- en steenkoolbronne wat na beraming oor 20 tot 30 jaar tot niet sal gaan, raak die rol wat hernubare bronne speel al hoe belangriker. Plantbiomassa bevat sellulose en hemisellulose wat tot die verskillende komponente afgebreek moet word voordat dit optimaal vir die vervaardiging van produkte aangewend kan word.

Alhoewel die ensieme wat vir die hidrolise van die xilaanruggraat benodig word, (xilanases en β -xulosidases) belangrik is, is die ensieme wat die sygroepe vanaf die polimeer verwyder ewe belangrik aangesien hulle die hidrolise deur xilanases en β -xulosidases bevorder. Wanneer hulle saam met die ander xilanolitiese ensieme gebruik word, sal hulle die beskikbaarheid van monomeriese suikers vir fermentasie verhoog. Baie van hierdie sygroepe is esters en hulle word deur die aksie van esterases verwyder, of direk van die ruggraat, of vanaf korter xilo-oligosakkariede.

'n Bestaande genoom DNA biblioteek van *Bacillus pumilus* in *Escherichia coli* is vir die teenwoordigheid van 'n asetielesterase-koderende geen gesif. Positiewe klone is deur die vorming van 'n sone op plate wat glukose pentaasetaat bevat, geïdentifiseer. Die DNA-invoeging van die positiewe *E. coli*-kloon se DNA-volgorde is bepaal en twee ooplesrame is gevind waarvan een vir 'n unieke esterase (*estA*) kodeer. Met behulp van verskillende inleiers is die geen met die polimerasekettingreaksie (PKR)

geamplifiseer en in 'n induseerbare promotor vir uitdrukking in *E. coli* gekloneer. Die plasmied is getransformeer in *E. coli* en aktiwiteit is bepaal deur α -naftielasetaat te gebruik. Vlakke van aktiwiteit het kort na induksie met IPTG weer gedaal en daarom was plasmied pAP4 vir ensiematiese toetse gebruik. *E. coli*-transformante met plasmied pAP4 het ekstrasellulêre aktiwiteit van 2.5 nkatal/ml gelever. Die pH en temperatuur optima sowel as die temperatuurstabiliteit van die ensiem was bepaal. Die ensiem toon optimale aktiwiteit by pH 6 en 'n temperatuur van 60°C. Aktiwiteitstoetse op verskillende substrate het aktiwiteit op metielumbelliferielasetaat en metielumbelliferielbutiraat bo-en-behalwe die glukosepentaasetaat en α -naftielasetaat getoon. Die *estA* geen is in uitdrukingskasette bevattende die *PGK*-promotor en-termineerder vir uitdrukking in *Saccharomyces cerevisiae* gekloneer. Dit is ook agter die MF α 1-sekresiesein gekoppel vir sekresie vanuit *S. cerevisiae*. Geen ekstrasellulêre aktiwiteit is gevind nie. Slegs intrasellulêre aktiwiteit van 0.26 nanokatal per mililiter was bepaal.

BIOGRAPHICAL SKETCH

Anton Pieterse was born in Kimberley, South Africa on 3 July 1974. He attended the D.F. Malherbe Primary School, Van der Bijl Park until standard two and the Charlo Primary School, Port Elizabeth until standard five. He matriculated at High School D.F. Malherbe, Port Elizabeth in 1992.

Anton enrolled at the University of Stellenbosch in 1993 and obtained a B.Sc degree in Biochemistry and Microbiology in 1995. In 1996 he completed a B.Sc.Hons degree at the same university.

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PREFACE

This thesis is made up out of five sections. Chapter 1 is a literature review written in the style of Applied Microbiology and Biotechnology, chapter 2 and 3 are publications and are written in the style of the journal for which each will be submitted. Chapter 4 follows as a general discussion.

Chapter 2: "Cloning and expression of a novel *Bacillus pumilus* carboxyl esterase (*estA*) in *Escherichia coli*", will be submitted for publication in Applied and Environmental Microbiology.

Chapter 3: "Cloning and expression of the novel carboxyl esterase (*estA*) from *Bacillus pumilus* in the yeast *Saccharomyces cerevisiae*", will be submitted for publication in Biotechnology Letters.

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Chapter 1

Literature Review

1: Esterase families

Esterases (enzymes that act on ester bonds), belong to a large hydrolase family of at least 256 enzymes (Enzyme Handbook). Their method of action is largely dependent on the substrate, for example phorbol-diester hydrolase will hydrolyse phorbol 12,13-dibutanoate to phorbol 13-butanoate and butanoate while pectinesterase will hydrolyse pectin to methanol and pectate (Enzyme Handbook). A few examples will be highlighted to illustrate the diversity in this family of enzymes. Ribonuclease F from *Escherichia coli* is involved in the processing of RNA molecules by introducing specific cleavage near the 3' end of tRNA (Gurevitz *et al.* 1982). Acyl-CoA thioesterases (PTE1 and YJR019C) from human and yeast are peroxisomal proteins that are involved in the oxidation of fatty acids contributing to growth on fatty acids (Jones *et al.* 1999). Ubiquitin hydrolase is important in the ubiquitin pathway as it maintains levels of free ubiquitin by freeing the protein from other molecules that it is bound to (Johnston *et al.* 1999; Rajesh *et al.* 1999). In snake venom, two esterases are believed to work in conjunction. The first, phospholipase A2 acts on phosphatidyl choline to release lysophosphatidyl choline. This destroys cell membranes. It also activates a phosphodiesterase found in the venom. The latter hydrolyses DNA and RNA (Mallipali *et al.* 1998). Phosphodiesterases are also involved in signal transduction and affect events such as glucose-induced insulin secretion (Han *et al.* 1999; Kakkar *et al.* 1999). In *Saccharomyces cerevisiae* acetyl-CoA hydrolase is involved in acetate utilisation and shows high levels of expression at the start of sporulation (Lee *et al.* 1996). In *Neurospora crassa* deletion of the acetyl-CoA hydrolase results in sensitivity to acetate (Connerton *et al.* 1992).

Serine esterase, vitamin A esterase and cocaine esterase, represent a subgroup of the esterases collectively known as carboxylesterases. They catalyse the reaction where a carboxylic ester is hydrolysed to an alcohol and a carboxylic acid anion in the presence of water. The substrates that can be hydrolysed by carboxylesterase include carboxylic esters, *p*-nitrophenyl acetate, methylumbelliferyl acetate, α -naphthyl butyrate, α -naphthyl acetate as well vitamin A esters (Enzyme handbook). Carboxylesterases of microsomes can also catalyse the reaction carried out by arylesterase, acylglycerol lipase, aryl-acylamidase, phospholipase A1, acylcarnitine hydrolase, amidase, lysophospholipase, cholinesterase and acetyl esterase (Enzyme Handbook). Carboxylesterase from pig liver can be used to determine the concentration of organophosphates when these are present in small quantities by looking at the decrease in enzyme activity (Heymann 1999).

Carboxylesterase activity in carrots is associated with the formation of the secondary cell wall (Melati *et al.* 1996). Rabbit liver carboxylesterase might find application in prodrug/enzyme therapy by converting the inactive form of the anticancer drug CPT-11 to the active form (Guichard *et al.* 1998). Another medical application of carboxylesterase is as indicator of the severity of acute pancreatitis. By monitoring the levels of carboxylesterase in the serum of patients doctors can differentiate between acute interstitial pancreatitis and necrotizing pancreatitis (Blind *et al.* 1991). Carboxylesterase produced by rubber trees may play a role in latex allergy (allergic reaction to products made from natural rubber). This is supported by the fact that people allergic to latex have antibodies specific for the carboxylesterase produced by the trees (Yagami 1998). Carboxylesterase might also play a role in delivery of

cysteine to cells by cleaving cysteine esters (Butterworth *et al.* 1993). Carboxylesterases are inhibited by substrates such as bis-(4-cyanophenyl) phosphate, paraoxon, and bis-(4-nitrophenyl) phosphate. Thus, these compounds can be used to characterise the type of carboxylesterase (Sertkaya and Gorrod 1988). Carboxylesterase can also be inactivated by sugars, this interaction of sugar and protein is called glycation (Yan and Harding 1999).

Esterases have wide applications. One of the areas that are becoming more important is the degradation of plant material. This material is an ideal abundant substrate for bioconversion to commodity products such as bioethanol. With coal and oil reserves steadily decreasing plant material as a renewable resource is coming into its own right. To utilise the plant material it has to be degraded to the individual building blocks. Esters are common substituents of these compounds and for complete degradation to be achieved these esters have to be removed by esterases. To understand the role esters and esterases play in this regard, one has to look first at the structure of the material that has to be degraded.

2: Esterases in wood degradation

Plant biomass is the most abundant renewable source of carbohydrates we have on Earth today (Linden *et al.* 1994b). Most of these, especially wood and agricultural by-products contribute an estimated 155 billion tons per annum to the amount of dry biomass available (Singh and Mishra 1995). Microorganisms proliferate in any environment where nutrients and conditions are favourable. Some of these microorganisms have specific methods of attacking plants and deriving their nourishment from the various components of the plant. Intensive studies have been

done on plant material degradation to gain a better understanding of the structure of plants and the microorganisms themselves. Mankind has come to realise that we can use the microorganisms and the various enzymes that they produce to our advantage. Many chemically driven processes can be performed much better by the use of appropriate organisms and/or their enzymes. The use of chemicals has come under sharp criticism for the harmful effects they have on the environment.

A major organism that has received much attention is the yeast *S. cerevisiae*. It is an organism with many advantages such as the fact that it has GRAS (Generally Regarded As Safe) status (Hadfield *et al.* 1993; Buckholtz and Gleeson 1991). It has been used in baking and brewing for numerous generations and is widely accepted as a food microorganism. One of its disadvantages, however, is that it can only metabolise a limited range of substrates and there is a concerted effort to improve the available range of substrates by providing *S. cerevisiae* with the necessary genes to metabolise these substrates.

The wood component that is important for this study is hemicellulose, an abundant cheap source of carbon for the growth of microorganisms and the production of commodity products. In the field of wood degradation much work has gone into the study of xylanases, but relatively little into the ancillary enzymes. Cloning and characterisation of esterases will not only have application in a number of areas as will be discussed later but will also further our knowledge of the action of esterases in wood degradation.

3: Wood composition

The three major compounds found in the cell wall of plants are cellulose (34 - 62%), hemicellulose (14 - 36%), lignin (18 - 39%) and pectin (McDermid *et al.* 1990a; Schäfer *et al.* 1996; Donaghy and McKay 1994). The cell walls of growing cells (primary cell wall) play an important role, not only in determining the size and shape of the cell, but also as a source of food for humans and animals and as a source of dietary fibre (Mcneil *et al.* 1984). Straw contains between 35 and 39% fibre while wood cells contain up to 90% fibre (Singh and Mishra 1995). The primary cell wall surrounds the growing plant cell (Ishii 1997) and provides structure for the expanding cell (Linden *et al.* 1994b). Polysaccharides account for 90% of the primary wall with the rest made up out of glycoproteins (Mcneil *et al.* 1984; Linden *et al.* 1994b). Besides these two groups plant cells also contain lipids, structural and non-structural phenolics and some contain cutin as well. The main polysaccharide in the primary cell wall is cellulose, which forms the skeletal framework of the cell wall (Ishii 1997). When the plant cell stops growing the properties and composition of the plant cell changes in part due to the formation of a second cell wall called the secondary cell wall (Ishii 1997, Linden *et al.* 1994b). This causes the protoplast to become diminished or even lost as can be seen in fibre cells, xylem tracheary and sclerenchyma. Figure 1 shows a simplified view of the cell wall of woody plants.

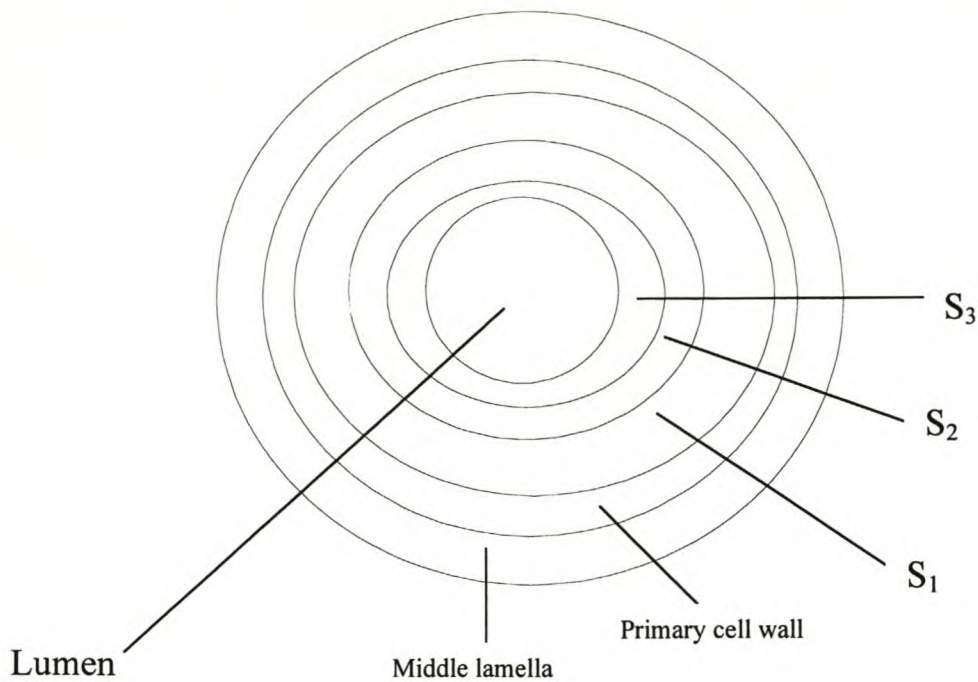


Fig. 1 Structure of plant cell wall (Erikson *et al.* 1990). S_1 , S_2 and S_3 are layers of secondary cell wall

Fibre cells, xylem tracheary and sclerenchyma have very thin primary cell walls, thick secondary cell walls consisting of more than one layer and occasionally a third layer (Ishii 1997). The generally rod-shaped hemicellulose surrounds the cellulose and this complex as a whole is imbedded in lignin (Singh and Mishra 1995). The lignin strengthens the secondary cell wall through covalent cross-linking to cell wall carbohydrate polymers such as cellulose and hemicellulose (Linden *et al.* 1994b). The secondary cell wall forms a considerable portion of the cell wall and consists of three layers, S_1 , S_2 and S_3 (Fig. 1). While the S_1 layer is closest to the primary cell wall, the S_3 layer is closest to the lumen with the S_2 layer in-between. There is also a middle lamella and together with the primary cell wall it forms the compound middle lamella which is situated between the secondary cell walls (Erikson *et al.* 1990; Singh and Mishra 1995). The arrangements of the microfibrular bundles consisting of cellulose and other components in the three different layers differ from each other. While these groups are arranged in alternately crossed helices in S_1 , they lie parallel

to the axis of the cell in S_2 . In S_3 they are still running parallel, but the direction makes a right angle with that of the fibrular groups found in the S_2 layer. The middle lamellae is heavily lignified containing 90% of lignin in hardwoods and 70% of lignin in softwoods while 70 to 80% of lignin is found in the secondary cell wall (Singh and Mishra 1995).

3.1: Major compounds of the plant cell wall

3.1.1: Cellulose

Cellulose is the most abundant polysaccharide in nature (Singh and Mishra 1995). It is a major component of wood and amounts to 40 - 45% of most wood species (Erikson *et al.* 1990). In woody plant cells the highest concentration of cellulose is found in the S_2 layer decreasing towards the middle lamella (Singh and Mishra 1995). About 20 - 30% of the cell wall comprises of cellulose while values of up to 45% have been found for tobacco callus cell walls (Mcneil *et al.* 1984). Plant material such as cotton is for all intents and purposes pure cellulose. The cellulose found in cotton is α -cellulose while the cellulose of plant and woody cell walls contain β -cellulose as well. A difference between α -cellulose and β -cellulose is that α -cellulose is not soluble in 17.5% NaOH, but β -cellulose is (Singh and Mishra 1995). Figure 2 shows the structure of cellulose.

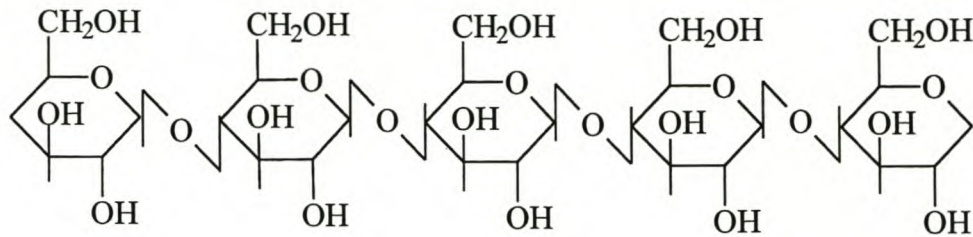


Fig. 2: Structure of a cellulose polymer (Tomme *et al.* 1996)

Cellulose is an unbranched polymer of D-glucose units linked by β -1,4-glycosidic bonds (Christov and Prior 1993; Biely 1993; Han *et al.* 1995b; Kleman-Leyer *et al.* 1996), but the basic repeat unit of the molecule is cellobiose and not glucose (Tomme *et al.* 1996, Erikson *et al.* 1990). The linear glucan chains have a two-fold axis of symmetry that is stabilised by the intra and intermolecular bonds. These include hydrogen bonds between hydroxyl groups of adjacent molecules and Van der Waal's forces (Mcneil *et al.* 1984; Kleman-Leyer *et al.* 1996; Singh and Mishra 1995). This causes the glucan chains to aggregate into what is known as microfibrils, which in turn form fibrils (Erikson *et al.* 1990). Amorphous and crystalline cellulose are intertwined in the plant cell wall (Erikson *et al.* 1990, Kleman-Leyer *et al.* 1996) and in total seven types of cellulose (I –IV) have been described with type I commonly found in plant cell walls (Erikson *et al.* 1990).

3.1.2: Lignin

Lignin (Fig. 3) is a complex polymer consisting of phenylpropane units linked by a variety of carbon-carbon and carbon-oxygen bonds (Donnelly and Crawford 1988; Borneman *et al.* 1986). The amount of lignin in hardwood is lower than that in softwood. It is distributed through the entire secondary cell wall and compound

middle lamella, the greatest concentration occurring in the middle lamella. The function of lignin is to give rigidity to plants, bind the fibre cell walls together, make the cell wall water proof, provide protection against enzymatic attack and act as a storage system (Singh and Mishra 1995; Erikson *et al.* 1990).

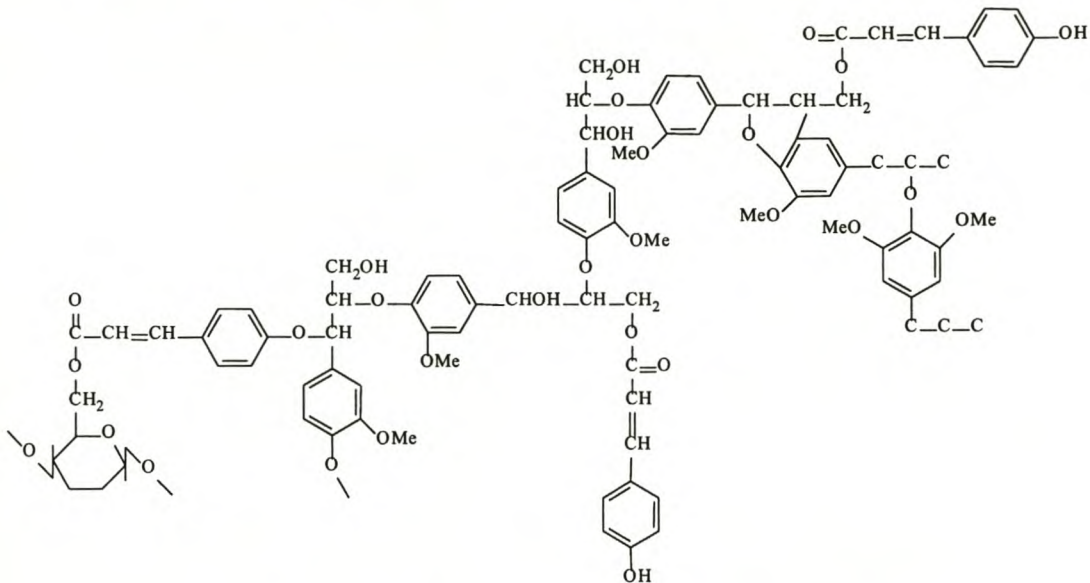


Fig. 3: Simplified lignin structure (Iiyama *et al.* 1990)

3.1.3: Pectin

Pectin is a complex polymer with a backbone consisting of 1,4-linked α -D-galacturonic acid interspersed with α -1,2-linked L-rhamnose. Pectin is a common constituent in the primary wall of dicots and makes out 33% (one third) of the dry weight of dicots and some monocots (Bordenave *et al.* 1995; Thakur *et al.* 1997). The highest concentration of pectin in the plant cell wall occurs in the middle lamella and it decreases steadily from the primary wall to the plasma membrane. Pectin is thought to play a role in controlling the movement of water and plant fluids in rapidly growing plant tissue as large amounts of it are found in soft plant tissue undergoing rapid growth or where the moisture content is elevated. It also contributes

to the firmness and structure of plant tissue (Thakur *et al.* 1997). Figure 4 shows the structure of pectin.

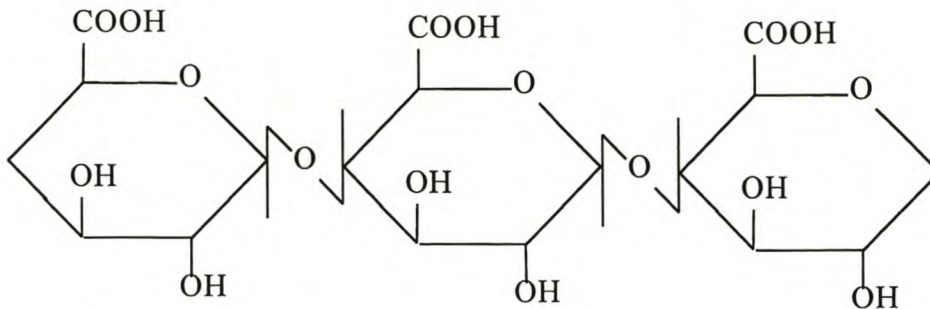


Fig. 4: Short stretch of a pectin molecule (Thakur *et al.* 1997)

3.1.4: Hemicellulose

Hemicellulose refers to a group of non-cellulosic and non-pectolytic heteropolysaccharides that include glucans, mannans, arabinans and xylans (Biely 1993; Linden *et al.* 1994b). The main sugars that can be found in these polysaccharides are D-xylose, L-arabinose, D-glucose, D-galactose and D-mannose (Singh and Mishra 1995). Figure 5 shows the structure of the different sugars along with that of glucuronic acid. Hemicellulose is distributed much in the same way as lignin with the largest proportion being found in the middle lamella. It surrounds the cellulose microfibrils, filling the space between the individual molecules (Erikson *et al.* 1990). Hemicellulose can hydrogen bond to cellulose as well as other hemicellulose components. It can also form linkages with lignin through ferulic acid. Ferulic acid covalently links the lignin to arabinose and 4-O-methylglucuronic acid

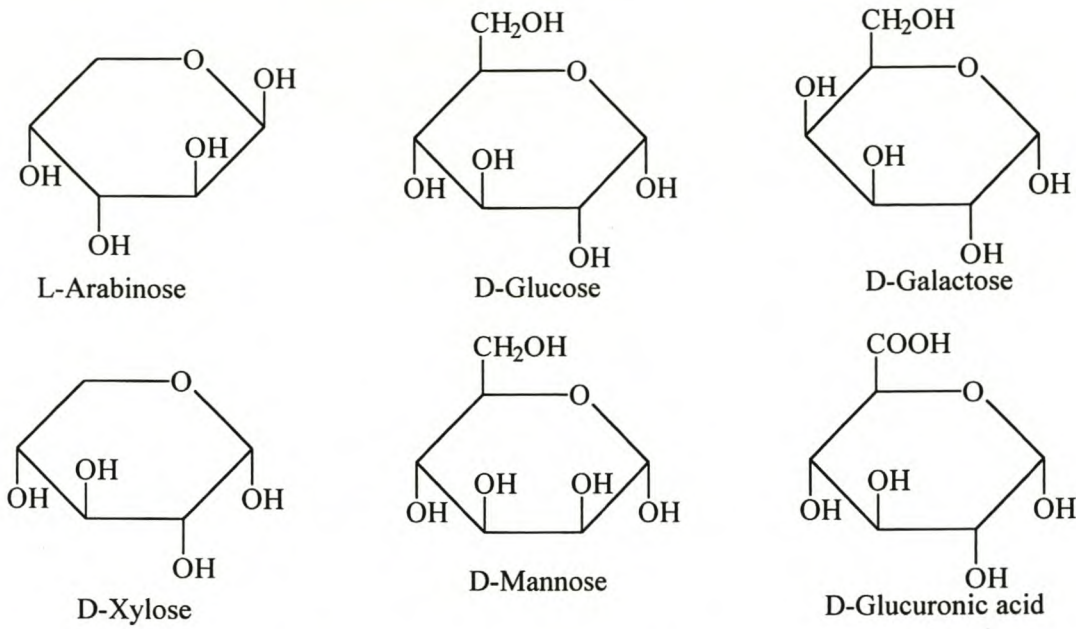


Fig. 5: Monosaccharides found in hemicellulose (Boons 1998)

bound to hemicellulose (Linden *et al.* 1994a). The association of the hemicellulose component with the cellulose and lignin stabilises the cell wall by making it more rigid. This association also prevents water from entering the matrix (Singh and Mishra 1995; Linden *et al.* 1994a). In hardwood hemicellulose represents 26%, in softwood it is 22% and in grasses and cereal it is 30%. There is a variation from simple to mixed polysaccharides and except for galactose based hemicellulose the units of the different hemicellulose backbones are joined by β -1,4-linkages.

3.1.4.1: Arabinogalactan

Arabinogalactan is found in low amounts in the cell walls of hardwood and softwood, but in the heartwood of larch it attains larger quantities (10 - 20%). Figure 6 shows the structure of arabinogalactan. The backbone of the arabinogalactan molecule consists of β -1,3-linked D-galactose units. Arabinogalactan shows a high degree of

substitution with substituents occurring at carbon six of nearly every galactose unit of the backbone. The substituent most commonly found linked to arabinogalactan is β -1,6-linked D-galactose with the second most common substituent

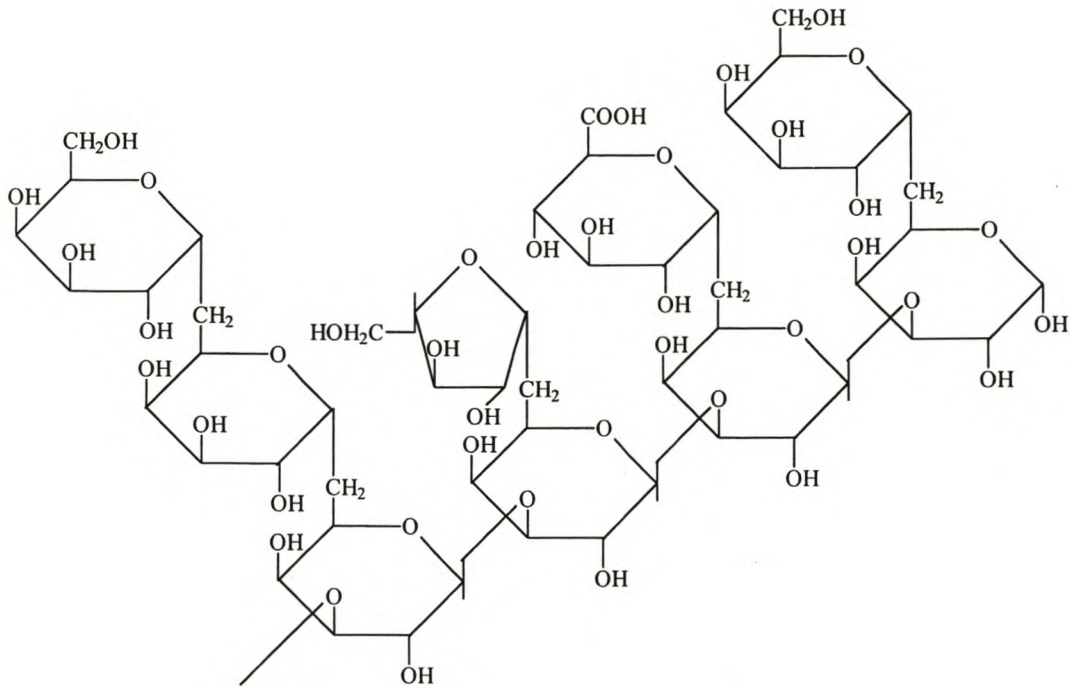


Fig. 6: Structure of arabinogalactan (Puls and Schuseil 1993)

being L-arabinose. Arabinogalactan is the only hemicellulose that can be directly extracted with water. When attempting to isolate the other types of hemicellulose the material must first be delignified and subsequently be treated with an alkali (Singh and Mishra 1995).

3.1.4.2: Xyloglucan

Xyloglucan is based on a linear backbone of β -1,4-linked D-glucose residues that can be highly substituted (up to 75%) with monosaccharide, disaccharide and

trisaccharide side chains (Coughlan *et al.* 1993; Mcneil *et al.* 1984; Levy *et al.* 1997; Thompson and Fry 1997). The first substituent linked to the glucan backbone is xylose. In the disaccharide sidechains, D-galactose is linked to the xylose while in the trisaccharide sidechain, fucose is linked in turn to the galactose residue. Xyloglucan often contains the fucosylated sidechain and in fact the presence of fucose is thought to be typical for dicots since xyloglucan of monocots in general lacks this sidechain. In fucose deficient strains of *Arabidopsis thaliana* it was found that the fucose residue was replaced by galactose (Vincken *et al.* 1997). While the role of the sidechains and the effect they have on the growth of the plant is not clearly understood, two functions are attributed to the different types of sidechains. The monosaccharide sidechains maintain the solubility of the xyloglucan molecule even though it cannot prevent the xyloglucan from associating with itself. Disaccharide sidechains can prevent this association from taking place. The fucosylated trisaccharide sidechains are involved in strengthening the cell wall since loss of this sidechain leads to a decrease in the strength of the affected cell wall (Levy *et al.* 1997)

The greatest amount of xyloglucan is found in the primary cell walls of dicots (25% of dry weight) with smaller amounts found in cell walls of monocots (2%) (Mcneil *et al.* 1984; Thompson and Fry 1997). Xyloglucan is found in close association with cellulose and hydrogen bonds with it (Vincken *et al.* 1997). These hydrogen bonds are very strong and very specific with the result that a cellulose-xyloglucan network is formed that has great influence on the shape of the plant cell. Xyloglucan hydrogen bonds with cellulose in the presence of other β -glucans, arabinogalactan and pectins and can even out compete other cellulose molecules for binding (Levy *et al.* 1997). Because of the length of the xyloglucan chain it can form hydrogen bonds with more

than one cellulose microfibril at a time and this in effect tethers the microfibrils. It prevents the individual microfibrils from separating and so limits expansion of the plant cell wall (Thompson and Fry 1997).

The xyloglucan is thought to be made up out of cellotetraose repeat units, which is borne out by the repetitive nature of the side chains on the glucan backbone. According to the branching pattern the xyloglucans are classed into two main groups. The first group of xyloglucan that will be discussed is named XXXG (Fig.7). In this xyloglucan the cellotetraose unit consists of three branched glucose units (designated X) and one unbranched glucose unit (designated G) (Yamagaki *et al.* 1997; Vincken *et al.* 1997). This group of xyloglucan is found in softwood and hardwood. Xyloglucan in the seeds of *Detarium senegalense*, *Tropaeolum majus* and *Tamarindus indica* that serve as a storage polysaccharide has only the monosaccharide and disaccharide sidechains. These molecules can be acetylated on carbon 2, carbon 3 or carbon 6 of the galactose residues of the sidechains. The galactose units can have either one or two acetyl groups linked to them. Acetylation of the sidechains has no limiting effect on the degradation of xyloglucan by endoglucanases. The role of acetylation is yet unknown (Vincken *et al.* 1997).

The second main group of xyloglucan is referred to as XXGG xyloglucan. This type of xyloglucan is found in solanaceous plants and unlike the XXXG type the cellotetraose unit of the XXGG xyloglucan consists of two branched glucose residues and two unbranched units. In xyloglucan of tobacco the xylose is further substituted mainly with arabinose while in potato and tomato the substituents linked to the xylose are arabinofuranose and galactose. It is not known whether this type of xyloglucan

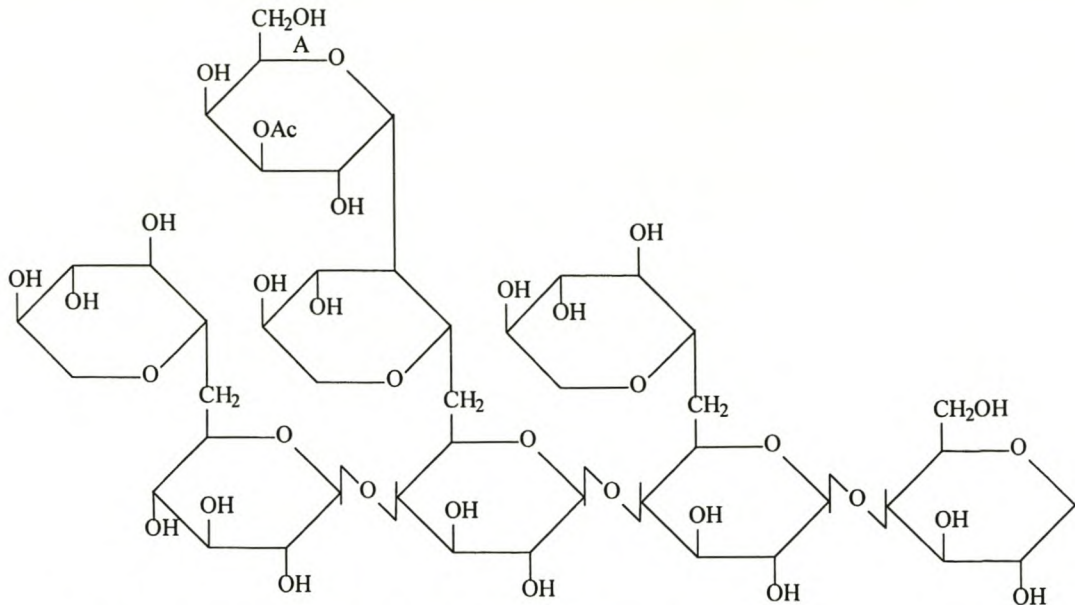


Fig. 7: Cellotetraose unit of XXXG xyloglucan (Yamagaki *et al.* 1997)

contains fucosylated sidechains. A trisaccharide sidechain consisting of arabinose → arabinose → xylose has been found in tomato and xylose → xylose sidechains are present in xyloglucan excreted by *Nicotinia plumbaginifolia*. Acetyl groups can be attached to carbon six of glucose residues of the glucan backbone as well as carbon five of terminal arabinose residues. Acetylation of the glucan chain reduces the number of endoglucanase cleavage sites (Vincken *et al.* 1997). This exerts a limiting effect on the degradation of this type of xyloglucan in comparison to the XXXG type of xyloglucan where acetylation of the xyloglucan molecule has no effect on degradation at all. Figure 8 shows the structure of a cellotetraose unit of XXGG xyloglucan.

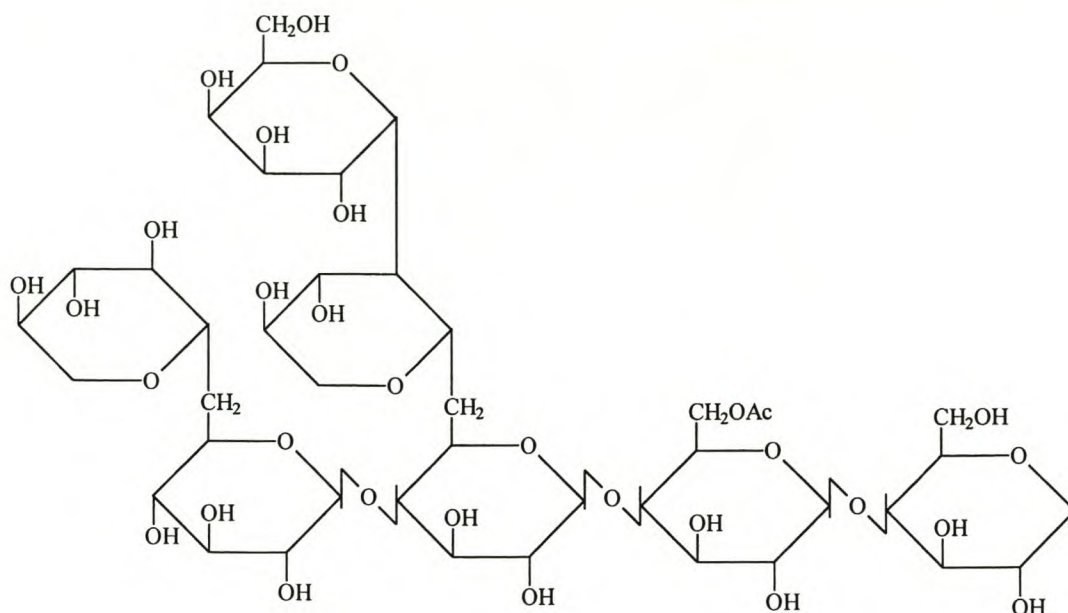


Fig. 8: Cellotetraose unit of XXGG xyloglucan (Vincken *et al.* 1997)

Besides the XXXG and XXGG xyloglucan other types of xyloglucan are known to exist. In tobacco a small portion of the xyloglucan fraction consists of a xyloglucan where the cellotetraose unit has only one branching point. Other than this xyloglucan there is also thought to exist three other types of xyloglucan. These three groups are XXG, XXGGG and XXXX xyloglucan. While the XXGGG type of xyloglucan is thought to exist in rice seedlings and immature barley its existence as well as that of the XXGGG type of xyloglucan is in doubt and these two types of xyloglucan will not be further discussed. XXXX xyloglucan is found in the coating of seeds of *Helipterum eximium* (Fig. 9). In this xyloglucan, as the name implies, every glucose residue of the cellotetraose unit is substituted. Each of the glucose units has two sidechains of different types linked to them. The first sidechain is a disaccharide sidechain consisting of galactose linked to xylose and the second type of sidechain is a single arabinofuranose unit α -1,2-linked to the glucan backbone. Because of the high degree of substitution this xyloglucan is very resistant to enzymatic degradation

by endoglucanases even after removal of the arabinofuranose residue (Vincken *et al.* 1997).

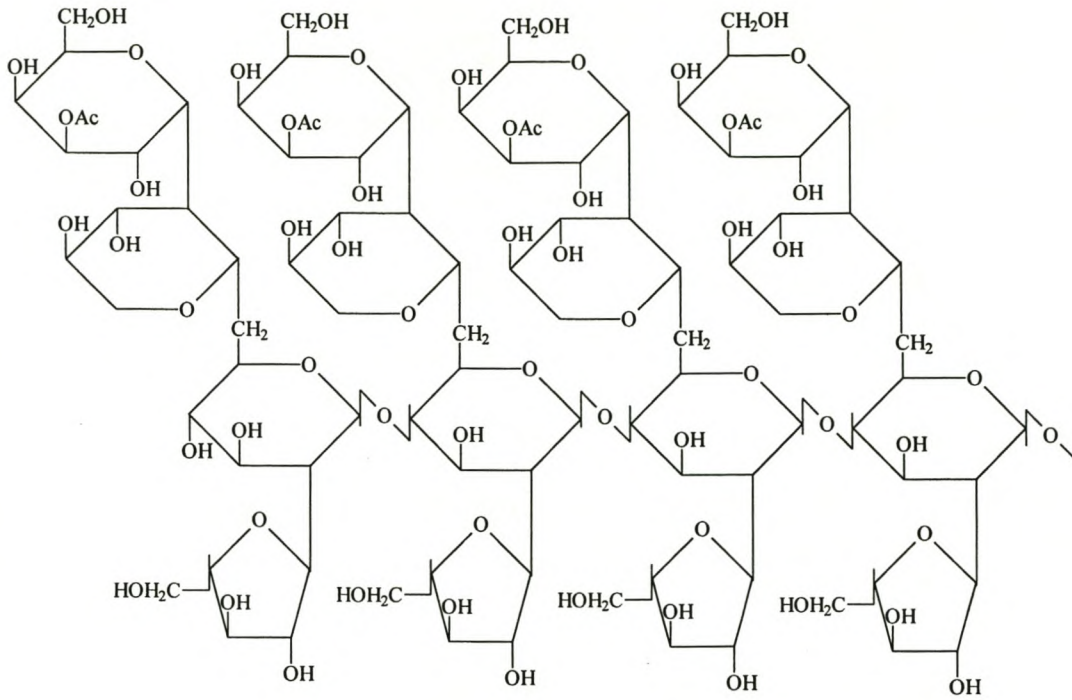


Fig. 9: Cellotetraose unit of XXXX xyloglucan (Vincken *et al.* 1997)

3.1.4.3: Mannan

The first type of mannan that will be discussed is galactomannan. It has a main chain of β -1,4-linked D-mannose substituted at carbon 6 with single galactose units (Fig. 10). Galactomannan is located mainly in the endosperm cell walls of legumes where it forms an important source of carbohydrate in these plants (Bewley *et al.* 1997).

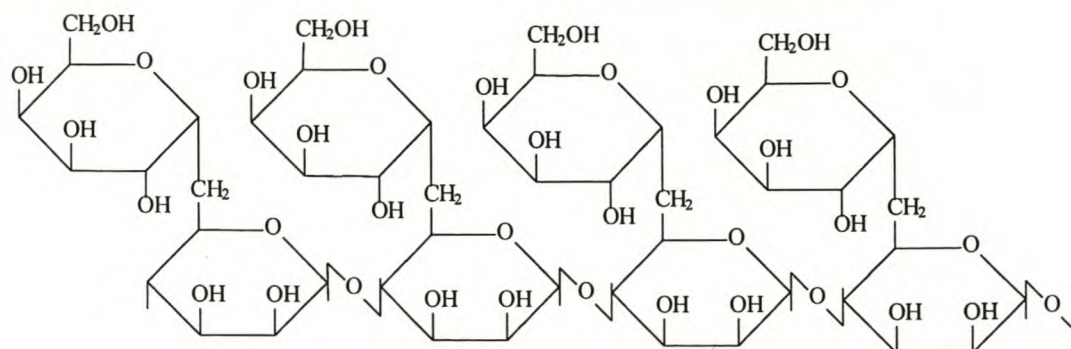


Fig. 10: Galactomannan (Bewley *et al.* 1997)

Glucomannan and galactoglucomannan on the other hand have a mixed backbone of β -1,4-linked D-mannose and β -1,4-linked D-glucose residues (Sims *et al.* 1997; Coughlan *et al.* 1993; Erikson *et al.* 1990). Galactoglucomannan is the main hemicellulosic component of softwood (12 - 15%) (Bewley *et al.* 1997). Single substitution with galactose can occur on the mannose residues (Erikson *et al.* 1990; Sims *et al.* 1997) (Fig. 11). There are two types of galactoglucomannan: one with a high galactose content with the ratio of mannose: galactose: glucose of 3:1:1 and one with a low galactose content (mannose: galactose: glucose = 4:0.1:1) (Erikson *et al.* 1990). Galactoglucomannan can be acetylated to a low degree with one in three or four hexose units (5.9 – 8.8%) being acetylated (Puls and Schuseil 1993; Poutanen *et al.* 1990; Poutanen and Sundberg 1988; Sims *et al.* 1997). While some researchers believe only the mannose residues to be acetylated on carbon 2 (C-2) and carbon 3 (C-3) others have found both mannose and glucose residues to be acetylated but only at C-3 (Puls and Schuseil 1993). In galactoglucomannan of the secondary cell wall the distribution of mannose and glucose in the backbone is not consistent and while two mannose residues may be found next to each other, the same is not true for glucose. Galactoglucomannan of the primary cell wall of *Nicotiana tabacum*

(tobacco) and that of *Rubus fruticosus* (blackberry) have alternating mannose and glucose residues in the backbone and thus have equal proportions of mannose, glucose and galactose. In the endosperm of asparagus seeds and that of the Judas tree the ratio of mannose: galactose: glucose is 5:1:4 and 11:2:1, respectively (Sims *et al.* 1997)

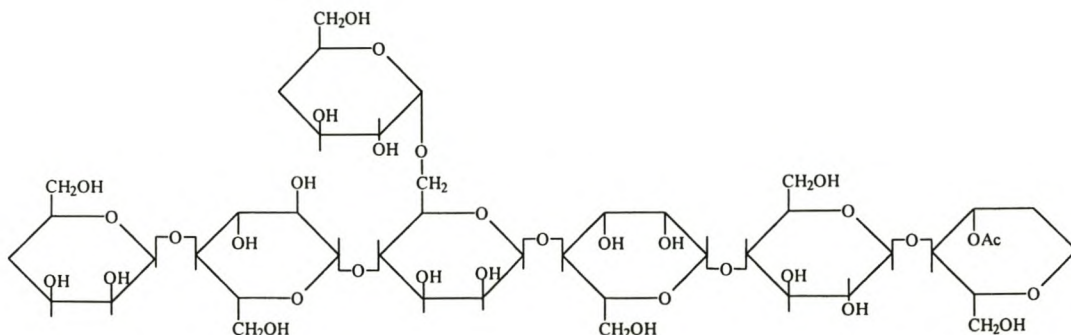


Fig. 11: Galactoglucomannan (Puls and Schuseil 1993)

Glucomanan is a small hemicellulosic constituent of hardwood making out 2 – 5% of hardwood (Fig. 12). Depending on the plant from which it has been isolated the ratio of mannose to glucose can vary from 2:1 to a ratio of 1:1 (Coughlan *et al.* 1993; Erikson *et al.* 1990).

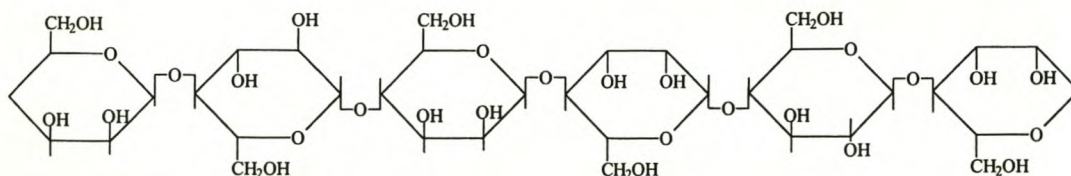


Fig. 12: Glucomanan (Puls and Schuseil 1993)

3.1.4.4: β -Glucan

β -Glucan refers to glucose polymers of high molecular weight where the glucose units are linked by both β -1,3- and β -1,4-linkages (Spilliaert *et al.* 1994; Lloberas *et al.* 1991; Lee *et al.* 1997; Becker *et al.* 1995) (Fig. 13). The arrangement of the two types of linkages are fixed with β -1,4-linkages occurring more than the β -1,3-linkages with ratios ranging from 2.3 to 2.7 (Becker *et al.* 1995). The structural arrangement of the β -glucan molecule is usually such that short stretches containing three or four β -1,4-linked glucose units are joined by a single β -1,3-linkage (Becker *et al.* 1995; Sundberg *et al.* 1996; van Rensburg *et al.* 1997). β -Glucan of barley contains longer stretches of β -1,4-linked glucose units with up to ten glucose units in one of these stretches (Becker *et al.* 1995). A similar polymer, lichenan, is formed by the lichen *Cetraria islandica*. β -Glucan forms part of the starchy endosperm and aleurone cell wall in cereals like *Hordeum vulgare* (barley), *Triticum aestivum* (wheat), *Secale cereale* (rye), *Zea mays* (maize), *Oryza sativa* (rice), *Sorghum bicolor* (sorghum), *Pennisetum americanum* (millet) and oats. Higher amounts are found in oats and barley than other cereals (Holthaus *et al.* 1996; Lee *et al.* 1997; Han *et al.* 1995a) with β -glucan forming 75 % of the endosperm cell wall of barley where it is uniformly distributed (Temelli 1997; Svihus *et al.* 1997). The function of β -glucan is to act as a carbohydrate reserve (Becker *et al.* 1995).

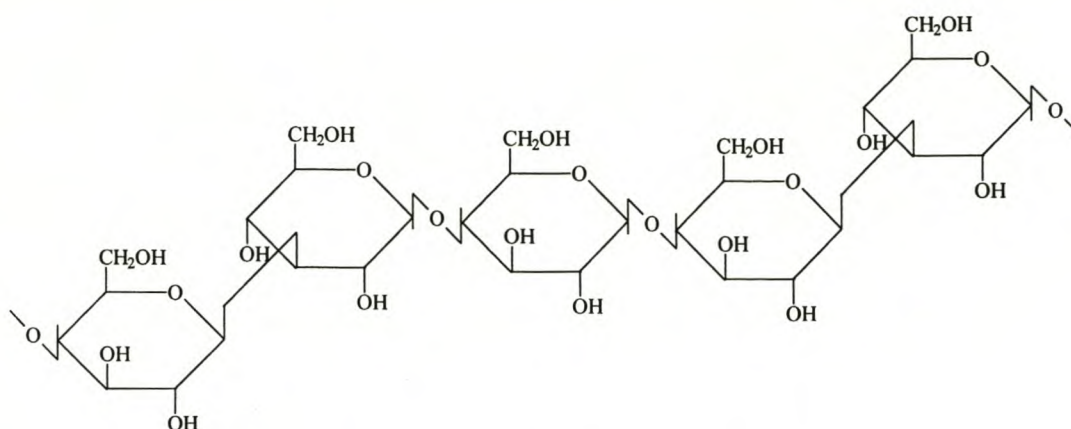
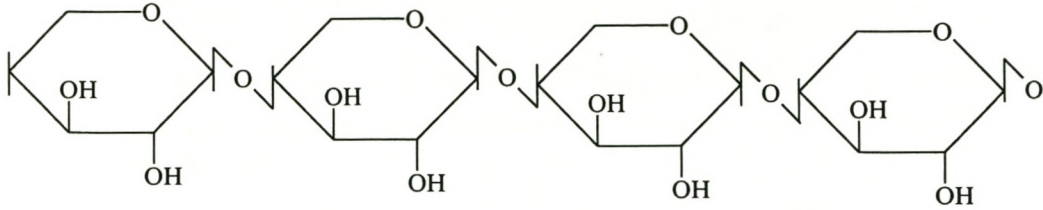


Fig.13: Short stretch of β -Glucan chain (Sunberg *et al.* 1996; Becker *et al.* 1995)

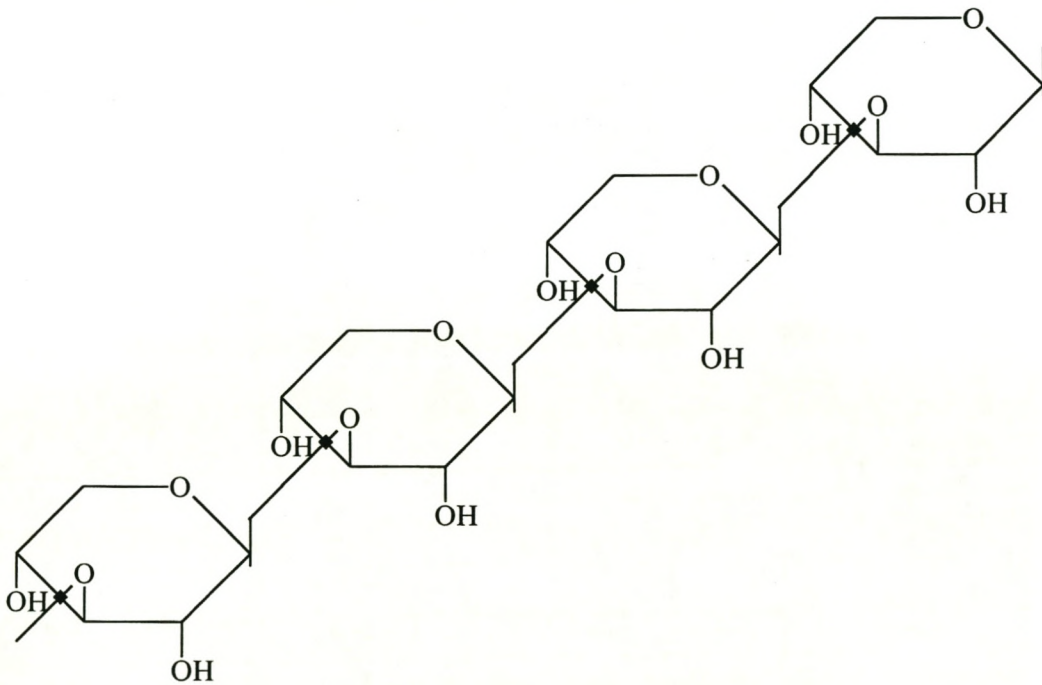
3.1.4.5: Xylan

Xylan is the most abundant polysaccharide in nature after cellulose and forms a major part of the hemicellulose fraction in all plants (Lorenz and Wiegel 1997; Fernández *et al.* 1995; Sunna and Antranikian 1997; Tokuda *et al.* 1997; Tsujibo *et al.* 1997). The xylan molecule can be anything from a simple homopolymer consisting only of D-xylose units to a complex heteropolymer with various substituents linked to various points of the xylan backbone (Biely 1985; Morrison 1975; Biely 1993; Hazlewood *et al.* 1993; Schäfer *et al.* 1996). Figure 14 shows the different types of xylan found in nature. Xylan linked by β -1,3 glycosidic bonds (Fig. 14 b) are found only in marine algae while a xylan containing a mixture of β -1,3- and β -1,4-linkages (Fig. 14 c) can be found in the seaweed *Rhodomenia palmata* (Coughlan and Hazlewood 1993; Coughlan *et al.* 1993). Homoxylans (Fig. 14a) are very rare and are found only in Esparto grass, tobacco stalks and guar seed husk (Sunna and Antranikian 1997). The xylan of hardwood, softwood, grasses and cereals (Fig. 14 d) is a β -1,4-linked

D-xylose polymer with a variety of substituents at various points of the xylan



(a) β -1,4-linked xylan



(b) β -1,3-linked xylan

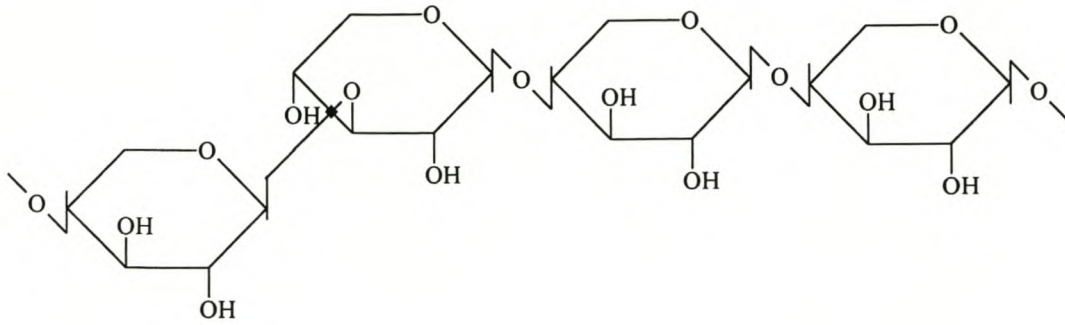
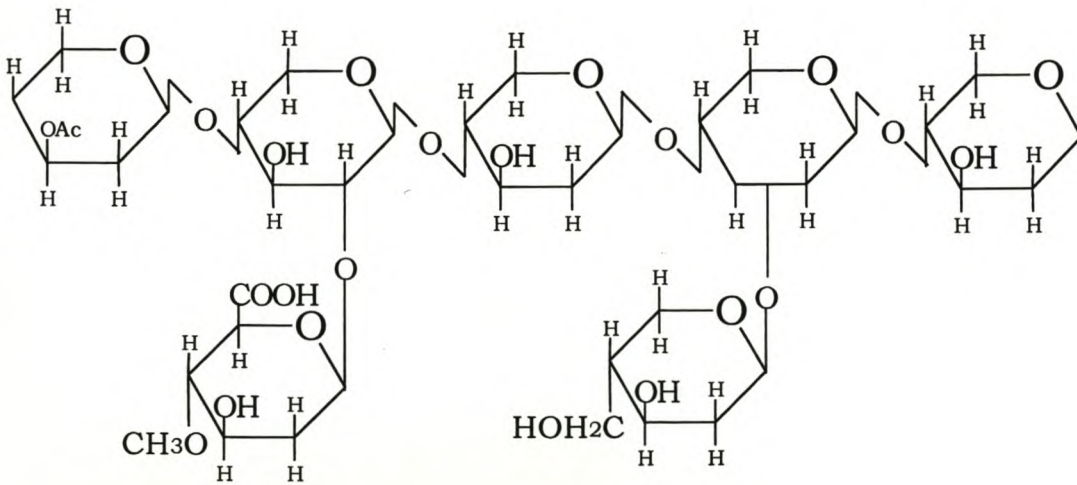
(c) β -1,3-1,4-linked xylan(d) β -1,4-linked xylan with substituents

Fig. 14 : Structure of different types of xylans found in (a) tobacco stalk, (b) marine algae (c) *Rhodomenia palmata* and (d) hardwood, grasses and cereals

backbone. These substituents can be arabinose, D-glucuronic acid or its methyl derivative, 4-O-methyl-D-glucuronic acid, acetyl groups (Shareck *et al.* 1995; MacKenzie *et al.* 1987; Borneman *et al.* 1990; Dalrymple *et al.* 1996) and some oligosaccharides such as mannose (Linden *et al.* 1994a; Tsujibo *et al.* 1997). Further substitution in the form of *p*-coumaric acid and ferulic acid can occur on the arabinose residues (Castanares *et al.* 1992; Donaghy and McKay 1995; Haltrich *et al.* 1993; Faulds and Williamson 1995a; Fernández *et al.* 1995). The arrangement, quantity and

type of substituents vary according to source (Biely 1993; Castanares *et al.* 1995). The different substituents and the type of bonds they form with xylose are shown in Table 1.

Table 1: Xylan substituents (Margolles-Clark *et al.* 1996; Fernández *et al.* 1995; Hazlewood *et al.* 1993)

Substituent	Position
Arabinose	C-2 / C-3
Glucuronic acid and methyl derivative	C-2
Acetyl group	C-2 / C-3
Phenolic acids	Secondary substitution on C-5 of Arabinose

Xylan is found in the primary walls of monocots and to a lesser extent in that of dicots. The xylan molecule can hydrogen bond to cellulose and the extent of this association is determined by the degree of substitution, which also influences the solubility of the xylan molecule. Highly substituted xylan, while very soluble, shows a very weak association with cellulose. Xylan with a lower degree of branching (substitution) is less soluble and shows a stronger association with cellulose (Mcneil *et al.* 1984; Linden *et al.* 1994b). Association of xylan and other components such as mannose protects cellulose against degradation as seen by the fact that removal of these components makes the cellulose fraction more sensitive to enzymatic degradation. Xylan may also play a role in cell wall cohesion. It acts as an important interface between lignin and other hemicellulosic components (Singh and Mishra 1995). The nature of these linkages between xylan, lignin and other hemicellulosic material will follow in section 4.3.

The xylan of hardwood is an acetyl-4-O-methylglucuronoxylan and comprises 10 – 35 % of the dry weight of hardwood and is the main hemicellulosic component of this type of wood (Biely 1993; Sunna and Antranikian 1997; Erikson *et al.* 1990; Singh and Mishra 1995). In white birch it reaches levels of more than 33% while in white elm it never goes above 25% (Singh and Mishra 1995). Glucuronoxylan has a degree of polymerisation of about 200 (Coughlan and Hazlewood 1993; Hazlewood *et al.* 1993; Sunna and Antranikian 1997) and up to 70% of the xylose residues can be acetylated at C-2, C-3 or both (Poutanen and Sundberg 1988; Poutanen *et al.* 1990; Puls *et al.* 1991; Puls and Schuseil 1993). Hardwood xylan has 10% α -1,2-linked 4-O-methyl-D-glucuronic acid substituents and contains small amounts of rhamnose and galacturonic acid as components of the main chain (Coughlan and Hazlewood 1993; Biely 1993; Hazlewood *et al.* 1993; Sunna and Antranikian 1997). Although low amounts of arabinose have been detected these might have originated from other hemicellulose components such as arabinogalactan and not from the hardwood xylan itself (Kormelink and Voragen 1993; Singh and Mishra 1995). Figure 15 shows the structure of hardwood xylan. The xylan molecule has a threefold screw axis with a rotation of 120 degrees and a repeating length of 1,5 nanometer (Singh and Mishra 1995).

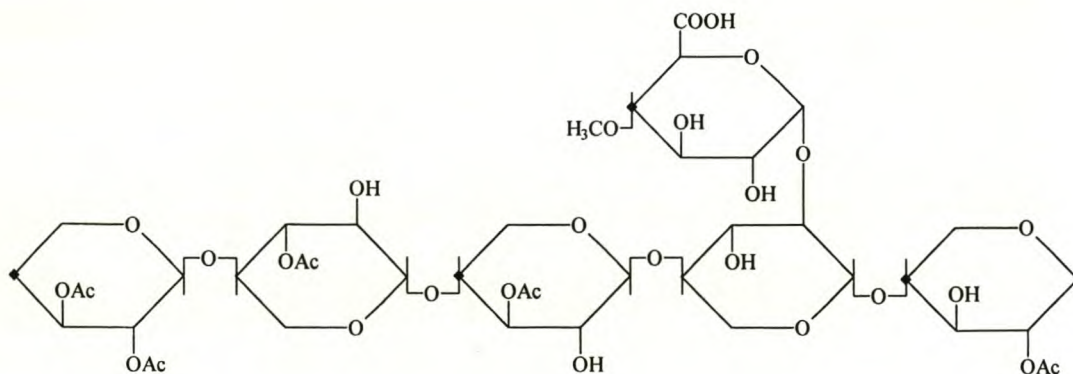


Fig. 15: Hardwood xylan

Arabino-4-O-methylglucuronoxylan makes out 10 – 15 % of softwood (Coughlan and Hazlewood 1993; Sunna and Antranikian 1997). Softwood xylan (Fig. 16) contains no acetyl groups, but it does have arabinose linked to C-3 of one in every eight or nine xylose residues (Puls and Schuseil 1993; Tenkanen and Poutanen 1992; Singh and Mishra 1995). With two glucuronic acid residues per ten xylose residues it has more glucuronic acid than hardwood (Erikson et al 1990; Sunna and Antranikian 1997), but with a degree of polymerisation (DP) of 70 – 130 it has a shorter chain length and is also less branched than hardwood xylan (Hazlewood *et al.* 1993).

Like the xylan of softwood, the xylan found in grasses and cereal is an arabino-4-O-methylglucuronoxylan (arabinoxylan) (Fig. 17). Unlike the xylan of softwood it is acetylated and has a shorter chain length (DP \approx 70) (Coughlan and Hazlewood 1993). It forms the main hemicellulosic component of grasses and cereals

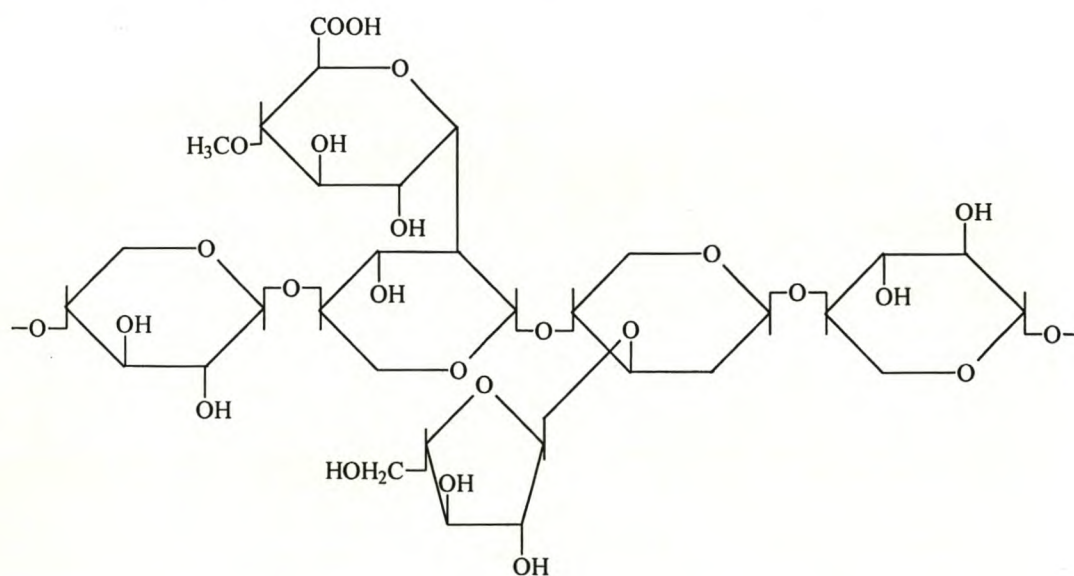


Fig. 16: Softwood xylan (Puls and Schuseil 1993)

whereas xylan in softwood is not the main hemicellulosic component (Wende and Fry 1997). The glucuronic acid content of arabinoxylan is lower than that of hardwood.

It contains a larger amount of arabinose, in many instance very high amounts of arabinose at C-2, C-3 or both though in many cases substitution at C-2 s favoured (Kormelink and Voragen 1993; Puls and Schuseil 1993; Coughlan and Hazlewood 1993; Biely 1993). Arabinoxylan from rice bran has an arabinose to xylose ratio ranging from 0.90 to 0.98, which is indicative of a high degree of branching. In wheat the ratio is much lower with a value of 0.51 (Kormelink and Voragen 1993). Rice bran arabinoxylan also contains larger amount of galactose (D- and even the rarer L-galactose) than the other xylans (Kormelink and Voragen 1993; Linden *et al.* 1994a). Grasses have 1 – 2 % phenolic acids linked to some of the arabinose substituents. In barley straw one in 31 arabinose (3%) is substituted with *p*-coumaric acid and one in 15 arabinose (6%) is substituted with ferulic acid (MacKenzie and Bilous 1988, Borneman *et al.* 1992, Mcallum *et al.* 1991, Singh and Mishra 1995).

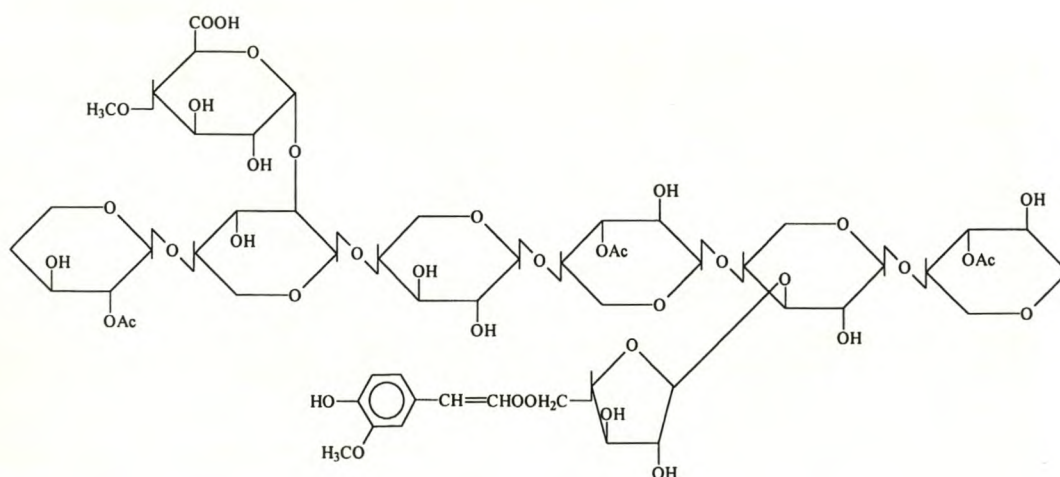


Fig. 17: Xylan from grasses and cereals (Puls and Schuseil 1993)

4: Ester groups of xylan

Ester linkages of acetyl groups and phenolic acids (more specifically, *p*-coumaric acid and ferulic acid) that occur on the xylan backbone will be discussed under special headings.

4.1: Acetyl groups

Up to 2% of the dry mass of higher plants can consist of acetic acid (Christov and Prior 1993). The acetyl content of cell walls of hardwood, softwood and grasses are 3 - 7%, 1 - 2% and 2 - 5%, respectively (Tenkanen and Poutanen 1992). Acetyl groups in softwood are not associated with the xylan fraction, but rather with the mannan fraction (Poutanen *et al.* 1990). Although the xylose residues can be acetylated at both C-2 and C-3 substitutions at C-3 seem to be favoured (Singh and Mishra 1995). A compounding factor in this is the fact that the acetyl groups can migrate between these two positions during and after isolation of the material (Tenkanen and Poutanen 1992). The ratio of substitution at C3: C2: both: none was found to be 22:24:10:44 for birch xylan (Christov and Prior 1993) while in other studies the ratio of substitution C2: C3: both was determined at 2:4:1, 2:2:1 and for a bracatinga xylan 3:3:1 (Tenkanen and Poutanen 1992). The ester bonds joining the acetyl groups to the xylose residues are easily broken under alkaline conditions (Poutanen and Sundberg 1988). It has been mentioned earlier that substitution of the xylan molecule has an influence on its properties. The effect of acetylation is to make the xylan molecule more soluble in water (Biely 1985) by preventing association of individual xylan molecules (Poutanen *et al.* 1990; Bacon *et al.* 1975). It is also

believed to have a negative influence on the gelating properties of pectins (Poutanen *et al.* 1990). Acetylation has an inhibiting effect on degradation of xylan (Khan *et al.* 1990; Linden *et al.* 1994a; Bacon *et al.* 1975). Xylanases are inhibited through steric hindrance, which may affect the ability of the enzyme to bind to the substrate or negatively affect the catalytic site of the enzyme (McDermid *et al.* 1990b). Another enzyme that is inhibited is α -glucuronidase and this is when the acetyl groups are adjacent to glucuronosyl residues (Sunna and Antranikian 1997).

4.2: Phenolic acids

Figure 18 shows five of the phenolic acids found in nature. Of these five, *p*-coumaric acid (4-hydroxy-cinnamic acid) and ferulic acid (4-hydroxy-3-methoxy-cinnamic acid), which are the oxidised forms of *p*-coumaryl alcohol and coniferyl alcohol respectively, form ester linkages (Christov and Prior 1993). Figure 19 shows the biosynthesis of these two phenolic acids in plants.

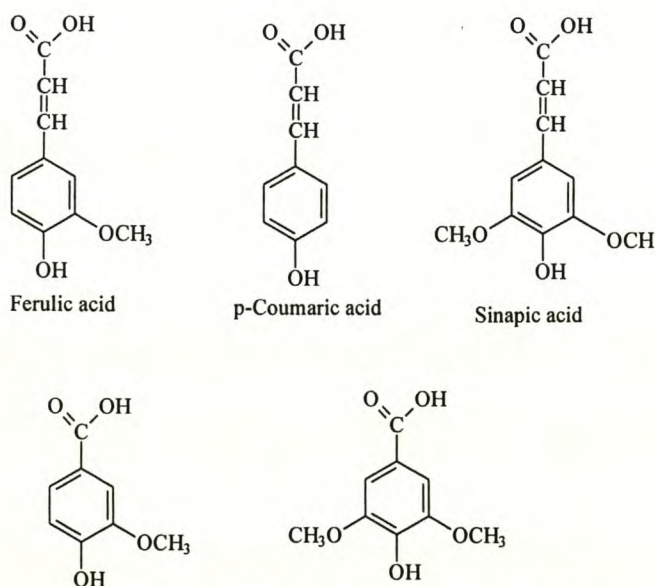


Fig. 18: Phenolic acids (Nazareth and Mavinkurve 1986; Ishii 1997)

Ferulic and *p*-coumaric acids are common cell wall components in *Poaceae* and graminaceous plants (Tenkanen and Poutanen 1992; Bacon *et al.* 1975; Faulds and Williamson 1995a) and occur mainly in the *trans* configuration (Borneman *et al.* 1993). Feruloyl esters are present in large quantities in mesophile cell walls while *p*-coumaric acid more represented in the secondary cell wall (Castanares *et al.* 1992). These two phenolic acids are normally ester linked to C-5 of arabinose of xylan (Borneman *et al.* 1993; Tenkanen and Poutanen 1992), but in wheat bran and sugar beet, ferulic acid have been found joined at other positions (Faulds and Williamson

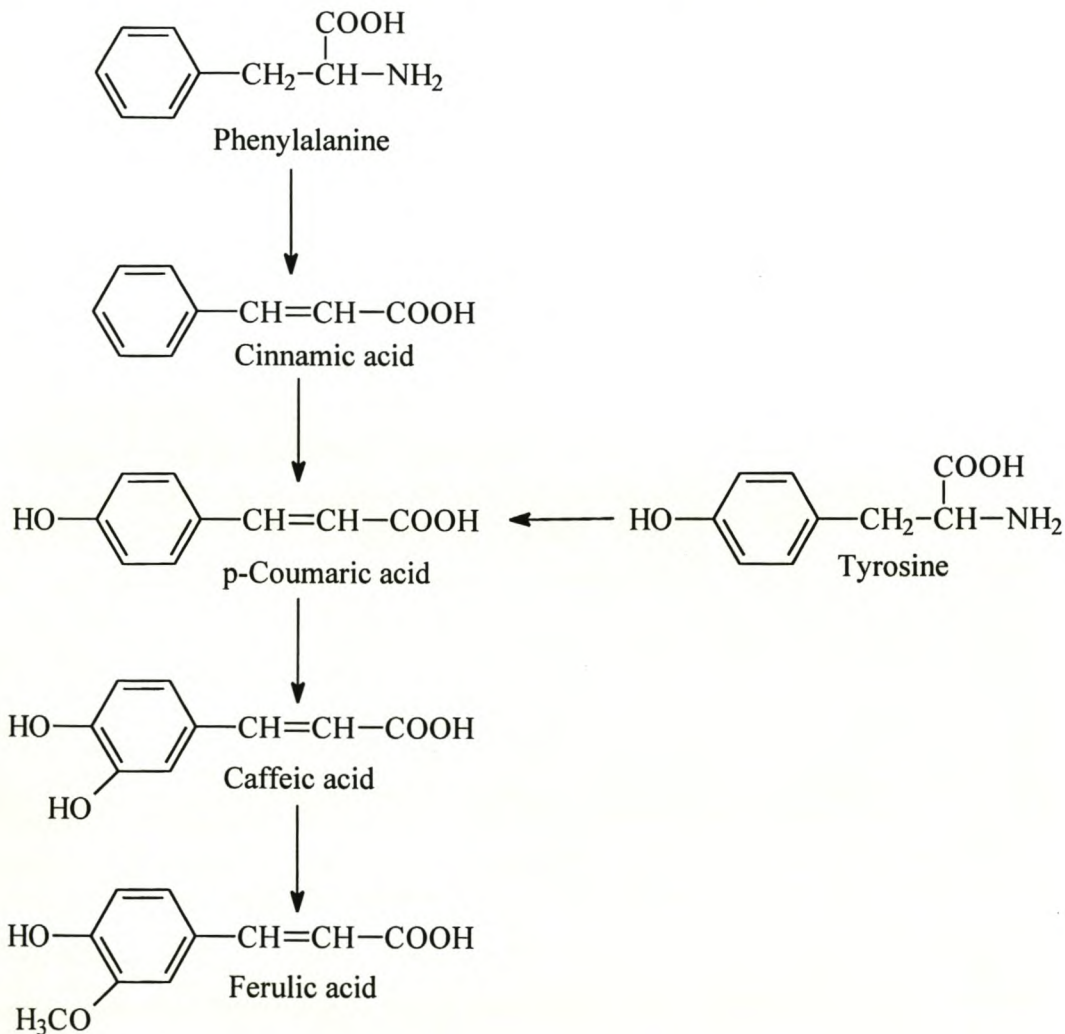


Fig. 19: Biosynthesis of ferulic and *p*-coumaric acids in plants (Kapulnik *et al.* 1996, Rice-Evans *et al.* 1996)

1995a). Ferulic acid can also bind to pectins (sugar beet), galactan and arabinan disaccharides (spinach) and lignin (bamboo and grass) (Christov and Prior 1993) and has also been involved in various types of linkages with proteins. This interaction plays a role during the mixing of dough and will influence the quality of the final product. All these can be attributed to the bifunctional nature of the phenolic acids. This allows them to form ester linkages by reaction of their carboxyl groups or ether linkages by reaction of their phenolic groups (Scalbert *et al.* 1985). Figure 20 shows the crosslinking of two polysaccharide chains

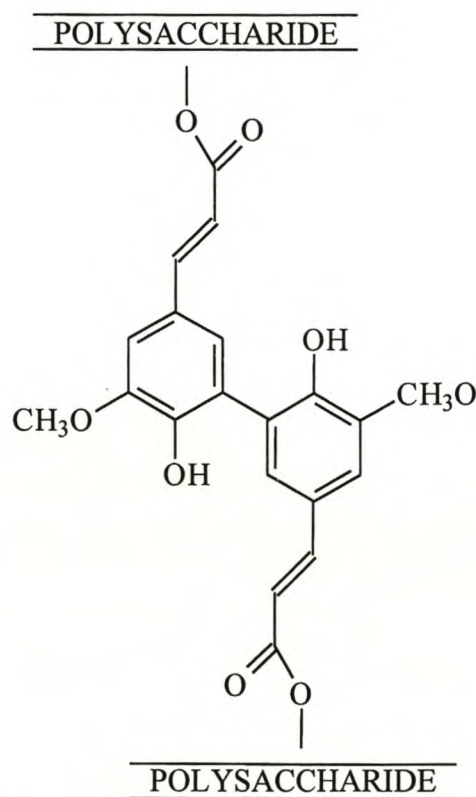
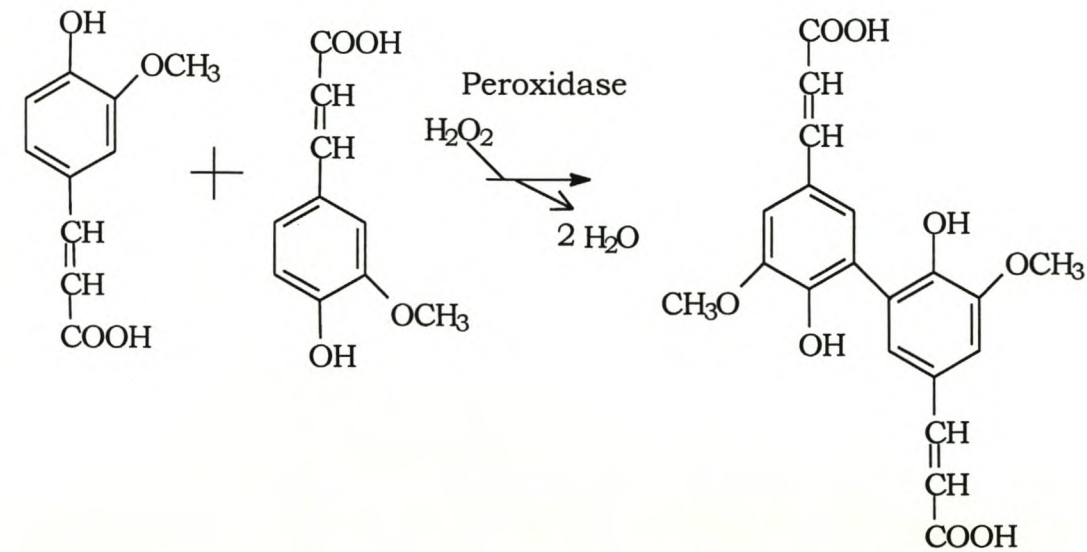


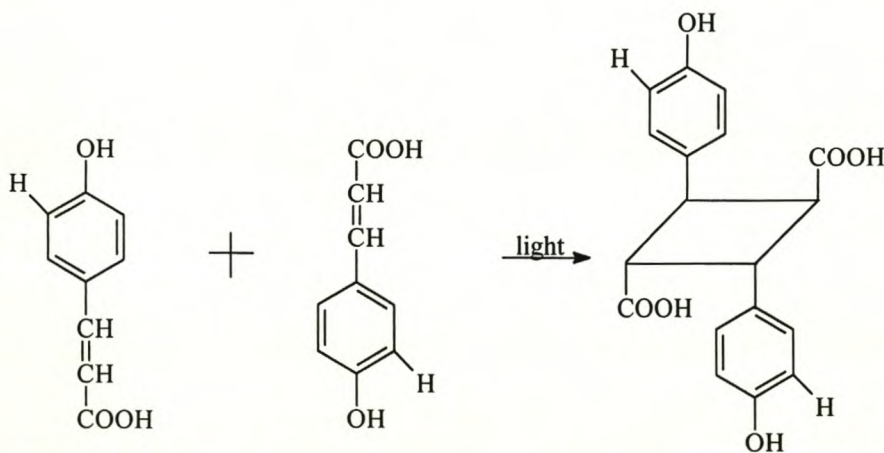
Fig. 20: Crosslinking of two polysaccharide chains by ferulic acid (Ishii 1997)

Dimerisation can occur between two phenolic acids cross-linking carbohydrate and lignin in the cell wall, linking different carbohydrates to each other and to lignin. The matrix stabilises the cell wall and provides integrity (McDermid *et al.* 1990b, McDermid *et al.* 1990a, Castanares *et al.* 1992). Although both chains are

polysaccharides one of them can be lignin. Cross-linking is one of the events that occurs during the mixing of dough in bread-making where ferulic acid is associated with the larger molecular weight portion of the arabinoxylan (Hoseney and Faubian 1981, Faulds and Williamson 1991) and is important in the gelling of wheat flour during dough formation in the making of bread (Hartley and Jones 1976). Dimerisation can either occur through peroxidase mediated oxidative coupling or by photodimerisation (Borneman *et al.* 1993, MacKenzie and Bilous 1988, Borneman *et al.* 1992). The two processes are shown in figure 21.



(a) Peroxidase mediated oxidative coupling



(b) Photodimerisation

Fig. 21: Dimerisation of phenolic acids (Christov and Prior 1993; McNeil *et al.* 1984)

Two types of dimers are formed by different reactions. The first is formed by the oxidative coupling of two ferulic acid residues with the loss of two hydrogens to form dihydroferulic acid. The second group is called truxillic acids (substituted cyclobutane dimers) and is formed from ferulic and *p*-coumaric acid in a sunlight driven dimerisation reaction where, unlike the oxidative coupling, there is no loss of hydrogen. The dimers that fall into this group can be: 4,4'-dihydroxy- α -truxillic acid (*p*-coumaric acid-*p*-coumaric acid dimer), 4,4'-dihydroxy-3,3'-dimethoxy- α -truxillic acid (ferulic acid – ferulic acid dimer) or 4,4'-dihydroxy-3-methoxy- α -truxillic acid (*p*-coumaric acid – ferulic acid dimer) (Borneman *et al.* 1993). Twelve dimers have been identified and four of these (all feruloyl dehydrodimers) are shown in the figure 22. Cross-linking is one of the ways in which the plant protects itself. In fungal

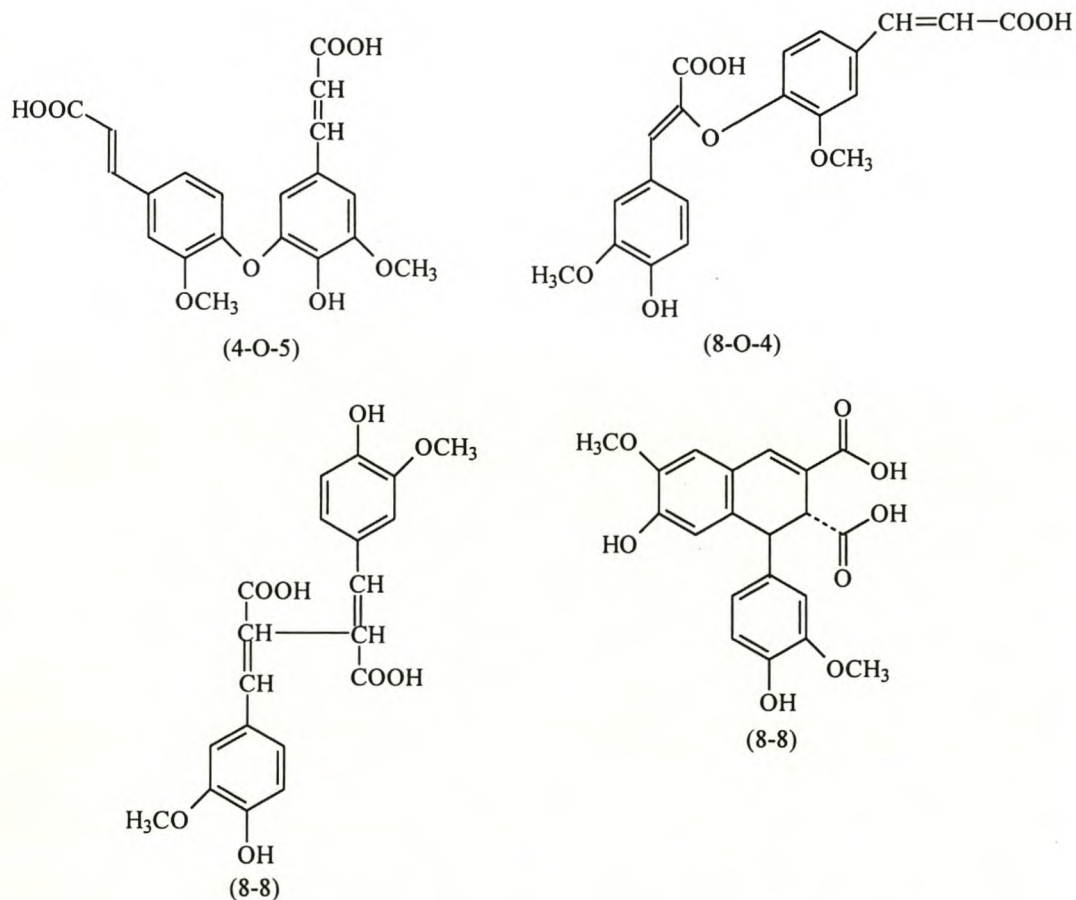


Fig. 22: Feruloyl dehydrodimers (Oosterveld *et al.* 1997, Ishii 1997)

infected plant tissue there is an increase in the amount of cell wall bound phenolics along with an increase in the peroxidase activity, increasing the level of oxidative coupling and thereby strengthening the cell wall making it impervious to fungal assault (Fry 1983). Due to the presence of these dimers biodegradability of the cell wall is diminished (Borneman *et al.* 1993; Kroon *et al.* 1996; Dalrymple *et al.* 1996; Donaghy and McKay 1994). In tests with *Digitaria decumbens* (pangola), *Setaria anceps*, sugar cane and barley straw biodegradability decreased as the levels of alkali soluble phenolics increased (McDermid *et al.* 1990a). Some of the functions that have been attributed to phenolics and their dimers are control of cell growth since cross-linking limits extension of the cell wall (Ishii 1997; Wende and Fry 1997), protection of the xylan molecule against hydrolysis of the glycosidic linkages (Wende and Fry 1997), stabilisation of the plant cell wall (Faulds and Williamson 1991), defence against pathogen attack and participation in mechanism of lignification (Tenkanen and Poutanen 1992).

4.2.1: Effect of phenolic acids on microorganisms

Phenolic acids inhibit degradation primarily through cross-linking of the cell wall. However, some phenolic acids are toxic or at the very least indigestible to microorganisms (Chauvaux *et al.* 1995; Borneman *et al.* 1986). Microorganisms that are negatively influenced by phenolic acids are bacteria (of importance for soil and ruminal bacteria), yeast, fungi and protozoa. In fact, phenolic acids have been used for a long time in the preservation of food (Borneman *et al.* 1986). The effect of the phenolic acids differs and different microorganisms are affected in different ways by the same phenolic acid. Experiments with the ruminal bacteria *Ruminococcus albus*,

Ruminococcus flavefaciens and *Butyrivibrio fibrosolvens* indicated that not all the phenolic acids have an inhibitory effect on the growth of microorganisms, some of them can actually stimulate the growth of microorganisms. The phenolic acids that have a stimulatory effect on the growth of microorganisms are hydrocinnamic acid, *p*-hydroxy-benzoic acid and syringic acid. Phenolics that had an inhibitory effect on bacterial growth are *p*-hydroxybenzaldehyde, *p*-coumaric acid and ferulic acid. Of these ferulic acid and *p*-coumaric acid have the most toxic effect on microorganisms while others had no or very little effect (Varel and Jung 1986; Borneman *et al.* 1986). The main effect experienced was an increase in the lag phase of these bacteria while in some instances, e.g. 10 mM of *p*-coumaric acid, growth was completely abolished. Hydroxybenzoic acid and *p*-coumaric acid also cause certain morphological changes in *R. flavefaciens* and *B. fibrosolvens*. In *R. flavefaciens* the cell size decreased, as did the size of the capsule that is formed to facilitate adhesion to surfaces or substrates. Gram variation, cell lysis and pleomorphism also occurred. As the concentration of *p*-coumaric acid increased *B. fibrosolvens* formed long chains with increasing length.

4.2.2: Degradation of *p*-coumaric acid and ferulic acid

As highlighted in previous sections, *p*-coumaric acid and ferulic acid in plant material are important because of their involvement in structures and the effect they have on the organisms degrading the cell wall. Since application of ferulic acid and *p*-coumaric acid esterases involves the release of these compounds it is important to look at the ways by which microorganisms can deal with the “threat” presented by ferulic and *p*-coumaric acid. Microorganisms can detoxify the phenolics or even use them as carbon source (Chauvaux *et al.* 1995). The conversion of ferulic and

p-coumaric acid has other consequences that will be dealt with in another section.

Figure 23 shows the pathway in *Bacillus pumilus* and *Fusarium solani*.

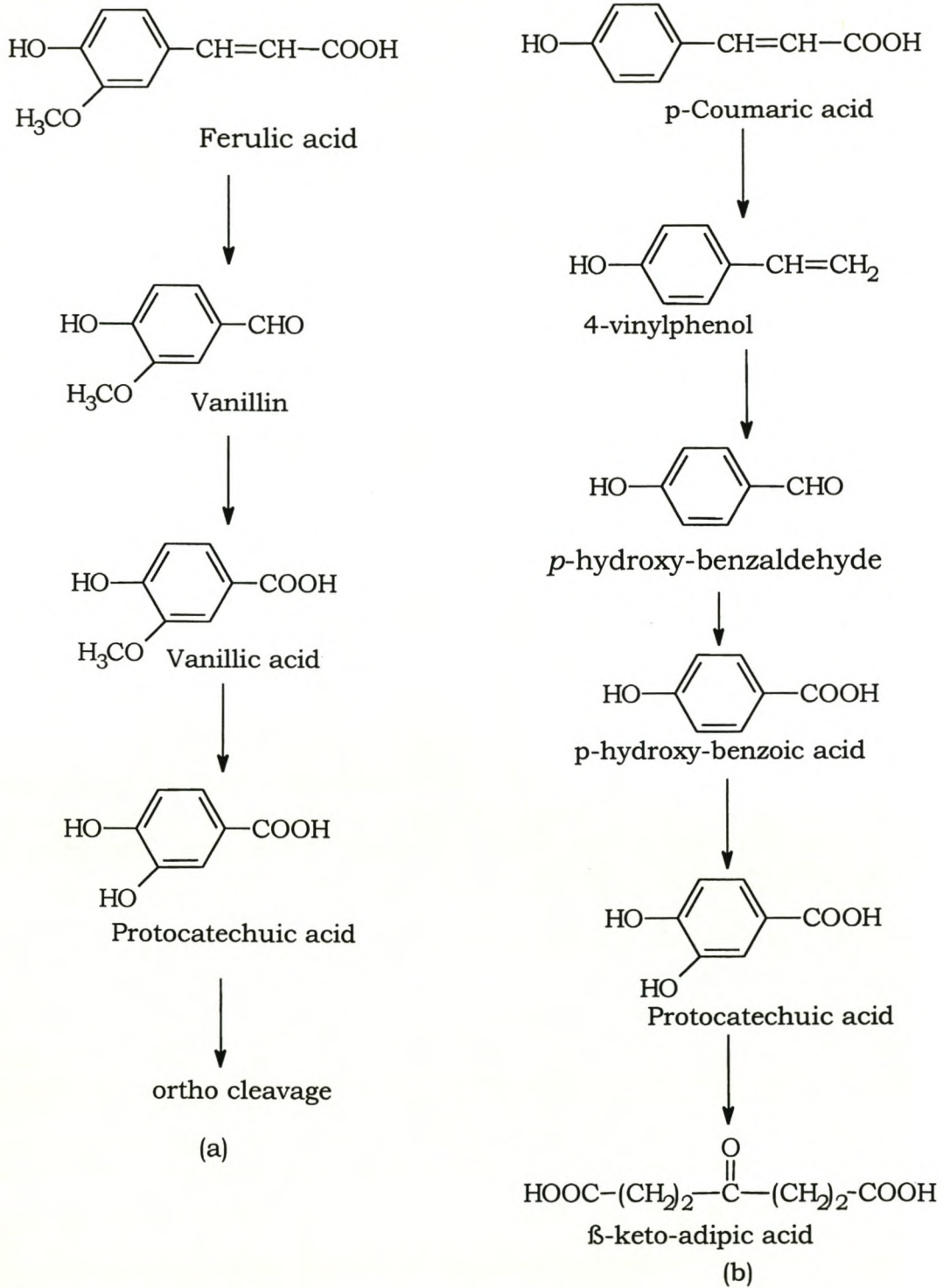


Fig. 23: Degradation of (a) ferulic acid by *Bacillus subtilis* and of (b) *p*-coumaric acid by *Fusarium solani* (Gurujeyalakshmi and Mahadevan 1987; Nazareth and Mavinkurve 1986)

Microorganisms that detoxify phenolic acids fall in genera such as *Bacillus*, *Pseudomonas*, *Candida*, *Hansenula*, *Saccharomyces*, *Rhodotorula*, *Streptomyces*, *Nocardia*, *Penicillium* and *Aspergillus*. They convert ferulic acid to 4-hydroxy-3-methoxy styrene (4-vinylguaiacol) and *p*-coumaric acid to 4-vinylphenol by way of decarboxylation (Zago *et al.* 1995; Huang *et al.* 1993). Those microorganisms that metabolise phenolics are *B. pumilus*, *F. solani* (Mart.) Sacc and methanogenic bacteria (Gurujeyalakshmi and Hahavedan 1987; Nazareth and Mavinkurve 1986). In each case the pathway is different. In methanogenic bacteria the degradation takes place under strict anaerobic conditions with methane and carbon dioxide as the end products (Healy *et al.* 1980).

5: Enzymatic degradation of xylan

Due to the complex nature of xylan, breakdown to its monomeric components requires the application of several enzymes, each with its own unique properties (Christov and Prior 1993, Kormelink and Voragen 1993, Linden *et al.* 1994a, Johnson *et al.* 1989). Degradation involves enzymes that remove the side-chains from the backbone and those that attack the main chain itself. During event one (Fig. 24) the main chain is randomly cleaved by endoxylanase, creating the shorter xylooligosaccharides (Haltrich *et al.* 1993, Biely 1993, Hazlewood *et al.* 1993) needed during the second event. At the same time the sidechain cleaving enzymes act

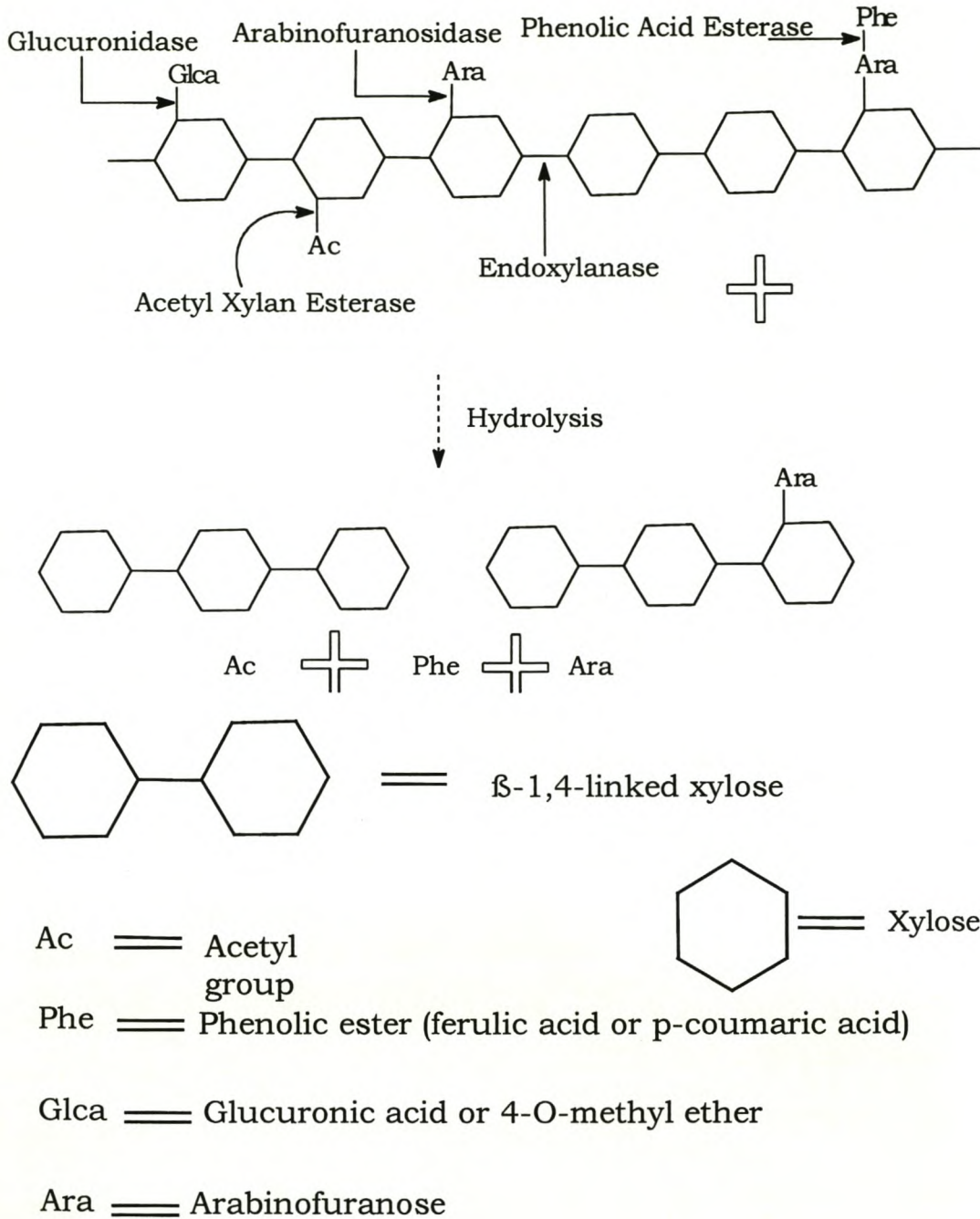


Fig 24: Event 1 of Xylan degradation (Haltrich *et al.* 1993; la Grange *et al.* 1997)

on their respective substrates. Phenolic acid esterases (ferulic and coumaric acid) remove their respective phenolics from the arabinose sidechains while acetyl xylan esterase liberates acetic acid directly from the xylan molecule (Donaghy and McKay 1995; Johnson *et al.* 1988). α -Glucuronic acid and its 4-O-methyl derivative are

removed by the action of α -glucuronidase. Arabinofuranosidase facilitates the removal of arabinose (Christov and Prior 1993, Biely 1993).

During the second event (Fig. 25) any remaining sidechains are removed as before, but the xylo-oligosaccharides are hydrolysed to xylose in an exo manner by β -xylosidase (Poutanen and Puls 1988, Lüthi *et al.* 1990, Anand and Vithayathil 1996).

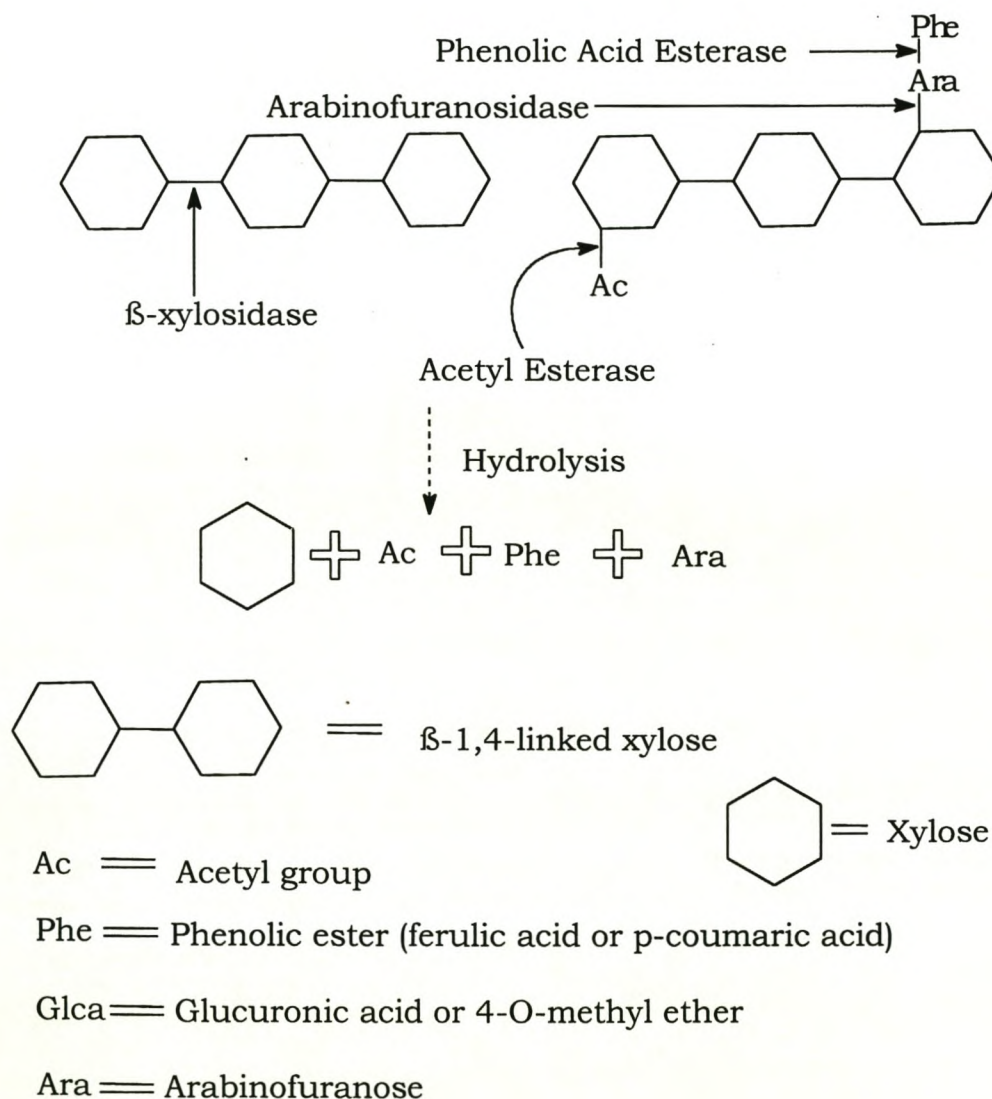


Fig. 25: Event 2 of Xylan hydrolysis (la Grange *et al.* 1997)

5.1: Synergism of esterases and xylanases

Degradation of xylan is a process where there is cooperation between different side chain-cleaving enzymes, different main chain-cleaving enzymes and between main and side chain cleaving enzymes (Coughlan and Hazlewood 1993; Sundberg and Poutanen 1991). When the different enzymes are applied in different combinations the extent of degradation varies according to the enzymes used (Christov and Prior 1993). Hydrolysis of acetylated xylan is greatly enhanced when esterases and xylanases are present in the reaction. The action of the acetyl xylan esterase creates new sites on the xylan molecule for endoxylanase cleavage (McDermid *et al.* 1990b; Poutanen *et al.* 1990). When acetyl esterase and xylanases are applied together there is also a synergism, but this time it is with respect to depolymerisation of the xylan molecule. While deacetylation provides new sites for endoxylanase cleavage the action of the endoxylanases also provides substrates for cleavage by the esterases (acetyl and phenolic). Synergism between the enzymes is very important for deacetylation of the xylan molecule. Aggregation and precipitation of the molecule occurs without treatment with xylanases that renders it unavailable for subsequent degradation (Kormelink *et al.* 1993).

5.1.1: Acetyl (xylan) esterase

Those enzymes that are responsible for cleavage of acetyl groups during xylan degradation can be either acetyl esterases or acetyl xylan esterases. Although both release acetic acid, acetyl esterases are specific for short xylooligosaccharides, while

the acetyl xylan esterases show preference for large molecular weight polymeric substrates (Poutanen *et al.* 1990). In Table 2 characterised acetyl (xylan) esterase enzymes are shown, some of which will be mentioned in the text.

Table 2: Characterised acetyl (xylan) esterases

Organism	Enzyme	pH*	Reference
<i>Schizophyllum commune</i>	AXE		Christov and Prior 1993
<i>Thermomonospora fusca</i>	AXE		Christov and Prior 1993
<i>Trichoderma reesei</i>	AE	5 – 6	Poutanen and Sundberg 1988
	AXE I	5 – 6	Poutanen and Sundberg 1988
	AXE II	5 – 6	Sundberg and Poutanen 1991
<i>Rhodotorula mucilaginosa</i>	AXE	5.5 – 9.2	Lee <i>et al.</i> 1987
<i>Fibrobacter succinogenes</i>	AXE	6 – 8	McDermid <i>et al.</i> 1990b
<i>Aspergillus niger</i>	AE	7.0	Linden <i>et al.</i> 1994a
	AXE	5.5 – 6	Kormelink <i>et al.</i> 1993
<i>Thermoanaerobacterium</i> sp.	AXE	7.0	Shao and Wiegel 1995
	AXE	7.5	
<i>Termitomyces clypeatus</i>	AXE	6.5	Mukhopadhyay <i>et al.</i> 1997
<i>Penicillium purpurogenum</i>	AXE I	5.3	Egaña <i>et al.</i> 1996
	AXE II	6.0	
<i>Bacillus pumilus</i>	AXE	8.0	Degrassi <i>et al.</i> 1998
<i>Streptomyces lividans</i>	AXE	7.5	Dupont <i>et al.</i> 1996

* pH range or optima

The acetyl xylan esterase of *Thermomonospora fusca* is an 80-kDa protein consisting of two 40-kDa subunits. However, a 20-kDa form of the protein has been observed. This may be formed by partial degradation of the native protein (Christov and Prior 1993; Bachman and McCarthy 1991). The acetyl xylan esterases of *Trichoderma reesei* are very similar and differ only with respect to pI and digestion patterns. Other than that they have similar pH and temperature ranges and molecular weights (Sundberg and Poutanen 1991). The last that will be discussed is that of the red yeast *Rhodotorula mucilaginosa*. This yeast produces an esterase capable of attacking the xylan molecule directly although it does not produce a xylanase. What function the enzymes have in the metabolic activities of this yeast is not known as yet (Lee *et al.* 1987).

5.1.2: Ferulic and coumaric acid esterases

Most of the ferulic and coumaric acid esterases release phenolic acids only in the presence of xylanases, but there are those that are capable of acting on plant cell walls in the absence of xylanases (Faulds and Williamson 1994). These enzymes can have a narrow substrate specificity hydrolysing only their specific phenolic acid or they can have a broader range releasing a range of phenolic acids. An enzyme from *Penicillium pinophilum* can release both *p*-coumaric acid and ferulic acid from wheat straw xylan (Castanares *et al.* 1992). *Aspergillus niger* produces three different ferulic acid esterases (FAE I, FAE II and FAE III). FAE I can liberate ferulic acid, *p*-coumaric acid and caffeic acid. FAE II also liberates ferulic acid, but also sinapic acid and has low activity towards *p*-coumaric acid. The third enzyme, FAE III, shows a second activity in that it can release acetyl groups from acetylated xylan, an activity

that is enhanced by the presence of xylanases (Faulds and Williamson 1993). By its ferulate action, *FAE III* can release arabinoxylan and β -glucan from the endosperm cell wall of barley (Moore *et al.* 1996). Table 3 shows characterised phenolic esterases.

Table 3: Characterised ferulic and coumaric acid esterases

Organism	Enzyme	pH*	Reference:
<i>Penicillium pinophilum</i>	CAE \ FAE	6.0	Castanares <i>et al.</i> 1992
<i>Neocallimastix MC-2</i>	FAE I	6.2	Borneman <i>et al.</i> 1993
	FAE II	7.0	
	CAE	7.2	
<i>Aspergillus niger</i>	FAEI	5	Faulds and Williamson 1993
	FAEII		Faulds and Williamson 1994
	FAEIII		
<i>Aspergillus oryzae</i>	FAE	3 – 7	Tenkanen <i>et al.</i> 1991
<i>Streptomyces olivochromogenes</i>	FAE	5.5	Faulds and Williamson 1991

* pH range or optima

FAE = ferulic acid esterase

CAE = coumaric acid esterase

6: Applications of esterases

While the genetic and biochemical characterisation of these enzymes and their genes are important, of equal importance is the potential application of these enzymes. One application of these esterases is that they can be used as a tool in research regarding cell wall structure, plant disease resistance and decomposition of plant material

(Christov and Prior 1993). Industrial applications will be highlighted in the following sections.

6.1: Pulp and paper industry

During the production of pulp the main aim is the removal of the lignin from the cellulose fibres, since the lignin imparts a brown colour to the pulp (Solomon 1996). The removal of lignin from the pulp is referred to as bleaching and until recently was routinely carried out using chlorine and chlorine dioxide combined with alkaline extraction (Coughlan and Hazlewood 1993, Rixon *et al.* 1996). Figure 26 shows the bleaching of wood.

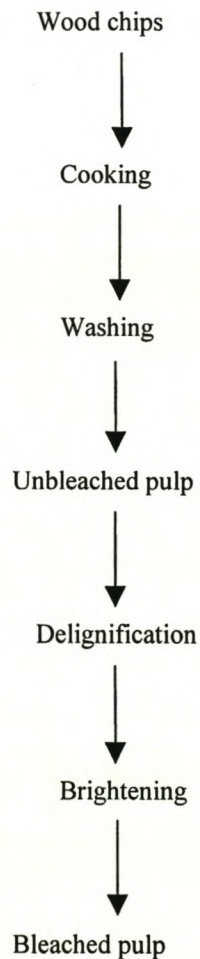


Fig. 26: The Kraft pulping process (Solomon 1996)

A side effect of the bleaching of wood is the production of organochlorines, which show properties such as persistence, bioaccumulation and toxicity. Some have properties similar to that of DDT (Solomon 1996), a chemical banned from the market. Growing concern over these compounds has led to the development of other strategies, one of which is the pre-treatment of pulp with enzymes such as xylanases (Patel *et al.* 1993). Use of enzymes in pre-treatment can have one of two objectives. The first is the breakdown of lignin by lignin-degrading enzymes while the second entails the breakdown of the hemicellulose component, mainly xylan (Ross *et al.* 1992). The beneficial effect of xylanases comes not as much from degradation of the xylan, but rather from reducing its chain length. This makes the lignin more mobile and makes for greater ease in removal by alkaline extraction (Paice *et al.* 1992). Here the esterases can play an important part by making the lignin-carbohydrate complex more vulnerable to enzymatic degradation through disruption and loosening of the cell wall. Overall use of enzymes will lead to a reduction in the amount of chemicals used along with improvement in the brightness of the pulp (Christov and Prior 1993; Ross *et al.* 1992, Bachman and McCarthy 1991).

6.2: Phenolics and beer

Lipid oxidation can lead to undesired flavours in beer. Malt contains a group of compounds known as phenolic acids that have been shown to be antioxidants (Bartolomé *et al.* 1996). Ferulic acid is one of the more effective phenolic acids in this regard and besides its antioxidant activity also has antimutagenic and anticarcinogenic properties. It has been implicated in the nitrite scavenging effect of wheat bran (Faulds and Williamson 1995b; Sharzad and Bitsch 1996). Despite all

these positive attributes the effectiveness of ferulic acid as an antioxidant is limited due to its low concentration in beer. By liberating ferulic acid, ferulic acid esterase will contribute to its beneficial effect.

6.3: Bioconversion

The pulp and paper industry along with agriculture and other industries generate huge amounts of lignocellulosic material yearly, much of which is regarded as waste. These lignocellulosic materials, with their available cellulose and hemicellulose, are the ideal (readily available and very cheap) substrate for conversion to useful products by microorganisms. Esterases used in conjunction with cellulases and hemicellulases can be used to convert this waste to fermentable sugars for the production of desired compounds (Christov and Prior 1993). One such product is fuel ethanol which has the advantage that it will not contribute to the greenhouse effect since it is CO₂ neutral (Ahring *et al.* 1996).

6.4: Animal nutrition

A large proportion of feedstock that is given to animals consists of grain or agricultural refuse. The composition of the feed can cause dietary problems for the animals that consume it. The application of esterases in animal nutrition will be discussed under two headings.

6.4.1: Animal digestion

Forage fed to animals contains acetyl groups and phenolic cross-linkings that inhibit the degradation of the materials by these animals (McDermid *et al.* 1990a). By breaking the cross-linkages, loosening the wall structure and releasing lignin, the esterases increase access of the other enzymes to the substrate thereby increasing the availability of carbohydrate to the animal (Chen *et al.* 1996; McDermid *et al.* 1990b). This is illustrated by the fact that the amount of ferulic acid released from the cell wall correlates with the amount of forage digested by the animals (MacKenzie *et al.* 1987).

6.4.2: Poultry

While β -glucans has beneficial effects when added to the diets of humans, their addition to the diets of especially monogastric animals cause antinutritional effects (Humphreys and Mather 1996). While β -glucan quantities is a problem in barley feed, other pentosans found in rye (e.g. arabinoxylan) can also be an antinutritional factor. These pentosans can exert a negative effect at levels lower than 50 g/kg (Choct and Annison 1992). The reason why these pentosans have a negative influence on the growth of the animals especially that of broiler chickens is that the animals are not capable of digesting the polysaccharide. Thus, the highly viscous material is not broken down and this causes problems such as hampering of nutrient diffusion. As it binds water it makes the excreta watery (Wong and Saddler 1993), which results in loss of fluids. Pigs are not as prone to this affliction as the longer retention time in their digestive tracts reduces the negative effect of the pentosans (Walsh *et al.* 1993). Besides creating what is referred to as the dirty egg problem in

laying hens it also causes sticky droppings and depressed growth (van Rensburg *et al.* 1997, Choct and Annison 1992). The interference with the uptake of nutrients is a serious matter since it impairs performance of the animal (Walsh *et al.* 1993). Use of xylanolytic and β -glucanases will lead to a decrease in the molecular weights of these pentosans, decreasing their viscosity and their gelling effect and so alleviate the problems caused by these pentosans (Wong and Saddler 1993, Walsh *et al.* 1993, Choct and Annison 1992). β -Glucanases will act by degrading the β -glucan. Esterases will not act directly, but will enhance the ability of the xylanases to break down the arabinoxylan. Correct application of these enzymes might enable the animals to utilise part or all of these pentosans that constitute a large part of the forage fed to them. This will allow the use of grain and other feed with higher amounts of β -glucan or arabinoxylan.

7: Esterases and yeast

The yeast *S. cerevisiae* has been used extensively for the making of bread and wine (Buckholz and Gleeson 1991), and is an ideal host for the production of bioethanol (van Rensburg *et al.* 1997). These positive characteristics of the organism are negated by the fact that *S. cerevisiae* is unable to degrade polysaccharides such as xylan (la Grange *et al.* 1997). As discussed, esters are present on many of these polysaccharides. For complete degradation of these substrates to the monomeric units for utilization by *S. cerevisiae*, esterases are needed. To date no esterases have been expressed in *S. cerevisiae*. The benefit of expressing esterases in yeast is two-fold: Firstly, it will increase our knowledge of heterologous production of especially esterases in yeast. Secondly, expression of esterases that are involved in the

degradation of hemicellulose, can only be of benefit for the effective and complete utilisation of these substrates by *S. cerevisiae*.

8: Aims of study

Esterases play an important role in nature. These include cellular function (Rajesh *et al.* 1999), plant defence (Yagami 1998), plant development (Melati *et al.* 1996) and the method of action of snake venom (Mallipali *et al.* 1998). Esterases are involved in the degradation of wood and wood components. Complete degradation of this substrate to the monomeric components will enable an organism such as *Saccharomyces cerevisiae* to ferment the sugars to commodities such as ethanol.

The primary aim of this study was to isolate an acetyl esterase from a genomic library of *Bacillus pumilus* and to express and characterise the esterase in *Escherichia coli*. The secondary aim of this study was to clone the esterase gene in different yeast expression cassettes, transform them into the yeast *S. cerevisiae* and to evaluate the production of the esterase in *S. cerevisiae*.

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Chapter 2

Cloning and expression of a novel

***Bacillus pumilus* carboxyl esterase (*estA*) in**

Escherichia coli

Cloning and expression of a novel *Bacillus pumilus* carboxyl esterase (*estA*)

Anton Pieterse, Ricardo R Cordero Otero and Willem H van Zyl
Department of Microbiology, University of Stellenbosch, Stellenbosch 7600,
South Africa,

A novel carboxyl esterase (*estA*) was cloned from the Gram-positive bacterium *Bacillus pumilus* and expressed in *Escherichia coli*. The *estA* gene contains an open reading frame of 834 bp and encodes a protein with a molecular weight of 30 kDa. Expression of the *estA* gene in *E. coli* yielded 2.5 nkatal/ml activity on α -naphthyl acetate after 15 h. The recombinant esterase produced in *E. coli* has a pH optimum of 6 and a temperature optimum of 60°C. It is thermostable at the optimum temperature and after 2 hours retains 90% of its activity. The esterase exhibited activity against glucose pentaacetate, α -naphthyl acetate, methylumbelliferyl butyrate and methylumbelliferyl acetate. It was therefore identified as a carboxyl esterase.

1: Introduction

Esterases are a large family of enzymes that are involved in the regulation of insulin secretion (Han *et al.* 1999), tRNA processing (Gurevitz *et al.* 1982), acetate utilization (Lee *et al.* 1996), fatty acid oxidation (Jones *et al.* 1999), protein degradation (Rajesh *et al.* 1999) and the inactivation of platelet-activating factor (Cao *et al.* 1998). They are also present in snake venom (Mamillapalli *et al.* 1998). Carboxylesterase catalyses the reaction where a carboxylic ester is reduced to an alcohol and a carboxylic acid anion. Carboxylesterase has very wide substrate specificity and can be used for the synthesis and hydrolysis of esters (Kim *et al.* 1997). Substrates such

as vitamin A esters, methylumbelliferyl acetate, α -naphthyl acetate, α -naphthyl butyrate, phenylthiovalerate, *p*-nitrophenylacetate and carboxylic esters are included in this spectrum (Enzyme Handbook). Carboxylesterases have been isolated from organisms such as *Bacillus acidocaldarius* (Manco *et al.* 1994), *Pseudomonas* (Sugihara *et al.* 1994, Kim *et al.* 1997) and *Yersinia pseudotuberculosis* (Goullet *et al.* 1989).

With oil and coal resources being depleted, the use of plant material as a renewable energy resource is becoming increasingly important. Hemicellulose is a major component of plant material and consists of a number of hetero-polysaccharides (Biely 1993, Schäfer *et al.* 1996). The XXXG (substituted:unsubstituted = 3:1) and XXGG (substituted:unsubstituted = 1:1) forms of xyloglucan (Vincken *et al.* 1997), galactoglucomannan (Sims *et al.* 1997) and xylan (Shareck *et al.* 1995) are acetylated. Acetic acid can limit degradation of XXGG xyloglucan by endoglucanases (Vincken *et al.* 1997) or xylan by xylanase and α -glucuronidase through steric hindrance (McDermid *et al.* 1990, Sunna and Antranikian 1997). In the XXXG form of xyloglucan, acetylation has no effect on degradation (Vincken *et al.* 1997). Phenolic esters such as *p*-coumaric acid and ferulic acid are found linked to xylan (Fernández *et al.* 1995). By binding to other cell wall components (Christov and Prior 1993), phenolic acids increase the integrity of the plant cell wall (Castanares *et al.* 1992).

In this paper we discuss the isolation of a novel esterase gene from a genomic DNA library of *Bacillus pumilus* and its cloning and characterisation in *Escherichia coli*.

2: Materials and Methods

2.1: Strains, plasmids, media and growth conditions

Escherichia coli XL1-Blue MRF' *endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* [F' *proAB laqI^ΔZΔM15 Tn10 (tet)*] (Stratagene) was used as bacterial host. Cultures were grown aerobically at 37°C. Liquid cultures were grown in TB (Terrific Broth), and on LB (Luria-Bertani broth) agar plates (Sambrook *et al.* 1987). Solid and liquid media was supplemented with ampicillin (100 µg /ml) to select for plasmids. Plasmid pGDL1 (this work) was isolated from positive *E. coli* transformants obtained after screening of a genomic DNA library of *B. pumilus* kindly prepared by Danie la Grange (la Grange *et al.* 1997). Plasmids pBluescript KS⁺ (Stratagene) and pUC18 (Yanisch-Perron *et al.* 1985) were used for subcloning of DNA fragments from plasmid pGDL1 for sequencing of the genomic fragment. Plasmid pAP4 (this work) was used for constitutive expression of *estA* and plasmid pKK223-3 (Amersham Pharmacia Biotech) for overexpression of the *estA* gene in *E. coli*.

2.2: DNA manipulation

Standard protocols were used for DNA manipulation (Sambrook *et al.* 1989). Restriction and ligation enzymes for DNA manipulation were purchased from Roche Molecular Biochemicals and used as recommended by the supplier. Digested DNA was isolated from agarose gels using the Agarose gel extraction kit (Roche Molecular Biochemicals). PCR products were purified using the High PureTM PCR Product Purification Kit (Roche Molecular Biochemicals). DNA used for sequencing

purposes was isolated with the Nucleon MiP plasmid purification kit (Amersham) according to the guidelines of the manufacturer. Sequencing was done by amplifying DNA fragments with the Big Dye Terminator Cycle sequencing Reader reaction using Amplitaq DNA polymerase F5 (Applied Biosystems kit). Reaction mixtures were electrophoresed on an Applied Biosystems automatic DNA sequencer (model ABI Prism™ 377). Data obtained was processed on computer using the PCGENE program (IntelliGenetics). PCR was done according to standard procedure using the Perkin Elmer 2400 machine using oligonucleotide primers obtained from Whitehead Scientific.

2.3: Plasmid construction

The construction of plasmid pAP4 is shown in Figure 1. Plasmid DNA isolated from a positive *E. coli* transformant (plasmid pGDL1) and plasmid pUC18 was digested with *EcoRI* and *Sall*. The 3-kb DNA insert containing the esterase gene was excised from plasmid pGDL1 and ligated into plasmid pUC18, generating plasmid pAP4. Plasmid pAP4 was introduced into *E. coli* and the transformants were plated on glucose pentaacetate to verify the esterase activity. After most of the DNA sequence of the 3-kb insert was determined, the *estA* gene was amplified as an 834-bp fragment by PCR using primers CE1-L and CE1-R (Table 1) and cloned into the corresponding *EcoRI* and *HindIII* restriction sites of plasmid pKK223-3, generating plasmid pOE1. The cassette is shown in Figure 2. Correct insertion of *estA* was confirmed through sequencing.

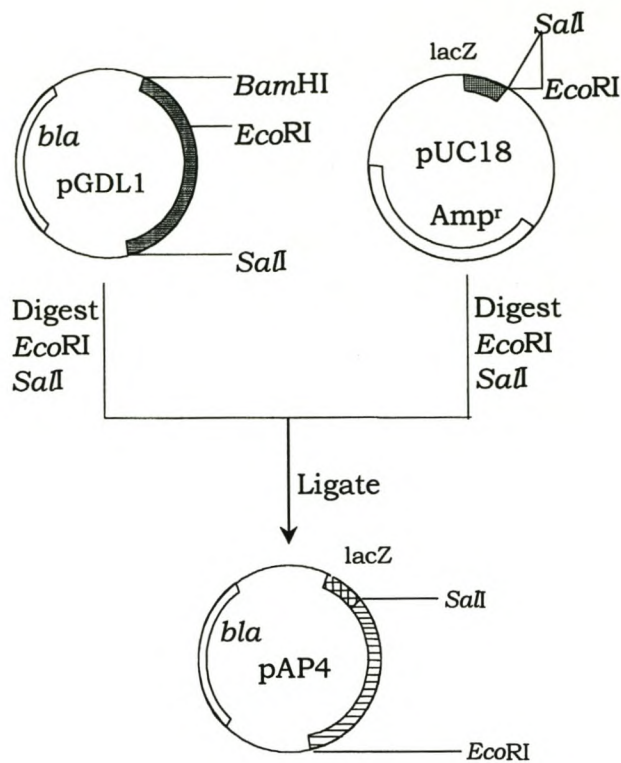


Fig. 1:Construction of pAP4. Open boxes are the β -lactamase gene (*bla*); parallel lined boxes represent the gene fragment and cross hatched boxes *lacZ*

TABLE 1. Deoxyribooligonucleotides used for amplification the of *estA* gene

Name	Sequence
CE1-L	5'- CCGGAATTCATGAACTTAGAAGAACAAATC-3'
CE1-R	5'-CCCAAGCTTAGATCTAAATCTCCGGCGTG-3'

Restriction sites are underlined

2.4: Screening and selection of positive clones

The genomic DNA library of *B. pumilus* was screened using a modification of the method used by Lee and co-workers (Lee *et al.* 1987). The screening plates consisted of two layers. The bottom layer contained LB agar with 100 μ l/ml ampicillin and was

buffered with phosphate buffer (50 mM final concentration). The top layer (3 ml) was similar to the bottom layer, but in addition contained 2% (m/v) glucose pentaacetate. Colonies forming a clear zone were selected and replated using sterile toothpicks. To link enzyme activity with the presence of the plasmid, plasmid DNA was isolated from colonies that formed a clear zone and retransformed in *E. coli*. The transformants were subsequently plated on screening plates to check for zone formation.

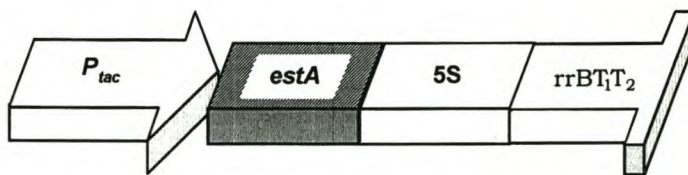


Fig. 2: Expression cassette in plasmid pOE1 for over-expression of *estA* in *E. coli*. P_{tac} is the inducible promoter. 5S is the *E. coli* 5S gene and $rrBT_1T_2$ is the T_1 and T_2 terminators.

2.5: Enzymatic assays

Unless stated otherwise, cell free extracts were used for enzyme assays. Assays were done using a combination of the methods of la Grange *et al.* (1997) and Poutanen and Puls (1988). To 890 μ l of 50 mM Na-phosphate buffer (pH6) and 100 μ l of enzyme solution 10 μ l of the substrate (500 mM α -naphthyl acetate in N-N-dimethylformamide) was added. The reaction was incubated at 50 $^{\circ}$ C for 10 min after which 1 ml of the colouring reagent (0.01% Fast Corrinth V salt in 1M sodium acetate buffer, pH 4.4, containing 10% Tween 20) was added. Absorbance was read at 535 nm after 10 min. Activity was expressed as nanokatal per ml (nmol α -naphthol ions produced $s^{-1} ml^{-1}$). α -Naphthol was used as standard (Poutanen and Puls 1988).

A modification of this procedure was used to test for substrate specificity. 250 mM stock solutions of methylumbelliferyl butyrate (Sigma) and methylumbelliferyl acetate (Fluka) and methylumbelliferyl ferulate were prepared in dimethylformamide. Twenty microliters of these substrates were used in the assay and the reactions were incubated for 10 min at 37°C after which a two-fold dilution of samples and control were made. *E. coli* containing plasmid pUC18 was used as negative control. Production of methylumbelliferone by catalysis of substrates was visualised by UV. Acetyl xylan esterase assays were done using a 2% solution of birchwood (Sigma) in sodium phosphate buffer as substrate. Two hundred microliters of the substrate were added to 700 µl buffer and 100 µl enzyme. The reaction was incubated for 15 min and released acetic acid was quantified using an acetic acid test combination (Roche Molecular Biochemicals). Thermostability was tested by heating the enzyme solution for pre-determined time intervals at various temperatures. Activity was assayed using α -naphthyl acetate, as described earlier. Assays at different temperatures were performed as described above within the range 25 - 77°C. Assays at different pH values were performed as described above, using two buffers: citrate buffer (pH 3.2 to 6.0) and phosphate buffer (pH 6.0 to 8.0). Different enzyme preparations were used for each assay.

2.6: Protein separation

A 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gel was used to separate proteins in the extracellular fraction. Both control and sample cultures were grown in modified M9 media. To 870 ml sterile ddH₂O the following was added:

100 ml 10X M9 salts (Sambrook *et al.* 1987), 1 ml magnesium sulphate (1 M), 10 ml glucose (20%), 1 ml thiamine and 10 ml calcium chloride (100 mM). *E. coli* cultures, pre-grown to OD₆₀₀, were induced with IPTG and after 4 hours growth was stopped by placing the cultures on ice. Proteins were precipitated using 75% ammonium sulphate. Excess salts were removed through dialysis using a 1,000 Da molecular weight cut-off membrane (Spectra/Por®).

3:Results:

3.1: Screening on glucose pentaacetate plates

A total of 726 *E. coli* transformants were screened on glucose pentaacetate of which one transformant, containing plasmid pGDL1, was found with esterase activity. A 3-kb DNA fragment from plasmid pGDL1 was inserted into plasmid pUC18 to generate plasmid pAP4. Acetyl esterase activity of *E. coli* transformants containing plasmid pAP4 was confirmed through the solubilisation of glucose pentaacetate to glucose and

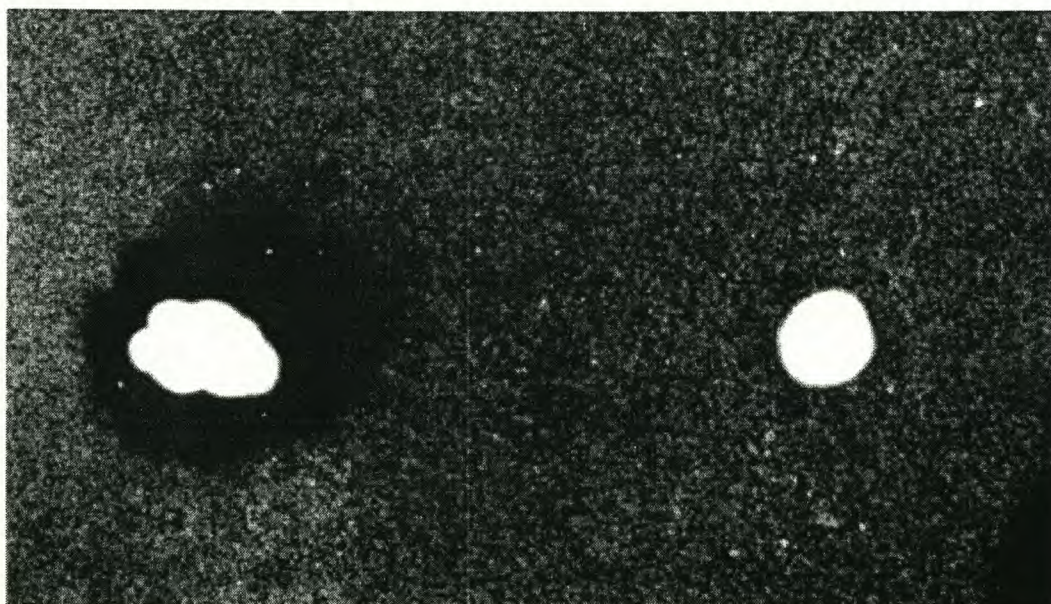


Fig. 3: Screening for acetyl esterase activity in *E. coli* transformants. *E. coli* containing plasmid pAP4 formed a clear zone (left colony). *E. coli* containing plasmid pUC18 (control) on the right formed no clear zone. This indicates that the esterase activity is associated with the plasmid.

acetate, forming a clear zone around the colony (Fig. 3).

3.2: Determining the *estA* gene sequence:

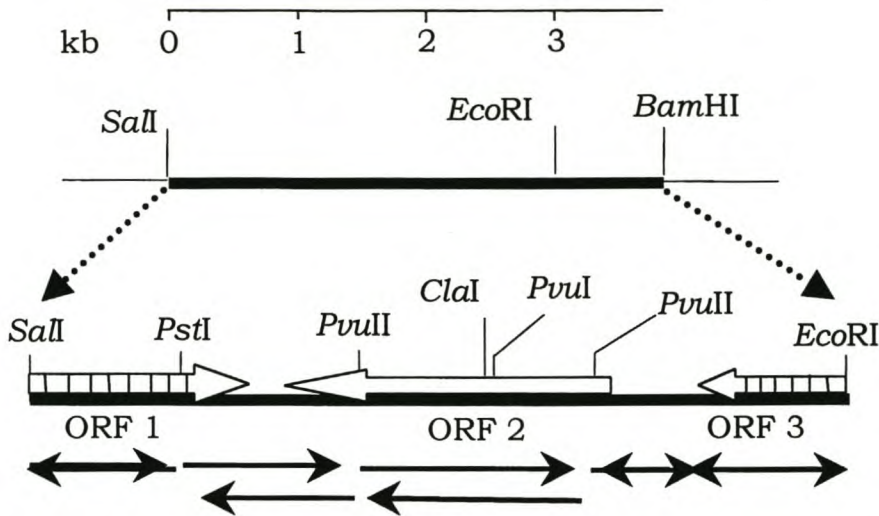


Fig. 4: Sequencing of genomic insert. Solid arrows indicate direction of sequencing. Boxes with vertical lines represents partial ORFs and the open box the complete ORF.

The 3-kb *EcoRI/SalI* DNA fragment was subcloned in pBluescript and pUC18 and the DNA sequence of the DNA insert determined (Figure 4). Sequencing revealed the presence of a complete and two partial open reading frames within the 3-kb insert. While ORF2 and 3 have the same orientation, ORF1 is orientated in the opposite direction. The sequence is shown in appendix A. The partial open reading frame 1 showed 86% identity on DNA level with the endo- β -1,3-1,4-glucanase from *Bacillus licheniformis*. ORF 3 showed 58% identity with the *lmrB* gene from *Bacillus subtilis*. This gene is responsible for lincomycin resistance. The complete open reading frame 2 showed identity with the carboxylesterase from *Archaeoglobus fulgidus* (38%), heroin esterase from *Rhodococcus* sp. (36%) and a lipase from *Pseudomonas* sp. B11-1 (37%) at the protein level. The nucleotide sequence and protein sequence of the *estA* gene is shown in Figure 5. A putative Shine-Delgarno region was located approximately 9 bp upstream from the start codon

and a rho-independent-like termination site was located downstream of the open reading frame.

TATCATAAGAGAAATCAAAGCACTGAGGACATAGCGCCGAGTGCTTTTTTATTTGCTCAATGCTTGT
 TTCAAAGAGAGGGAAAAAGGGGAGATAGGTAAAAAGTGAACCAATATAAGTTGCTGTTGATTTCGGTAGG
 AGGAAAAGAGGAAAAGGAAACGAATGAGTAACTAAGAAATGAATAAGATAGGAGTCTGAGATC

ATG	AAC	TTA	GAA	GAA	CAA	ATC	AAA	ATC	GCT	GCT	TCA	TTA	CGC	CAG	CCA	TCT
M	N	L	E	E	Q	I	K	I	A	A	S	L	R	Q	P	S
GAA	GGT	TCA	TTA	GCG	AGT	CAA	TCG	GAA	CTA	AAG	CCA	GTA	AAT	CCT	CCT	GAA
E	G	S	L	A	S	Q	S	E	L	K	P	V	N	P	P	E
GTG	AAC	AAA	ATG	GAA	TAT	GAC	ATT	CCA	ACA	AGT	GCT	GGC	GAA	ACA	AAG	GTG
V	N	K	M	E	Y	D	I	P	T	S	A	G	E	T	K	V
TGG	GTA	TTT	AAG	CCA	GTG	AAC	ACA	CAA	AAA	CAG	CTG	CTT	CCT	GTT	TTT	GTG
W	V	F	K	P	V	N	T	Q	K	Q	L	L	P	V	F	V
AAT	TTA	CAT	GGC	GGC	GGA	TTT	ATC	ATG	GGC	AGT	GCT	GAA	ATG	GAT	AAT	CCC
N	L	H	G	G	G	F	I	M	G	S	A	E	M	D	N	P
TGG	TGT	CCA	GTC	ATT	GCA	GAC	CGG	GCG	CAA	TGT	ATC	GTC	GTC	AAT	GTC	GAG
W	C	P	V	I	A	D	R	A	Q	C	I	V	V	N	V	E
TAT	CAG	CTT	GCA	CCC	GAG	CAC	CCT	TTC	CCA	GCC	GCT	CTT	CAT	GAA	TGC	TAC
Y	Q	L	A	P	E	H	P	F	P	A	A	L	H	E	C	Y
GAT	GTA	CTG	AAG	TGG	CTG	TAT	GAA	CAT	CCT	GAG	GAG	CTT	CAA	ATC	GAT	CGG
D	V	L	K	W	L	Y	E	H	P	E	E	L	Q	I	D	R
AAT	GCA	TTA	GCC	ATT	GGC	GGA	CAT	AGC	GCA	GGA	GGA	AAT	TTG	GCA	ACG	GCT
N	A	L	A	I	G	G	H	S	A	G	G	N	L	A	T	A
GCT	TGT	CTA	TTG	AAT	ATT	CAA	AGA	GGA	AAT	ACA	CTC	CCA	ATT	GCC	TAT	CAA
A	C	L	L	N	I	Q	R	G	N	T	L	P	I	A	Y	Q
GTG	CTT	GAT	TAT	CCG	CCG	CTT	GAT	CTA	GCC	ACT	GAT	CCA	GCA	CAA	AAG	CCA
V	L	D	Y	P	P	L	D	L	A	T	D	P	A	Q	K	P
GCG	TTT	GAA	GAA	GCG	ATC	CCA	GTT	GAA	TTG	GCG	AGG	CTC	TTT	AAT	TCC	TTC
A	F	E	E	A	I	P	V	E	L	A	R	L	F	N	S	F
TAT	CTG	CAA	GGC	CAA	GAT	CCG	CAC	AAT	CCG	CTC	GTT	TCT	CCG	GTT	TTT	GCA
Y	L	Q	G	Q	D	P	H	N	P	L	V	S	P	V	F	A
GAT	CGT	TCA	TCC	TTA	GCA	CAA	ATG	CCT	CCA	GCT	CTC	GTC	ATC	ACA	GCT	GAA
D	R	S	S	L	A	Q	M	P	P	A	L	V	I	T	A	E
AGA	GAT	TCG	CTA	GCT	CAA	GAA	GCC	GAC	CAA	TAT	GCG	AAG	AAG	TTA	AAA	GAA
R	D	S	L	A	Q	E	A	D	Q	Y	A	K	K	L	K	E
GCA	GGG	GTA	GAT	GTC	ACG	TAC	AGC	AGT	TTA	AAG	GAG	TTC	CTC	ACG	GCT	TCA
A	G	V	D	V	T	Y	S	S	L	K	E	F	L	T	A	S
CAC	ACG	CCG	GAG	ATT	TAG	CATAGCTGAAGAGGCTTGGCATTGATGGGGGATCAACTAAAG										
H	T	P	E	I	-											

AAAGCGTTTGAATAAGAAAAGTAGAGTGATTCAAAGCACAGGAGACAATGGGCCTGTGCTTTTATTAC
 TGTCTGAAAAGAAAAGTGAATGTA AAAAGGGT CAGATATCTTTCCTCGATATAATA

Fig. 5: Nucleotide sequence of *estA* ORF and flanking regions. The protein sequence is shown beneath the nucleotide sequence. Opposing arrows indicate the inverted repeats of a possible rho-independent terminator. A putative Shine-Dalgarno region is boxed

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EstA  -----MNLEE
Ceaf  -----MLDMPIDPVYYQLAEYFDSL
Lp    -----MPLDKQIAAVLQQFSEL
Her   -----MTTFPTLDPELAAALTML
Cea   MTAfVQTIQEVLEKGHGPAARALDKLPSfVQESIAKVLGYPYQYPQLDSfIKCLMAVQIK

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EstA  QIKIAASLRQPSEGLA SQSELKPVNP-P EVNKMEYDIPTSAG-ETKVVWFKPVNTQKQL
Ceaf  PKFDQFSS--AREYREA INRIYEERNRQLSQHERVERVEDRTIKGRNGDIRVRVYQQKPD
Lp    PAPDFSQLD-AAQYRQFCNLLPAIPGDPMIEVRNLRVAAAAG---ELDARLYRPLEEDN
Her   PKVDFADLPNARATYDALIGAMLADLSFDGVSLRELSAPGLDGDPEVKIRFVTPDNTAGP
Cea   QGQTGFIGSDVEKSRLAFETQMESIILRKP TAITFVEDIRLPLQSGTIFARHYHPAPNKK-

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```

EstA  LPVfVNIHGgGFIMGSAEMDNpWCpVIA DRAQCIvVNV EYQLAPEHPFPAA LHECYDVLK
Ceaf  SPVlVYYHGgGFVICSESHDALCRRlARLSNSTvVsvDYRLAPEHKFPAAVYDCYD ATK
Lp    LPllVFFHGgGFVMGNLDTHDNLcRSlASQTEAVvVsvAYRLAPENHFPAA PLDCY AATC
Her   VPVllWiHGgGFaIGTAESSDPFcVEVA RELGFAVANVEYRLAPEttFP GPVNDCYAAL L
Cea   LPMIvfyHGgGFvVGNVDTHDEACRLlAKYANAQvLSIDYPLAPEvSPQRlIQSCEDALA
      P      HGgGF          C      A      V      Y LAPE          C

```

```

EstA  WLYEHPEELQIDRNALAIgGHsAGGNlATAACLLNIQRGNTLPiAYQVLdYpPlDLATDP
Ceaf  WVAENAEELRIDPSKIFVGGDSAGGNlAAVSIMARDSG-EDFtKHQILiYpVvNFVAPT
Lp    WLVEHAAELGVDGRRLA LAGDSAGGNlAAVSRLAAQRQ-GPKiSYQCLfYpVtDARCDS
Her   YIHAHA EELGIDPSRIAVGGESAGGGLAAGTVLkARDEG-VVPVAFQfLEiPELDRLET
Cea   WVYQNKRFKlLKNQIAVAGDSAGGN IStVVAQRaIGKV--YAQDAQfLiYpVvDFKSRH
      G SAGG          Q L P

```

```

EstA  AQKPAfEEA-----IPVEL---ARLFNS FYLQGDpHNPLVSPVFADRSSLAQMPPALVI
Ceaf  PSLLEfGEGl-WILDQKIMSWFSEQYfS REEDKFN---PLASVI fAD---LENLPPALI I
Lp    QSYEEfAEG--YFLTGAMMYWfWQQYlQDTGQGDD---PLASPLRAET--LADLPPtTLI
Her   VSMTNfVDTPLWHRPNAILSWKYyLGES YSGPEDpDVS IYAAPSRATD--LTGLPPtYLS
Cea   PSFYAYKDG----LVLTGNDVDYvTDYyATKHAVHLDDPiISpTYGN---FKKLAPAYIV
      P

```

```

EstA  TAErDSLAEADQYAKKlKEAGVdVtYSSlKEFLtASHTPEI-----
Ceaf  TAEYDPLRDEGEVfGQMLRRAGVEASIVRYRGVlHGfINyYpVlKAARDAINQIAALLVf
Lp    TAEfDPLRDEGEAFALRLQAGVsvRVQRCEGMiHGfISMAPfVERAAHALSDAAADLRR
Her   TMElDPLRDEGIEYALRLlQAGVsvELHSfPGTFHGSalVATAGVRERGAAKPHCDPERV
Cea   TAGHDVlHDEGEIYSHKlRQAGVKIHfEEYLDQTHGfINlTPVSHKARANLIQMSKsFRK
      T   D L E          L AGV

```

```

EstA  -----
Ceaf  D-----
Lp    ALN----
Her   AFAVAVS
Cea   FWNKYA-

```

Fig. 6: Alignment of EstA with the carboxyl esterase of *Archaeoglobus fulgidus* (Ceaf), lipase of *Pseudomonas* sp. B11-1 (Lp), heroin esterase of *Rhodococcus* sp. (Her) and carboxyl esterase of *Acinetobacter calcoaceticus*. Consensus areas are indicated beneath the sequence. Three, four and five amino acid identity with EstA is shown in red, green and blue respectively.

Alignment of EstA with the carboxyl esterase of *Archaeoglobus fulgidus* (Ceaf), the lipase of *Pseudomonas* sp. B11-1 (Lp), heroin esterase of *Rhodococcus* sp. (Her) and carboxyl esterase of *Acinetobacter calcoaceticus* is shown in Figure 6. There are a number of conserved regions as well as areas of identity between EstA and the other proteins. These areas of conserved and identical amino acids appear to cluster together in defined regions.

3.3: Expression of the carboxyl esterase in *E. coli*

Transformants of *E. coli* containing plasmids pAP4 and pUC18, respectively, were grown in liquid media and esterase activity determined in the extracellular fraction (Fig. 7). The esterase activity increased with time to an activity of 2.6 nkatal per millilitre after 15h.

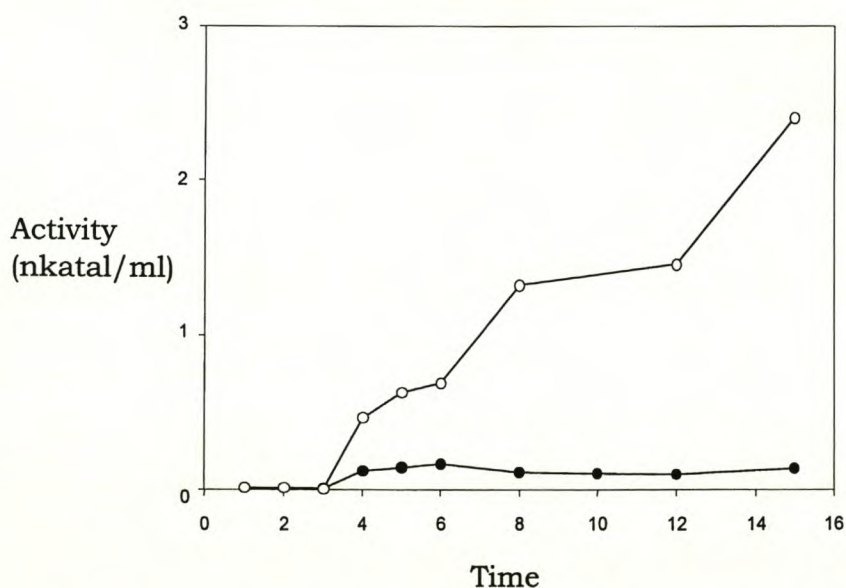


Fig. 7: Expression of carboxyl esterase. Open circles represent *E. coli* Transformants with plasmid pAP4 and closed circles *E. coli* transformants with plasmid pUC 18. Assays were done in duplicate.

3.4: Temperature and pH optima and temperature stability

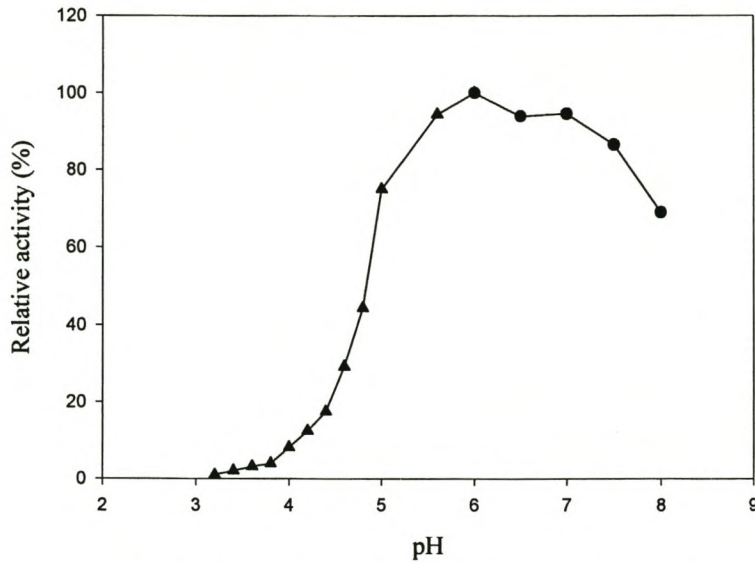


Fig. 8: Effect of pH on enzyme activity. 50mM Citrate buffer was used for pH 3.2 to 6 and 50 mM phosphate buffer was used for pH 6 to 8. Assays were done in triplicate.

Figure 8 shows the effect of pH on the activity of the esterase and Figure 9 the effect of temperature. EstA reaches optimum activity at pH 6 (1.26 nkatal/ml) and at a

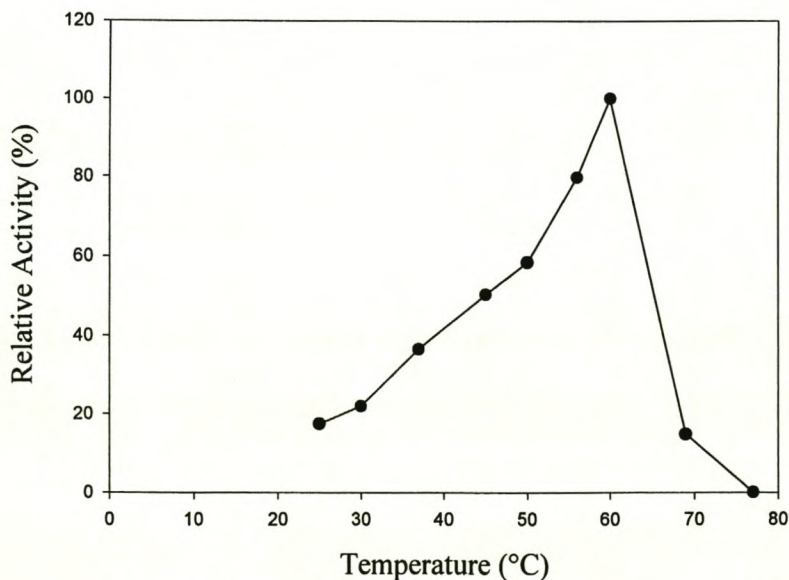


Fig. 9: Effect of temperature on esterase activity. Assays were done in triplicate.

temperature of 60°C (0.53 nkatal/ml). EstA was found to be very stable at both 50°C and 60°C, retaining 90% of activity after 2 h (Fig. 7). At a temperature of 70°C only 40% of the enzyme activity was present after 2 h. At 80°C loss of activity was more rapid with more than 90% loss in activity after only 10 min. As with pH and temperature, the maximum activity was taken as 100%. All other values are given as percentages of the maximum value. For the temperature stability assay activity at time zero (1.22 nkatal/ml) was taken as the maximum activity. Differences in maximum activity levels are due to different enzyme preparations being used.

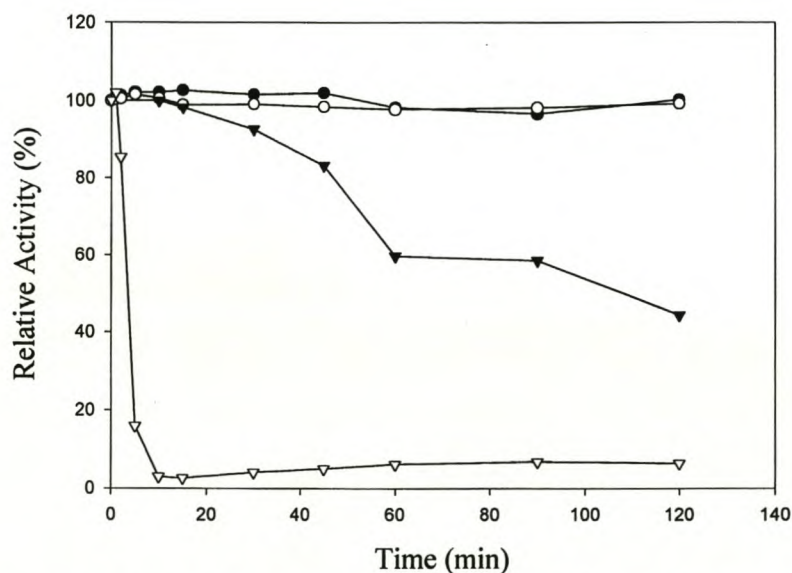


Fig. 10: Stability of the esterase at 50°C, 60°C, 70°C and 80°C. Black circles indicated 50°C, open circles 60°C, black triangles 70°C and open triangles 80°C. Assays were done in triplicate.

3.5: Substrate specificity:

Esterase activity on three methylumbelliferyl substrates: methylumbelliferyl acetate, methylumbelliferyl butyrate and methylumbelliferyl ferulate are shown in Figure 11.

The degree of fluorescence (brightness) is an indicator of the amount of methylumbelliferone released. With both methylumbelliferyl butyrate and methylumbelliferyl acetate the amount of fluorescence of samples where EstA is present was greater than the samples (control) where EstA was absent. This was ascribed to the hydrolysis of methylumbelliferyl butyrate and methylumbelliferyl acetate to acetate, butyrate and fluorescent methylumbelliferone by EstA. With methylumbelliferyl ferulate the amount of fluorescence released was the same and thus EstA presumably did not contribute significantly to the hydrolysis of this substrate.

3.6: Protein separation in SDS PAGE

Extracellular fractions of *E. coli* transformants containing plasmids pOE1 and pKK223-3, respectively were fractionated with 10% SDS PAGE. The arrows indicated an additional protein species present in *E. coli* containing pOE1, but not present in *E. coli* containing pKK223-3 (Fig. 12). This protein species has a molecular weight of about 30 kDa corresponding with the predicted molecular weight of EstA.

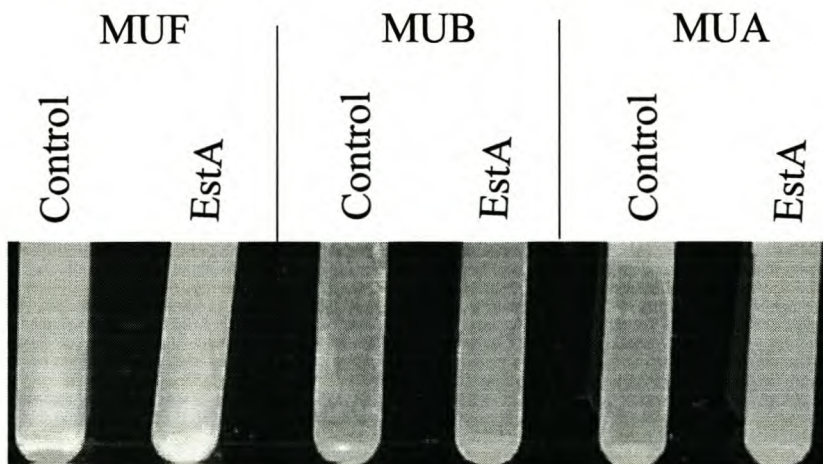


Fig. 11: Activity of ESTA on methylumbelliferyl substrates. The three substrates tested are methylumbelliferyl acetate (MUA), methylumbelliferyl butyrate (MUB), and methylumbelliferyl ferulate (MUF). Equal amounts of activity was observed for MUF for control and EstA. For both MUB and MUA, EstA showed more activity than the control.

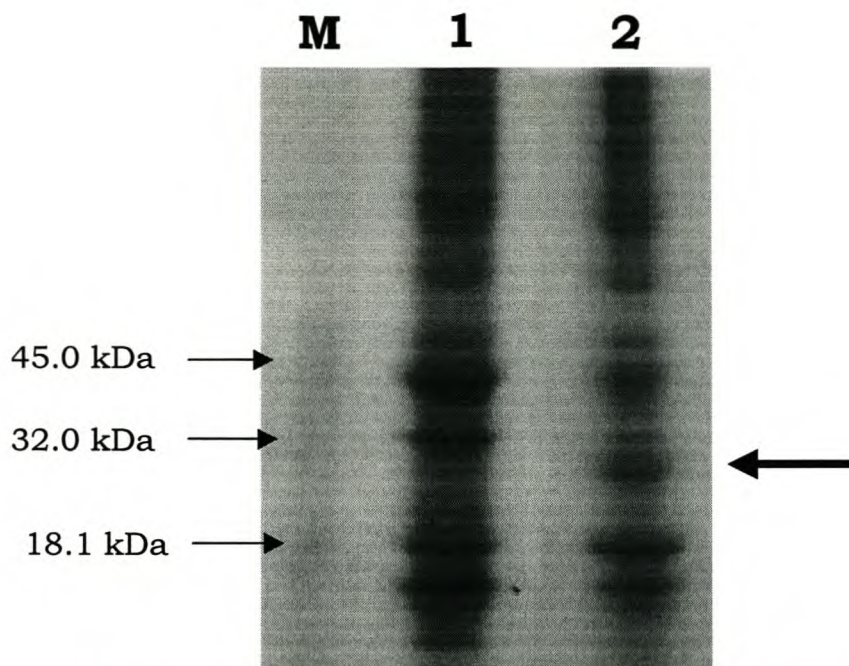


Fig. 12: Fractionation of extracellular protein of *E. coli* transformants containing plasmid pKK223-3 (1) and pOE1 (2) in 10% SDS PAGE. Lane M contains protein markers. The right hand arrow indicate the presence of an extra protein species in lane 2

4: Discussion

An esterase gene was isolated from a genomic DNA library of the Gram-positive bacterium *B. pumilus* by screening for acetyl esterase activity on plates containing glucose pentaacetate. The gene (*estA*) contained an ORF of 834 base pairs, encoding a protein with an estimated molecular mass of 30,426 kDa. A Shine-Dalgarno sequence was identified approximately 10 bp upstream of the *estA* start codon and a rho-independent-like terminator approximately 76 bp downstream from the termination codon. At the DNA level *estA* did not show significant homology with other known genes. However, at the protein level it showed 38% homology with a carboxylesterase from *Archaeoglobus fulgidus*, 36% homology with a heroin esterase from *Rhodococcus* sp and 37% homology with a lipase from *Pseudomonas* sp. B11-1. Compared to this, the partial ORF 1 showed 86% homology with endo- β -1,3-1,4-glucanase of *Bacillus licheniformis*. The lack of DNA homology

between *estA* and other genes, especially those of the genera *Bacillus*, and its relatively low level of homology at the protein level would indicate that EstA is a novel esterase of *Bacillus pumilus* not yet found in other *Bacillus* species.

The *estA* gene was successfully expressed in *E. coli* under control of its native promoter and terminator sequences. Activity levels of 2.5 nkatal/ml were observed. Very little background activity was observed in control strains, indicating that *E. coli* secreted the protein relatively free of other enzymes with acetyl esterase activity. The extracellular protein fraction from *E. coli* producing EstA from plasmid pOE1 contained an additional protein species of about 30 kDa, corresponding to the predicted molecular weight of the encoded EstA protein. Even though fractionation of the extracellular proteins showed an extra protein species at the expected molecular weight, this method is not ideal for the visualisation of EstA on SDS PAGE gel. Expression of a foreign gene can cause changes in the expression of other host or plasmid genes resulting in a change in protein profile that is not easily explained. Different types of overlay gels were done, but no satisfactory results were obtained. With overlays containing methylumbeliferyl substrates, fluorescence was observed over the entire gel, obscuring any possible bands. In overlays containing glucose pentaacetate, the crystals dissolved over the entire gel. The protein gel could have caused this. Overlay gels containing α -naphthyl acetate were tested, but the colouring reagent reacted with the gel, again obscuring any possible bands. The ideal would have been to purify the protein through column or affinity chromatography and run it on a gel as a pure sample. This will eliminate background protein and misinterpretation of the data. Another possibility is to raise antibodies against the protein. This was, however outside the scope of the study.

EstA has a temperature optimum of 60°C and a pH optimum of 6 and is stable at 60°C. A β -xylosidase (*xynB*) isolated from the same genomic library by la Grange *et al.* (1997) had a comparable pH optimum but was inactivated at 60°C after only 5 min. EstA shows activity on methylumbelliferyl acetate, methylumbelliferyl butyrate, glucose pentaacetate and α -naphthyl acetate. No activity was found on either acetylated xylan (data not shown) or methylumbelliferyl ferulate. A number of carboxyl esterases have been characterised. The carboxyl esterase from *Bacillus stearothermophilus* showed optimal activity at pH 7 and a temperature optimum over 60°C. It was also stable at 60°C. The enzyme had a molecular mass of 50 kDa and was able to hydrolyse a number of *p*-nitrophenyl substrates such as *p*-nitrophenyl laurate and N-CBZ-glycine *p*-nitrophenyl ester (Wood *et al.* 1995). A carboxyl esterase from *Bacillus coagulans*, showed optimum activity at pH 7 and 65°C. The enzyme had a molecular mass of 70 - 73 kDa and showed activity towards α -naphthylbutyrate, β -naphthylbutyrate, α -naphthylacetate and α -naphthylcaprylate (Molnari *et al.* 1996). The carboxyl esterase from *Arthobacter viscosus* showed optimum activity at pH 7.4 and 40°C. The carboxyl esterase had a Mr of 16.7 kDa and hydrolysed xanthan, glucose pentaacetate, naphthyl acetate, triacetin, alginate cellobiose octaacetate and isopropenyl acetate (Cui *et al.* 1999). It is notable that while the molecular mass, temperature optima of these four carboxyl esterases may differ, their pH optima are all in the pH 6 to pH 8 range. Compared to EstA, these carboxyl esterases showed some differences as can be expected. The substrates that are hydrolysed by these enzymes are very similar, suggesting that these enzymes are similar in their method of action. The protein alignment in Figure 6 shows conserved regions between EstA and the proteins from *A. fulgidus*, *Rhodococcus*, *Pseudomonas*

and *Acinetobacter calcoaceticus*. The conserved areas and regions of similarity between EstA and two or three of the proteins suggested that they are arranged in clusters. It is possible that these regions might be involved in the catalytic domains of the enzymes. On the basis of sequence homology, alignment data and substrate activity the esterase was classified as a carboxyl esterase and named *estA*.

5: Acknowledgements

Methylumbelliferyl ferulate was kindly supplied by Prof. Marc Claeysens, Laboratorium voor Biochemie, Rijks Universiteit van Gent, Ghent 9000, Belgium.

The authors thank Danie la Grange for use of the genomic DNA library of *B. pumilus* PLS.

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Chapter 3

**Cloning and expression of the novel carboxyl
esterase (*estA*) from *Bacillus pumilus* in the yeast
*Saccharomyces cerevisiae***

CLONING AND EXPRESSION OF THE NOVEL CARBOXYL ESTERASE (*estA*) FROM *Bacillus pumilus* IN THE YEAST *Saccharomyces cerevisiae*

A. Pieterse, R.R. Cordero Ortero and W.H. van Zyl

Department of Microbiology, University of Stellenbosch, Stellenbosch 7600,
South Africa,

The carboxyl esterase gene from *Bacillus pumilus* (*estA*) was cloned in the yeast *Saccharomyces cerevisiae* under control of the *PGK* promoter and terminator sequences. The *estA* open reading frame was also fused to the MF α 1 secretion factor of *S. cerevisiae* to facilitate secretion. Insignificant intracellular activity levels were observed in the absence of the MF α 1 secretion factor (0.33 nkat/ml) and with the MF α 1 secretion factor (0.25 nkat/ml). No extracellular activity was observed even with the MF α 1 secretion factor present. The *estA* gene product was most probably not active in *S. cerevisiae*.

1: Introduction

S. cerevisiae has been widely used in the making of wine and bread (Buckholtz and Gleeson 1991). Hemicellulose and cellulose materials (discussed earlier) are a cheap source of substrate that could be used by *S. cerevisiae* for fermentation. *S. cerevisiae*, though, has a very narrow substrate range (Pretorius 1997). The broader focus of the research in our laboratory is the complementation of the genetic potential of *S. cerevisiae* to degrade polymeric xylan and ferment its monomeric substituents. The degradation of this polymer requires the action of several enzymes that act in concert. Several genes for hemicellulose enzymes have been successfully expressed in *S. cerevisiae* (van Rensburg *et al.* 1994, Crous *et al.* 1996, la Grange *et al.* 1996, la Grange *et al.* 1997). An important aspect of xylan degradation is the presence of

acetyl groups on the xylan backbone (Sunna and Antranikian 1997). While a lot of work has been done on the esterases that remove acetyl groups from xylan (Christov and Prior 1993), no esterases have been expressed in *S. cerevisiae* to date. A carboxylesterase from *Aspergillus oryzae* has already been shown to deacetylate glucosides (Yoshioka *et al.* 1997). EstA showed acetyl esterase activity (chapter 3) and its expression in *S. cerevisiae* might enhance the degradation of xylan by recombinant yeast.

In this short paper we show the construction of two expression cassettes containing the *estA* gene and the subsequent expression of the *estA* gene in *S. cerevisiae* under the transcriptional control of the *PGK* promoter, with or without the MF α 1 secretion signal.

2: Materials and Methods

S. cerevisiae strain CEN.PK2-1C (K24) (MATa *ura3 leu2 trp1 his3*) was used as host. Liquid cultures were grown at 30°C in SC^{-URA} and SC^{-leu} medium. Plasmid pJC1 (Crous *et al.* 1995) was used for the expression of the *estA* gene under the transcriptional control of the *PGK* promoter sequence, in the absence of the mating factor secretion signal (Fig. 1). pAM100 was supplied by Ancha Zietsman, Department of Microbiology, University of Stellenbosch, Stellenbosch, South Africa and was used to evaluate expression of *estA* when fused to the MF α 1 secretion signal (Fig. 1). PCR primers (Whitehead Scientific) used to amplify the *estA* gene are shown in Table 1.

Table 1: Deoxyoligonucleotides used for amplification of the *estA* gene by PCR

Name	Sequence
CE1-L	5'- <u>CCGGAATTC</u> ATGAACTTAGAAGAACAAATC-3'
CE1-R	5'-CCCA <u>AAGCTT</u> AGATCTAAATCTCCGGCGTG-3'
CE1XHO1-L	5'- <u>GCTCGAGAT</u> GAACTTAGAAGAACAAATC-3'
CE1XHO1-R	5'- <u>GCTCGAG</u> CTAAATCTCCGGCGTG-3'

Restriction sites are underlined. They are *EcoRI* (CE1-L), *HindIII* (CE1-R) and *XhoI* (CE1XHO1-L and CE1XHO1-R)

Standard protocols were used for DNA manipulations (Sambrook *et al.* 1989). Restriction and ligation enzymes for DNA manipulation were purchased from Roche Molecular Biochemicals and used as recommended by the supplier. Digested DNA was isolated from agarose gels using the Agarose gel extraction kit (Roche Molecular Biochemicals). PCR was done according to standard procedure using a Perkin Elmer 2400 machine. PCR products were purified using the High PureTM PCR Product Purification Kit (Roche Molecular Biochemicals). The PCR product obtained with primers CE1-L and CE1-R was cloned between the *PGK* promoter and terminator of pJC1 as an 834 bp *EcoRI/HindIII* insert. The PCR product obtained using primers CE1XHO1-L and CE1XHO1-R was cloned behind the MF α 1 secretion signal of pAM100 as an 834 bp *XhoI/XhoI* insert. The expression cassettes are shown in Figure 1.

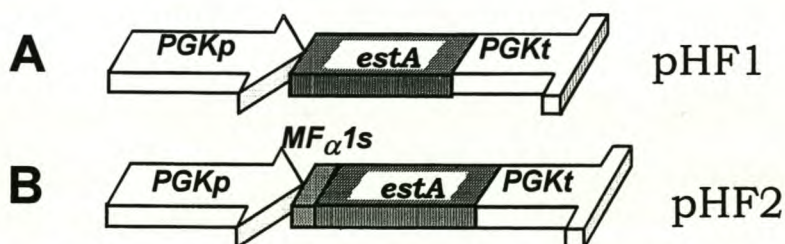


Fig. 1: Cassettes for expression of *estA* in *S. cerevisiae*. Plasmids pHF1 and pHF2 are plasmids pJC1 and pAM100 containing the *estA* gene of *Bacillus pumilus*. *PGKp* and *PGKt* represent the *PGK* promoter and terminator respectively.

Insertion of *estA* into plasmids pJC1 and pAM100 generated plasmids pHF1 and pHF2. The recombinant plasmids as well as the original host plasmids were transformed into *S. cerevisiae* using the lithium acetate method (Hill *et al.* 1991). To determine activity liquid cultures of *S. cerevisiae* containing plasmids pHF1, pHF2, pAM100 and pJC1 were grown at 30°C in SC^{-URA} (selection for plasmids pJC1 and pHF1) and SC^{-LEU} (selection for plasmids pAM100 and pHF2). Samples were taken at regular time intervals. Cell free culture solutions were obtained by lysing the yeast cells with glass beads (la Grange *et al.* 1996). The cell free extract and supernatant were used for activity assays. Assays were done using a combination of the methods of la Grange *et al.* (1997) and Poutanen and Puls (1988). To 890 µl of phosphate buffer (200mM) and 100 µl of enzyme solution, 10 µl of the substrate (500 mM α-naphthyl acetate in N-N-dimethylformamide) was added. The reaction was incubated at 50°C for 10 min after which 1 ml of the colouring reagent (0.01% Fast Corrinth V salt in 1M sodium acetate buffer, pH 4.4, containing 10% Tween 20) was added. Absorbance (OD) was determined at 535 nm after 10 min. Activity was expressed as nanokatal per ml (nmol α-naphthol ions produced s⁻¹ ml⁻¹). α-naphthol was used as standard (Poutanen and Puls 1988).

3: Results and discussion

Plasmids pHF1 and pHF2, expressing the *estA* gene within the *PGK* expression cassette, were introduced into *S. cerevisiae* K24. Intracellular acetyl esterase activity was determined for the transformants (Figures 2 and 3). Assays were done to determine extracellular activity, but no significant activity was found (data not shown) even when the gene was fused to the MFα1 mating factor secretion signal. As can be

seen from Figures 2 and 3, the standard deviation in activity observed with pHF1 and

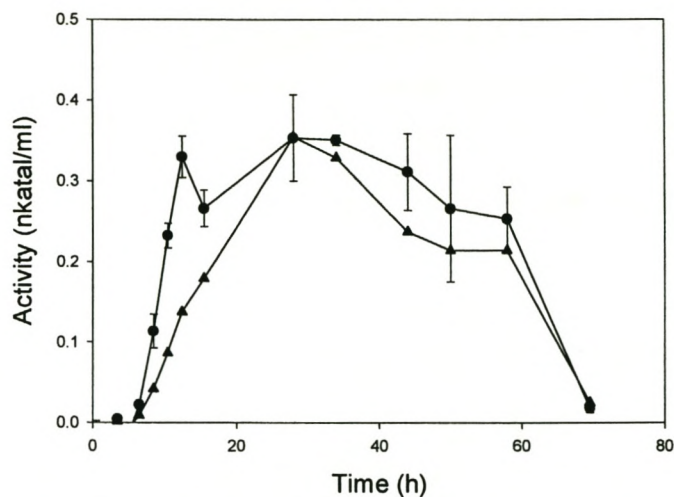


Fig. 2: Intracellular activity by *S. cerevisiae* transformants containing pJC1 (triangle) and pHF1 (circle). Error bars are shown for pHF1

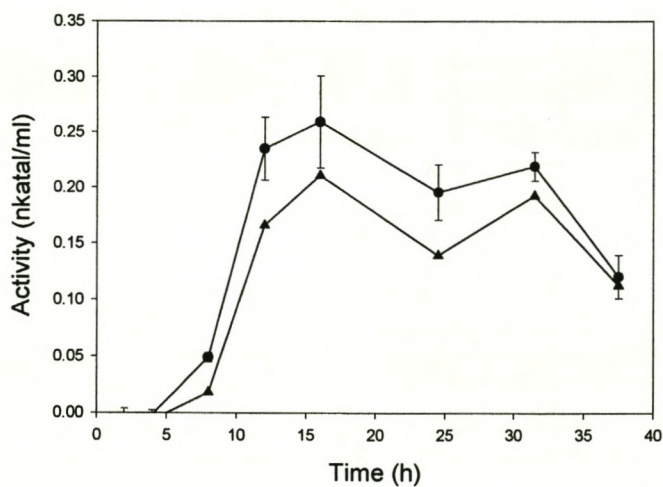


Fig. 3: Intracellular esterase activity *S. cerevisiae* containing pAM100 (triangles) and pHF2 (circles). Error bars are shown for pHF2

pHF2 cultures is as much as 0.1 nkatal/ml. No standard deviation is shown for the pJC1 and pAM100 as they were not done in triplicate. Although acetyl esterase activity was detected on α -naphthyl acetate, comparable results were obtained for control strains not expressing the *estA* gene, therefore these results were not conclusive. *estA* has an estimated codon bias index (Sharp and Cowe 1991) of 0.083 in *S. cerevisiae*. A comparison of codon usage between *S. cerevisiae* and *estA* is shown in appendix B. While there were similarities in codon usage, there were also some severe imbalances. For example, for the amino acid glycine *S. cerevisiae* preferred the GGU codon, which it uses almost 60% for yeast mRNA. *estA* used this codon only 7.8%. *EstA* contained the GGC and GGA codons which are not used to a great extent in yeast. Of the 278 codons in *EstA* only 118 were preferred codons in yeast. This very likely resulted in lower expression of the *estA* gene in *S. cerevisiae* due to tRNA shortages. Other factors would include instability of either mRNA or protein. Incorrect folding of the protein might also explain lack of extracellular activity and coupled with this, a misfolded protein may be problematic as the exposed hydrophobic areas can interact and form aggregates (Netzer and Hart 1998). The *estA* gene was most probably not expressed efficiently in *S. cerevisiae* or the recombinant *EstA* protein was not functional in yeast.

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Chapter 4

General Discussion

1: General discussion

A novel esterase gene (*estA*) was isolated from a genomic library of *Bacillus pumilus* and successfully expressed in *Escherichia coli*, producing extracellular enzyme at levels of 2.5 nkatal/ml. The DNA sequence of the insert was found to contain an open reading frame that encoded a protein of 834 amino acids. In a 10% SDS PAGE gel, an extracellular protein species was observed with a molecular mass corresponding to the predicted molecular weight for EstA of 30 kDa.

The screening method employed was originally used for the detection of acetyl xylan esterases by different yeast (Lee *et al.* 1987). It is, however, not surprising that an enzyme with acetyl esterase activity was isolated through this screening procedure. The basis of the screening is solubilisation of glucose pentaacetate, a low molecular weight substrate. Acetyl esterases are able to deacetylate low molecular weight substrates while only acetyl xylan esterases can normally act on the high molecular weight xylan. Thus, an enzyme with acetyl esterase activity can solubilise the glucose pentaacetate even though it is not capable of deacetylating polymeric xylan. This does not preclude this substrate for the isolation of acetyl xylan esterases, but activity has to be confirmed on polymeric xylan. The alternative would be to develop a quick and reliable method to screen large numbers of transformants containing a genomic library on polymeric xylan. This might involve an indicator sensitive enough to detect the acetic acid formed by deacetylation of the xylan. Existing assays for acetyl xylan esterase activity rely on the detection of acetic acid released after incubation of the substrate (acetylated xylan) in the presence of the enzyme. These methods include gas liquid chromatography (Hespell and O'Brian-Shah 1988), enzymatic assays

(Poutanen and Sunberg 1988), gas chromatography (Khan *et al.* 1990) and HPLC analysis (McDermid *et al.* 1990). Although these methods can be used for enzyme assays, they are not adequate for the rapid screening of a genomic DNA library.

The EstA protein exhibited acetyl esterase activity with a pH optimum of 6 and a temperature optimum of 60°C, with no noticeable difference between enzyme stability at 50°C and 60°C. EstA showed 38% homology with a carboxylesterase from *Archaeoglobus fulgidus*. Alignment of EstA with the carboxyl esterase of *Archaeoglobus fulgidus* (Ceaf), lipase of *Pseudomonas* sp. B11-1 (Lp), heroin esterase of *Rhodococcus* sp. (Her) and carboxyl esterase of *Acinetobacter calcoaceticus* shows a number of consensus areas as well consensus areas between EstA and the carboxyl esterases of *Archaeoglobus* and *Acinetobacter*. As was discussed in chapter 2, the activity of EstA corresponds with that of carboxylesterases. Based on sequence alignment, protein homology and the spectrum of substrates utilised, EstA has been classified as a carboxylesterase.

As discussed in section 4 of chapter 2, the substrate specificity of the *B. pumilus* carboxyl esterase is comparable with that of *B. stearothermophilus*, *B. coagulans* and *A. viscosus*. Carboxylesterases play a role in the detoxification of drugs and toxins. Two drugs that are de-esterified are heroin and cocaine. The metabolites can be excreted in urine. Carboxyl esterases are involved in numerous biological processes and have application in many medical fields as well as industry. Carboxyl esterases are therefore important enzymes and may have a big role to play in biotechnology.

Expression of the EstA carboxylesterase was attempted in yeast. A β -xylosidase had already been cloned from the same genomic library and had been successfully expressed in *S. cerevisiae* (la Grange *et al.* 1997). Expression would allow comparison of the expression of *B. pumilus* genes in *Saccharomyces*. Secondly, it would give more information on expression of esterases in yeast. The *estA* gene was successfully cloned in a yeast multicopy vector under the transcriptional control of a *PGK* promoter and terminator sequence as well as a similar vector containing the MF α 1 mating factor secretion signal to facilitate secretion from yeast. Activity was lower than that found with *E. coli* and intracellular levels of 0.25 to 0.3 nkatal/ml were observed. These results are, however, not conclusive as comparable values were shown for the control samples. This is in stark contrast to expression in *E. coli* where very little background activity was observed. Failure to secrete may be as result of the size or tertiary structure of the protein. Chaperone proteins are important in protein production and secretion. They prevent aggregation of the protein product and keep the protein in a state where it can fold properly. It is likely that *S. cerevisiae* lacks the chaperones needed for correct folding of the bacterial esterase or that the chaperones are not present under the growth conditions used. Often the way in which prokaryotic and eucaryotic proteins fold, differ and this will have an effect on the folding of a prokaryotic protein in a eucaryotic host (Netzer and Hart 1998).

The codon bias index is a measure of the availability of tRNA for mRNA translation (Sharp and Cowe 1991). The genetic code is degenerate and organisms show a preference for a certain set of anticodons. When *S. cerevisiae* is used to express a gene showing a preference to a different set of codons this will result in a lower rate

of translation and lower amounts of protein being formed. The *estA* gene has a low codon bias index and this might slow the rate of protein production in *S. cerevisiae*.

The β -xylosidase that was expressed in *S. cerevisiae* was mainly associated with the cell wall and then in the periplasmic space. No extracellular activity was detected and activity levels were not higher than 0.09 nkatal/ml (La Grange *et al.* 1997). These two results would indicate that *B. pumilus* bacterial proteins are not effectively produced by *S. cerevisiae*

A number of subjects exist that can be addressed in future. Further activity assays can be done to determine the full extent of EstA's substrate spectrum. Knowing what substrate can be utilised by EstA will show possible applications of the enzyme. Inhibition studies will also enable the classification of EstA into one of three carboxyl esterase groups (A, B and C) (Sertkaya and Gorrod 1988, Manco *et al.* 1994). Calculation of pI value of EstA will aid in isolation of pure protein. This can then be used in enzyme studies or to visualise the protein by SDS PAGE gel. With a pure protein sample the crystal structure of EstA can be determined and this will add to the knowledge of various protein structures.

Further work needs to be done on expression of EstA in *S. cerevisiae*. Using tagged proteins the localisation of EstA can be determined. The levels of mRNA formed can be calculated by way of Northern analysis. Efficient expression in *S. cerevisiae* might not be possible, but this would depend on whether the hitches in the way of successful expression can be removed. Codon bias can be addressed by optimising codon usage or an alternative promoter might be used to promote expression of *estA*.

Integration of the gene into the genome of *S. cerevisiae* might also alleviate low expression. Instability of EstA in *S. cerevisiae* can be circumvented by secreting the protein into the extracellular environment. If the problem lies in folding, secretion or secondary alterations to the protein by *S. cerevisiae*, it might be better to consider the use of an alternative host.

The DNA fragment isolated from the genomic DNA library contained part of an endoglucanase. Screening on plates containing azo-barley glucan (megazyme) lead to the isolation of four different constructs with varying endoglucanase activity on azo-barley glucan. Future work would include the sequencing, characterisation and expression of these genes and their proteins. Further screening for acetyl xylan esterase can also be done. With a β -xylosidase isolated from the library and evidence for an endoglucanase, it is most probable that *B. pumilus* also possesses genes for the other hemicellulose-degrading enzymes. Isolation of these genes will increase knowledge of enzymes involved in the degradation of plant material. It will also increase the number of enzymes available for processes involving the degradation of plant material.

A third ORF with homology to the lincomycin resistance gene of *Bacillus subtilis* was also identified. This gene is normally found as part of an operon. Isolation and sequencing of this operon will add to existing knowledge on the genetic make-up of *B. pumilus*.

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Appendix ASequence of *EcoRI/SalI* fragment of pAP4.

1 CAGCGATTATGAATACATTGCAGCAAGTGTCCAGGAGCCATTGGTACTGCGGTCCGGTATCT
 61 CGATCCTTTCTTCAGGCGCCCGCAGCTTTATGGAAAATGTGTCTGATCCCGCAAATCCGG
 121 TGAAT^{PvuII}CAGCTGCTCGCCTTTACAAATGGCGTCCAGC^{NsiI}ATGCATTTATCTTTGCAGTAATCA
 181 TGACCATCGTTGGTTTAATTATTGCTTTCTTCATTAACGAGTGAAGGTAGAACAGCAAG
 241 TATCATAAGAGAAATCAAAGCACTGAGGACATAGCGCCGAGTGCCTTTTTTATTGCTC
 301 AATGCTTGTTCAAAGAGAGGGAAAAAGGGGAGATAGGTAAAAAGTGAACCAATATAAGT
 361 TGCTGTTGATTCCGTAGGAGGAAAAGAGGAAAAGGAAACGAATGAGTAACTAAGAAATGA
 421 ATAAGATAGGAGTCTGAGATCATGAACTTAGAAGAACAAATCAAATCGCTGCTTCATTA
 481 CGCCAGCCATCTGAAGGTTTATTAGCGAGTCAATCGGAACTAAAGCCAGTAAATCCTCCT
 541 GAAGTGAACAAAATGGAATATGACATTCCAACAAGTGTGGCGAAACAAAGGTGTGGGTA
 601 TTAAGCCAGTGAACACACAAAAA^{PvuII}CAGCTGCTTCTGTTTTTGTGAATTTACATGGCGGC
 661 GGATTTATCATGGGCAGTGCAGAAATGGATAATCCCTGGTGTCCAGTCATTGCAGACCGG
 721 GCGCAATGTATCGTCGTCAATGTGAGTATCAGCTTGCACCCGAGCACCCCTTTCCCAGCC
 781 GCTCTTCATGAATGCTACGATGTACTGAAGTGGCTGTATGAACAT^{SauI}CCTGAGGAGCTTCAA
 841 ATCGATCG^{PvuI}GAATGCAT^{ClaI}TAGCCATTGGCGGACATAGCGCAGGAGGAAATTTGGCAACGGCT
 901 GCTTGTCTATTGAATATT^{SspI}CAAAGAGGAAATACACTCCCAATTGCCTATCAAGTGCCTTGAT
 961 TATCCGCCGCTTGATCTAGCCACTGATCCAGCACAAAAGCCAGCGTTTGAAGAAGCGATC
 1021 CCAGTTGAATTGGCGAGGCTCTTTAATTCTTCTATCTGCAAGGCCAAGATCCGCACAAT
 1081 CCGCTCGTTTTCTCCGGTTTTTGCAGATCGTTCATCCTTAGCACAAATGCCTCCAGCTCTC
 1141 GTCATCACAGCTG^{PvuII}AAAGAGATTGCTAGCTCAAGAAGCCGACCAATATGCGAAGAAGTTA
 1201 AAAGAAGCAGGGGTAGATGTCACGTACAGCAGTTTAAAGGAGTTCCTCACGGCTTCACAC
 1261 ACGCCGGAGATTTAG[→]CCATAGCTGAAGAGGCTTGGCATTGATGGGGGATCAACTAAAGA
 1321 AAGCGTTTGAATAAGAAAAGTAGAGTGATTCAAAGCACAGGAGACAATGGGCCTGTGCT
 1381 TTTATTACTGTTTCTGAAAAGAAAAGTGAATGTAAAAAGGGTCAGATAT^{EcoRV}CTTTCCCTCGT
 1441 CGATATAATAGAAAAGAAATAGATGATCTCTCAAAGGAGCTAATGAAATGAAAAAGAACA

1501 GCTACAGGCATTTTGTCTTTCTTTAAAAGGAACAACCCATGATTATTAGCCGGAATGGCA
BglI
 1561 GGCAGATCGTTATCATATTGGCGGAAAGATGTTTGCTATGATGGGCGGAGATGCGAATCG
NsiI
 1621 AAAGCCTGTCATCACATTAAAATGTGACCCTCAACGTGCTGAAGAACTAAGAGAGATGCA
 1681 TGAAGGAATTATCCCTGGCTACTATATGAATAAGACTCATTGGAATT
 300 bp of which the sequence was not confirmed
 1 ClaI SspI
 ATCGATTTTCGGTCTTGTGGTTCTGTAAATATTGGCATAAAGATATAACCAAATACGAGTGCAGCCATT
 71 AATAAAGGAAGTGAATCCAGAAAATCCAATGCCACGTGCGCATTTTCAGAATAAGACCG
ClaI
 131 GAGACGGTTGGACCGACTGCTGGTCAAACATGATCACAAGACCAATAATCCCCATCGAT
 191 CTGCCTCGTTTATGAGGCGGGATGATGACCAAATCGTATTAACATCAATGGCAGAAGC
 251 AATGCTGTACCGACTGCCTGCACGACACGTGCTACCATTAAAATACTAAAGACCGGGCGC
 311 AACCGCCGCAATAAATGTTCCGATGATCGAAAAGACAAGCGATGTAATGAAAAGCTGTCT
 371 TGTCTAACCATTGAAGAATGAGTCTGAAATCGGTACGAGAATACCAAGTGTAAATAA
 431 TATCCTGTTGTCCAGCCACTGAGCAGTCGTCGCTTCAATTCCAAAGTCTAACGTTAAACT
 491 TCTCAGCGCCATGTTTAAATGCCGTTTCACTGAACAGGCCAATAAATCCAGCCGTGATAAA
 551 AGAAATAATTATGGGCAGCGTTCCAATGTCAGAAGCTGCTGCTTTCTGTTTGTTTCATATT
SauI
 611 AAGTTGGCTCCTTTTCTTTTTCAATTTAGATGAACCTTAGGAAGTCAGGGTGGTCAGAAA
PstI
 671 TGAGCCACTTTTCATGTTCTATTGAAAGACGGATAAAATCTGTTTTCTGCGAGCAAATTGAG
SacI
SstI
 731 CGATTCTTTTCGATTGAATAGCCGGATTCATGTATATAAAACATGAGCTCAGGCCTCATTC
 791 GAGAGGACATAACGTTGTTCAGTTCATATGTCCTGTATGTTTCGCTTCTCCCTTTTCTC
 851 TCGCTTCTTCAAGCAAATCACGAATGATTTGTCGTAAGTGATTGACTGTAATGACTTCAT
 911 ACACTTTACTTGCTCTGCTCTAAACTCCAGAGATTGTGAGCATCCGAATCCAGTCTAAAT
BstEII
 971 GGTGACC GACGATGAGATGAATTTCTTCAAGAACGTAGCGCAAACGTTACGGACCGCT
SalI
 1031 GATCCGTCGAC

The open reading frames (ORFs) are indicated in bold. The arrows indicate the orientation of the ORFs. For a stretch of 300 bp located between ORF 1 and 2 the sequence was not confirmed in both orientations. Enzyme restriction sites are indicated in blue and red above the sequence. The first base pair of each segment is numbered one.

Appendix B**Comparison of codon usage between *S. cerevisiae* and *estA***

Amino Acid	Codons	<i>S. cerevisiae</i>	<i>EstA</i>
Phe	UUU	1.08	1.33
	UUC	0.92	0.66
Leu	UUA	1.60	1.60
	UUG	2.08	0.60
	CUU	0.66	1.20
	CUC	0.28	1.00
	CUA	0.79	0.80
	CUG	0.59	0.80
Ile	AUU	1.47	1.38
	AUC	0.89	1.62
	AUA	0.63	0.00
Met	AUG	--	--
Val	GUU	1.73	0.84
	GUC	0.96	1.26
	GUA	0.66	0.84
	GUG	0.65	1.05
Ser	UCU	1.83	0.80
	UCC	1.09	0.80
	UCA	1.16	1.60
	UCG	0.51	0.80
Pro	CCU	1.18	0.96
	CCC	0.54	0.32
	CCA	1.88	1.76
	CCG	0.39	0.96
Thr	ACU	1.5	0.40
	ACC	0.97	0.00
	ACA	1.06	2.00
	ACG	0.47	1.60
Ala	GCU	1.73	1.41
	GCC	0.97	0.65
	GCA	0.95	1.16
	GCG	0.35	0.77

Tyr	UAU	1.02	1.56
	UAC	0.98	0.44
Ter	UAA	1.56	0.00
	UAG	0.60	3.00
	UGA	0.84	0.00
His	CAU	1.20	1.14
	CAC	0.80	0.86
Gln	CAA	1.46	1.63
	CAG	0.54	0.37
Asn	AAU	1.11	1.54
	AAC	0.89	0.46
Lys	AAA	1.05	0.66
	AAG	0.95	1.33
Asp	GAU	1.25	1.53
	GAC	0.75	0.46
Glu	GAA	1.46	1.45
	GAG	0.54	0.55
Cys	UGU	1.34	1.5
	UGC	0.66	0.50
Trp	UGG	--	--
Arg	CGU	0.99	0.86
	CGC	0.29	0.86
	CGA	0.30	0.00
	CGG	0.17	1.71
	AGA	3.20	1.71
	AGG	1.05	0.86
Ser	AGU	0.84	1.33
	AGC	0.57	0.67
Gly	GGU	2.35	0.31
	GGC	0.65	1.84
	GGA	0.64	1.53
	GGG	0.37	0.31

Values are given as relative synonymous codon usage. e.g. tyrosine has two codons. In yeast the UAU codon is used 1.02 times out of every two times and the AUC codon only 0.98 times. No values are given for methionine and tryptophane as they have only one codon. Codons that are predominantly used in yeast are in bold as well as the corresponding codons for *estA*