# Production, characterisation and application of a recombinant ferulic acid esterase from *Aspergillus tubingensis*

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# DECLARATION

By submitting this dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

This dissertation includes one original paper published in peer-reviewed journals or books and two unpublished publications. The development and writing of the papers (published and unpublished) were the principal responsibility of myself and, for each of the cases where this is not the case, a declaration is included in the dissertation indicating the nature and extent of the contributions of co-authors.

Signature: Date: 12 November 2014

# SUMMARY

Ferulic acid esterase (FAE) is involved in the release of ferulic acid from xylan and is an important enzyme for the extraction of ferulic acid from plant biomass, whilst also reducing plant cell wall recalcitrance for biofuel production and improving the digestibility of animal feed. The production of FAE was investigated in strains of *Aspergillus tubingensis, Aspergillus carneus, Aspergillus niger* and *Rhizopus oryzae*. The *A. tubingensis* T8.4 strain showed the highest activity on triticale bran, producing a type A FAE active against methyl *p*-coumarate, methyl ferulate and methyl sinapate. The native *A. tubingensis* ferulic acid esterase gene (*faeA*) was subject to glucose inhibition and substrate induction by maize bran. The results also indicated a combined action of endoglucanase, endoxylanase and ferulic acid esterase for the utilisation of maize bran.

The *A. niger* D15#26 strain, which has reduced protease activity and does not acidify the growth medium (thus promoting high-level expression of recombinant enzymes) was used as host for the expression of a genomic copy of the *A. tubingensis faeA* gene under transcriptional control of the *A. niger gpdA* promoter. The *A. niger* D15[*AtfaeA*] strain produced 13.5 U/ml FAEA after 5 days on autoclaved maize bran (3-fold higher than the *A. tubingensis* donor strain) and was able to extract 50% of the available ferulic acid from non-pretreated maize bran.

The recombinant *At*FAEA was purified 7-fold with anion-exchange chromatography and its identity confirmed with peptide mass fingerprinting. The physical properties of the recombinant *At*FAEA were similar to that of the native enzyme; enzyme activity peaked at pH 6 and 50°C. It was stable at pH 3 to 7 and 30°C to 60°C, with a $K_m$ of 0.43 mM, $K_{cat}$ of 0.48/min and $K_{cat}/K_m$ of 1.1/min.mM. These properties suggest that *At*FAEA would be suitable for the food, pulp and paper, and animal feed industries where important phytochemicals could be released from the hemi-cellulosic backbone.

Culturing *A. niger* D15[*AtfaeA*] in a bioreactor significantly improved *At*FAEA production, with fed-batch fermentation yielding 2-fold higher FAE activity than batch fermentation. Fed-batch conditions resulted in a higher biomass yield, volumetric productivity and volumetric activity than batch fermentation, suggesting that fed-batch conditions are better suited for large-scale production of *At*FAEA in *A. niger*.

A crude preparation of the *A. niger* D15[*AtfaeA*] enzyme cocktail extracted 531 mg/l and 177 mg/l ferulic acid from maize bran and triticale bran, respectively, as well as 77 mg/l *p*-coumaric acid from triticale bran. This confirmed that *At*FAEA could increase the ferulic acid content and nutritional value of maize and triticale bran, which can add nutritional value to animal feed. The enzyme cocktail was also able to extract 0.2 g ferulic acid/100 g soluble solids from *Aspalathus linearis* (rooibos) leaves and stems, indicating the potential of *At*FAEA for the extraction of polyphenolics from other plant substrates.

# OPSOMMING

Feruliensuuresterase (FSE) is by die vrystelling van feruliensuur vanaf xilaan betrokke en dus 'n belangrike ensiem vir die onttrekking van feruliensuur uit biomassa, terwyl dit ook plantselwande se weerstandigheid vir biobrandstofproduksie verlaag en die verteerbaarheid van veevoer verbeter. Die produksie van FSE is in stamme van *Aspergillus tubingensis*, *Aspergillus carneus*, *Aspergillus niger* en *Rhizopus oryzae* ondersoek. Die *A. tubingensis* T8.4 stam het die hoogste aktiwiteit op korogsemels getoon met die produksie van 'n tipe A FSE aktief teen metiel-*p*-kumuraat, metielferulaat en metielsinapaat. Die natuurlike *A. tubingensis* feruliensuuresterase geen (*faeA*) was onderhewig aan glukose-onderdrukking en substraat-induksie deur mieliesemels. Die resultate het op ‘n samewerking tussen endoglukanase, endoxilanase en feruliensuuresterase vir die benutting van mieliesemels gedui.

Die *A. niger* D15#26 stam, wat oor verlaagde protease-aktiwiteit beskik en nie die groeimedium versuur nie (en dus hoë-vlak uitdrukking van rekombinante ensieme bevorder), is as gasheer vir die uitdrukking van 'n genoom-kopie van die *A. tubingensis faeA* geen onder transkripsionele beheer van die *A. niger* ***gpdA*** promotor gebruik. Die *A. niger* D15[*AtfaeA*] stam het 13.5 U/ml FAEA na 5 dae op ge-outoklaveerde mieliesemels gelewer (3-voudig hoër as die *A. tubingensis* skenkerstam) en kon 50% van die beskikbare feruliensuur uit onbehandelde mieliesemels onttrek.

Die rekombinante *At*FAEA is 7-voudig met anioon-uitruilingschromatografie gesuiwer en sy identiteit met peptiedmassavingerafdrukke bevestig. Die fisiese eienskappe van die rekombinante *At*FAEA was soortgelyk aan dié van die natuurlike ensiem; die ensiemaktiwiteit was die hoogste by pH 6 en 50°C. Dit was stabiel by pH 3 tot 7 en 30°C tot 60°C, met ‘n $K_m$ van 0.43 mM, $K_{cat}$ van 0.48/min en $K_{cat}/K_m$ van 1.1/min.mM. Hierdie eienskappe het aangedui dat *At*FAEA geskik vir die voedsel, pulp en papier, en voerbedrywe kan wees waar belangrike plantchemikalieë uit die hemisellulose-ruggraat vrygestel kan word.

Kweking van *A. niger* D15[*AtfaeA*] in 'n bioreaktor het FSEA produksie aansienlik verbeter, met voerlot-fermentasie wat 2-voudig meer FAE aktiwiteit as die lot-fermentasie gelewer het. Die *A. niger* D15[*AtfaeA*] stam het 'n hoër biomassa-opbrengs, volumetriese

produktiwiteit en volumetriese aktiwiteit onder voerlot- as lot-fermentasie getoon, wat daarop dui dat voerlot-toestande meer geskik is vir die grootskaalse produksie van *At*FAEA deur *A. niger*.

'n Ongesuiwerde preparaat van die *A. niger* D15[*AtfaeA*] ensiemmengsel het onderskeidelik 531 mg/l en 177 mg/l feruliensuur uit mielie- en korogsemels onttrek, asook 77 mg/l *p*-kumuraat vanuit korogsemels. Dit bevestig dat *At*FAEA die feruliensuur-inhoud en voedingswaarde van mielie- en korogsemels kan verhoog, wat voedingswaarde tot veevoer kan toevoeg. Die ensiemmengsel het ook 0.2 g feruliensuur/100 g oplosbare vastestowwe vanuit *Aspalathus linearis* (rooibos) blare en stingels onttrek, wat dui op die potensiaal van *At*FAEA vir die onttrekking van polifenole uit ander plantsubstrate.

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*The difference between a successful person and others is not a lack of strength, not a lack of knowledge, but rather a lack in will.*

***Vince Lombardi***

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**Declaration by the candidate:**

With regard to Chapters 2 to 4, the nature and scope of my contribution were as follows:

| Nature of contribution | Extent of contribution |
|---|---|
| Planning and execution of experimental work, data analysis and preparation of draft manuscript | 90% of Chapter 2<br>80% of Chapter 3<br>92.5% of Chapter 4 |

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The undersigned hereby confirm that

1. the declaration above accurately reflects the nature and extent of the contributions of the candidate and the co-authors to Chapters 2 to 4,
2. no other authors contributed to Chapters 2 to 4, besides those specified above, and
3. potential conflicts of interest have been revealed to all interested parties and that the necessary arrangements have been made to use the material in Chapters 2 to 4 of this dissertation.

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# Motivation

The Twentieth century has brought about dramatic changes in energy supply, in particular a shift from coal to oil. The concern for improved security of oil supply and the negative environmental impact of fossil fuels have put pressure on society to find renewable fuel alternatives, such as biofuels from plant biomass. First-generation biofuels are produced primarily from food crops such as grain and sugar cane, while second-generation biofuels are produced from lignocellulosic materials.

Lignocellulosic biomass is considered to be the most promising feedstock for bioethanol production given its abundance and low cost, but large-scale commercial production of second-generation biofuel is considered too expensive to effectively compete with fossil fuels (Balat 2011). It has been proposed that a biorefinery concept, where biomass feedstock is converted to a range of additional products that can add value to low-value feedstock and thus decrease the production cost of second-generation biofuels (Carvalheiro et al. 2008).

Lignocellulosic material consists of cellulose, hemicellulose and lignin, as well as other compounds, including phenolics (such as ferulic acid, syringic acid and *p*-coumaric acid) that are esterified to $\alpha$-L-arabinose moieties in arabinoxylan or ether-linked to lignin. These ether and ester bonds contribute to the recalcitrant nature of lignocellulose and therefor limit the microbial production of bioethanol (Wang and Sun 2010). Detailed knowledge of the phenols in lignocellulosic hydrolysates is important from both a scientific and technological point of view when considering value addition and second-generation biofuel production from plant biomass.

Among the phenolic compounds associated with lignocellulosic materials, ferulic acid is the major cinnamic acid with substantial industrial significance (Faulds 2010; Hasyierah et al. 2008). Ferulic acid (FA) is best known for its antioxidant properties due to its radical scavenging ability; it is widely used as a food preservative and is an active ingredient of skin lotions and sunscreens (Graf 1992). Ferulic acid can also be converted enzymatically to various value-added products, such as the flavouring agents vinyl guaiacol and vanillin for use in foods, beverages and perfumes (Hasyierah et al. 2008). Although commercial application of FA has been limited by its availability and cost, but the abundance of FA in

nature has stimulated research on the extraction of this antioxidant for alternative food, cosmetic, medical and veterinary applications (Graf 1992).

To date, the commercial production of natural ferulic acid has mainly been from γ-oryzanol in rice bran oil. Although plant cell walls contain a significant amount of ferulic acid, the complex binding of ferulic acid to hemicellulose and lignin renders it difficult to extract. Alkaline hydrolysis releases the total ferulic acid content from the plant cell wall, but purification of ferulic acid has proven to be difficult as the dark-brown hydrolysate contains many components (Faulds 1999). A more natural and specific way of extracting ferulic acid from lignocellulosic material is with enzymes that specifically release ferulic acid from its bound constituents without any adverse side reactions (Fazary and Ju 2007). One such group of enzymes are ferulic acid esterases (FAEs) and by virtue of being a biological treatment, the use of FAEs is regarded as a safer and more environmentally friendly method for FA extraction.

Ferulic acid esterases represent a diverse group of hydrolases that can release FA from the plant cell wall and thus act as accessory enzymes for the complete saccharification of plant cell wall hemicellulose (Mathew and Abraham 2005). Interest in FAEs as key enzymes for cell wall hydrolysis and the extraction of phenolic acids from agricultural crops in particular is increasing as it can break the bond between the arabinose and ferulic acid releasing the covalently bound lignin from hemicelluloses, ferulic acid and other phenolic phytochemicals. In this manner, FAEs can provide a clean and ‘green’ route for the extraction of phenolic compounds (e.g. ferulic acid and other antioxidants), whilst also reducing enzyme recalcitrance in animal feed and assisting with the hydrolysis of plant substrates for bio-ethanol production (Champagne et al. 2010).

# Aims of the study

Enzyme treatment of plant material with FAE is currently not considered a practical way of producing commercial ferulic acid due to the high costs associated with the microbial production of the enzyme, as well as the long reaction time required to release the bound ferulic acid (Hasyierah et al. 2008). It has therefore become very important to find and/or develop microbial strains that will produce FAE at high levels to render their production and subsequent application cost-effective.

The objectives of this study were therefore to:

- screen selected fungal strains for FAE production and characterise their crude enzyme cocktails;
- clone the relevant gene from the best FAE producer and express it in *Aspergillus niger*;
- optimise expression of the recombinant FAE; and
- evaluate potential value-addition to different plant substrates through treatment with the recombinant FAE.

# Structure of dissertation

This dissertation is presented as follow:

1. **Chapter 1** provides an overview of the relevant literature related to the structure of plant biomass, the nature and function of ferulic acid and FAEs, and their potential to provide a clean and 'green' route to extract high-value phenolic compounds (e.g. ferulic acid and other antioxidants) from plant biomass.
2. **Chapter 2** describes the screening, cloning and over-expression of the *Aspergillus tubingensis faeA* gene in *A. niger*. This chapter was published in its entirety in the Journal of Industrial Microbiology and Biotechnology (Zwane et al. 2014) and was therefore written in the style specified by the journal.
3. The purification and characterisation of the recombinant *At*FAEA is described in **Chapter 3**, as well as *At*FAEA production in a bioreactor under either batch or fed-batch conditions. This chapter was prepared for submission to Enzyme and Microbial Technology and thus written in accordance with the journal specifications.
4. **Chapter 4** reports the evaluation of the recombinant *At*FAEA on different plant substrates with the focus on its ability to release ferulic acid and/or other antioxidants from maize bran, triticale bran and rooibos (an herbal tea rich in antioxidants). The data will form the basis of a third manuscript to be submitted for publication (journal not yet specified).
5. A summary and the main conclusions are provided in **Chapter 5**.

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# Table of Contents

# CHAPTER 1

# Literature Review

## 1. Biofuels

Biofuels are predominantly produced from plant biomass and can be classified as liquid, gas or solid fuels. Biomass presents the opportunity to produce a variety of fuels such as ethanol, methanol, biodiesel, Fischer-Tropsch diesel, hydrogen and methane (Demirbas 2007). In recent times, biofuels have been considered an important step towards limiting greenhouse gas emissions, improving air quality and securing new energy resources (Nigam and Singh 2011).

The current use of bioethanol includes direct application as a gasoline improver or gasoline substitute, or in the form of ethyl tertiary butyl ether for use as a synthetically produced octane enhancer and in bioethanol-diesel blends for the reduction of exhaust gasses (Pejin et al. 2009). Bioethanol is routinely blended with gasoline in a 1:9 ratio, known as E10 and nicknamed gasohol. Under the European Union (EU) Quality Standard EN 228, bioethanol can be blended with gasoline at a concentration of 5%, which does not require any engine modifications and is covered by vehicle warranties. Higher bioethanol concentrations can be used (e.g. E85) after the required engine modification (Balat 2009; Balat 2011).

### 1.1 A South African perspective

An analytical study by Smeets et al. (2007) identified South Africa as an area with extensive biofuel potential due to a climate suitable for high levels of biomass production. It is mostly the deciduous dry forests covering vast areas of Southern Africa, especially the Moimbo Woodlands stretching over much of the Central African Plateau, that are most likely to be a target for biofuel production due to the favourable climate and low population density, although fertilizers will be required to overcome low soil fertility.

Although South Africa seems to have the best potential for biofuel production in Southern Africa, a high population density and high intensity of land-use implicates limited land for crop production for the biofuel industry. However, there are pockets of under-utilised land and some commercial farmers are not producing at full capacity due to the poor food crop market. The three key drivers for biofuel production in South Africa are (1) producing adequate levels of biofuel to ensure fuel security; (2) ensuring national development by reducing foreign exchange expenditures and creating local employment for South Africans; and (3) enhancing rural development (Von Maltitz and Brent 2008).

Commercial farmers in South Africa have shown great interest in the establishment of a biofuel industry. Farmers have stated that they can produce 14 million tonnes of maize per annum, whereas the South African market only requires 9 million tonnes. They cannot afford to overproduce as this will drive market prices downwards, resulting in small profit margins. The production of bioethanol from maize could therefore help to stabilise the local maize market (Spadavecchia 2008).

The beneficial effects of biofuel production will only be appreciated if they are cultivated in a sustainable way with both biodiversity and the food versus fuel debate in mind (Nigam and Singh 2011). Selected food crops such as maize, sugarcane an soybeans are currently used for biofuel production in different parts of the world. The question is whether to designate these food crops for biofuel and/or food production. Since available land for agricultural purposes is limited, Escobar et al. (2009) suggested that it is necessary to identify portions of land that is amenable for biofuel production.

Cereals are a very important global source of nourishment (Escobar et al. 2009) for both human consumption and feeding livestock, and a variation in its availability and price has significant impacts for the world's food supply. The potential use of farmland and grains that were historically used for human consumption for biofuels is therefore raising alarm bells in some parts of the world. However, the growing world population and a significant increase in the number of vehicles on the roads, necessitate an alternative source of transport fuel. Recent technology advancements have made it possible to produce biofuel from non-edible substrates such as the stems, stalks and leaves of plants (Nigam and Singh 2011).

South Africa appears to be less sensitive to the potential impacts of biofuel on food prices, as it has extensive tracts of land that could theoretically be earmarked for the biofuel industry. It may therefore be possible to establish large farms dedicated to biofuel crops without having

to sacrifice land currently used for food production (Haywood et al. 2008). In fact, even with a large biofuel industry, South Africa could still be totally food self-sufficient and be a net food exporter (Von Maltitz and Brent 2008).

## 1.2 Different types of biofuels

Biofuels are found in solid form (such as fuel wood, charcoal and wood pellets); liquid form (ethanol, biodiesel and pyrolysis oils); or in gaseous form (biogas or methane) (Nigam and Singh 2011). Biofuels are categorized as primary or secondary biofuels. The primary biofuels are in an unprocessed form (fuel wood, wood, chips and pellets, etc.) and used as such for heating, cooking or electricity production. Secondary biofuels (ethanol, biodiesel, dimethyl ether, etc.) are produced from plant biomass and are primarily used for transport fuels and various industry processes. Secondary biofuels are further divided into first, second and third generation biofuels depending on the raw material and technology used for their production. Biofuels classification includes the source and type of feedstock, as they can be derived from forest, agricultural products or municipal wastes.

### 1.2.1 First-generation liquid biofuels

First-generation biofuels are primarily produced from sugars (Banat et al. 1992; Brady et al. 1996, 1997a, b; Love et al. 1998; Nigam et al. 1997; Riordon et al. 1996), grains or seeds (Suresh et al. 1999; Turhollow and Heady 1986; Zhao et al. 2009) through a relatively simple process. The best-known first-generation bioethanol is produced by the fermentation of sugar extracts from food crop plants and starchy crops such as maize kernels (Larson 2008). These starchy crops are high in polysaccharides that can be hydrolysed to sugars, which can be fermented by yeast that are able to convert six-carbon sugars (mainly glucose) to bioethanol. Following distillation and dehydration, the ethanol can be used as is or blended with fossil-derived fuels (Nigam and Singh 2011). Although some countries produce commercial first-generation biofuels in significant quantities, the viability of this type of biofuels is questionable due to the potential conflict with food supply, which contributes to high production cost. The rapid increase of global biofuel produced from grain, sugar and oilseed crops has also raised the market price of certain crops and foodstuffs (Giselrod et al. 2008). These limitations therefore support the search of non-edible biomass as feedstock for the production of biofuels, therefore the focus on second-generation biofuel in this dissertation

### 1.2.2 Second-generation liquid biofuels

Second-generation liquid biofuels are generally produced in two different ways, i.e. via a biological or thermochemical processingof lignocellulosic biomass from agriculture and forestry. The biomass can be either non-edible wastes from food crop production or non-edible whole plant biomass such as grasses or trees grown specifically for this purpose. The most important advantage of second-generation biofuel is circumventing the food versus fuel debate associated with first-generation biofuels. The future of bioethanol production is expected to include both traditional grain/sugar crops and lignocellulosic materials (Aggarwal et al. 2001; Singh et al. 1995; Stevens et al. 2004; Verma et al. 2000).

The feedstock for second-generation liquid biofuels can be cultivated specifically for biofuel production, resulting in higher production yield per area unit, and thus a more efficient use of land relative to first-generation biofuel. According to Stevens et al. (2004), the production of second-generation biofuels requires sophisticated processing production equipment, increased investment per unit production, as well as larger-scale facilities. The cost-effective production of biofuels therefore requires more research and development focused on feedstock production and conversion technologies (Nigam and Singh 2011).

## 1.3 Advantages and challenges

There are a number of advantages associated with biofuel as an alternative energy source, including energy security, economics and environment impacts. To turn biofuel production into an economically viable process, there are several significant challenges that need to be overcome. The key advantages of utilising renewable sources in biofuel production is the use of natural bioresources that are geographically more evenly distributed than fossil fuels, and the independence and security of energy supply. The usage of agricultural residues and wastes substrates will also decrease the potential conflict between food and fuel. Biofuels produced from lignocelluloses materials have the potential to decrease GHG emissions, hence reducing environmental impacts (Nigam and Singh 2011).

There are several challenges associated with biofuel production, including a need for an improved biomass waste collection network and storage; strong policy supported by government for organic waste collection; and the blending of biofuels at a higher ratio. A government-approved subsidy for the establishment of commercially viable biofuel plants is needed to accelerate the production process, whilst tax credits will support the development

of a strong market for biofuels. Technological advances are also required to improve production system efficiencies to allow the production of value-added co-products that will reduce the production costs related to biofuel production (Nigam and Singh 2011).

To render bioethanol cost-effective, it is necessary to increase current production yields and reduce operational costs. Real technological advances are necessary to make this process economically viable in order to compete with fossil fuels, but most of the incremental improvements will come from the commercial learning curve. According to Wyman (1995), increasing the production of bioethanol yields to more than 100 gallons/ton of dry cellulosic biomass, would be a major step forward. The application of cellulase enzymes for the hydrolysis of cellulose into glucose is therefore a far more promising option than traditional acid hydrolysis (Brennan 1986; Grethlein and Converse 1991; Wooley 1999a; Wright 1998).

A substantial decrease in the use of chemicals, nutrients and other additives would significantly decrease operational costs, as would the use of enzymes with greater specific activity (Wooley 1999b). Process cost can also be reduced by using technologies that require less heat energy and that could render lignin available for other value-added products, thus enhancing revenues. A process is thus required that reduce the number of steps, simplifies operations, substantially speeds up reaction rates and reduces the overall use of energy and chemicals (Lynd et al. 1996).

## 1.4 Biorefinery concept for lignocellulosics

The biorefinery concept is an emerging concept that is based on using biomass as a feedstock for the production of fuels and industrially significant chemicals. This biomass biorefinery concept is similar to petroleum refineries that produce numerous fuels and products from petroleum. To ensure the economic viability of bioethanol production, industrial biorefineries might be the most promising route to create a new domestic bioethanol-based industry. The production of multiple products would take advantage of the differences in biomass components and intermediates and therefore maximize the value of the overall bioethanol production process. By way of example, a biorefinery could produce low value liquid fuels, but at high volumes, while generating electricity and heat for its own use and some surplus for sale.

Some of the valuable compounds in lignocellulose material, such as phenolic compounds, have an inhibitory effect on the growth of microorganisms responsible for hydrolysis and

fermentation of the polysaccharides. These phenolic compounds are thought to disrupt the cell membrane function by changing the protein to lipid ratio (Franden et al. 2013; Jonsson et al. 2013; Sakai et al. 2007).The extraction of high value products would enhance profitability as a secondary need, while the high-volume fuel would help meet national energy needs as a primary need. This would reduce the costs of bioethanol production process and greenhouse-gas emissions (Balat 2011). In order to benefit from value-added products produced through the biorefinery concept, there should be efficient technologies to allow for the extraction of these products prior to further processing of the substrate. One of the most common chemicals extracted in high yields from biomass such as corn fibre using various extracting techniques, is ferulic acid. Ferulic acid has various industrial applications and is thus of significant economic importance (Fitzpatrick et al. 2010).

## 2. Lignocellulosic substrates for bioethanol production

Lignocellulose materials can be classified into different categories, namely forest residues, municipal solid waste, waste paper and crop residues (Balat 2011). According to Bohlmann (2006), lignocellulose plant materials could produce a maximum of 442 billion litres of bioethanol per annum. These plant materials include but are not limited to lignocellulosic residues from wood (e.g. poplar trees), herbaceous (e.g. switch grass), agricultural (e.g. corn stover, and wheat straw), forestry (e.g. sawdust, thinnings and mill waste), municipal solid wastes (e.g. wastepaper) and various industrial wastes (Sánchez 2009). Table 1 summarises the lignocellulosic material generated globally.

**Table 1**. Lignocellulosic residues (dry weight) generated from agricultural sources (Sanchez 2009)

| Lignocellulosic residues | Million tons per annum |
|---|---|
| Sugar cane bagasse | 317-380 |
| Maize straw | 159-191 |
| Rice shell | 157-188 |
| Wheat straw | 154-185 |
| Soya straw | 54-65 |
| Yuca straw | 40-48 |
| Barley straw | 35-42 |
| Cotton fibre | 17-20 |
| Sorghum straw | 15-18 |
| Banana waste | 13-15 |
| Mani shell | 9.2-11.1 |
| Sunflower straw | 7.5-9.0 |
| Bean straw | 4.9-5.9 |
| Rye straw | 4.3-5 2 |
| Pine waste | 3.8- 4.6 |
| Coffee straw | 1.6-1.9 |
| Almond straw | 0.4-0.49 |
| Hazelnut husk | 0.2-0.24 |

## 2.2 Chemical composition of lignocellulosic materials

The chemical composition of lignocellulosic materials differs due to genetic and environmental influences, and this serves as the key factor that affects the efficiency of biofuel production (Balat 2011; Lee 2007). Typically, lignocellulosic material is composed of 48% (w/w) carbon, 6% hydrogen and 45% oxygen, together with minor inorganic matter component (Molina-Sabio and Rodriguez-Reinoso 2004). About 90% of lignocellulosic dry matter consists mainly of cellulose (35-50%), hemicellulose (20-35%) and lignin (25%) (Balat 2011; Hasyierah et al. 2008) (Figure 1), but this varies depending on the biomass type (Hasyierah et al. 2008). The rest of the lignocellulose material is represented by proteins, oils, extractives and ash (Wyman 1994).

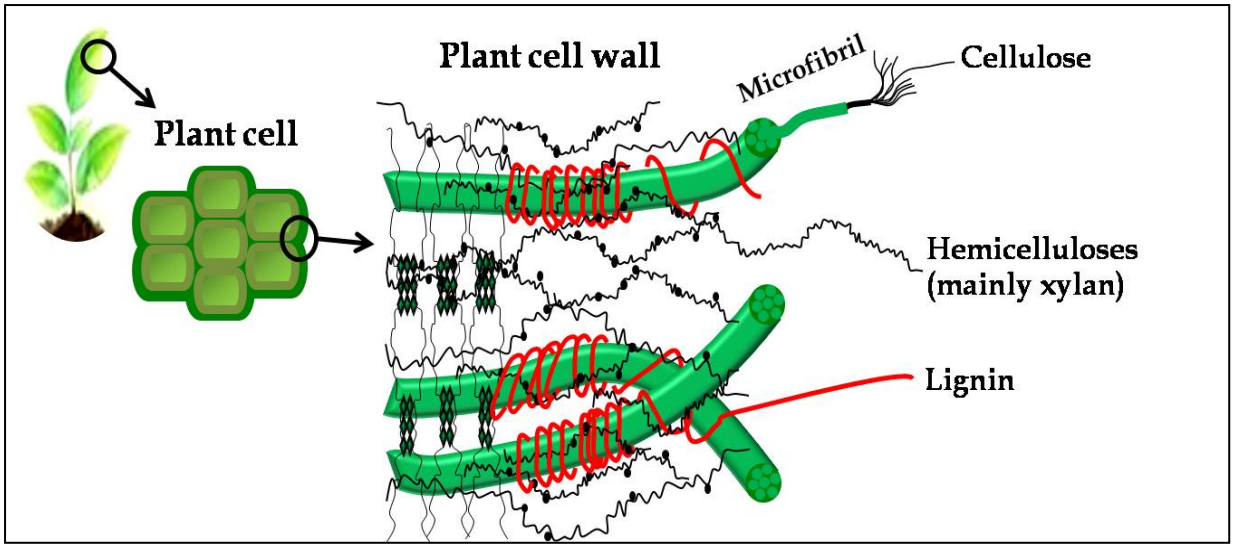



**Figure 1.** The structure of lignocellulosic plant biomass (adapted from Tomme et al. 1995).

Cellulose is a high-molecular weight linear polymer of $\beta$-1,4-linked D-glucose units that can have a crystalline appearance (Fan et al. 1982). Hemicellulose is a branched polysaccharide made up of pentoses (D-xylose and L-arabinose) and hexoses (D-mannose, D-glucose, D-galactose and uronic acids) (Saka 1991). The lignin component is an aromatic polymer synthesized from phenylpropanoid precursors (Adler 1977). Softwoods generally contain more lignin than hardwoods (Saka 1991).

Lignocellulosic substrates can be grouped into four categories: agricultural residues (straws, hulls, stems and stalks), deciduous and coniferous woods, waste from pulp and paper industry and herbaceous energy crops. Extensive research has gone into these various lignocellulose materials as feedstock for various products, including bioethanol and ferulic acid (Hasyierah et al. 2008).

The rigidity of the lignocellulose material renders it resistant to both biological and chemical degradation, termed recalcitrance (Zhang 2008). There are several factors that have been attributed to the recalcitrance of the lignocellulose materials (Himmel et al. 2007), but the most significant factors are the low accessibility of microorganisms and/ or their enzymes to the crystalline cellulose fibres and the presence of lignin and hemicellulose on the surface of cellulose. Both these factors restrict enzymes' access to the substrate and thus prevent enzymes from working effectively (Hong et al. 2007; Pan et al. 2005; Zhang and Lynd 2006; Zhang et al. 2006).

Cellulose is a linear biopolymer of anhydro-glucopyranose connected by $\beta$-1,4-glycosidic bonds. It is the coupling of adjacent cellulose chains by orderly hydrogen bonds, together with the Van der Waal's forces that lead to a parallel alignment and crystalline structure that restricts accessibility (Zhang 2008). The hemicellulose is located between the lignin and the cellulose fibres underneath. In the hemicellulose, the xylan is interspersed, interweaved and esterified at various points and has an overlay of lignin, but also produces a coat around the underlying strands that is hydrogen bound cellulose. This form of binding is thought to be significant in maintaining the structural integrity of the plant cell wall and resist any form of enzymatic degradation (Ding and Himmel 2006). In a study by De Vries and Visser (2001), two forms of covalent cross-links between the hemicellulose and lignin were identified, namely ester bonds between lignin and glucuronic acid attached to xylan, as well as diferulic acid bridges, which further contribute to the recalcitrance nature of lignocellulose materials. Table 2 summarizes the main bonds within the different components.

One of the most important considerations is the need to fractionate, solubilize, hydrolyse and separate the cellulose, hemicellulose and lignin components, for which a number of pretreatment methods have been described in literature (Bungay 1992; Dale and Moreira 1982; Weil et al. 1994).

**Table 2**. Overview of linkages between lignin, cellulose, hemicelluloses in lignocellulose (Harmsen et al. 2010)

| Bonds within different components (intrapolymer linkages) | |
|---|---|
| Ether bond | Lignin, (hemi)cellulose |
| Carbon to carbon bond | Lignin |
| Hydrogen bond | Cellulose |
| Ester bond | Hemicellulose |
| **Bonds connecting different components (interpolymer linkages)** | |
| Ether bond | Cellulose-lignin |
| | Hemicellulose-lignin |
| Ester bond | Hemicellulose-lignin |
| Hydrogen bond | Cellulose-hemicellulose |
| | Hemicellulose-lignin |
| | Cellulose-lignin |

## 2.3 Pretreatment of lignocellulosic materials

Pretreatment is a very important step for lignocellulose conversion processes and its significance has long been recognized (McMillan 1994). The goal is to degrade the lignocellulose structure by breaking the lignin shield and disrupting the crystalline structure of cellulose as shown in Figure 2 (Hasyierah et al. 2008). The main purpose of pretreatment is not only to separate the different lignocellulose material components, but also to increase the porosity of the materials. The different pretreatment methods can be divided into physico-chemical, and biological methods.

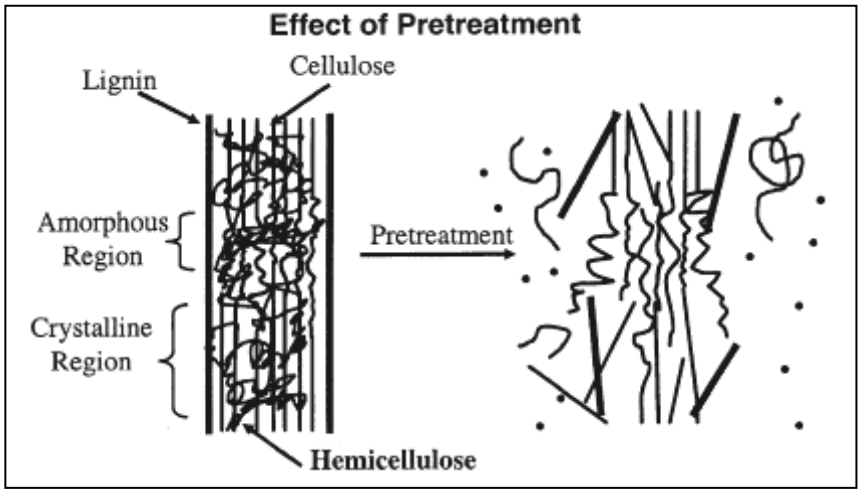



**Figure 2**. Schematic representation of the impact of pretreatment on lignocellulosic materials (Balat 2011).

### 2.3.1 Physico-chemical treatment

Physical pretreatment involves breaking down the lignocellulose material by grinding, chipping or milling to reduce the crystalline structure of cellulose. The energy requirement for the pretreatment is dependent on the final particle size and waste biomass characteristics (Hasyierah et al. 2008).

Physico-chemical pretreatment can be divided into three categories: steam explosion, ammonia fibre explosion and $CO_2$ explosion.

**Steam explosion** is the most commonly used method for lignocellulose materials (Saka 1991). It entails the use of $H_2SO_4$ (or $SO_2$) or $CO_2$ for removal of hemicelluloses to improve

enzyme hydrolysis, decrease the production of inhibitory compounds and for the complete removal of hemicellulose (Morjanoff and Gray 1987). It is initiated by treating chipped biomass with high-pressure saturated steam and then swiftly reducing the pressure to allow the materials to undergo an explosive decompression. The material is usually exposed to temperatures of 160 to 260°C for several minutes before being subjected to atmospheric pressure.

The four factors that influence steam explosion pretreatment are residence time, temperature, chip size and moisture content (Hasyierah et al. 2008). According to Duff and Murray (1996), optimal hemicellulose solubilisation and hydrolysis can be achieved with a residence time of 10 min at a temperature of 270°C or 1 h at temperature of 190°C.

**Ammonia fibre explosion** is very similar to steam explosion, but differs in that liquid ammonia replaces the steam. Typically, 1 to 2 kg of liquid ammonia is used at 90°C with a residence time of 30 minutes. It can be used for the pretreatment of a vast array of lignocellulose materials (Holtzapple et al. 1992; Mes-Hartree et al. 1988; Reshamwala et al. 1995; Tengerdy and Nagy 1988; Vlasenko et al. 1997), but is not be very effective for biomass with a high lignin content, such as newspaper (18-30% lignin) and aspen chips (25% lignin) (Hasyierah et al. 2008; McMillan 1994). The major disadvantage of this pretreatment method is the need for recycling of ammonia to reduce costs and to protect the environment (Hasyierah et al. 2008).

**$CO_2$ explosion** is thought to produce carbonic acid and thus increases the hydrolysis rate for lignocellulose materials (Hasyierah et al. 2008). Zheng et al. (1998) concluded that this was a more practical method than ammonia explosion as it was cost-effective and did not result in inhibitory compounds associated with steam explosion.

**Ozonolysis** requires a large amount of ozone, which renders the process very expensive (Hasyierah et al. 2008). Its main advantages are its ability to effectively remove lignin, there are no toxic residues that affect downstream processing and the reaction can be carried out at room temperature (Hasyierah et al. 2008; Vial and Molinier 1988).

**Acid hydrolysis** involves the use of concentrated acid such as $H_2SO_4$ and HCl are often used to ensure complete hydrolysis, although a dilute sulphuric acid pretreatment also achieved high reaction rates and significantly improved cellulose hydrolysis (Esteghlalian et al. 1997). In literature, two types of dilute acid pretreatment processes have been described:

high temperature (>160°C) for continuous-flow process and low solids loading (Brennan et al. 1986; Converse et al. 1989), and low temperature (<160°C) for batch processes with high solid loading (Cahela et al. 1983; Esteghlalian et al. 1997). The use of concentrated acids has been found to be very toxic, corrosive and hazardous and require specialized reactors that are corrosive resistant. Added to that, concentrated acid must be recovered after hydrolysis to make the process economically feasible (Sivers and Zacchi 1995), and pH neutralization is necessary for downstream enzymatic hydrolysis and fermentation (Hasyierah et al. 2008).

**Alkaline hydrolysis** uses dilute NaOH to swell the lignocellulose material, increase the internal surface area, decrease the degree of polymerisation and crystallinity, separate the structural linkages between lignin and carbohydrates and disrupt the lignin structure (Fan et al. 1987). Bjerre et al. (1996) observed that this form of pretreatment was effective for the hydrolysis of straws with a low lignin content (10-18%), but not for softwood with a lignin content higher than 26% (Millet et al. 1976).

**Organosolv** utilises an organic or aqueous organic solvent mixture with inorganic acid catalysts (HCl or $H_2SO_4$) and is used to rupture the internal lignin and hemicellulose bonds. Methanol, ethanol, acetone, ethylene glycol, triethylene glycol and tetrahydrofurfuryl alcohol are organic solvents used in this pretreatment (Chum et al. 1988; Thring et al. 1990). Following pretreatment, the solvent needs to be removed to prevent inhibition of microbial growth.

### 2.3.2 Biological pretreatment

Biological pretreatment implies the use of microbial organisms for the degradation of lignin and hemicellulose in waste materials. Enzyme hydrolysis can be carried out by any fungi or bacteria that produce hydrolytic enzymes. The most effective fungi for pretreatment of lignocellulosic materials are fungi like *Penicillium chrososporium*. White-rot fungi are able to produce lignin-degrading enzymes, lignin peroxidases and manganese-dependent peroxidases for the delignification of wood and the decomposition of lignin polymers to yield vanillin, vanillic acid, ferulic acid, coniferyl aldehyde, guaiacylglycerol (Fan et al. 1987).

Cellulase systems are generally made up of three kinds of extracellular enzymes, namely endoglucanase (EC 3.2.1.4), exocellobiohydrolase (EC 3.2.1.91) and $\beta$-glucosidase (EC 3.2.1.12) enzymes (Figure 3A).

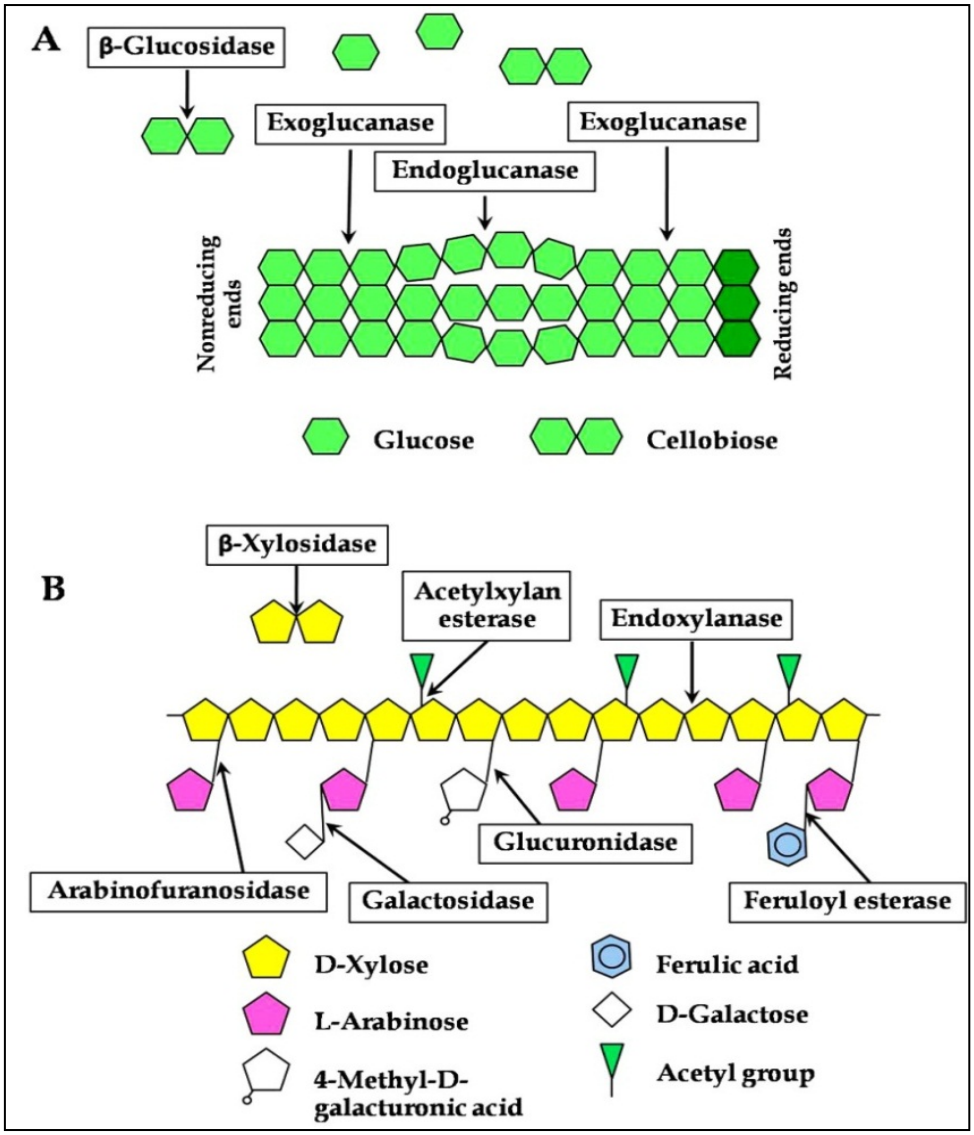



**Figure 3.** Enzyme systems involved in the degradation of cellulose (A) and xylan (B) (adapted from Aro et al. 2005).

Endoglucanases are able to hydrolyse the internal glycosidic bonds of cellulose. The exocellobiohydrolase releases cellobiose units from the non-reducing ends of the polymer, whereas the glucosidase hydrolyses cellobiose to glucose (Hasyierah et al. 2008). The hemicellulase system comprises enzymes that break down both the $\beta$-1,4-xylan (xylanases EC 3.2.1.8 and $\beta$-xylosidases EC 3.2.1.37) and a number of other side chain enzymes ($\alpha$-L-arabinofuranosidases EC 3.2.1.55, $\alpha$-glucuronidases EC 3.2.1.139, acetyl xylan esterases EC 3.1.1.72, ferulic acid esterases EC 3.2.1.73 and $\alpha$-galactosidases EC 3.2.1.22) (Figure 3B).

Figure 4 shows the different cellulose microfibrils that can be attacked by cellulase producers (Hasyierah et al. 2008). Previously, Bisaria (1991) reported biological treatment as

being the most environmentally friendly form of pretreatment for the removal of lignin from lignocellulose materials. However, biological pretreatment is a slow process and the microorganisms tend to consume carbohydrates, which may result in lower carbohydrate yields (Gray et al. 2006).

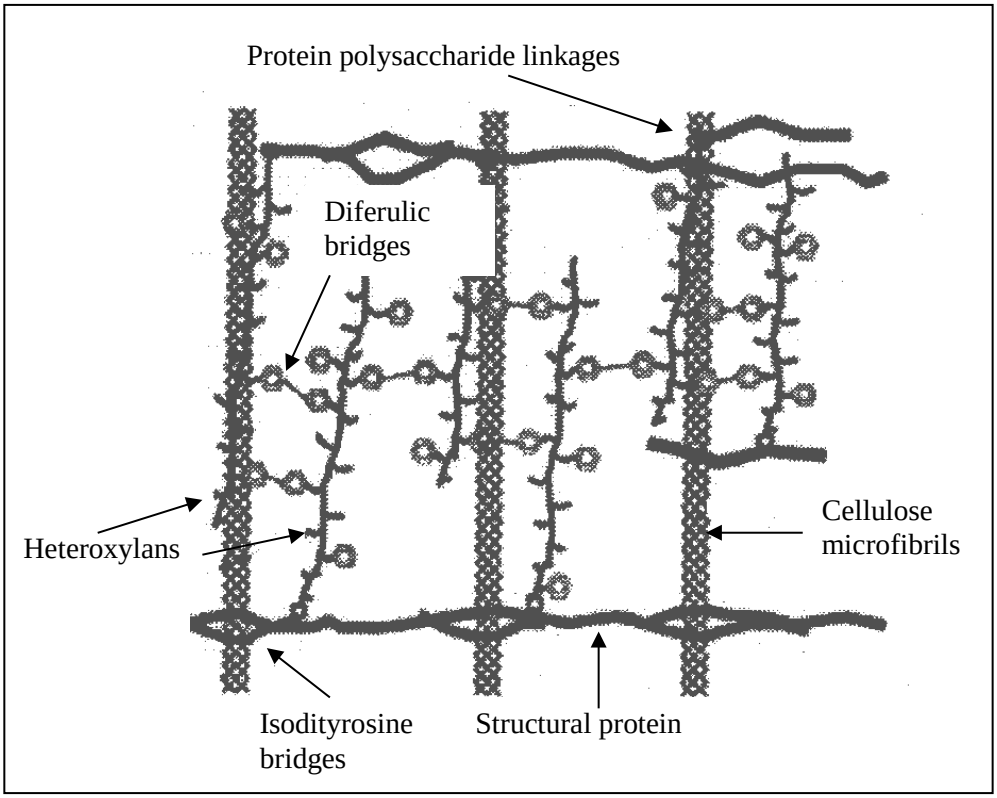



**Figure 4.** Graphic model of the structural components of corn fibre cell walls (Hasyierah et al. 2008)

# 3. Plant phenolics as natural antioxidants

## 3.1 Sources of plant antioxidants

Food has been suggested to have protective effects against oxidative damage in cells. For example, in a study to examine the antioxidant activity of bran extracts growing in three locations in Colorado, it was found that wheat was able to significantly reduce lipid peroxidation *in vitro*. The results suggested that wheat-based products show great potential in suppressing oxidation of biological substrates *in vivo* (Yu et al. 2005c). Another study on green tea antioxidants using both chronic oral feeding and topical application of green tea

polyphenols, resulted in significant protection against UVR-induced cutaneous oedema and erythema, lipid peroxidation and depletion of epidermal antioxidants defence enzyme system (Katiyar and Elmets 2001; Svobodova et al. 2003; Wei et al. 1999).

In plants, the photosynthetic system is exposed to high levels of oxygen and singlet oxygen can be formed by the transfer of high-energy photons from chlorophyll, which acts as a photo sensitizer within the chloroplast (Halliwell and Gutteridge 1999). This singlet oxygen is very toxic to plants and they therefore had to evolve strategies to eliminate this toxin (Benzie 2000; Fridovich 1998), such as the production of antioxidants to quench singlet oxygen and break cycles of oxidation.

Plants antioxidants include carotenoids, flavonoids, cinnamic acids, benzoic acids, folic acids, vitamin C, tocopherols and tocotrienols, which are localized and concentrated at the oxidation-prone sites of the plant (Gonzalez et al. 2008; Hollman 2001). Examples of antioxidants found in various food sources include (Kandall 2000):

- **Vitamin C**: This water-soluble vitamin is found in body fluids and represents one of the body's first lines of defence. It is present in various important food sources, including citrus fruits, green leafy vegetables and strawberries, amongst others.
- **Vitamin E**: This lipid-soluble vitamin is thought to be important in delaying aging and also plays a role in healing sunburn. Important sources include wheat germ, nuts, seeds, whole grains and vegetable oil.
- **Beta-carotene**: This antioxidant protects dark green, yellow and orange vegetables from solar radiation damage. Important sources include carrots, squash, broccoli, sweet potatoes, tomatoes, kale, collards, peaches and apricots.
- **Selenium**: It is suggested that this mineral helps fight cell damage caused by oxygen-derived compounds and thus may facilitate protection against cancer. The most important sources of selenium include fish, shellfish, red meat, grains, eggs, chicken and garlic.
- **Phenols**: Phenols are the most abundant antioxidants in food. The most important sources of polyphenols are fruit juices, tea, coffee, red wine, vegetables, cereals, chocolate and dry legumes (Scarlbert et al. 2005).

## 3.2 Reaction mechanisms of phenolic antioxidants

Initially, the term antioxidant was used to refer specifically to a chemical that prevented the consumption of oxygen. Currently, the term is used to refer to any substance that, in small quantities, is able to prevent oxidation of easily oxidisable substrates, such as lipids, protein and DNA (Laguerre et al. 2007). Oxidation can be described as a redox chemical reaction involving the transfer of electrons from a particular substrate to an oxidizing agent. The process of oxidation can have detrimental effects, resulting in the formation of free radicals that can disrupt biological systems. Antioxidants can terminate these reactions by removal of radical intermediates or by being oxidized themselves. Thus, antioxidants are often reducing agents, as is the case for phenols (Laguerre et al. 2007).

The continuous exposure of cells to various types of oxidative stress from a number of sources has led to the evolution of various mechanisms for protection against Reactive Oxygen Species (ROS), such as the DNA repair system. Amongst these defence mechanisms, antioxidants are the most significant as they can directly remove oxidative compounds such as free radicals, thereby offering maximum protection to biological sites (Kohen and Nyska 2002; Laguerre et al. 2007).

Antioxidants can be classified into two broad groups, depending mainly on their solubility in water. In general, water-soluble antioxidants react with oxidants in the cell cytoplasm and the blood plasma, while lipid-soluble antioxidants protect cell membranes from lipid peroxidation (Sies 1997). These compounds are synthesized by the body or can be obtained from dietary sources (Gonzalez et al. 2008).

Various antioxidants can co-exist in cells and may interact with each other. The interaction between antioxidants is complex, with the various metabolites and enzyme systems having synergistic and interdependent effects on one another (Chaudiere and Ferrari-Iliou 2001; Sies 1993). Furthermore, the level of protection provided by any one antioxidant depends on its concentration and reactivity towards a particular ROS (Vertuani et al. 2004).

The antioxidant activity of phenolic compounds varies according to their chemical structure (Moure et al. 2001), and the relationship between structure and activity of phenolic compounds has been used as a theoretical method of predicting antioxidant activity. Both the configuration and the total number of hydroxyl groups influence the activity of phenolic antioxidants (Heim et al. 2002). The activity of an antioxidant is determined by (Gonzalez et al. 2008; Rice-Evans et al. 1997; Rietjens et al. 2006) the following:

- reactivity as a hydrogen or electron-donating agent, which relates to its reduction potential;
- fate of the resulting antioxidant-derived radical, which is governed by its ability to stabilize and delocalize the unpaired electron;
- reactivity with other antioxidants; and
- transition metal-chelating potential.

Phenolic compounds are divided into various structural groups, namely phenolic acids and derivatives, flavonoids, stilbenes and lignans. The ROS scavenging ability of phenols is primarily attributed to the high reactivity of their hydroxyl components. The hydroxyl groups on the ring donate hydrogen or an electron to ROS, stabilizing them and giving rise to a relatively stable phenolic radical (Heim et al. 2002). Polymeric polyphenols have been found to be more potent than their monomers.

Phenolic compounds can stabilize ROS using two major mechanisms, namely hydrogen atom transfer (HAT) and single electron transfer (SET). The result, whichever mechanism is employed, is the same (Heim et al. 2002).

**Free radical quenching via hydrogen electron transfer (HAT)** involves phenolic compounds quenching ROS by a hydrogen atom transfer reaction. By definition, phenolic compounds have at least one hydroxyl group on an aromatic ring (ArOH). The relative weakness of the OH bond in the phenol determines how fast the phenolic antioxidant will react with the ROS. To be effective, a phenolic antioxidant should react slowly with the non-radical substrates, but rapidly with ROS. In principle, the weaker the OH bond in ArOH, the faster it reacts with free radicals and hence the greater its antioxidant activity (Wright et al. 2001).

**Free radical quenching via single electron transfer (SET)** implies that the phenolic compound quenches the reactive oxygen species by donating an electron to it, which results in spontaneous and reversible deprotonation in solution (Gonzalez et al. 2008; Wright et al. 2001). An example of such a reaction is represented below:

$$RO_2^{\bullet} + ArOH \rightarrow ROOH + ArO^{\bullet}$$

### 3.2.1 Measuring antioxidant activity *in vitro*

A wide variety of antioxidant assays can be used to assess the radical scavenging ability of antioxidants *in vitro.* In general, SET-based assays entail two components in the reaction mixture, i.e. the test antioxidant and the oxidant (ROS). The antioxidant donates an electron to the oxidant, resulting in a colour change of the oxidant. The degree of the colour change is directly proportional to the concentration of the antioxidant.

**2, 2-Diphenyl-picrylhydrazyl (DPPH) assay:** The DPPH radical is one of the few available stable organic nitrogen radicals and it has a characteristic deep purple colour. The assay is based on measuring the ability of antioxidants to reduce the colour of the DPPH radical. The reaction can be observed spectrophotometrically and the percentage of the DPPH radical remaining is directly proportional to the antioxidant activity (Bondet al. 1997; Brand-Williams et al. 1995).

**2, 2'-azinobis (3-ethylbenzothiazoline 6-sulfonic acid) (ABTS) assay:** This assay is based on the generation of the ABTS radical that is used to determine the antioxidant activity of various solutions. When reacted with potassium persulphate, ABTS produces a blue/green ABTS chromophore, which has absorption maxima at wavelengths 645 nm, 734 nm and 815 nm and 415 nm (Miller et al. 1993; Miller and Rice-Evans 1996). Adding an antioxidant to the ABTS radical reduces the ABTS on a time scale dependant on the antioxidant activity. Therefore, the extent of decolourisation relates to the percentage inhibition of the ABTS radical as a function of concentration and time and is calculated based on Trolox (6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid) as a standard under the same assay conditions (Miller and Rice-Evans 1996).

## 3.3 Ferulic acid

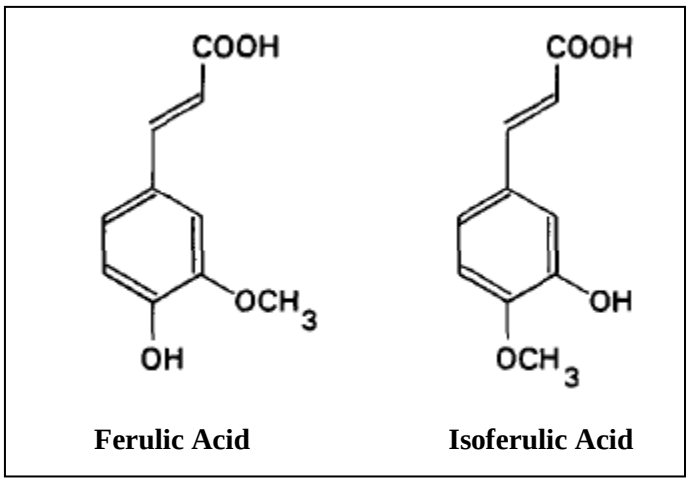
In 1866, Hlasiwetz and Barth isolated a protocatechuic acid and resorcinol from the commercial resin of *Ferula foetida*, an umbrelliferous fennel-like plant (Mathew and Abraham 2004). The addition of divalent lead to an alcoholic resin solution yielded a light yellow precipitate; when the lead salt precipitate was washed with alcohol, the free acid was reconstituted and determined to be $C_{10}H_{10}O_4$. This new compound was named ferulic acid and was found to be a dibasic acid consistent with its predicted chemical structure (Graf 1992). It was only in 1925 that ferulic acid was chemically synthesized for the first time through the amine-catalysed condensation of vanillin with malonic acid (Dutt 1925;

Robinson and Shinoda 1925). By 1927, the chemistry of ferulic acid was fairly well understood; the *cis* and *trans* isomers separated (Graf 1992), its stereochemistry ascertained by $^{13}$C-nuclear magnetic spectroscopy (Kelley et al. 1976) and its chemical structure confirmed by X-ray crystallographic analysis (Nethaji et al. 1988).

Ferulic acid exhibits better inhibition of lipid and protein oxidation compared to other naturally occurring phenolic antioxidants such as gallic acid, caffeic acid, malvidin and epicatechin, among others (Graf 1992; Heinonen et al. 1998). Due to its low toxicity, ferulic acid has been approved for use as an antioxidant in cosmetics, food and beverages (Graf 1992; Tada et al. 1999). In sport foods, ferulic acid is used as an ergogenic substance (Headley and Massad 1999). In cosmetics, ferulic acid is believed to decrease skin damage associated with prolonged ultraviolet light exposure that may result in precancerous and cancerous skin lesions or accelerating skin ageing (Carbonare and Pathak 1992; Podda et al. 1998). Ferulic acid can potentially be used in the synthesis of vanillin through chemical synthesis or microbial biotransformation (Priefert et al. 2001; Rao and Ravishankar 2000).

### 3.3.1 Chemical structure and synthesis

Ferulic acid is generally known as 3-(4-hydroxy-3-methoxyphenyl)-2-propenoic acid, although 3-methoxy-4-hydroxycinnamic acid, caffeic acid 3-methyl ester and coniferic acid are also used to describe the compound. Plants are able to produce isoferulic acid or ferulic acid, shown in Figure 5 (Graf 1992). Storage of *cis*- or *trans*-ferulic acid in water at room temperature results in the slow isomerization, resulting in an equilibrium ratio of 23% *cis* and 77% *trans* isomers after 2 weeks (Fenton et al. 1978).




**Ferulic Acid** **Isoferulic Acid**

**Figure 5**. Chemical structure of ferulic and isoferulic acid (Graf 1992).

The *cis*-isomer of ferulic acid does not exist in crystalline form and forms a yellow oil in ethanol with a spectrophotometric absorbance peaking at 316 nm. Trans-ferulic acid can be crystallized from hot water (Hiramoto and Watanabe 1939) and has a molecular weight of 194 Da and melting point of 174°C. It is readily soluble in ethanol, ethyl acetate and hot water; sparingly soluble in benzene and petroleum ether; and moderately soluble in ether. Ferulic acid is a strong dibasic acid, where the first proton dissociation creates the carboxylate anion and the second generating a phenolate anion. The *trans*-form of ferulic acid strongly absorbs at 284 and 307 nm in aqueous solution at pH 6 (Mendez and Lolo 1968). When dissolved in organic solvents, ferulic acid is fairly stable, but may undergo slow thermal decarboxylation in water to form vinylguaiacol (Hiramoto and Watanabe 1939).

In 1932, Dalal and Dutt showed that melonic acid condensed relatively easily with aromatic and aliphatic aldehydes in the presence of small quantities of pyridine and piperidine (Dalal and Dutt 1932). This reaction was executed in pyridine alone, but it was found that adding small quantities of piperidine or other secondary and tertiary amines significantly accelerated the reaction. Condensing malonic acid with vanillin in the presence of quinolone at 80°C for 8 hrs resulted in the production of ferulic acid with a theoretical yield of 50.3%. In one study, the yield for ferulic acid production was increased to 73% by carrying out the condensation at room temperature for a period of 3 weeks (Nethaji et al. 1988). In another route to synthesize ferulic acid, the Perkin condensation of vanillin with acetic anhydride was carried out. In this reaction, vanillin, acetic anhydride and anhydrous sodium acetate were heated at 150-170°C for 5h. To stop the reaction, water was added, the precipitate filtered, all the ether extracted and the ferulic acid recrystallized from hot water (Hiramoto and Watanabe 1939).

### 3.3.2 Ferulic acid as a natural antioxidant

One of the most abundant phenolic antioxidants, ferulic acid occurs in rice, wheat, barley, oats, sorghum, forage, tree bark, poplar buds, roasted coffee, tomatoes, asparagus, olives, berries, peas, vegetables, citrus fruits and leaves, and many other plants. Ferulic acid is also found in the bran fraction of seeds. A great deal of the ferulic acid is found esterified to plants, covalently conjugated to the mono and disaccharides, glycoproteins, lignin,

betactanins as well as other insoluble carbohydrates biopolymers of cell walls. Another form of ferulic acid is found in the form of steryl ferulates in maize, corn, wheat, rye and triticale (Kroon et al. 1996).

In monocots (cereals, bamboo, sugar cane, wheat and maize), hydroxycinnamic acids are esterified to the arabinofuranoses. Ferulic acid is the most abundant hydroxycinnamic acid found in monocots and is usually esterified at the *O*-5 position of the *α*-L-arabinofuranosyl side chains in arabinoxylans (Mueller-Harvey et al. 1986), at the *O*-6 position of the *β*-D-galactopyranosyl residues in the pectic rhamno-galacturonans (Colquhoun et al. 1994), or at the *O*-4 position of the *α*-D-xylopyranosyl residues in xyloglucans (Ishii et al. 2003). Ferulic acid represents about 0.66% (w/w) of wheat bran (Smith and Hartley 1983), 1.24% of wheat straw (Benoit et al. 2006), 3.1% of maize bran (Saulnier et al. 1995), 0.9% of rice endosperm cell wall (Shibuya 1984), 0.14% of barley grains (Nordkvisk et al. 1984), 0.32% of barley spent grain (Bartolome et al. 1997a,b), 2.2 to 3.8% of oat hulls (Garleb et al. 1988; Yu et al. 2002a; 2002b) and 1.4% of barley hulls (Tenkanen et al. 1991).

In dicots (spinach and sugar beet), ferulic acid is esterified at the *O*-2 position of the *α*-L-arabinofuranosyl in arabinans or in the *O*-6 position of the *β*-galactopyranosyl residues in pectin rhamnogalacturonans (Colquhoun et al. 1994). In sugar beet pulp, ferulic acid represents up to 0.87% of the dry weight (Kroon et al. 1996).

Due to its antioxidant and antimicrobial properties, ferulic acid has been used as a food preservative to prevent auto-oxidation in foods such as linseed oil, lard and soybean oil. In comparison to other phenolic compounds, ferulic acid has strong antioxidant properties.Ferulic acid is not too affected by pH changes like other phenols such as as chlorogenic acid, caffeic acid and gallic acid, making it suitable for the food industry where food is subjected to alkaline processing to recover proteins from food. Also, ferulic acid is well-known for its antimicrobial nature and can inhibit bacterial, fungal and yeast growth. The antioxidant action of glucoside esters of ferulic acid is greater than that of ferulic acid (Ou and Kwok 2004). Ferulic acid is used as an ingredient in sport drinks as it has the ability to stimulate hormone secretion in the human body. Its addition to skin lotions is motivated by its ability to protect the skin from photo damage as a result of prolonged sun exposure.

### 3.3.3 Extraction of ferulic acid from plant material

Prior to ferulic acid extraction from lignocelluloses materials, a preparative step is necessary to enhance the accessibility of the biomass, which may vary greatly depending on the lignocellulosic substrate. The first step usually involves drying and grinding the plant material into fine particles to disrupt the cells walls. Different types of mills are used, such as ball milling, jet milling and high-pressure micronization, which allow for the milling of large quantities of biomass (Berberousse et al. 2008). There are two recognized processes for extracting ferulic acid from lignocellulosic materials, namely chemical and enzymatic extraction.

**Chemical hydrolysis** for the extraction of ferulic acid from biomass can be achieved by cleaving the ester and ether linkages that bind the polysaccharide and lignin, respectively. This chemical hydrolysis can be carried out with an alkaline or acidic agent. Acid hydrolysis involves inorganic acid such as hydrochloric or sulphuric acid to treat the plant material. The disadvantage of acid hydrolysis is that it reportedly breaks glycosidic bonds and solubilizes sugars, but leave the ester and ether bonds intact (Berberousse et al. 2008). Since acid hydrolysis also decomposes hydroxycinnamic acids, alkaline hydrolysis with NaOH is regarded as more appropriate for ferulic acid extraction. The ferulic acid yield with chemical extraction is generally regarded as the total extractable ferulic acid content for the respective plant material and is therefore used as a benchmark for enzyme hydrolysis (Berberousse et al. 2008).

**Biological or enzymatic extraction** of ferulic acid is regarded as a mild hydrolysis as it is less denaturing than chemical hydrolysis. However, the synergistic action of a mixture of cellulosic and hemicellulosic enzymes naturally secreted by bacteria, yeast and fungi is required to recover most of the ferulic acid from biomass. An array of crude preparations of these enzymes are commercially available, including Depol 740L and Depol 686L (Biocatalysts), Ultraflo L (Novozymes) and Pentopan 500BG (Novozymes), which contain various lignocellulases in combination with ferulic acid esterases to allow the extraction of ferulic acid, among other components, from lignocellulose (Berberousse et al. 2008).

# 4. Ferulic acid esterases

Ferulic acid esterases FAE; (EC 3.1.1.73), also known as feruloyl esterases, cinnamic acid or cinnamoyl esterases, are able to release ferulic acid and other cinnamic acids from plant cell wall polysaccharides. Due to their ability to specifically extract cinnamic acids from complex plant cell wall polysaccharides, they have industrial significance where they can target the linkage between ferulic acid and hemicellulose, which generally regarded as one of the hurdles in polysaccharide hydrolases (Benoit et al. 2008).

## 4.1 Catalytic mechanism

The catalytic mechanism of FAE is similar to that of serine proteases, lipases and other esterases that involve a covalent acyl-enzyme intermediate; they all display the classic constellation of the serine-histidine-asparagine (Ser-His-Asp) triad as depicted in Figure 6 (Wong 1995). The first step is acylation facilitated by the catalytic His residue in a general base catalysis; it involves the nucleophilic attack by the catalytic Ser-OH on the carbonyl carbon of the scissile bond of the substrate, and yields a covalent tetrahedral intermediate. Collapsing of the intermediate to an acyl-enzyme is followed by the His-catalysed protonation of the ester-oxygen, resulting in the liberation of the carbohydrate moiety as the product. The deacylation step is characterized by the nucleophilic attack by $H_2O$ aided by the general base catalysis involving the His, which leads to a second tetrahedral intermediate.The subsequent His-catalysed protonation of the Ser-O results in the breakdown of the intermediate and thus liberates ferulic acid as a product (Daggett et al. 1991; Warshel and Russel 1986; Warshel et al. 1989).

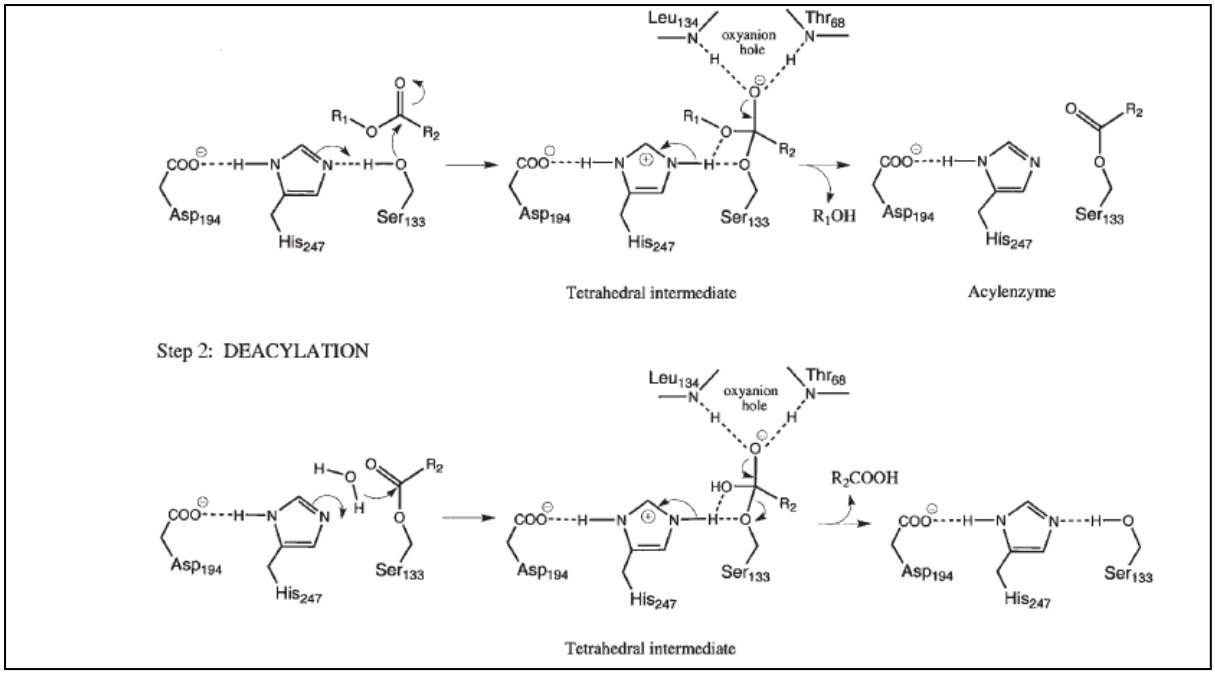



**Figure 6**. The proposed mechanism of feruloyl esterase involves the formation and subsequent breakdown of an acyl-enzyme via a tetrahedral intermediate (numbering refers to the *A. niger* AnFaeA). $R_1$, carbohydrate moiety; $R_2$, phenolic acid moiety (Wong 2006).

Ferulic acid esterases are classified according to their substrate specificity for aromatic substrates and their ability to release diferulic acids from lignocellulosic materials, as shown in Table 3 (Crepin et al. 2003a). Crepin et al. (2004b) proposed a broad classification of ferulic acid esterases based on the type of substrate it utilized:

- **Type A** FAEs are active against methyl ferulate (MFA), methyl *p*-coumarate (M*p*CA) and methyl sinapate (MSA), but not methyl caffeate (MCA), as well as synthetic ferulate dehydrodimers.Their amino acid sequences are related to that of lipases.
- **Type B** FAE are able to hydrolyse MFA, M*p*CA and MCA, but not MSA. Type B FAE, which show an amino acid sequence similarity to Carbohydrate Esterase Family 1 acetylxylan esterases, are unable to release diferulic acid from lignocellulosic material.
- **Type C and D** ferulic acid esterases are able to hydrolyse all four substrates and has an amino acid sequence similarity to chlorogenate esterases and xylanases respectively. Type D is able to release ferulate dehydrodimers, but not type C (Wong 2006).

**Table 3.** Classification of feruloyl esterases

| Criteria | Type A FAE | Type B FAE |
|---|---|---|
| **Example of native host** | *Aspergillus niger* | *Penicillium funiculosum* |
| **Preferential induction medium** | WB | OSX SBP |
| **Hydrolysis of methyl esters** | MFA, MSA, M*p*CA | MFA, MCA, M*p*CA |
| **Release of free diferulates from plant cell walls** | 5,5′-diferulic acid only | No |
| **Amino acid sequence similarity** | Lipase | Carbohydrate Esterase Family 1 acetyl xylan esterase |
| **Criteria** | **Type C FAE** | **Type D FAE** |
| **Example of native host** | *Talaromyces stipitatus* | *Pseudomonas fluorescens* |
| **Preferential induction medium** | SBP-WB | WB |
| **Hydrolysis of methyl esters** | MFA, MSA, M*p*CA, MCA | MFA, MSA, M*p*CA, MCA |
| **Release of free diferulates from plant cell walls** | No | 5,5′-diferulic acid only |
| **Amino acid sequence similarity** | Chlorogenate esterase tannase | Xylanase |

*MCA, methyl caffeate; MFA, methyl ferulate; M*p*CA, methyl *p*-coumarate; MSA, methyl sinapate; OSX, oat spelt xylan; SBP, sugar beet pulp; WB, wheat bran

## 4.2 Microbial sources of ferulic acid esterases

Ferulic acid esterases are produced by several fungal and bacterial microorganisms (Table 4). The release of FAEs by *Streptomyces olivochromogenes* was first induced by wheat bran (McKenzie et al. 1987), which led to FAEs been recognized as necessary components of hemicellulolytic enzymes systems. Under submerged cultivation, *Aspergillus flavipes* (Mathew and Abraham 2005) and *A. niger* (Johnson et al. 1989) strains were found to be the most active producers using lignocellulose-derived carbon sources such as maize bran and de-starched wheat bran.

A number of ferulic acid esterases have been purified and characterized from various microorganisms, such as *C. stercorarium* (Donaghy et al. 2000), *Clostridium thermocellum* (Blum et al. 2000), *S. thermophile* (Topakas et al. 2004), *S. olivochromogenes* (Topakas and Christakopoulos 2004), *A. awamori* (Shin and Chen 2007), *F. oxysporum* (Topakas and Christakopoulos 2004; Faulds and Williamson 1991), *F. proliferatum* (Shin and Chen 2006),

*N. crassa* (Crepin et al. 2003a; Crepin et al. 2004a), *Aspergillus nidulans* (Shin and Chen 2007), *A. pullulans* (Rumbold et al. 2003), *A. niger* (Dzedzyulya et al. 1999), *Piromyces equi* (Fillingham et al. 1999), *Cellvibrio japonicus* (Smith et al. 1991) and *T. stipitatus* (Crepin et al. 2003b; Garcia-Conesa et al. 2004).

**Table 4**. Microbial production of feruloyl esterases (Fazary and Ju 2007)

| **Microorganism** | **Induction conditions (temperature, induction period, carbon source, assay technique)** | **FAE activity** | |
|---|---|---|---|
| | | **(mU/mg$_{protein}$)** | **(mU/ml)** |
| *Streptomyces* C254 | 37ºC, 3 d, DSWB; DSWB | 300 | 80 |
| *Streptomyces* C248 | 37ºC, 3 d, DSWB; DSWB | 380 | 130 |
| *Streptomyces olivochromogenes* NRCC 2258 | 37ºC, 3 d, DSWB; DSWB | 2350 | 1200 |
| | 37ºC, 3 d, SCB; DSWB | 1800 | 830 |
| *Sporotrichum thermophile* | 50ºC, 7 d, WS; DSWB | 156 | – |
| *Streptomyces avermitilis* | 37ºC, 2 d, DSWB; DSWB | 16.8 | – |
| *Schizophyllum commune* ATCC 38548 | 30ºC, 14 d, DSWB; DSWB | 41.2 | 7 |
| | 30ºC, 14 d, OSX; DSWB | 9.5 | 20 |
| | 30ºC, 14 d, cellulose; DSWB | 28 | 28 |
| *Streptomyces avermitilis* UAH 30 | 37ºC, 4 d, DSWB; DSWB | 16.82 | 1.75 |
| | 37ºC, 4 d, OSX; DSWB | 11.15 | 1.55 |
| | 37ºC, 4 d, SCB; DSWB | 2.61 | 0.44 |
| *Neocallimastix* MC-2 | 39ºC, 5 d, cellulose; FAXX | 55 | – |
| *Penicillium brasilianum* IBT 20888 | 30ºC, 8 d, BSG; MFA | 1542 | – |
| *Penicillium pinophilum* CMI 87160ii | 30ºC, 12 d, OS+WB; MFA | 156 | – |
| *Trichoderma reesei* QM 9414 | 30ºC, 7 d, MFG+Glu; MFA | – | 3000 |
| *Streptomyces* sp. S10 | 30ºC, 4 d, DSWB; DSWB | 15.45 | 2 |
| *T. stipitatus* CBS 375.48 | 25ºC, 7 d, WB; MCA | – | 27 |
| *Piromyces* MC-1 | 39ºC, 5 d, CBG+S; MFA | 560 | – |
| *Penicillium funiculosum* IMI-134756 | 25ºC, 6 d, SBP; M*p*CA | – | 120 |
| *Piromyces brevicompactum* | 26ºC, 4 d, MFA; MFA | – | 32 |
| *Piromyces expansum* | 26ºC, 4 d, MFA; MFA | – | 45 |
| *Aspergillus awamori* IFO4033 | 30ºC, 3 d, WB; DSWB-SFO | 6900 | – |
| *Aspergillus awamori* VTTD-71025 | 30ºC, 7 d, SFC; WS | – | 10 |

**Table 4**. (continued)

| Microorganism | Induction conditions (temperature, induction period, carbon source, assay technique) | FAE activity (mU/mg) | FAE activity (mU/ml) |
|---|---|---|---|
| *Aspergillus foetidus* VTTD-71002 | 30ºC, 7 d, WB; WS | – | 12 |
| | 30ºC, 7 d, SFC; WS | – | – |
| *Aspergillus niger* | 26ºC, 4 d, MFA; MFA | – | 28 |
| *Aspergillus niger* CBS 120.49 | 25ºC, 4 d, OSX+Glu; MFA | 130 | 1250 |
| | 25ºC, 4 d, WB+Glu; MFA | 340 | – |
| *Aspergillus niger* CBS 120.49 | 24ºC, 4 d, SBP; MFA | 10 | – |
| *Aspergillus flavipes* | 28ºC, 5 d, WB; MFA | 6980 | 33180 |
| | 28ºC, 6 d, MB; MFA | 10570 | 6820 |
| *Aspergillus niger* CS 180 (CMICC 298302) | 25ºC, 5 d, SBP; MCA | 413 | 10.3 |
| *Aspergillus oryzae* VTTD-85248 | 30ºC, 7 d, WB; WS | – | 72 |
| *Aspergillus niger* VTTD-77050 | 30ºC, 7 d, WB; WS | – | 96 |
| | 30ºC, 7 d, SFC; WS | – | 132 |
| *Aspergillus niger* NRCC 401127 | 37ºC, 4 d, DSWB; DSWB | 13 2250 | 10580 |
| *Aspergillus niger* NRRL3 | 30ºC, 5 d CB; MFA | – | 13.9 |
| *Aspergillus niger* CS 180 (CMICC 298302) | 25ºC, 5 d, MCA; MFA | 413 | 10.3 |
| *Aspergillus niger* 1-1472 | 30ºC, 5 d, CB; MFA | – | – |
| *Aureobasidium pullulans* NRRLY 23311-1 | 30ºC, 2.5 d, BX; NPh-5-Fe-Araf | 1600 | 347.5 |
| *Bacillus subtilis* ATCC 7661 | 30ºC, 1 d, MFA; MFA | 1.3 | – |
| *Bacillus subtilis* FMCCDL1 | 30ºC, 1 d, MFA; MFA | 19.9 | – |
| *Bacillus subtilis* NCIMB 3610 | 30ºC, 1 d, MFA; MFA | 3.4 | – |
| *Clostridium stercorarium* NCIMB 11754 | 30ºC,3 d CC; DSWB | 3.4 | – |
| *Neurospora crassa* STA (74 A) | 30ºC, 3 d,WB; MSA | 9000 | – |
| *Orpinomyces* PC-1 | 90ºC, 5 d, CBG+S; MFA | 220 | – |
| *Orpinomyces* PC-2 | 90ºC, 5 d, CBG+S; MFA | 360 | – |
| *Orpinomyces* PC-3 | 90ºC, 5 d, CBG+S; MFA | 380 | – |
| *Fibrobacter succinogenes* S851 | | 10 | – |
| *Fusarium oxysporum* F3 | 30ºC,3 d, CC; DSWB | – | 98 |
| *Fusarium proliferatum* NRRL | 30ºC, 5 d, CB; MFA | – | 33.46 |

A, Avicel; BSG, brewer's spent grain; BX, birchwood xylan; CB, corn bran; CBG, coastal Bermuda grass; CC, corn cobs; DSWB, de-starched wheat bran; FAX,2-*O*-[5-*O*-(*trans*-feruloyl)-*β*-L-arabino-furanosyl]-D-xylopyranose; FAXX, *O*-[5-*O*-(*trans*-feruloyl)-*α*-L-arabino-furanosyl]-(1→3)-*O*-*β*-D-xylopyranosyl-(1→4)-D-xylopyranose;Glu, glucose; MB, maize bran; MCA, methyl caffeate; MFA, methyl ferulate; MFG, meadow fescue grass; M*p*CA, methyl *p*-coumarate; MSA, methyl sinapate; NPh- 5-Fe-Araf, 4-nitrophenyl 5-*O*-*trans*-feruloyl-*α*-L-arabinofuranoside; OSX, oat spelt xylan; S, sisal; SBP, sugar beet pulp; SCB, sugar cane bagasse; SFC, Solka-Floc cellulose; WB, wheat bran; WS, wheat straw

Biophysical and chemical studies of FAEs will contribute to our knowledge of the possible application of FAEs in food processing, agricultural industries and biomedical field (Fazary and Ju 2007). However, no correlation has yet been found between the biochemical characteristics and their optimal reaction conditions. The chemical characteristics of FAEs, such as the reported molecular weight (27-210 kDa), show significant variation and the optimal pH and temperature can range from pH 5 to 8 and 30°C to 65°C, respectively.

### 4.2.1 Recombinant ferulic acid esterases

A number of recombinant ferulic acid esterases have been produced from fungi; cinnamoyl esterase (FAEA) was expressed in *Pichia pastoris* at 300 mg/l (Juge 2001); the *fae*-1 gene was overexpressed in *P. pastoris* at 297 mg/l (Crepin et al. 2003b). The *fae*B gene was expressed in a protease-deficient strain of *A. niger* with a secretion yield of 100 mg/l (Levasseur et al. 2004), whereas the NcFaeD-3.544 was expressed in *P. pastoris* and showed an activity against MFA of 85 mU/ml (Crepin et al. 2004a). An FAEA was cloned from *A. awamori* that resulted in a secretion yield of 30 mg/l (Koseki et al. 2005); the catalytic domain EstA was produced in *Trichoderma reesei* resulting in a secretion yield of 33 mg/l (Poidevin et al. 2009); and the *A. niger* feruloyl esterase gene (*faeA*) was expressed in *Saccharomyces cerevisiae* with a secretion yield of 2 mg/l (Wong et al. 2011).

## 4.3 Commercial production of ferulic acid esterases

**Solid-state fermentation (SSF)** has lower production costs compared to submerged fermentation, as simpler equipment is needed (Roche at al. 1994). It utilizes various substrates like bran, bagasse and paper pulp; with the main advantage that nutrient-rich material is utilized slowly and steadily, allowing for lengthy incubation periods. This technique is best suited for fermentations involving fungi that require limited moisture content, since bacteria require a high water activity (Babu and Satyanarayana 1996).

**Submerged fermentation (SMF) and Liquid Fermentation (LF)** utilize free-flowing liquid substrates such as molasses and broths, which are utilized rapidly and constantly need nutrients supplementation. Due to its high moisture content, this fermentation technique is best suited for bacteria and allows easy purifying of the products (Subramaniyam and Vimala 2012).

For the optimal production of FAE, the choice of substrate is of great importance as it provides a carbon and energy source and also serves as an inducer for the microbial production of FAEs. Due to catabolite repression, monosaccharides and disaccharides (glucose, xylose, lactose, maltose) have generally not been efficient at inducing FAEs.

The best induction of FAEs was observed with complex substrates that have esterified ferulic acid bonds such as wheat bran (Crepin et al. 2003a; Donaghy and McKay 1995; Faulds et al. 1997; Garcia-Conesa et al. 2004; Koseki et al. 1998; Mathew and Abraham 2005; Tenkanen et al. 1991), de-starched wheat bran (Ferreira et al. 1999; Garcia et al. 1998a, b; Johnson et al. 1988, 1989; MacKenzie and Bilous 1988; Mukherjee et al. 2007), maize bran (Bonnina et al. 2001; Mathew and Abraham 2005), brewer's spent grain (Bartolome et al. 2003; Panagiotou et al. 2006) or sugar beet pulp (Asther et al. 2002; Bonnina et al. 2001; Crepin et al. 2003b; Donaghy and McKay 1995; Faulds and Williamson 1994; Faulds and Williamson 1995; Ferreira et al. 1999; Garcia-Conesa et al. 2004).

In a study using *Fusarium oxysporum*, it was observed that de-esterified corncobs decreased FAE production 5.5-fold. The addition of ferulic acid to de-esterified corncobs did not significantly increase FAE production, thus it was concluded that the esterified bond, rather than free ferulic acid, is necessary for efficient induction of FAEs (Faulds et al. 1997).

## 4.4 Isolation, purification and quantification

To study protein structure, it is important to obtain the enzyme in its homogeneous form in the absence of other cellular components such as lipids, nucleic acids, sugars, etc. For homogeneous purification of the enzyme, the physical and chemical properties of the protein need to be taken into consideration to ensure that the protein retains all its native biological characteristics, in particular its structure and activity. The general steps for protein purification include chromatography, precipitation and/or extraction, but this may vary depending on the nature of the particular protein (Reed et al. 2007).

### 4.4.1 Ion exchange chromatography

The net charge of proteins can be used to separate proteins using ion-exchange chromatography. Positively charged proteins can be separated on negatively charged

carboxymethyl-cellulose columns, while negatively charged proteins can be successfully separated by positively charged diethylaminoethyl-cellulose columns (Berg et al. 2002).

A net positive charge allows the proteins to bind to a column packed with beads that contain carboxymethyl-cellulose. Proteins with a negative charge will not bind, whereas elution of bound proteins is achieved with an increase in sodium chloride concentration due to competition with the positively charged proteins. Proteins that elute first are those with a low charge density, followed by those with a higher charge density.

### 4.4.2 Hydrophobic interaction

This method of protein separation takes advantage of hydrophobic amino acid residues on the protein surface. The separation is based on the interactions of the hydrophobic stationary phase, the hydrophobic protein molecules and the aqueous mobile phase (Reed et al. 2007). In the aqueous environment, interaction between the hydrophobic groups cause binding of hydrophobic proteins to the stationary phase. The strength of the binding is linked to the degree of hydrophobicity of the protein. The presence of certain salts, such as ammonium sulphate, promotes the strength of the binding. Elution of the bound protein can be achieved by reducing the ammonium sulphate concentration of the mobile phase to decrease the elution, also known as the salting-out effect.

### 4.4.3 Affinity chromatography

Another generally applicable and powerful method of purifying proteins is affinity chromatography, which is based on the affinity of proteins for specific chemical groups. When a protein mixture is passed through an affinity chromatography column, proteins with a high affinity for the specific chemical group will be bound to the column, while the rest is eluted with a washing buffer. The bound protein is eluted by adding a highly concentrated solution of the chemical group bound to the column, to ensure a decrease in the binding affinity of the protein to the column (Berg et al. 2002).

### 4.4.4 High-pressure liquid chromatography (HPLC)

There is a number of advantages offered by HPLC relative to the above-mentioned chromatographic techniques. The column material is more finely divided, providing more

interaction sites and increased resolving power. Given the finely divided nature of the column material, it is necessary to apply pressure to the column to obtain adequate flow rates, resulting in high resolution as well as rapid separation (Berg et al. 2002).

### 4.4.5 Quantification of ferulic acid esterase activity

Various methods are available to measure ferulic acid esterase activity, most of which are HPLC-based using enzymatic hydrolysis of ferulic acid esters (Barbe and Dubourdieu 1998; Kroon et al. 1997a), plant polysaccharides (Borneman et al. 1990; Bartolome et al. 1995; Garcia et al. 1998a, b), polysaccharides fragments (Dalrymple et al. 1996) and fragment analogues (Hatfield et al. 1991), as well as chlorogenic acid or hydroxycinnamic tartrate materials (Barbe and Dubourdieu 1998). The disadvantages of HPLC are linked to the need for expensive equipment, being time-consuming and therefore not suitable for rapid analysis of a large number of samples. Capillary zone electrophoresis (Donaghy and McKay 1997) and gas chromatography (Borneman et al. 1990) have also been used to determine ferulic acid activity using natural substrates, in particular their hydroxycinnamic methyl esters analogues, but were found to pose similar disadvantages as HPLC.

Spectrophotometric methods used for ferulic acid esterase activity analysis rely on differences in the spectral properties of free ferulic and its natural esters (Okamura and Watanabe 1982; McCallum et al. 1991) or their analogues (Hatfield et al. 1991). The 4-nitrophenyl ferulate (4NPF) spectrophotometric method (Mastihubová et al. 2001; Mastihuba 2002) measures the release of 4-nitrophenol through ferulic acid esterase action on the relatively cheap 4NPF substrate. The major disadvantage of this method and most other spectrophotometric methods using methyl esters, is the low solubility of the substrate in aqueous buffer solutions. Dimethyl sulphoxide and Triton X-100 have therefore been used to create a transparent emulsion, which is stable for several hours allowing the spectrophotometrical measurement of FAE activity (Kordel et al. 1991). Other substrates include feruloylated oligosaccharides (Blum et al. 2000; Fillingham et al. 1999), destarched wheat bran (O'Neill et al. 1996), synthetic esters of various cinnamic acids and short chain alcohols (Faulds and Williamson 1997; Kroon et al. 1997b). In these assays, the rate of hydrolysis of the model substrate is measured and FAE activity is defined as the amount of enzyme required to release 1 µM ferulic acid/min at pH 6, 37°C.

Nuclear magnetic resonance (NMR) is used to identify compounds using the differences in magnetic states of atomic nuclei, which are charged and therefore have spin. The typical magnetic nuclei, i.e. $^{1}H$, $^{13}C$, $^{14}N$, $^{15}N$, $^{19}F$ and $^{31}P$, interact with a uniform magnetic field spin to align parallel or antiparallel to the field. These two energy forms have different energy levels with the parallel having a lower energy state than the antiparallel. The differences in the energy levels correspond to a precise electromagnetic frequency such that when a sample containing an isotope with a magnetic nucleus is passed through a magnetic field with an appropriate radiofrequency, an exact match in energy gap and applied frequency results in a resonance. The difference in resonance frequencies and the reference value is known as a chemical shift (Reed et al. 2007).

When a sample is passed through Mass Spectrophotometry (MS), the protein is disintegrated into fragment ions in a gas phase that are accelerated to specific velocities in an electron field and eventually separated based on their respective masses. Each fragment with a particular mass is detected sequentially with time (Reed et al. 2007).

## 4.5 Industrial applications of FAEs

The potential biotechnological application of FAEs has led to significant interest from the chemical, fuel, animal feed, textile and laundry, pulp and paper, food and agriculture and pharmaceutical industries. There has also been considerable interest in the extraction of ferulic acid from agro-industrial waste materials such as those from the milling, brewing and sugar industries. The broad application of FAEs has fuelled research efforts in the screening and characterization of FAEs from numerous microorganisms (Fazary and Ju 2007).

### 4.5.1 Pulp and paper industry

In the pulp and paper industry, enzymes are used for the delignification of non-woody plant material due to their ability to hydrolyse the ester bond between the lignin and carbohydrate in the plant cell wall of the monocots and dicots. Using FAE together with xylanases and other xylan-degrading enzymes for biopulping and -bleaching of pulp, would allow the esterase to partially disrupt the cell wall, which would render the complex more susceptible to enzymatic attack. Lignin extraction can improve brightness of the pulp and thus reduce the use of chlorine during the bleaching stage (Evangelos et al. 2007).

### 4.5.2 Food industry

In the food industry, ferulic acid is among others used as a precursor for the production of vanillin, which has significant use as a flavouring agent. Ferulic acid as the most abundant hydroxycinnamic acid in plant cell wall and when naturally extracted using FAEs, provides an opportunity for the natural production of vanillin, which is important for consumer acceptance (Priefert et al. 2001). According to studies by Mathew and Abraham (2004) and Topakas et al. (2004), FAEs are good catalysing agents for the synthesis of sugar-phenolic esters, which will be beneficial for the addition of phenolic derivatives on natural biopolymers.

### 4.5.3 Farming

In the farming industry, the dimer and trimer forms of ferulic acid that are either esterified or etherified to plant residues, have been reported to inhibit digestion of these residues in ruminants (Yu et al. 2005a). The addition of a multi-enzyme cocktail that included purified *Aspergillus* FAE (13 mU FAE, 4 096 U xylanase, 1 024 U cellulase, 256 U endoglucanase I and II and 64 U $\beta$-glucanase), increased the cell wall digestibility of oat hulls by 86% (Yu et al. 2005b).

### 4.5.4 Pharmaceutical industry

Phenolic acid sugar esters have been reported to have antitumor activity and show significant potential towards their use in formulating antimicrobial, antiviral and anti-inflammatory agents (Fazary and Ju 2008; Garcia-Conesa et al. 2004). In the pharmaceutical industry, specific FAEs can be used to tailor-make pharmaceutical products (Garcia-Conesa et al. 2004; Kondo et al. 1990; Malherbe and Cloete 2002), including the release of ferulic acid and other plant phenolics with antioxidant characteristics.

# 5. Conclusions

The availability of large quantities of agro-industrial waste requires the development of new technologies that will add value beyond the bioethanol production process. The development of cost-effective techniques to degrade lignocellulose to its core components will support research towards the efficient use of feedstock using a biorefinery concept for producing various sugars and value-added chemicals with different industrial applications. The use of microbial enzymes, such as FAEs for the partial hydrolysis of lignocellulose, offers a natural process for products that will be acceptable to the consumer market.

The first report of FAE from *S.olivochromogenes* resulted in FAEs being recognized as common components of hemicellulases in many microorganisms (Faulds and Williamson 1991). FAE-producing microorganisms show vast differences with respect to FAE activities, and many have been purified and characterized to date. The ability to degrade plant biomass with a combination of FAEs and other hemicellulases presents an opportunity to extract novel bioactive compounds such as ferulic acid to be used in industry and/ or to synthesize other industrially significant compounds. It will add value to commercial products by increasing the availability of dissolved beneficial polyphenols for human consumption, and make nutrients from animal feedstock more readily available. The continued discovery of novel FAEs therefore remains an active part of research that includes investigations into the range of substrates that these enzymes can work on, as well as structural studies to understand their mechanism and to improve their biocatalytic abilities.

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# CHAPTER 2

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## Over-expression of *Aspergillus tubingensis faeA* in a protease-deficient *Aspergillus niger* enables ferulic acid extraction from plant material

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## Abstract

The production of ferulic acid esterase involved in the release of ferulic acid side groups from xylan was investigated in strains of *Aspergillus tubingensis, Aspergillus carneus, Aspergillus niger* and *Rhizopus oryzae*. The highest activity on triticale bran as sole carbon source was observed with the *A. tubingensis* T8.4 strain, which produced a type A ferulic acid esterase active against methyl *p*-coumarate, methyl ferulate and methyl sinapate. The activity of the *A. tubingensis* ferulic acid esterase (*At*FAEA) was inhibited two-fold by glucose and induced two-fold in the presence of maize bran. An initial accumulation of endoglucanase was followed by the production of endoxylanase, suggesting a combined action with ferulic acid esterase on maize bran. A genomic copy of the *A. tubingensis faeA* gene was cloned and expressed in *A. niger* D15#26 under control of the *A. niger gpdA* promoter. The recombinant strain has reduced protease activity and does not acidify the

media, promoting high-level expression of recombinant enzymes. It produced 13.5 U/ml FAEA after 5 days on autoclaved maize bran as sole carbon source, which was 3-fold higher than for the *A. tubingensis* donor strain. The recombinant *At*FAEA was able to extract 50% of the available ferulic acid from non-pretreated maize bran, indicating potential application thereof for the release of ferulic acid from lignocellulosic plant material.

**Keywords:** *Aspergillus tubingensis*, ferulic acid esterase, *Aspergillus nig*er, ferulic acid extraction

# 1. Introduction

With the increased demand for fuel ethanol and the predicted decline in global crude oil reserves, lignocellulosic biomass currently offers the most sustainable alternative source of transport fuel. However, large-scale commercial cellulosic biofuel production is still unable to compete effectively with fossil fuels on a cost basis and has therefore not yet made a significant breakthrough in the market [4]. A biorefinery concept is considered the most promising to reduce the production cost of cellulosic biofuels [8] due to value-addition through a range of valuable products in addition to bioethanol (similar to a petroleum refinery).

Lignocellulosic plant material consists of cellulose, hemicellulose and lignin, as well as a range of other compounds, such as phenolics (ferulic acid, syringic acid and *p*-coumaric acid) that are either esterified to α-L-arabinose moieties in arabinoxylans or ether-linked to lignin. This complex interaction is important to protect the structural intergrity of the plant cell wall against enzymatic degration. Knowledge of the type of phenolic ester bonds present in a given feedstock is thus important to inform the choice of hydrolytic enzymes and the general strategy for the production of high-value commodities from a specific feedstock.

Ferulic acid is the major cinnamic acid among the phenolic compounds associated with lignocellulosic materials that are released through pretreatment of biomass [11, 17, 31]. Ferulic acid is best known for its antioxidant properties due to its radical scavenging ability and is widely used as a food preservative and active ingredient in skin lotions and sunscreens [16]. Ferulic acid can also be converted enzymatically to various value-added products, e.g. vinyl guaiacol and vanillin, which are well known flavouring agents in foods, beverages and

perfumes [17]. However, the commercial application of ferulic acid has been limited by its availability and high production cost [16].

Although plant cell walls contain a significant amount of ferulic acid, the complex binding of ferulic acid to hemicellulose and lignin renders it difficult to extract. To date, the commercial production of natural ferulic acid has mainly been from the chemical extraction of ferulic acid from rice bran oil using food grade n-hexane, but this poses potential health and environmental hazards [13, 26]. Although alkaline hydrolysis releases the total ferulic acid content from the plant cell wall, purification of ferulic acid from the hydrolysate has also proven difficult due to the presence of many other components. To benefit from ferulic acid production within a biorefinery concept, efficient technologies will therefore be required to enable ferulic acid extraction prior to processing of the lignocellulose. A more natural and specific way of extracting ferulic acid from lignocellulosic material would be based on hydrolytic enzymes that can specifically release ferulic acid from its bound constituents without any adverse side reactions [13].

There is growing interest in ferulic acid esterases (also known as feruloyl esterase) as key enzymes for cell wall hydrolysis and in particular the extraction of phenolic acids from agricultural crops, as they are able to hydrolyse the bond between arabinose and ferulic acid, releasing the covalently bound lignin from hemicellulose. Extraction of ferulic acid also releases other carbohydrates that may be useful for fermentation processes, including bioethanol production. Ferulic acid esterases (FAEs) are classified as type A, B, C or D depending on their substrate specificity. Type A FAEs tend to be induced during growth on cereal-derived substrates, and are active against methyl ferulate (MFA), methyl sinapate (MSA) and methyl *p*-coumarate (MpCA), but not methyl caffeate (MCA). Type B FAEs are active against methyl ferulate, methyl caffeate and methyl *p*-coumarate, but not methyl sinapate. Types C and D are able to act on all four hydroxycinnamic acid methyl esters, but only type D enzymes are able to hydrolyse ferulic acid dimers [13].

A number of recombinant organisms have been developed for the production of FAE, in particular those from *Aspergillus* species. Expression of the *Aspergillus niger* ferulic acid esterase gene in *Saccharomyces cerevisiae* yielded 2 mg/l protein with a specific activity of 8.2 U/µg [40], whereas expression of the *A. niger* cinnamoyl esterase yielded 300 mg/l in *Pichia pastoris* [20] and 1 g/l in *A. niger* [30]. Production of the *A. tubingensis* ferulic acid esterase A (*At*FAEA) in *Aspergillus vadensis* was significantly higher than in the donor *A. tubingensis* strain due to the low proteolytic activity of *A. vadensis* [9]. Furthermore,

expression of only the catalytic domain of the *Piromyces equi* cinnamoyl esterase (EstA) in *Trichoderma reesei* yielded 33 mg/l of ferulic acid [13]. The *faeA* genes from *A. niger* and *A. tubingensis* were cloned and overexpressed in *A. niger* and *A. tubingensis* strains [10], with expression of the *A. niger faeA* gene in *A. tubingensis* (3.16 U/ml) yielding the best results. It was concluded that the *At*FAEA was more sensitive to the acid proteases in *A. niger*, as overexpression in an *A. niger* protease-deficient strain yielded 7.71 and 0.97 µM/min for the respective *A. niger* and *A. tubingensis* enzymes.

The application of FAE is currently not considered a practical way of producing commercial ferulic acid in view of the high costs associated with microbial enzyme production and the long reaction time required to release the bound ferulic acid [17]. It has therefore become important to find and/or develop strains that will be able to produce FAE at high levels to ensure the cost-effective extraction of ferulic acid from plant material. The objectives of this study were to identify a FAE gene for heterologous expression in *A. niger* D15#26, a protease-deficient strain that doesn't acidity the growth media, which is desirable for the large-scale production of recombinant FAEs. Four fungal strains representing *Rhizopus oryzae, Aspergillus carneus, Aspergillus nidulans* and *Aspergillus tubingensis* were evaluated for FAE activity, with *Rhizopus oryzae* and *A. tubingensis* producing higher levels of FAE than the other two strains. The *A. tubingensis* FAE released the highest levels of ferulic acid from triticale and the *A. tubingensis* FAE gene was therefore selected for cloning and over-expression in *A. niger*.

# 2. Materials and Methods

## 2.1 Strains and chemicals

The fungal strains *Rhizopus oryzae* MP1, *Aspergillus carneus* ABO374, *Aspergillus nidulans* IFO4342, *Aspergillus tubingensis* T8.4 (PPRI 13401, hereafter referred to as *A. tubingensis*) and *A. niger* D15#26 (*cspA1*, *pyrG1*, *prtT13*, *phmA*, a non-acidifying mutant of AB1.13, ATCC 9029 [15], hereafter referred to as *A. niger* D15) were obtained from the culture collection at Department of Microbiology, Stellenbosch University, South Africa. Unless stated otherwise, all chemicals were of analytical grade and were obtained from Merck (Darmstadt, Germany).

## 2.2 Culturing conditions

Fungal strains were cultivated in minimal media containing 5 g/l yeast extract (Difco), 0.4 g/l $MgSO_4.7H_2O$, 2 g/l casamino acids, 20 mL 50 x AspA (300 g/l $NaNO_3$, 26 g/l KCL, 76 g/l $KH_2PO_4$, pH 6), 1 ml/l of 1000 x trace elements [32], as well as 10 g/l glucose (only where specified) and 0.01 M uridine for cultivation of *A. niger* D15. *Escherichia coli* DH5α was cultured at 37°C in Luria Broth [35] with 100 µg/ml ampicillin added for the selection of transformants.

Triticale (Department Process Engineering, Stellenbosch University) was pretreated (steam exploded, 205°C, 15 min, 25 Bar) and used at 10 g/l as lignocellulosic substrate for quantification of enzyme activities in the native strains, whereas 10 g/l untreated maize bran (Sasko, Paarl, South Africa) was used for characterisation of the recombinant enzymes. Optimisation of FAE expression was done in minimal media without glucose with 10 g/l maize bran prepared at different pH values (pH 4 to 8) and with different autoclaving periods (30, 60 or 90 minutes). The growth media was inoculated with $1x10^6$ spores/ml and incubated on a rotary shaker at 125 rpm at 30°C. The cultures were checked daily by microscopy to ensure that there was no bacterial contamination. Following incubation, samples were centrifuged at 8 000 rpm at 4°C for 15 min and the supernatant used for further analysis.

## 2.3 Enzyme assays

Fungal strains were cultivated in 50 ml minimal media with either 10 g/l triticale or 10 g/l maize bran in Erlenmeyer flasks for 5 days (10 g/l glucose was added where indicated). The 4-nitrophenyl ferulate (4NPF) substrate was synthesized and purified as described by Hedge *et al.* [18]. The reaction mixture contained nine volumes 0.1 M potassium phosphate buffer solution (pH 6.5), 2.5% Triton X-100, and one volume of 11 mM 4NPF in DMSO, followed by immediate vortexing. Enzyme assays consisted of 0.1 ml supernatant and 2 ml 4NPF solution incubated at 37°C for 1 hr, with the release of 4NP measured spectrophotometrically at 410 nm (xMark™ Microplate Spectrophotometer, Bio-Rad, San Francisco, USA). One unit (1 U) of FAE activity was defined as the amount of enzyme that released 1 µmol of 4NP from 4NPF in 1 min.

Ferulic acid was quantified by HPLC analysis [23] on a Nucleosil C18 column (5 µM particle size, Supelco, Bellefonte, USA) with 70% acetonitrile as mobile phase at a flow rate

of 1 ml/min at room temperature, using analytical grade ferulic acid (Sigma-Aldrich, UK) as reference and a Surveyor Plus UV/VIS detector (Thermo Electron Corporation, Elandsfontein, South Africa) at 320 nm. Ferulic acid extraction was expressed as a percentage relative to the total available ferulic acid extracted by chemical means. For the latter, maize bran and triticale (100 mg) was suspended in 1 M NaOH (2 ml) at 55°C for 5 h. The soluble fraction was then centrifuged (3,600×g, 15 min) and acidified to pH 2 with HCl for HPLC analysis [1].

Endoxylanase and endoglucanase activities were quantified with the reducing sugar assay [2] using 10 g/l Birchwood xylan and carboxymethyl cellulose (CMC) in 0.05 M sodium citrate buffer (pH 5) as respective substrates. One unit (1 U) was defined as the amount of enzyme releasing 1 µmol of reducing sugar per minute after incubation at 50°C for 30 min [25]. Total protein concentrations were quantified with the Qubit assay and Qubit 2.0 fluorometer (Invitrogen, Carlsbad, USA) as per the supplier's specification.

Substrate specificity assays were performed with 20 µl supernatant from triticale-induced cultures and 40 µl substrate solutions of 1 mg/ml hydroxycinnamic substrates (Table 1) prepared in MOPS buffer, pH 6. The decline in spectrophotometric absorbance at 335 nm was monitored with the sample compartment kept at 37°C. One unit of FAE activity (1U) is defined as the amount of enzyme releasing 1 µmol of free phenolic acid per minute under these conditions [24, 30].

## 2.4 Cloning of *A. tubingensis* ferulic acid esterase gene and expression in *A. niger*

The *AtfaeA* gene [GenBank Y09331] was amplified from *A. tubingensis* genomic DNA using gene specific primers ('-ATGAAGCAATTCTCCGCAAAATACGCCATCGC-3' (AtfaeA-left), 5'-CTTACCATGTACAATGTCCGCTCGTCATCC-3' (AtfaeA-right) and TaKaRaExTaq polymerase (Takara Bio Inc, Otsu, Japan). The open reading frame was cloned as a blunt-end fragment into the blunted *Not*I site of the *A. niger* expression vector pGTP, generating pGTP-AtfaeA. Recombinant plasmids were transformed into electro-competent *Escherichia coli* DH5α cells [35] and transformants were selected on LB-Amp agar plates. The DNA sequence of the cloned fragment was verified using the ABI PRISM$^{TM}$ 3100 Genetic Analyzer. Plasmids pGTP and pGTP-*AtfaeA* were transformed to *A. niger* D15 [32] and transformants were selected on minimal medium plates lacking uridine, yeast extract

and casamino acids. Seventy transformants were selected after three purification cycles and screened in minimal medium with maize bran for FAE production.

### 2.5 Protein analysis

SDS-PAGE was used to separate the denatured protein samples, followed by renaturing of the proteins for 4 h at 4°C using 0.1 M sodium phosphate buffer (pH 6.5). The buffer was replaced with freshly prepared 0.1 M sodium phosphate buffer (pH 6.5) containing 25 µl of a 20 mg/ml Fast Blue solution (Sigma-Aldrich, South Africa). The addition of 50 µl α-naphthyl acetate (1.2 mg/ml acetone) would yield a purple-coloured band in the presence of esterase activity.

## 3. Results

### 3.1 Partial characterisation of native enzymes

After 3 days' culturing in the presence of triticale and glucose, the *A. tubingensis*, *R. oryzae*, *A. nidulans* and *A. carneus* strains respectively yielded 3.1 U/ml, 2.8 U/ml, 2.05 U/ml and 2.05 U/ml of extracellular FAE (Fig. 1a). The release of ferulic acid was evaluated in the *A. tubingensis* and *R. oryzae* strains (Fig. 1b), with approximately 31% and 26% of the ferulic acid respectively extracted (relative to NaOH treatment) after 2 days. However, the *A. tubingensis* strain utilised most of the released ferulic acid by day 5.

In minimal media with triticale and glucose, the endoxylanase activity in the *A. tubingensis* and *R. oryzae* supernatant reached 206 U/ml and 211 U/ml on day 5 (Fig. 1c). The endoglucanase activity was much lower in both the *A. tubingensis* and *R. oryzae* supernatants, reaching only 0.9 U/ml and 1.3 U/ml on day 3, respectively (Fig. 1d).

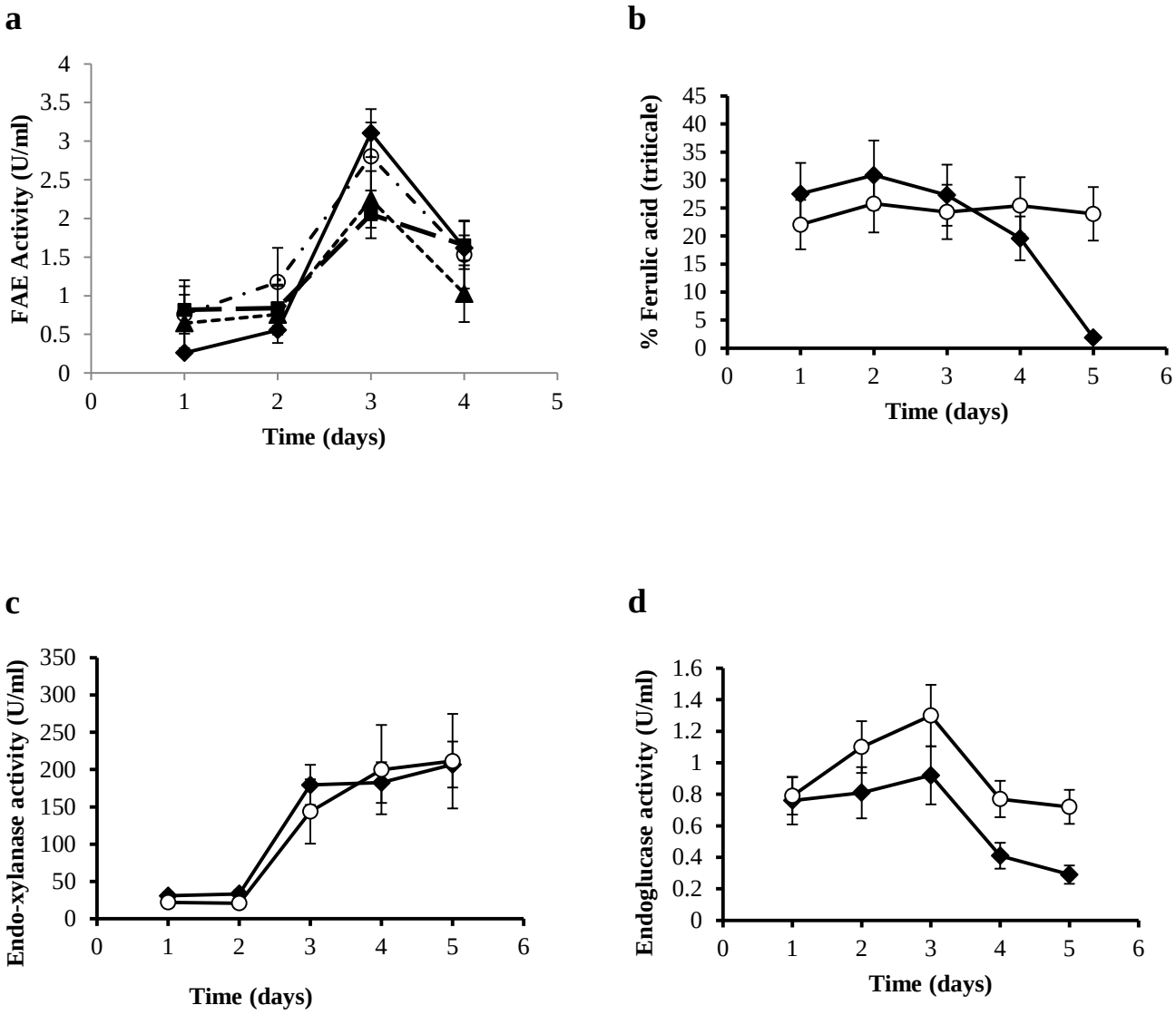



**Fig. 1 a** Extracellular FAE activity, **b** ferulic acid accumulation (as a percentage of the available ferulic acid extracted by 1 M NaOH), **c** endo-xylanase and **d** endoglucanase activity displayed by *A. tubingensis* (*filled diamonds*), *A. nidulans* (*closed square*), *A. carneus* (*closed triangle*) and *R. oryzae* (*open circles*) in minimal media containing 10 g/l triticale

The hydrolysis efficiency patterns observed for the methyl ester substrates were MpCA>MFA>MSA for *A. tubingensis*, and MFA>MSA>MpCA>MCA for *R. oryzae* (Table 1). This indicated that *A. tubingensis* produced a type A FAE that could hydrolyse C-5 feruloylated substrates, whereas the *R. oryzae* strain produced a type C or D ferulic acid esterase able to hydrolyse C-5 and C-2 feruloylated substrates.

**Table 1** Substrate specificity of ferulic acid esterase produced by *A. tubingensis* and *R. oryzae*

| Substrate | Abbreviation | Enzyme activity (U/ml) | |
|---|---|---|---|
| | | *A. tubingensis* | *R. oryzae* |
| methyl sinapate | MSA | 0.2 (±0.21) | 1.04 (±0.63) |
| methyl caffeate | MCA | 0 | 0.2 (±0.55) |
| methyl *p*-coumarate | MpCA | 1.5 (±0.42) | 0.3 (±0.18) |
| methyl ferulate | MFA | 1.1 (±0.11) | 1.6 (±0.33) |

## 3.2 Cloning and expression of *A. tubingensis faeA* in *A. niger*

Since the *A. tubingensis* strain displayed the highest FAE activity among the four fungal strains, it was selected for recombinant expression of its FAE in *A. niger* strain D15#26, a protease-deficient strain that does not acidify the medium [15]. Amplification of the genomic copy of *AtfaeA* generated a 931 bp fragment, which correlated with the published *AtfaeA* open reading frame (ORF) [10]. The fragment was subcloned into plasmid pGTP under control of the constitutive *A. niger gpdA* promoter [32] and 70 *A. niger* D15 transformants were evaluated in liquid cultures. The *A. niger* D15[pGTP] strain, containing the empty vector, served as negative control. The *A. niger* D15[*AtfaeA*] transformant that displayed the highest activity in liquid cultures (data not shown), was selected for further study.

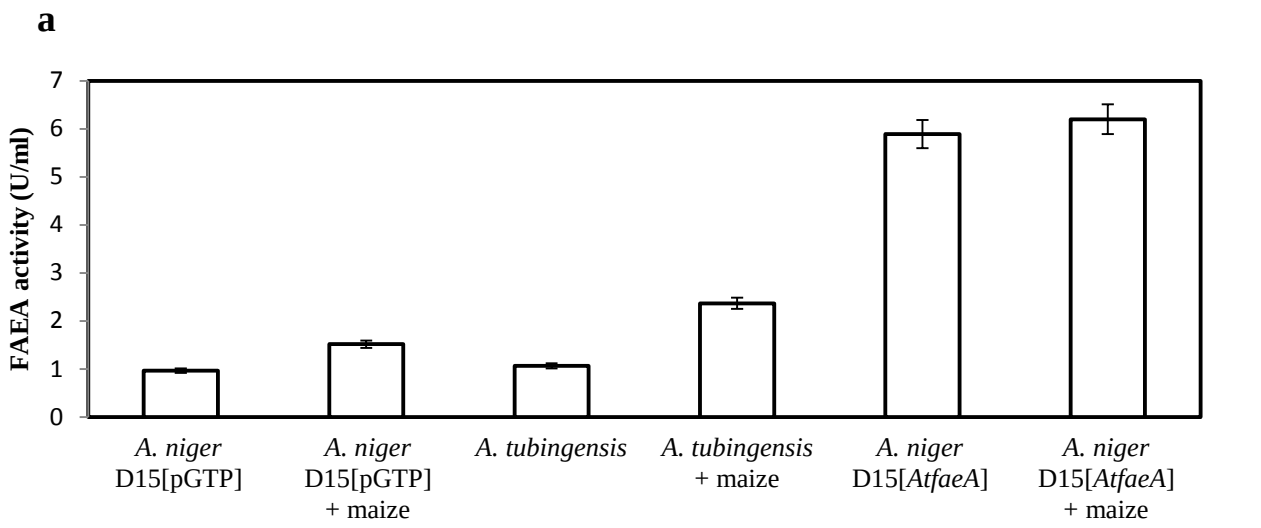
## 3.3 Production and partial characterisation of recombinant *At*FAEA

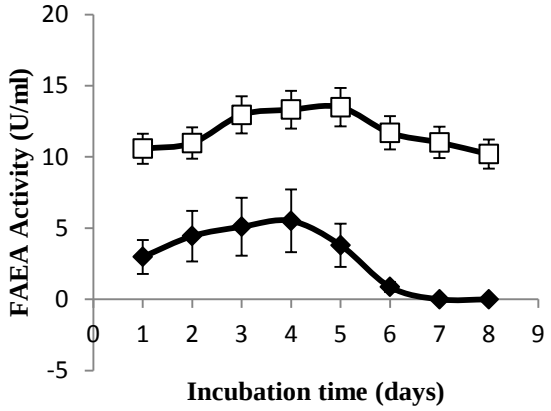
Maize bran contains more bound ferulic acid than triticale (31.28 mg/g versus 2.29 mg/g as determined by alkali extraction) [36] and was therefore used in subsequent experiments to ensure detectable levels of ferulic acid. The wild type *A. tubingensis* and *A. niger* strains displayed similar levels of FAE activity on minimal medium + 10 g/l glucose, with significant induction of both in the presence of 10 g/l maize bran (Fig. 2a). Under uninduced conditions (i.e. no maize bran), the *A. niger* D15[*AtfaeA*] strain displayed more than 5-fold higher FAE activity than the native *A. tubingensis* and *A. niger* D15[pGTP] strains, i.e. 5.89

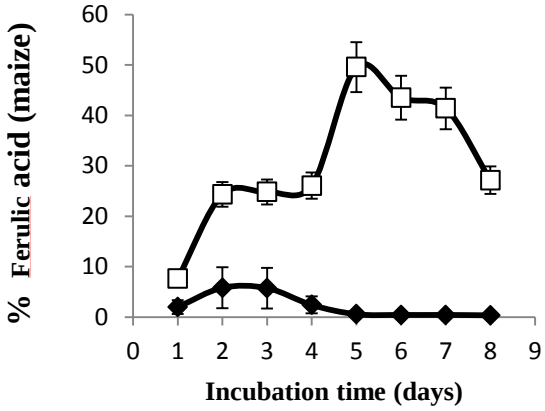
U/ml versus 1.07 and 0.97 U/ml FAE, respectively. No induced expression was observed for *A. niger* D15[*AtfaeA*] as the constitutive *A. niger gpdA* promoter was used for expression.

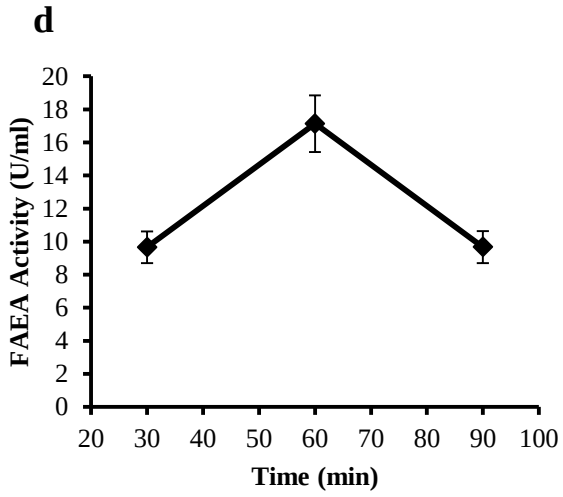
Extracellular FAE activity peaked at 5.5 U on day 4 for the wild type *A. tubingensis* and at 13.5 U on day 5 for *A. niger* D15[*AtfaeA*] (Fig. 2b), with a significant decline in activity for *A. tubingensis* towards day 6, probably due to cell lysis. After 5 days, *A. niger* D15[*AtfaeA*] was able to extract 50% of the available ferulic acid in untreated maize bran (Fig. 2c). Although there was some ferulic acid accumulation after 3 days, the *A. tubingensis* strain quickly consumed or degraded this to leave no residual ferulic acid by day 5. A similar trend was observed when the native *A. tubingensis* was grown on triticale (Fig. 1b), with very little ferulic acid detected on day 5.

Pretreatment of maize bran by means of autoclaving for 60 min (Fig. 2d) increased FAE activity in *A. niger* D15[*AtfaeA*] almost two-fold relative to 30 and 90 min. Although an initial pH of 6 for the culture medium yielded the highest FAE activity (22.6 U/ml) for *A. niger* D15[*AtfaeA*], this was not significantly higher than for the other pH values (Fig. 2e).



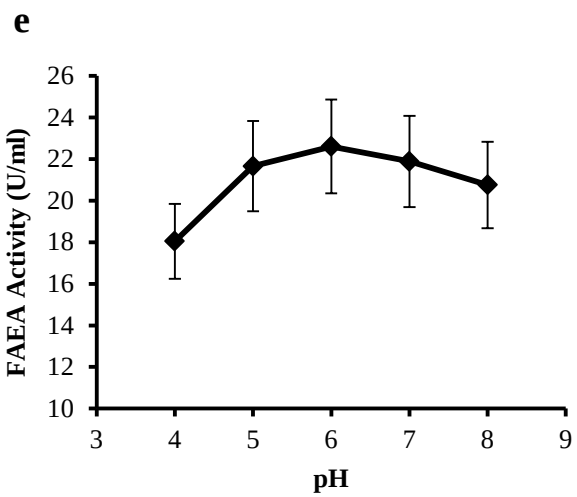



**Fig. 2 a** Extracellular FAE activity produced by *A. tubingensis*, *A. niger* D15[pGTP] and *A. niger* D15[*AtfaeA*] after 5 days of cultivation in minimal media containing 10 g/l glucose with or without 10 g/l maize bran. **b** FAE activity and **c** ferulic acid accumulation (as a percentage of available ferulic acid extracted by 1 M NaOH) by *A. tubingensis* (*filled diamonds*) and *A. niger* D15[*AtfaeA*] (*open squares*) grown in minimal media with 10 g/l maize bran. The effect of **d** autoclaving and **e** pH of the growth medium on ferulic acid esterase production by *A. niger* D15[*AtfaeA*] when grown in minimal media with 10 g/l maize bran for 5 days

Following SDS-PAGE analysis and renaturation of the recombinant *At*FAEA, a Zymogram with α-naphthyl acetate revealed a purple halo (confirming FAE activity) that corresponded to a molecular weight of 36 kDa (Fig. 3).

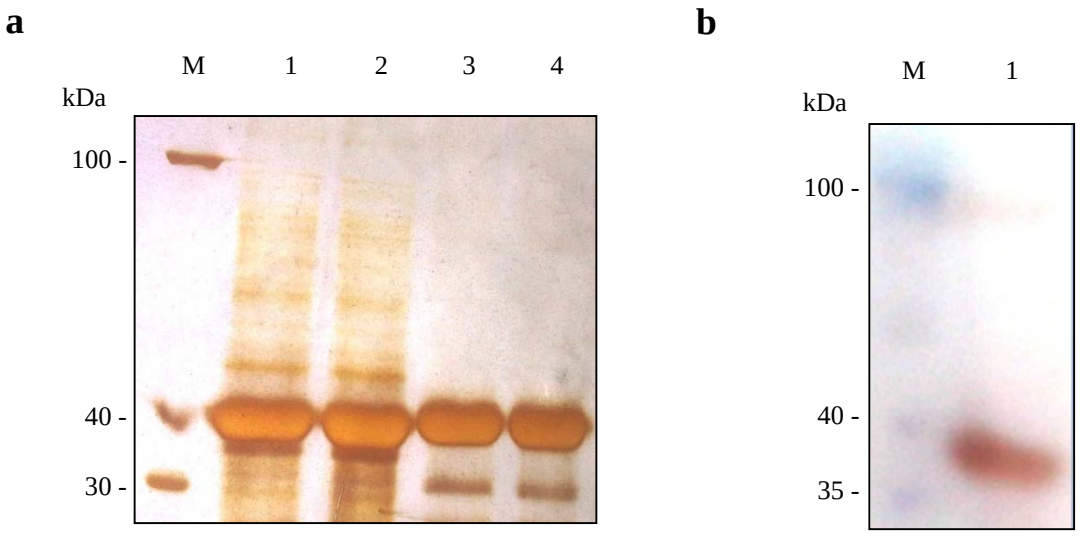



**Fig. 3 a** SDS-PAGE analysis of the extracellular FAE obtained fom *A. niger* D15[*AtfaeA*]. *Lane M* molecular weight marker; extracellular proteins (sampled in duplicate) produced in minimal media with maize bran (*lanes 1 and 2*) or maize bran plus glucose (*lanes 3 and 4*). **b** Zymogram of the extracellular proteins produced by *A. niger* D15[*AtfaeA*] in minimal media with maize bran (*lane 1*). *Lane M* pre-stained molecular weight marker

# 4. Discussion

The production of ferulic acid esterase by *Aspergillus* strains (*A. nidulans*, *A. carneus* and *A. tubingensis*) is well-documented [6, 13]. Previous reports indicated that the soft-rot fungus *R. oryzae* produce significant levels of laccase, endoglucanase and endoxylanase activity [27], suggesting that it should be able to also produce ferulic acid esterases. The four strains evaluated in this study produced 2 to 3 U/ml FAE in liquid minimal medium with triticale, with *A. tubingensis* and *R. oryzae* being able to extract approximately 31% and 26% of the available ferulic acid after 2 days. Note that these percentages should be treated with caution as alkaline treatment releases both ester and ether-bound ferulic acid, whereas enzymatic extraction only releases ester-bound ferulic acid.

Different types of FAEs have been identified based on their substrate specificity, which is determined by the substitutions on the aromatic ring that can be accommodated by the respective enzymes [41]. The *A. tubingensis* strain was able to hydrolyse all the methyl esters evaluated in this study, except for MCA, indicating that it is a type A FAE, similar to those reported for *A. niger*, *R. oryzae* and *A. awamori* [14, 34]. The *R. oryzae* strain was able to hydrolyse all the methyl esters, similar to the type C or D FAE reported for *Clostridium stercorarium* [37]. The substrate preferences of *A. tubingensis* (MpCA>MFA>MSA) suggested that the *At*FAEA would only be able to release ferulic acid from monocots (wheat, oats, maize and barley), as ferulic acid is esterified to the C-5 hydroxyl group of some arabinopyranose residues of arabinoxylans in monocots. In contrast, the *R. oryzae* strain (MFA>MSA>MpCA>MCA) should be able to release ferulic acid from both monocots and dicots where the ferulic acid is esterified to the C-2 hydroxyl group of arabinofuranose.

Access to cellulose is hampered by the complexity of the lignocellulose structure; fungal strains therefore typically produce an array of hydrolytic enzymes, including endoglucanases and endoxylanases when grown on a complex lignocellulosic substrate [4]. In a study by Kumar and Wyman [22], the addition of xylanases to an enzyme cocktail improved hydrolysis and released glucose and xylose in a linear fashion. With triticale as substrate, the endoglucanase and endoxylanase activities of *A. tubingensis* and *R. oryzae* were comparable to other endoglucanase producers [21]. The endoxylanase activity displayed by *A. tubingensis* and *R. oryzae* also correlated with other reports on endoxylanase-producing fungi such as *Penicillium canescens*, *Trichoderma viride* and *Aspergillus tamarri* grown on different carbon sources [3]. The levels of endoglucanase (Fig. 1d) and ferulic acid esterase

(Fig. 1a) in both strains peaked on day 3 and then declined, whereas the endoxylanase activities continued to increase up to day 5 (Fig. 1c). The co-production of the ferulic acid esterase, endoxylanase and endoglucanase supports previous reports on the cooperation between these lignocellulose degrading enzymes [39].

Since the *A. tubingensis* strain showed the highest FAE activity and release of ferulic acid from 10 g/l triticale, its ferulic acid esterase gene (*AtfaeA*) was cloned and successfully expressed in *A. niger* D15. The FAE activity of the recombinant enzyme reached 13.5 U/ml on day 5, which was 3-fold higher than for the *A. tubingensis* donor strain (Fig. 2b). The *At*FAEA was produced as a significant portion (estimated at 20 mg/l) of the total extracellular proteins secreted by *A. niger*, which would simplify downstream processing and purification of the recombinant protein.

Induction of FAE is often observed when fungi are grown on complex substrates, most likely exerted by specific components of the hemicellulosic material [12, 24]. A two-fold induction of the native FAE activity was observed in the *A. tubingensis* strain after 5 days when 10 g/l maize bran was added as an inducer to glucose-containing minimal medium (Fig. 2a). Both *A. niger* D15[pGTP] and *A. niger* D15[*AtfaeA*] strains displayed an increase of about 0.5 U/ml in FAE activity in the presence of maize bran relative to the absence thereof (Fig. 2a), suggesting that the native *A. niger* FAE was subject to mild substrate induction. However, FAE activity in *A. niger* D15[*AtfaeA*] was two-fold higher in minimal medium + maize bran than in glucose-containing medium (Fig. 2b), indicating glucose repression of the native *A. niger* FAE.

The available carbon source influences FAE production and activity and some fungal strains produce more than one type of FAE, which can contribute to the total FAE activity measured under a specific set of conditions [24]. The total FAE activity observed for *A. niger* D15[*AtfaeA*] exceeded those previously reported for type A FAE on maize bran [5, 13, 24], suggesting that the *A. tubingensis* FAE performs particularly well in *A. niger* D15. Added to that, using the *A. niger* protease-deficient strain and a constitutive promoter resulted in a significant increase in extracellular enzyme levels with no significant degradation, in contrast to FAE expression systems previously reported [10].

HPLC analysis indicated that 50% of the available ferulic acid was extracted from untreated maize bran by *A. niger* D15[*AtfaeA*] at day 5, i.e. 10-fold more than the native *A. tubingensis* strain's maximum at day 3 (Fig. 2c). The ferulic acid released was

significantly higher than the 40% reported for a native *A. niger* FAE on autoclaved maize bran [7, 28], 30% for the *Thermoanaerobacter tengcongensis* thermostable FAE on triticale bran [1], and the 7.5% for expression of the *P. equi* cinnamoyl esterase (*estA*) gene in *Trichoderma reesei* Rut C30 [28].

Release of ferulic acid from maize bran was further enhanced through autoclaving, with treatment of 60 min at 121$^{o}$C and pH 6 yielding the best results. Autoclaving of maize bran improves the solubilisation of feruloylated oligosaccharides [7], whilst an optimum of pH 6 corresponds to previous reports on the optimal performance of the *A. niger* D15 host strain [33]. The native *A. tubingensis* strain was able to extract 30% of the available ferulic acid from steam-exploded triticale (Fig. 1a), but only 5% from autoclaved maize bran (Fig. 2c). This can be expected as autoclaving is a milder form of pretreatment and maize bran has a more branched structure, which is more recalcitrant than triticale [37]. However, there was a significant decrease in free ferulic acid during prolonged cultivation (Fig. 1a and 2c), which could be ascribed to oxidative coupling of ferulic acid to form larger and more complex molecules, or the metabolism of ferulic acid to various other products [17, 19, 31].

A mixture of extracellular enzymes is required to work synergistically with FAE to liberate ferulic acid from lignocellulosic material, which can induce the expression of the relevant enzymes. On 10 g/l triticale + glucose media, a two-fold induction of the native *A. tubingensis* FAE was observed, together with a significant increase in endoglucanase and endoxylanase activities (Fig. 1). Furthermore, the recombinant *A. niger* D15[*AtfaeA*] strain was an effective producer of FAE when compared to the native *A. tubingensis* and *A. niger* strains. *Aspergillus* species tend to acidify the medium and a drop in pH is difficult to control even in automatized fermenters. In the study by De Vries *et al.* [10], lower levels of ferulic acid esterase activity were obtained due to the *A. tubingensis* FAE being highly sensitive to acid proteases. A low pH also triggers the production of extracellular acid proteases, which in turn leads to the degradation of heterologous proteins. The *A. niger* D15 strain used is this study does not acidify the medium, thus representing a better host for recombinant expression of *At*FAEA [32, 33].

We were able to produce extracellular *At*FAE at high levels in an *A. niger* host that is known for its reduced protease activity and that does not acidify the media. The extracellular enzyme cocktail from the recombinant strain extracted 50% of the available ferulic acid from non-pretreated maize bran - a very recalcitrant substrate - without a pretreatment step. This extraction efficiency was higher than some other feruloyl esterases reported in literature after

a pretreatment step. This confirms that enzymatic ferulic acid extraction is a feasible alternative to chemical processes, but the enzyme production system and conditions need to be further optimised to increase enzyme production by the recombinant *A. niger* D15[*AtfaeA*] strain.

## 5. Acknowledgements

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## 6. Declaration

SHR constructed the recombinant *A. niger* D15[*AtfaeA*] strain, with all subsequent analysis conducted by ENZ. WHvZ, KR and MVB participated in the design of the study and revision of the manuscript. All authors read and approved the final manuscript.

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# CHAPTER 3

## Purification, characterisation and evaluation of a recombinant *Aspergillus* ferulic acid esterase in bioreactors

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**ABSTRACT**

A recombinant *Aspergillus tubingensis* ferulic acid esterase Type A (*At*FAEA) expressed in *Aspergillus niger*D15#26 was purified 7-fold with anion-exchange chromatography and its identity confirmed with peptide mass fingerprinting. The 35-kDa *At*FAEA protein showed maximum ferulic acid esterase activity at pH 6 and 50°C and was stable at 30°C to 60°C and at pH 3 to 7. The specific activity of *At*FAEA was significantly higher than previously reported for other ferulic acid esterases, but high concentrations of synthetic methyl ferulate displayed substrate inhibition of the purified *At*FAEA ($K_m$ = 0.43 mM, $K_{cat}$ = 0.43/min and $K_{cat}/K_m$ = 1.1/min.mM). In bioreactors, the recombinant *A. niger* D15[*AtfaeA*] strain produced an extracellular FAEA with 2-fold higher enzyme activity in fed-batch relative to batch fermentation. The fed-batch fermentation yielded a higher biomass (10.57 g/l), volumetric productivity (3.95 U/ml/h) and volumetric activity (184 U/ml) than batch fermentation. Fed-batch production of *At*FAEA yielded a FAE activity of 546 U/mg, as opposed to 11.1 U/mg in shake flasks, supporting the potential of the *A. niger* D15[*AtfaeA*] strain for up-scaled production of *At*FAEA for potential application in both the food and pulp and paper industries.

**Keywords**: *Aspergillus niger*, *Aspergillus tubingensis*, ferulic acid esterase, fed-batch

## 1. Introduction

Ferulic acid esterases (FAEs, also known as feruloyl esterases, cinnamic acid or cinnamoyl esterases; EC 3.1.1.73) belong to a diverse group of esterases that can release ferulic acid (4-hydroxy-3-methoxycinnamic acid) from its bound constituents in the plant cell wall. Ferulic acid esterases have evolved to attack ester linkages between hydrocinnamic acids and carbohydrates or lignin in the plant cell wall. Interest in FAEs as key enzymes for the extraction of phenolic acids from agricultural crops is increasing as ferulic acid is one of the two major aromatic acid building blocks in lignocellulosic materials [1].

Following the first report on a FAE from *Streptomyces olivochromogenes* [2], FAE was recognized as a general component of a hemicellulolytic enzyme system in various microorganisms [3,4,5,6] where they act synergistically with other carbohydrate-degrading enzymes for the complete degradation of lignocelluloses [7,8,9]. Commercial interest in FAEs lies in its potential application for the extraction of phenolic acids from agro-industrial materials to be used as antioxidants and the production of useful industrial compounds such as vanillin [10,11,12]. It is also applicable in the pulp and paper industry for removing substitutions and linkages between polymers to solubilise lignin-carbohydrate complexes [13,14,15], or for catalysing the synthesis of sugar-phenolic esters [16,17]. This broad application range requires the production of FAEs that can operate under varying working conditions, with pH, temperature and stability profiles that are compatible with those of other hemicellulases. The successful application of FAEs also requires production of the pure enzyme in high volumes with high specific activity, for which the potential can be evaluated in bioreactors [18].

A genomic copy of the *A. tubingensis faeA* gene was previously cloned and expressed in *A. niger* D15#26 under control of the *A. niger gpdA* promoter [19]. The *A. niger* D15 [*AtfaeA*] strain displayed more than 5-fold higher FAE activity (5.89 U/mL) than the native *A. tubingensis* and *A. niger* D15[pGTP] strains, and more than 2-fold higher (13.5 U/mL) than the wild type *A. tubingensis* in the presence of 10 g/L maize bran. This emphasised the value of *A. niger* D15#26 as host strain, previously also used for expression of various recombinant genes, including the *A. niger* *β*-1,4-endoglucanase [20], *Trichoderma reseei* β-

1,4-xylanase and *T. reseei* endo-1,4-glucanase [21]. However, in the latter cases, the expression levels were less than 50% higher in the recombinant *A. niger* D15 strain than in the native host.

In this study, the *A. niger* D15[*AtfaeA*] was cultured in shake-flasks with maize bran as sole carbon source, followed by purification and characterisation of the recombinant *At*FAEA. The strain was subsequently evaluated in 2-litre bioreactors using either the batch or fed-batch mode.

## 2. Materials and Methods

### *2.1 Strain and culture conditions*

The *A. niger* D15#26 host and recombinant *A. niger* D15[*AtfaeA*] strains [19] were cultivated in minimal medium (5 g/L yeast extract, 0.4 g/L $MgSO_4 \cdot 7H_2O$, 2 g/L casamino acids, 20 mL 50 x AspA (300 g/L $NaNO_3$, 26 g/L KCl, 76 g/L $KH_2PO_4$, pH 6), 1 mL/L 1000 x trace elements [21] and 10g/L maize bran with no additional glucose added. All chemicals were of analytical grade and unless stated otherwise, sourced from Merck (Darmstadt, Germany).

For enzyme purification, shake flasks containing 250 mL growth medium was inoculated with $1x10^6$ spores/mL and incubated on a rotary shaker at 125 rpm at 30°C for 5 days. Cultures were centrifuged at 8 000 rpm (Sorval RC-6 Plus, Thermo Scientific) at 4°C for 15 min and the supernatant used for purification.

### *2.2 Substrate preparation*

Quantification of FAE activity was based on 4NPF (4-nitrophenyl ferulate) as substrate to measure 4-nitrophenol (4NP) released by the enzyme [22]. The synthetic 4NPF substrate was synthesised through dehydrative coupling of ferulic acid and 4NP using dicyclohexylcarbodiimide (DCC) as a catalyst [23]. Ferulic acid (1g, 5.15 mM) was dissolved in 10 mL dry dioxane, with dropwise addition of 4NP (0.716g, 5.15 mM dissolved in dioxane). Equal volumes (1 mL each) of pyridine and triethylamine were stirred for 10 minutes and solid DCC (1.18 g, 5.7 mM) was added in 200 mg aliquots at 3 min intervals under a nitrogen atmosphere at 25$^o$C. Dicyclohexyl urea (DCU) started appearing in the reaction mixture as a precipitate after 15 min. The reaction progress was monitored by silica

thin-layer chromatography (TLC) using 10% v/v methanol in chloroform as solvent; the reaction was considered completed with the disappearance of the reactants (ferulic acid and 4NP). The reaction mixture was filtered to remove the DCU precipitate and the filtrate was evaporated under reduced pressure.

Column chromatography using silica gel (100-200 mesh size) with 10% v/v methanol in chloroform as solvent was used to purify 4NPF [24]. Fractions with a Retention Factor ($R_f$) value of 0.72 (new 4NPF product) were pooled, evaporated and quantified by HPLC on a Nucleosil C18 column (5 µM particle size, Supelco, Bellefonte, USA) with 70:30:0.1 methanol:water:trifluoroacetic acid as mobile phase at a flow rate of 1 mL/min at room temperature. The 4NPF product was detected by a Surveyor Plus UV/VIS detector (Thermo Electron Corporation, Elandsfontein, South Africa) at 320 nm. Unless stated otherwise, all chemicals were of analytical grade and were obtained from Sigma-Aldrich, UK.

The 4NPF sample was dissolved in *d*-methanol for $^1$H-NMR analysis with *d*-chloroform at 500 MHz [26] and recorded on a BrukerAvance Full Scan Liquid Chromatography-Electron Spray Mass Spectrophotometry (LC-ESMS, capillary voltage 3 kV, cone voltage 15 V) performed in the negative mode (0 to 1000 mV) where the 4NPF sample (1µl) was injected directly into a stream of 50% (v/v) acetonitrile, 0.1% (v/v) formic acid and analysed with a Waters UPLC at flow rate of 0.2 mL/min.

For the extraction of ferulated oligosaccharides, 10 g of maize bran was hydrolysed with 500 mL of 50 mM trichloro-acetic acid at 100°C for 2 h. Following centrifugation, the supernatant was filtered through a 10 µm cellulose nitrate filter (Sartorius Stedim Biotech, Germany), dried at 40°C under vacuum and redissolved in 200 mL $H_2O$ [25]. Two aliquots of 25 mL of the redissolved extract were injected on a XAD-2 column (Sigma-Aldrich) and eluted with 150 mL $H_2O$, followed by 150 mL 50% (v/v) MeOH-$H_2O$ and 150 mL 100% v/v MeOH. The 50% v/v MeOH eluate was dried at 40°C under vacuum, redissolved in 6 mL of a 25% v/v MeOH-$H_2O$ mixture and separated on a Sephadex LH-20 column (71 x 2.6 cm; 377 mL) (Sigma-Aldrich). Fractions eluted with 25% v/v MeOH at 40 mL/h were separated on a thin-layer chromatographic (TLC) plate (25 Aluminium sheet silica gel 60 F254, Merck) using 50:25:25 1-butanol:acetic acid:$H_2O$ as solvent. The different phenolic compounds were visualized using a Transilluminator-26 (Ultraviolet Products Inc., San Gabriel, CA, USA) [26,27] and samples that contained a single spot of FAXX (*O*-{5-*O*-((*E*)-feruloyl)-*α*-L-arabinofuranosyl}-(1,3)-*O*-β-D-xylopyranosyl-(1,4)-D-xylopyranose) with an $R_f$ of 0.84,were dried with a rotary evaporator (Büchi Rotavapor, Switzerland).

### *2.3 Purification and characterisation of AtFAEA*

The recombinant *At*FAEA was purified with anion-exchange chromatography using an ÄKTAPrime system and PrimeView 1.0 software (GE Healthcare Life Sciences, Uppsala, Sweden), followed by separation on a DEAE-Sepharose column (GE Healthcare Life Sciences) pre-equilibrated with 50 mM sodium phosphate buffer (pH 6.5). After washing with five column volumes of 50 mM sodium phosphate buffer (pH 6.5), the bound *At*FAEA was eluted with 10 column volumes of a 0 to 500 mM NaCl gradient in 50 mM sodium phosphate buffer (pH 6.5). The eluted fractions were heated for 3 minutes at 100°C in the presence of Protein Dye (Sigma-Aldrich) and separated on a 12% w/v SDS-polyacrylamide and 5% w/v stacking gel [47], using 5 µl peqGOLD as protein marker (Sigma-Aldrich). Electrophoresis was carried out at 100 V at ambient temperature and the proteins visualized following overnight staining and destaining with PAGE blue dye (Sigma-Aldrich). Protein sequencing and identification were performed on bands provisionally identified as *At*FAEA by the Centre for Proteomic and Genomic Research (Cape Town, South Africa).

For enzyme activity assays, the 4NPF substrate solution was prepared by vortexing 0.1 M potassium phosphate buffer (pH 6.5) containing 2.5% v/v Triton X-100 and 11 mM 4NPF in DMSO in a 9:1 ration. Standard FAE assays [29] were performed with 0.1 mL enzyme and 2 mL 4NPF substrate solution incubated at 37°C for 1 h, with the release of 4NP measured spectrophotomerically at 410 nm (xMark Microplate Spectrophotometer, Bio-Rad, San Francisco, USA). Due to instability, fresh 4NPF solution was prepared, but the DMSO solution was prepared 24 h in advance and stored at room temperature. One unit (1 U) of FAE activity was defined as the amount of enzyme that released 1 µmole 4NP from 4NPF in 1 min [24]. Total protein concentrations were quantified with the Qubit assay and Qubit2.0 fluorometer (Invitrogen, Carlsbad, USA) as per the supplier's specification.

The standard FAE assay conditions with 4NPF as substrate were used to quantify enzyme activity under different pH conditions, using either 100 mM citrate-phosphate buffer (pH 3 to 7) or 100 mM Tris-HCl (pH 7 to 9). For pH stability, the recombinant *At*FAEA was incubated in the respective buffers at 4°C and the residual activity measured after 24 h. For the temperature optimum, FAE activity was determined at 30°C to 70°C in 0.1 M potassium phosphate buffer (pH 6), whilst temperature stability was determined at 30°C to 70°C for 1 h in 0.1 M potassium phosphate buffer (pH 6, without substrate).

Substrate specificity was determined with 1.7 µg purified *At*FAEA in the presence of either 2 mg/mL hydroxycinnamic acid (methyl ferulate [MFA], methyl p-coumarate [M*p*CA], methyl sinapate [MSA] or methyl caffeate [MCA]) or FAXX prepared in 0.1 M potassium phosphate buffer, pH 6. The reaction was incubated at 37°C for 1 h and terminated by the addition of 500 µl methanol and 400 µl distilled water. Kinetic parameters ($K_m$, $K_{cat}$ and $K_{cat}/K_m$) for MFA were calculated from initial rate data determined for 50 mU *At*FAEA at substrate concentrations ranging from 0.2 to 0.8 mM at 50°C, pH 6 for 15 min. The corresponding hydroxycinnamic acids, i.e. ferulic acid (FA), sinapic acid (SA), coumaric acid (*p*CA) and caffeic acid (CA), were quantified by HPLC with a Nucleosil C18 column with a 5 µM particle size (Supelco, Bellefonte, USA) at a flow rate of 1 mL/min at room temperature with 70% v/v acetonitrile as mobile phase. Detection was done by a Surveyor Plus UV/VIS detector (Thermo Electron Corporation, Elandsfontein, South Africa) at 320 nm [30].

### *2.4 Batch and fed-batch fermentations*

The recombinant *A. niger* D15[*AtfaeA*] strain was cultivated on minimal agar medium (2 g/L neopeptone, 18 g/L agar, 1 g/L yeast extract, 0.4 g/L $MgSO_4.7H_2O$, 10 g/L glucose, 2 g/L casamino acids, 20 mL 50 x AspA [300 g/L $NaNO_3$, 26 g/L KCl, 76 g/L $KH_2PO_4$, pH 6], 1 mL of 1000x trace elements) at 30 °C for 120 h and harvested with physiological saline solution (NaCl, 0.9% w/v). All chemicals were of analytical grade and sourced from Merck (Darmstadt, Germany), unless stated otherwise.

The batch and fed-batch fermentations were carried out in a 2-litre bench-top bioreactor (Inforsag, Switzerland) with a working volume of 1.5 litres. Approximately $10^8$ spores/mL were inoculated in 500 mL shake flasks with 100 mL inoculum medium (10 g/L $NaNO_3$, 0.8 g/L $MgSO_4.7H_2O$, 2 g/L $KH_2PO_4$, 0.1 g/L $CaCl_2.2H_2O$, 5 g/L yeast extract, 5 g/L tryptone, 10% v/v antifoam (Struktol J673) and 1 mL/L trace elements [21]. The inoculum flasks were cultivated overnight at 30 °C at 125 rpm on a rotary shaker. Duplicate batches of both the batch and fed-batch fermentations were inoculated with 100 mL of the *A. niger* D15[*AtfaeA*] pre-culture minimal medium. The fermentation temperature was controlled at 30 °C (±0.1 °C), aeration at 0.8 volume per volume per minute (vvm) and pH at 6 with 4 M KOH and 2 M $H_2SO_4$. The dissolved oxygen was maintained above 30% saturation by adjusting the agitation between 400 and 800 rpm. An initial glucose concentration of 30 g/L and 10 g/L

glucose was used for batch and fed-batch fermentations, respectively, while the glucose feed for fed-batch fermentation was 50 g glucose in 250 mL water, initiated after depletion of the initial glucose charge and fed over 7 h.

Samples from batch fermentations were taken every 12 h and centrifuged at 15 000 rpm (Sorval RC-6 Plus, Thermo Scientific) for 5 min and the supernatant stored at -80°C for quantification of enzyme activity, extracellular protein and residual glucose. Ferulic acid esterase assays were done as described above. Total biomass was based on 10 mL samples taken at the end of the fermentation, vacuum-filtered using pre-weighed filters (Whatman no. 1) and dried to constant weight at 105 °C for 24 to 48 h. Total protein content was measured by the Bradford method (BioRad Protein Assay, Hempstead, UK). Glucose concentration was quantified using HPLC (CarboPac™ PA1 column, Dionex, MA, USA) and Accutrend glucose strips (Roche Diagnostics, East Sussex, UK).

## 3. Results

### *3.1 Synthesis of 4NPF and FAXX*

Thin-layer chromatography of the products from the dehydrative coupling of ferulic acid and 4-nitrophenol showed $R_f$ values of 0.39, 0.56 and 0.72 for ferulic acid, 4NP and 4NPF, respectively (data not shown). The size of the new 4NPF product ($R_f$ = 0.72) was confirmed as 314 kDa by LC-ESMS. The $^1$H NMR profile showed peaks at 3.93, 6.45, 6.97, 7.07, 7.12, 7.28 (corresponding to4NPF), 7.40, 7.88 (ferulic acid) and 8.28 (4NP). The absence of a peak at 9.56 (representing the carboxyl-proton of ferulic acid) confirmed coupling of ferulic acid and 4NP.

After extraction of FAXX from maize bran, the 25% v/v methanol fractions showed two spots with different $R_f$ values with TLC analysis (results not shown), one corresponding to the ferulic acid standard and a new product (FAXX) with an $R_f$ value of 0.84. Following drying, 7.7 mg FAXX was recovered.

### *3.2 Purification and characterization of recombinant AtFAEA*

Two overlapping peaks were observed for the *At*FAEA fractions eluted from the DEAE-Sepharose column (data not shown). The first peak (fractions 18 to 22) displayed a single band of 36 kDa on SDS-PAGE (Fig. 1) with 6.5 U/mL FAE activity, whereas no activity or

protein was observed for the second peak (fractions 23 to 32). Anion exchange chromatography enabled a 48.2% recovery of FAE activity (Table 1). Peptide mass fingerprinting (PMF) confirmed the protein as the *A. tubingensis* FAEA with a protein score of 146 and a 100% confidence interval (data not shown).

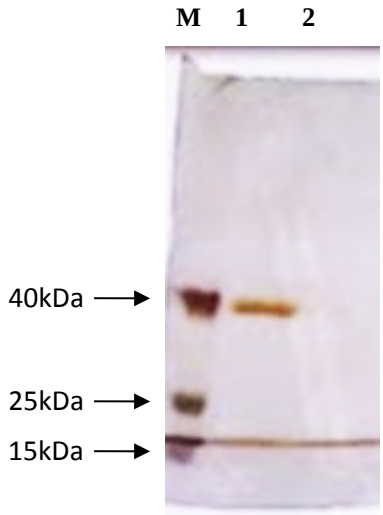


**Fig. 1.** SDS-PAGE analysis of extracellular *At*FAEA fractions 18-22 (Lane 1) and 23-32 (Lane 2) following DEAE-Sepharose fractionation. Lane M, peqGOLD protein marker.

**Table 1**

Purification of ferulic acid esterase from *A. niger* D15[*AtfaeA*].

| Purification step | Total FAE activity (U)[a] | Specific activity (U/mg) | Purification | Recovery (%) |
|---|---|---|---|---|
| Culture filtrate | 675.0 ± 0.5 | 11.10 ± 0.7 | 1-fold | 100.0 |
| Anion exchange | 325.0 ± 0.8 | 77.6 ± 0.3 | 7-fold | 48.2 |

[a]Determined with 4NPF as substrate

With 0.2 to 0.8 mM MFA as substrate, $K_m$, $K_{cat}$, $K_{cat}/K_m$ values for the purified *At*FAEA were 0.43 mM, 0.48 $min^{-1}$and 1.1 $min^{-1}$ $mM^{-1}$, respectively (Table 2). In a similar study by De Vries et al. [29], expression of the *A. tubingensis fae*A in *A. niger* NW154 (*prt*F mutant) yielded a $K_m$ of 0.76 mM, almost two-fold higher than in this study. The purified *At*FAEA

displayed varying affinities for the different substrates, with 9937 U/mg recorded for FAXX extracted from maize bran (Table 3) and 3 487 U/mg for the synthetic MFA substrate.

**Table 2**

Substrate specificities of FAEA from different fungal sources against MFA.

| FAEA source | Host | $K_m$ (mM) | $K_{cat}$ ($min^{-1}$) | $K_{cat}/K_m$ ($min^{-1}$ $mM^{-1}$) | Reference |
|---|---|---|---|---|---|
| *A. tubingensis* | *A. niger* D15 | 0.43 ± 0.11 | 0.48 ± 0.05 | 1.10 ± 0.21 | This study |
| *A. niger* | *P. pastoris* | 0.78 ± 0.05 | ND [a] | ND | [62] |
| *A. tubingensis* | *A. niger (NW154;; pIM3208.5)* | 0.76 | ND | ND | [29] |

[a]Not determined

**Table 3**

Comparison of substrate specificity for FAEA from different fungal strains.

| Substrate | | Specific activities (U/mg) | | |
|---|---|---|---|---|
| | | *A. niger* D15[*AtfaeA*] | *T. stipitatus* [55] | *A. niger* [55] |
| Synthetic | MFA[a] | 3 487 ± 0.5 | 4.3 | ND |
| | M*p*CA | 446 ± 0.1 | 1347 | 1560 |
| | MSA | 432 ± 1.5 | 17 | 1 |
| | MCA | 0 | 8434 | 672 |
| Natural | FAXX | 9 837 ± 5.3 | 3020 | 96 |

[a]MFA, methyl ferulate; M*p*CA, methyl *p*-coumarate; MSA, methyl sinapate; MCA, methyl caffeate; FAXX, *O*-{5-*O*-((*E*)-feruloyl)-*α*-L-arabinofuranosyl}-(1,3)-*O*-β-D-xylopyranosyl-(1,4)-D-xylopyranose. ND (not determined)

The *At*FAEA protein displayed optimum activity at pH 5 to 6 (Fig. 2A) and was most stable at pH 5 to 7 (retaining 100% activity after 24 h), with only 80% activity retained at pH 3 and 4. The highest *At*FAEA activity was observed at 50°C (Fig. 2B), with 100% activity

retained after 1 h between 30°C and 50°C. The *At*FAEA was heat stable up to 60°C, but lost 84% of its activity at 70°C.

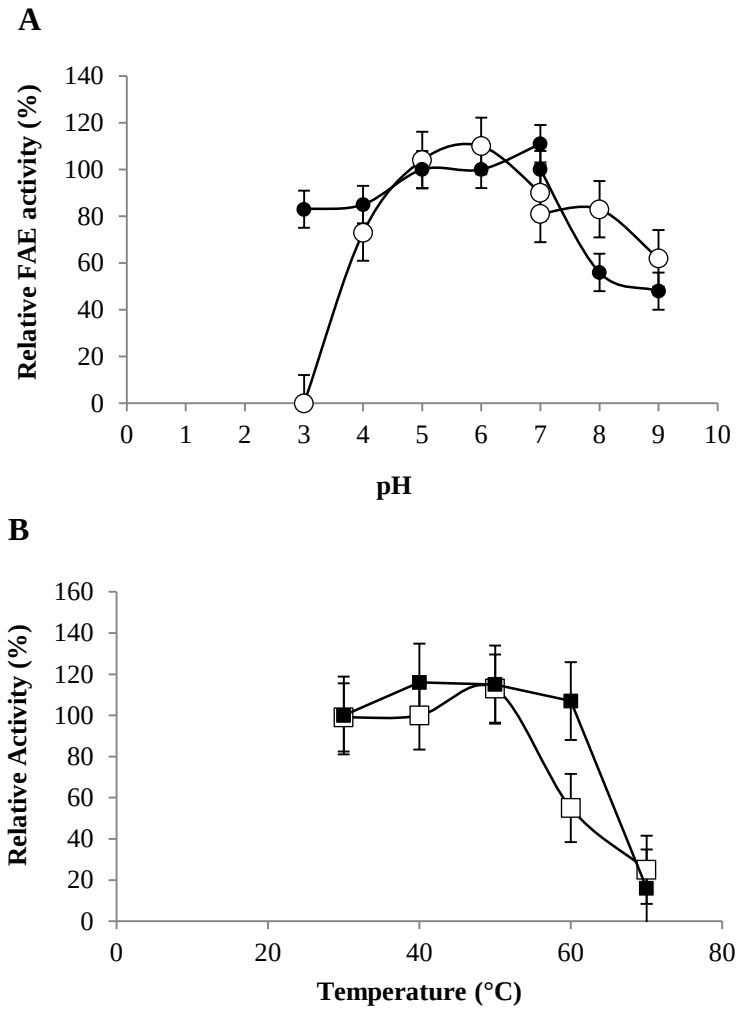


**Fig. 2.** Effect of pH and temperature on activity and stability of the *At*FAEA expressed in *A. niger* D15. (A) Activity at different pH values (○) and residual activity after 24 h (●). (B) Activity of *At*FAEA at different temperatures (□) and residual activity after 1 h (■). Error bars indicate standard deviations for duplicate measurements.

### *3.3 Batch and fed-batch fermentations*

To increase the levels of FAEA production, the recombinant *A. niger* D15[*AtfaeA*] strain was grown in minimal media using batch or fed-batch fermentations, with protein concentration, enzyme activity and glucose consumption monitored. The depletion of the

initial 30 g/L glucose in the batch fermentation (Fig. 3A) coincided with an increase in dissolved oxygen (data not shown).

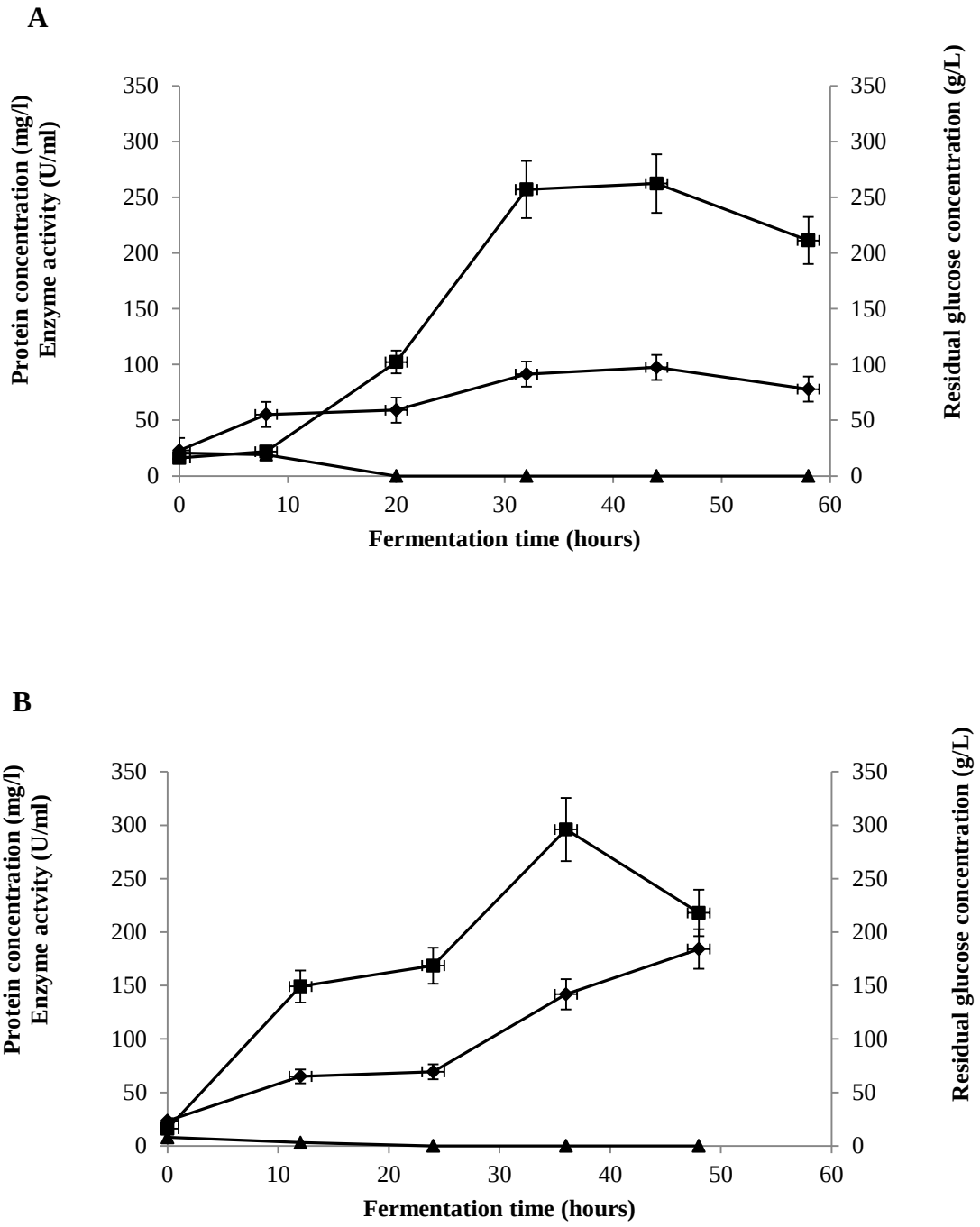


**Fig. 3.** Time course for the production of FAEA from *A. niger* D15[*AtfaeA*] using a 2-litre bioreactor under (A) batch and (B) fed-batch conditions. (◆) FAE activity (U/mL); (■) protein concentration (mg/L) and (▲) residual glucose concentration (g/L). Error bars indicate standard deviations for duplicate measurements.

After 48 hrs, FAE activity peaked at 97.4 U/mL (262 mg/L total proteins) and the biomass increased by 20% (8.68 g/L) with a volumetric productivity of 2.96 U/mL/h (Table 4). The specific activity on 4NPF was 578 U/mg with a yield of 0.29 g FAEA/$g_{glucose}$.

**Table 4**

Comparison of *A. niger* D15[*AtfaeA*] performance in batch and fed-batch fermentations.

| | Batch fermentation | Fed-batch fermentation |
|---|---|---|
| Biomass (g/L) | 8.68 ± 0.30 | 10.57 ± 0.50 |
| Specific activity (U/mg) | 578.36 ± 0.14 | 546.23 ± 0.18 |
| Volumetric productivity (U/mL/h) | 2.96 ± 0.25 | 3.95 ± 0.01 |
| Yield (g FAE/$g_{glucose}$) | 0.29 ± 0.13 | 0.18 ± 0.01 |
| Volumetric activity (U/mL) | 97.4 ± 0.11 | 184.2 ± 0.09 |

Similar to the batch fermentation, the depletion of the initial 10 g/L glucose in the fed-batch fermentation was indicated by a rise in dissolved oxygen (data not shown), in which case a dissolved oxygen-controlled glucose feeding profile was initiated. The initial rapid consumption of glucose was again mirrored by an increase in protein and enzyme production (Fig. 3B). After 20 hours of cultivation, the FAE activity (546 U/mg) in the fed-batch fermentation was similar than for the batch fermentation, but increased almost 2-fold (184.2 U/mL, 296 mg/L total protein) after 48 hrs. The fed-batch mode yielded 10.57 g/L biomass, a volumetric productivity of 3.95 U/mL/h and 0.18 g FAEA/$g_{glucose}$ (Table 4).

## 4. Discussion

Ferulic acid esterases have been reported in a variety of microorganisms, most of which are filamentous fungi [13] with varying molecular weights, DNA sequence and substrate specificity [31,32]. The current study describes the purification and characterization of the *A. tubingensis* FAEA expressed in *A. niger* D15#26 to evaluate its potential for increased production of *At*FAEA for biotechnological applications.

A number of natural and synthetic substrates have been used for the characterization of FAEs, including FAXX [3], phenolic acid methyl esters [4], 4-nitrophenyl-5-*O*-*trans*-feruloyl-*α*-L-arabinofuranoside (NPh-5-Fe-Ara*f*) and 4-nitrophenyl-2-*O*-*trans*-feruloyl-*α*-L-arabinofuranoside (NPh-2-Fe-Ara*f*) [8]. More recently, chromogenic and fluorogenic compounds have been synthesized for the spectrophotometric detection of hydrolytic enzyme activities [14], including 4NPF as substrate for the quantification of 4NP released by ferulic acid esterase activity [22]. As the 4NPF and FAXX substrates were not commercially available, this study required the *de novo* synthesis of 4NPF through the dehydrative coupling of ferulic acid and 4-nitrophenol, whereas FAXX was extracted from maize bran.

Different chromatographic techniques such as anion exchange, gel filtration and hydrophobic interaction, have previously been used for the purification of ferulic acid esterases [4,33,34]. In this study, *At*FAEA was 7-fold purified in a single step with anion exchange chromatography, with *At*FAEA representing 48% of the total protein in the supernatant (Table 1). The recombinant *At*FAEA had a molecular weight of 36 kDa under denaturing conditions, which is similar to the FAEA reported for *Sporotrichum thermophile, A. niger* and *Talaromyces stipitatus* [31]. The optimum FAE activity at pH 6 was comparable to that of *A. niger* [35,36], *Aspergillus oryzae* [29], *Neocallimastix* [37], *Neurospora crassa* [38,39], *Penicillium brevicompactum* [40] and *T. stipitatus* [41,42]. Stability of *At*FAEA at pH 3 to 7 (retaining more than 80% of its activity) resembles the higher stability in acidic conditions reported for the FAE from *Aspergillus awamori* G-2 [41] and *A. oryzae* [43].

The *At*FAEA displayed maximum enzyme activity at 50°C (Fig. 2), which is in agreement with the temperature optima of 35°C to 55°C reported for most fungal ferulic acid esterases, including that of *A. niger* [35,36], *Piromyces equi* [44] and *S. thermophile* [42]. The *At*FAEA was stable at 30°C to 60°C, but rapidly lost activity above 60°C. Enzyme stability at 60°C was also reported for FAEs from *Aureobasidium pullulans* [45], *P. equi* [46], *Cellvibrio japonicas* [46] and *T. stipitatus* [47], whereas the native *A. niger* FAE tolerated 80°C [48].

Different types of ferulic acid esterases are classified based on their substrate specificity for methyl esters (MFA, MSA, MCA, M*p*CA) and oligosaccharides (e.g. FAXX). Type AFAE typically has a preference for substrates with a methoxy substitution in the meta position (C3 and/or C5) of the aromatic ring (e.g. MSA and MFA) and substrates with a hydroxyl group at the C4 position of the aromatic ring (e.g. M*p*CA), such as those reported for *S. thermophile* [49,50], *A. awamori* [51], *F. oxysporum* [4,14], *A. niger* [52,53] and *T. stipitatus* [47]. The purified recombinant *At*FAEA exhibited a high affinity for MFA (3487

U/mg) and a hydrolysis profile of MFA>M*p*CA>MSA (Table 3), which is in agreement with previous results [19]. Since MCA has no methoxy or hydroxyl substitutions [52], it was a poor substrate for the *At*FAEA. Borneman et al. [37] also reported the highest specific activity on FAXX for a *A. niger* FAEA, whereas MCA was preferred by *T. stipitatus* FAEA. Except for MSA, the substrate affinities of the recombinant *At*FAEA were in general higher than reported for *T. stipitatus* [54,55] and *A. niger* [55].

Natural feruloylated oligosaccharides have been used in various studies to characterize ferulic acid esterases [3,56,57,58,59,60]. As shown in Table 3, the *At*FAEA displayed a 3-fold and 10-fold higher specific activity on maize bran FAXX than reported for *T. stipitatus* and *A. niger*, respectively [37]. The *At*FAEA also displayed a higher affinity for FAXX compared to the synthetic methyl ester substrates. Since FAXX is a feruloylated oligosaccharide, this indicates that the sugar moiety influence the substrate affinity, which in turn suggests the possible presence of sugar binding sites on the *At*FAEA [32]. With MFA as substrate (Table 2), the purified *At*FAEA displayed a $K_m$ of 0.43 mM on MFA, compared to0.78 mM reported for a recombinant *A. niger* FAEA expressed in *P. pastoris* [62]. De Vries et al. [29] reported a higher $K_m$ (0.76 mM) for expression of the *A. tubingensis* FAEA in *A. niger*, but this could be ascribed to the different donor and host strains that were used. The lower $K_m$ (0.43 mM) and higher $K_{cat}$ (0.48 /min) values obtained in this study, suggests that the *A. niger* D15#26 strain represents a better host for over-expression of *At*FAEA.

The recombinant *A. niger* D15[*AtfaeA*] strain produced an extracellular FAEA with 7- and 11-fold higher enzyme activity in batch and fed-batch bioreactors, respectively, relative to shake flasks (Table 4). The higher enzyme activity and protein production in the bioreactor systems can be attributed to better mixing and oxygen transfer, resulting in increased biomass and therefore an increase in enzyme activity and protein production. The fed-batch fermentation was superior as it produced 2-fold higher FAEA activity than the batch fermentation, coinciding with only a 15% increase in protein production (Fig. 3). There was 1.2-fold more biomass produced in the fed-batch (10.57 g/L) compared to the batch fermentation (8.68 g/L), with biomass production limited in the latter due to less glucose being available [63].

Limited work has been reported on the expression of heterologous feruloyl esterases in bioreactor systems. Huang et al. [64] reported expression of a thermostable esterase from *Thermobifida fusca* NTU22 in *Yarrowia lipolytica* P01g that yielded total esterase activity of 41 U/mL, which is much less than the FAE activity 184 U/mL reported in this study. Our

results also showed that upscaling from shake flasks to bioreactor improved both the cell growth and enzyme production by the recombinant *A. niger* D15[*AtfaeA*] strain. The *A. niger* D15[*AtfaeA*] strain allowed for the overproduction of ferulic acid esterase, i.e. 546 U/mg (Table 4), as opposed to 11.1 U/mg in shake flasks.

The benefits of upscaling to a bioreactor system are also evident when looking at FAEA enzymes that have been expressed in *A. niger* D15 and other strains (*A. tubingensis* NW241, *A. niger* NW154, *P. pastoris*, *T. reseei* and *S. cerevisiae*) cultured in shake flask conditions (Table 5). Enzyme activities that were reported ranged from 0.97 U/ml to 3.16 U/ml with protein concentrations ranging from 2 mg/L to 300 mg/L. The reported activities were were much lower than the 184 U/mL observed in this study in a bioreactor system while the protein production (260.6 ± 0.60 mg/L) was comparable to the 300 mg/L observed when using the *P. pastoris* recombinant, emphasising the need to upscale enzyme production processes for better enzyme productivity.

**Table 5**

Enzyme activities for a selection of recombinant strains and FAEA proteins.

| Enzyme | Activity/ Protein | Recombinant host | Reference |
|---|---|---|---|
| *A. tubingensis* FAEA | 184 U/mL | *A. niger* D15#26 | This study |
| *A. tubingensis* FAEA | 260.6 ± 0.60 mg/L | *A. niger* D15#26 | This study |
| *A. niger* FAEA | 3.16 U/mL | *A. tubingensis* NW241 | [29] |
| *A. tubingensis* FAEA | 0.97 U/mL | *A. niger* NW154 | [29] |
| Cinnamoyl esterase (FAEA) | 300 mg/L | *Pichia pastoris* | [62] |
| *Aspergillus awamori* FAEA | 30 mg/L | | [66] |
| Catalytic domain EstA | 33 mg/L | *T. reesei* | [65] |
| *A. niger* FAEA | 2 mg/L | *S. cerevisiae* | [67] |

## 5. Conclusions

Ferulic acid - known for its antioxidant properties due to radical scavenging - is generally found esterified to arabinose in various plant polysaccharides, e.g. arabinoxylans and pectins. Ferulic acid esterases, responsible for the release of ferulic acid from lignocellulosics, together with other lignocellulases play a significant role in the complete hydrolysis of plant xylans. Although FAE is of importance to the animal feed, pulp and paper and bioethanol industry, commercial preparations of FAE are expensive and render the production of ferulic acid via enzymatic treatment of plant biomass uneconomical.

The high expression level of the *A. niger* D15[*AtfaeA*] strain enabled the production of the recombinant *A. tubingensis* FAEA against a limited background of other secreted proteins, and allowed for a single step 7-fold purification with anion exchange chromatography. Characterisation of the recombinant *At*FAEA confirmed its potential for industrial application: the relative stability at pH 3 to 7 poses an advantage for applications in the food industry, whilst its stability at 50°C suggests potential application in the pulp and paper industry's bleaching process. Based on the hydrolysis profile of the methyl esters (MFA>MpCA>MSA), the recombinant *At*FAEA only has affinity for the C-5 feruloylated substrates and would thus be best at releasing ferulic acid from monocots (wheat, oats, maize, barley, etc.) where the ferulic acid is esterified to the C-5 hydroxyl group of the arabinopyranose residues.

In the current study, we achieve the highest FAEA activity per volume ever reported. Fed-batch fermentation proved to be superior for the upscaled production of *At*FAEA as it produced 2-fold higher FAEA activity than the batch fermentation, with a higher volumetric activity and biomass yield, accompanied by a specific activity 546 U/mg, as opposed to 11.1 U/mg in the culture filtrate (Table 1).

## 6. Acknowledgements

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## 7. Declaration

ENZ performed the experimental work, analysed the data and drafted the manuscript. KR and PVZ supervised experimental work and data analyses and assisted with drafting of the manuscript. SHR, WHVZ and MVB participated in the design of the study and revision of the manuscript. All authors read and approved the final manuscript.

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# CHAPTER 4

## Recombinant ferulic acid esterase increases ferulic acid extraction from plant material

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## Abstract

Plant material contains phenolic compounds with antioxidant characteristics, but its association with the lignocellulosic structure limits its bioavailability. A recombinant *Aspergillus tubingensis* ferulic acid esterase Type A (FAEA) expressed in *Aspergillus niger* D15#26 was evaluated for its ability to improve the release of phenolic compounds from different plant substrates. The *A. niger* D15[*AtfaeA*] cocktail extracted 531 mg/l and 177 mg/l ferulic acid from maize bran and triticale bran, respectively, as well as 77 mg/l *p*-coumaric acid from triticale bran. Treatment with FAEA can thus increase the ferulic acid content and nutritional value of maize and triticale bran routinely used as animal feed. The cocktail was also able to extract 0.2 g ferulic acid /100 g hot water soluble solids from *Aspalathus linearis* (rooibos) plant material (leaves and stems), indicating the potential value of *At*FAEA for the treatment of a variety of plant materials for the release of valuable phytophenolics.

**Keywords**: Ferulic acid esterase, *Aspergillus niger*, *Aspergillus tubingensis*, triticale, maize, rooibos

# 1. Introduction

The growing global food industry implies also an increase in agricultural by-products that requires the development of new technologies to add value and create new market opportunities for the agricultural sector. The use of microbial enzymes for the fractionation of lignocellulose is the most natural process to develop new products from agricultural by-products. In the food industry, ferulic acid is, among others, used as a precursor for the production of vanillin, a flavouring agent. Ferulic acid is the most abundant hydroxycinnamic acid in plant cell walls (Priefert et al. 2001; Mathew and Abram 2004; Topakas et al. 2004) and exhibits better inhibition of lipid and protein oxidation compared to other naturally occurring phenolic antioxidants such as gallic acid, caffeic acid, malvidin and epicatechin, among others (Graf 1992; Heinonen et al. 1998). Due to its low toxicity, ferulic acid has been approved for use as an antioxidant additive in cosmetics, foods and beverage industry (Graf 1992).

Ferulic acid esterases (FAEs; EC 3.1.1.73), also known as feruloyl esterases and cinnamic acid or cinnamoyl esterases, are able to release ferulic acid and other cinnamic acids from plant cells. The first report on a FAE was from *Streptomyces olivochromogenes* (McKenzie et al. 1987) and since then, a variety of FAE have been isolated and characterised, with varying enzyme activities and specificities reported (reviewed in Chapter 1). Due to their ability to release bound cinnamic acids from complex polysaccharides, ferulic acid esterases can potentially be applied to cleave the linkages between ferulic acid and hemicellulose, generally regarded as one of the hurdles in cell wall hydrolysis (Benoit et al. 2008). The possibility to degrade plant biomass with a combination of FAEs and other hemicellulases presents an opportunity to extract novel bioactive compounds - such as ferulic acid - for use by industry and/or to synthesize other industrially significant compounds (Fazary and Ju 2008; Garcia-Conesa et al. 2004).The potential applications of FAEs have led to interest from the chemical, fuel, animal feed, textile and laundry, pulp and paper, food and agriculture and pharmaceutical industries.

In the farming industry, the dimeric and trimeric forms of ferulic acid that are either esterified or etherified to plant residues have been reported to inhibit digestion of these residues in ruminants (Yu et al. 2005). However, the addition of a multi-enzyme cocktail that included purified *Aspergillus* FAE (13 mU FAE, 4 096 U xylanase, 1 024 U cellulase, 256 U endoglucanase I and II and 64 U β-glucanase), increased the cell wall digestibility of oat hulls

by 86%. The accumulation of agro-industrial waste from the milling, brewing and sugar industries has made it important to find better ways of using this waste, especially given the costs incurred during the treatment and disposal of the waste, which is mostly used as animal feed (Varga and Kolver 1997).

In maize bran, the phenolic acids, representing about 4-5% of the cell wall on a mass basis, are mostly ferulic acid and lower concentrations of *p*-coumaric acid and other phenolic acids esterified to the cell wall polymers (Lapierre et al. 2001). Triticale, a hybrid of wheat and rye, also contains a variety of cinnamic acids, including ferulic acid and *p*-coumaric acid. The phenolic content of maize bran and triticale bran has made these by-products a good potential source of antioxidants that may add some health benefits (Hosseinian and Mazza 2009).

Another primary source of antioxidants is *Aspalathus linearis*, an endemic South African fynbos plant, whose leaves and stems are used for the production of rooibos tea, a caffeine-free herbal drink and nutraceutical supplement with a high antioxidant and low tannin content (Joubert and de Beer 2011; Joubert et al. 2004; Von Gadow et al. 1997). The therapeutic potential of several of its phenolic compounds as hypoglycaemic agents, including aspalathin (Kawano et al. 2009; Muller et al 2012; Son et al 2013), its flavone analogue isoorientin (Andrade-Cetto and Wiedenfeld 2001; Mazibuko 2014) and the phenylpropenoic acid derivative, enolicphenylpyruvic acid-2-*O*-glucoside (PPAG) (Muller et al. 2013; Mathijs et al. 2014), has received particular attention in recent years. However, “fermentation” of the plant material, an open-air oxidation process to develop the characteristic sweet flavour and red-brown colour of rooibos tea, substantially decreases the levels of aspalathin and nothofagin compared to the green, unfermented tea (Joubert 1996). Pre-treatment of rooibos plant material with a combination of cellulase, ferulic acid esterase and/or pectinase increased the soluble solid yield, whereas ferulic acid esterase and β-glucanase/β-xylanase increased the total polyphenol yield (Coetzee et al. 2013).

Given the potential commercial importance of plant-derived ferulic acid and antioxidants, it is imperative to develop enzymatic treatment processes that are both efficient and cost-effective, particularly given the high cost of enzyme production. Fungal enzyme cocktails containing various celluloses and hemicellulases can cleave the chemical bonds between phytochemicals and the cellulosic backbone of plant material and thus release additional soluble matter and/or antioxidants, depending on the nature of the enzymes. In Chapter 2, we reported the construction of a recombinant *Aspergillus niger* D15[*AtfaeA*] strain over-

expressing the *Aspergillus tubingensis* FAE Type A enzyme (Zwane et al. 2014). In the current study, the crude *A. niger* D15[*AtfaeA*] enzyme cocktail was evaluated for the release of phytochemicals from triticale and maize bran, as well as green (unfermented) and fermented rooibos.

# 2. Materials and Methods

## 2.1 Plant material

Triticale (*X Triticosecale* Wittmack ex A. Camus cultivar US2007) bran was provided by the Department Process Engineering (Stellenbosch University, South Africa), while commercial maize bran was donated by Sasko (Pioneer Foods, Paarl, South Africa). Dried green and fermented rooibos (*A. linearis*) were obtained from Rooibos Ltd. (Clanwilliam, South Africa).

## 2.2 Strain and culture conditions

The *A. niger* D15#26 and recombinant *A. niger* D15[*AtfaeA*] strains over-expressing the *A. tubingensis faeA* were cultivated in minimal medium (5 g/l yeast extract, 0.4 g/l $MgSO_4 \cdot 7H_2O$, 2 g/l casamino acids, 20 ml 50 x AspA (300 g/l $NaNO_3$, 26 g/l KCl, 76 g/l $KH_2PO_4$, pH 6), 1 ml/l 1000 x trace elements [Rose and van Zyl 2002] and 10 g/l maize bran) without glucose. The growth media were inoculated with $1x10^6$ spores/ml and incubated on a rotary shaker at 125 rpm at 30°C for 5 days. Following incubation, samples were centrifuged at 8 000 rpm for 15 min at 4°C and the supernatant used as crude enzyme cocktail. All chemicals were of analytical grade and unless stated otherwise, were obtained from Merck (Darmstadt, Germany).

## 2.3 Enzyme treatment of triticale and maize bran

Duplicate batches of 20 mg triticale or maize bran were treated with 0.3 mg/ml of the crude enzyme cocktail from *A. niger* D15#26 or *A. niger* D15[*AtfaeA*] in 50 mM potassium phosphate buffer (pH 6) for 24 hrs at 40°C, while incubated on a rotary platform at 250 rpm (Shin et al. 2006).The control experiments were treated with growth medium alone (i.e. no enzyme cocktail).The supernatant was collected by centrifugation (13 000 g, 10 min) and

analysed for ferulic acid, *p*-coumaric acid and caffeic acid with reverse-phase HPLC using a modification of the method described by Kim et al. (2007).

The crude extracts were filtered through a 0.45 µm polytetrafluoroethylene (PTFE) syringe filter (Sigma Aldrich, UK) prior to injection into the HPLC system, which consisted of a Waters 1525 binary HPLC pump, YMC-Pack ODS AM-303 (250 mm x 4.6 mm internal diameter, 5 µm particle size) column and 2487 dual wavelength absorbance detector (Waters Associates, Milford, MA, USA). The injection volume of 10 µl was run at a flow rate of 0.8 ml/min and monitored at 280 nm. The mobile phase consisted of 0.1% acetic acid in HPLC-grade water (solvent A) and 0.1% acetic acid in acetonitrile (solvent B). The linear gradient of solvent B was increased from 8% to 10% in 2 min, then to 30% in 25 min, followed by an increase to 100% in 1 min, kept at 100% for 4 min, and returned to the initial conditions. The total running time was 38 min and the column temperature was kept at 25˚C during the run. Breeze software (Waters Associates) was used for data acquisition and analysis.

HPLC-grade pure standards of caffeic acid, *p*-coumaric acid and ferulic acid (Sigma Aldrich) were prepared in 0.1% acetic acid in acetonitrile at concentrations of 200, 150, 100, 50, 25 and 0 mg/l. Aliquots of 10 µl were chromatographed individually and as a mixture and regression equations showed a high degree of linearity ($R^2$>0.99) for each phenolic acid. Phenolic acids in the triticale and maize bran extracts were identified by comparing its retention time with that of the standards and the concentrations determined using the regression equations (expressed as mg/l of sample).The total phenol (TP) content and total antioxidant capacity (TAC) of extracts were determined using the Folin-Ciocalteau and DPPH radical scavenging assays (Arthur et al. 2011) and expressed as Trolox equivalents (TE), respectively.

## 2.4 Enzyme treatment of green and fermented rooibos

Duplicate batches of 10 g green or fermented rooibos plant material were treated for 2 hrs at 40˚C with 15 ml of the crude enzyme cocktails from *A. niger* D15[pGTP] and *A. niger* D15[*AtfaeA*]. The control experiments were treated with growth medium alone (i.e. no enzymes). The rooibos material was dried overnight at 40˚C and duplicate infusions were prepared: 2.5 g of leaves infused for 5 min with 200 ml freshly boiled deionised water without stirring, decanted immediately through a tea strainer and filtered warm using Whatman #4 filter paper (Coetzee et al. 2013).

The soluble solids (SS) of each infusion were determined gravimetrically by evaporating and drying 20 ml filtered infusion in duplicate in pre-weighed nickel moisture dishes (Joubert and De Beer 2012). The soluble solids content was expressed as a percentage of the plant material mass and of the infusion volume. The total phenol (TP) content and total antioxidant capacity (TAC) of infusions were determined using the Folin-Ciocalteau and DPPH radical scavenging assays (Arthur et al. 2011), respectively. The results were expressed as gallic acid equivalents (GAE) and Trolox equivalents (TE) per 100 g soluble solids.

The concentration of 13 individual phenolic compounds in the infusions was determined using HPLC conditions as previously described (Beelders et al. 2012). Standard curves for the major compounds were prepared by injecting standard mixtures at different injection volumes. Values reported are the means of duplicate measurements on duplicate treatments expressed as a percentage of the soluble solids.

# 3. Results

## 3.1 Release of phenolic acids from maize and triticale bran

Treatment with the crude *A. niger* D15[pGTP] cocktail reduced the ferulic, *p*-coumaric acid and caffeic acid contents in the maize bran when compared to the control that received no fungal cocktail (Table 1). However, the *A. niger* D15[*AtfaeA*] cocktail increased both the ferulic acid and *p*-coumaric content 10-fold relative to *A. niger* D15[pGTP], but only the ferulic acid was significantly higher (3.5-fold) than the control treatment. Both the *A. niger* D15[pGTP] and *A. niger* D15[*AtfaeA*] cocktails significantly increased the yield of all three phenolic acids from triticale bran, with *A. niger* D15[*AtfaeA*] yielding the best results with a 304-fold increase in *p*-coumaric acid, 54-fold in ferulic acid and 2-fold in caffeic acid content.

The higher extraction efficiency of *A. niger* D15 [*AtfaeA*] was also reflected in the total antioxidant capacity (TAC) and total polyphenol content (TPC) observed after treatment of both maize and triticale bran (Table 1). When compared to the control treatment, the *A. niger* D15[*AtfaeA*] enzyme cocktail increased the TAC and TPC of the maize bran by 32% and 23%, respectively, whereas triticale bran displayed 33% and 15% higher TAC and TPC values, respectively. The *A. niger* D15[pGTP] cocktail slightly raised the TAC of the maize

bran extract, but slightly reduced both the TAC and TPC of the triticale bran relative to the control.

**Table 1.** Selected phenolic acids, Total antioxidant capacity (TAC) and Total polyphenol content (TPC) after treatment of maize and triticale bran with fungal enzyme cocktails

| Treatment | Phenolic acids (mg/l) | | | TAC | TP |
|---|---|---|---|---|---|
| | Ferulic acid | *p*-Coumaric acid | Caffeic acid | (µM Trolox/g bran) | (mg FA/100g bran) |
| **Maize bran** | | | | | |
| Control | 153 ± 0.41 [a] | 20 ± 0.82 | 53 ± 11.2 | 1661 ± 1.51 | 0.67 ± 0.20 |
| *A. niger* D15[pGTP] | 54 ± 0.50<br>*36%* [b] | 1 ± 0.60<br>*6.9%* | 23 ± 11.33<br>*44%* | 1943 ± 0.10<br>*117%* | 0.71 ± 0.03<br>*106%* |
| *A. niger* D15[*AtfaeA*] | 531 ± 21.2<br>*3.5-fold* | 12 ± 0.29<br>*59%* | 24 ± 0.63<br>*45%* | 2196 ± 841<br>*132%* | 0.83 ± 0.05<br>*123%* |
| **Triticale bran** | | | | | |
| Control | 3 ± 0.10 | 3 ± 0.15 | 10 ± 1.03 | 1126 ± 1.02 | 0.56 ± 0.05 |
| *A. niger* D15[pGTP] | 75 ± 3.02<br>*25-fold* | 5 ± 0.19<br>*176%* | 20 ± 1.02<br>*200%* | 1070 ± 10.05<br>*95%* | 0.50 ± 0.08<br>*90%* |
| *A. niger* D15[*AtfaeA*] | 177 ± 1.03<br>*59-fold* | 77 ± 5.01<br>*26-fold* | 69 ± 2.02<br>*7-fold* | 1492 ± 18.08<br>*133%* | 0.64 ± 0.05<br>*115%* |

[a]Values are the means of duplicate measurements on duplicate treatments, together with standard deviations.

[b]Values as a percentage or fold increase relative to control.

## 3.2 Release of phenolic acids from rooibos

Neither the *A. niger* D15[pGTP] nor *A. niger* D15[*AtfaeA*] cocktails significantly impacted on the SS content of the green and fermented rooibos (Table 2), but aside from the *A. niger* D15[*AtfaeA*] treatment of fermented rooibos, the TAC and TPC values were lower than for the control treatment. The green rooibos turned brown in colour due to oxidation, which typically reduced the TAC and in particular, the aspalathin content (Coetzee et al. 2013).The *A. niger* D15[pGTP] cocktail significantly decreased the aspalathin, nothofagin, isoorientin and ferulic acid content of both the green rooibos and fermented rooibos SS (Table 3); it also decreased the quercetin-3-robinobioside content of fermented rooibos SS. The *A. niger*

D15[*AtfaeA*] treatment overcame most of the losses incurred by *A. niger* D15, and increased the ferulic acid content by 53% and 250% in the fermented and green rooibos SS, respectively, when compared to the respective control treatments. A significant increase in the SS levels of isoquercitrin and luteolin-7-glucoside was also evident for treatment of fermented rooibos with either *A. niger* D15[pGTP] or *A. niger* D15[*AtfaeA*]. In spite of these increased levels, their content in the soluble solids remained low.

**Table 2.** Soluble solids (SS), total antioxidant capacity (TAC) and total polyphenol content (TPC) of infusions prepared from control and enzyme-treated rooibos

| Sample | Soluble solids (g/100 g plant material) | Soluble solids (g/100 ml infusion) | TAC (µmol TE/ g SS)[a] | TP (g GAE/100 g SS)[b] |
|---|---|---|---|---|
| **Fermented samples** | | | | |
| Control | 14.01 ± 0.12 | 0.18 ± 0.01 | 1405 ± 125.35 | 16.47 ± 0.05 |
| *A. niger* D15[pGTP] | 14.98 ± 1.11 | 0.18 ± 0.01 | 1059 ± 154.15 | 14.86 ± 0.03 |
| | *117%* | *106%* | *75%* | *90%* |
| *A. niger* D15[*AtfaeA*] | 14.99 ± 0.07 | 0.19 ± 0.0 | 1407 ± 2.83 | 17.42 ± 0.21 |
| | *107%* | *107%* | *100%* | *106%* |
| **Green samples** | | | | |
| Control | 11.45 | 0.14 | 1692 | 19.62 |
| *A. niger* D15[pGTP] | 11.67 ± 0.76 | 0.15 ± 0.01 | 1315 ± 51.62 | 18.07 ± 0.66 |
| | *102%* | *102%* | *78%* | *92%* |
| *A. niger* D15[*AtfaeA*] | 11.37 ± 0.53 | 0.14 ± 0.01 | 1422 ± 90.51 | 17.17 ± 0.22 |
| | *99%* | *99%* | *84%* | *88%* |

[a]µmol Trolox equivalents per 100 g soluble solids; [b]g gallic acid equivalents per 100 g soluble solids;

**Table 3.** Phenolic composition of rooibos hot water soluble solids (SS) following treatment with enzyme cocktails

| | [compound] (g compound/100 g SS) | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Sample** | **PPAG[a]** | **Aspalathin** | **Nothofagin** | **Isoorientin** | **Orientin** | **Ferulic acid** | **Quercetin-3-rob[b]** | **Vitexin** | **Hyperoside** | **Rutin** | **Isovitexin** | **Isoquercitrin** | **Luteolin-7-glc** |
| **Fermented rooibos** | | | | | | | | | | | | | |
| Control | 0.50 | 0.11 | 0.03 | 0.83 | 0.75 | 0.15 | 0.35 | 0.18 | 0.11 | Nd | 0.17 | 0.03 | 0.02 |
| *A. niger* D15[pGTP] | 0.54±0.04[c] | 0.04 | 0.0 | 0.68±0.14 | 0.68±0.07 | 0.04±0.02 | 0.25±0.05 | 0.18±0.00 | 0.1±0.01 | Nd | 0.18± 0.03 | 0.05±0.01 | 0.04±0.02 |
| | *108%* [d] | *36%* | *0%* | *82%* | *91%* | *27%* | *71%* | *100%* | *91%* | | *106%* | *167%* | *200%* |
| *A. niger* D15[*AtfaeA*] | 0.53±0.00 | 0.12±0.00 | 0.03±0.00 | 0.84±0.00 | 0.76±0.01 | 0.23±0.02 | 0.37±0.01 | 0.18±0.00 | 0.12±0.00 | Nd | 0.20±0.01 | 0.04±0.00 | 0.05±0.00 |
| | *106%* | *109%* | *100%* | *101%* | *101%* | *153%* | *106%* | *100%* | *109%* | | *118%* | *133%* | *250%* |
| **Green rooibos** | | | | | | | | | | | | | |
| Control | 0.37 | 3.02 | 0.44 | 1.28 | 1.02 | 0.06 | 0.29 | 0.18 | 0.08 | 0.11 | 0.21 | 0.10 | traces |
| *A. niger* D15[pGTP] | 0.44±0.01 | 0.4± 24 | 0.2±0.06 | 1.04±0.04 | 0.95±0.03 | nd | 0.31±0.01 | 0.17±0 | 0.07±0.01 | 0.13±0.01 | 0.17±0.01 | 0.07±0.01 | traces |
| | *119%* | *14%* | *50%* | *81%* | *93%* | | *107%* | *94%* | *88%* | *118%* | *81%* | *70%* | |
| *A. niger* D15[*AtfaeA*] | 0.41±0.08 | 3.38±0.47 | 0.46±0.06 | 1.24±0.16 | 1.00±0.13 | 0.21±0.07 | 0.28±0.04 | 0.18±0.02 | 0.07±0.01 | 0.11±0.02 | 0.22±0.03 | 0.07±0.01 | traces |
| | *111%* | *112%* | *105%* | *97%* | *98%* | *350%* | *97%* | *100%* | *88%* | *100%* | *105%* | *70%* | |

[a]Phenylpyruvic acid glucoside.
[b]Rutin equivalents; glc = glucoside; nd = not detected; rob = robinobioside.
[c]Values are the means of duplicate measurements on duplicate treatments.
[d]Values expressed as a percentage relative to control.

# 4. Discussion

Value-addition to biomass presents a significant challenge due to the complexity of the cellulosic structure, which requires an array of enzymes with different catalytic functions for hydrolysis thereof. Fungal cells that produce a full complement of the appropriate mix of extracellular enzymes provide the opportunity for the development of more efficient and economical methods for biomass hydrolysis (Shin et al. 2006). In this study, the fungal enzyme cocktails produced by *A. niger* D15[pGTP] and *A. niger* D15[*AtfaeA*] were evaluated for the release of phenolic acids from triticale and maize bran, as well as from green and fermented rooibos plant material (leaves and fine stems).

When compared with the control strain, the *A. niger* D15[*AtfaeA*] cocktail extracted more ferulic acid, *p*-coumaric acid and caffeic acid from both triticale and maize bran, with triticale bran yielding better results. This suggests that the phenolic acids in triticale bran are more accessible and/or susceptible to enzymatic attack (Poidevin et al. 2009). This is in agreement with the observation that the tightly bound heteroxylan and high level of cross-linkages via diferulic bridges esterified to the arabinoxylan render maize bran highly resistant to enzymatic attack (Faulds et al. 1995). However, it is noteworthy that the *A. niger* D15[pGTP] cocktail reduced the levels of all three phenolic acids investigated, which could be ascribed to oxidative coupling of ferulic acid to form larger and more complex molecules, or the metabolism of ferulic acid to a variety of products (Hasyierah et al. 2008; Huang et al. 1993; Rosazza et al. 1995).

An important consideration is that the *A. niger* D15[*AtfaeA*] cocktail was able to release additional phenolic acids from maize and triticale bran without any pretreatment, which will have a significant impact on the production cost of fermentable sugars and/or value-added chemicals for application in various industries (Benoit et al. 2006, 2008; Fazary and Ju 2007; Koseki et al 2009;Topakas et al. 2007; Wong 2006).The *A. niger* D15[*AtfaeA*] cocktail was in particular effective in the release of *p*-coumaric and ferulic acid, with a respective 304- and 54-fold increase relative to the untreated control. It was interesting that more *p*-coumaric acid was extracted than ferulic acid, as ferulic acid is the most abundant phenolic acid in monocotyledonous plants (Balasundram et al. 2006). In a previous study where *p*-coumaric and ferulic acid were extracted from rye grass, 90% of the p-coumaric acid was found to be ester bound as opposed to 48% of ferulic acid that was ester bound and the rest ether bound (Kondo et al. 1990). Since the enzyme cocktail consists mainly of ferulic acid esterase which

hydrolyses only ester bonds and not ether bonds, this suggests that the ferulic acid extracted in this study was only ester bound and that the rest was left ether bound.

The antioxidants of cereal plants has significant health benefits in protecting the body against oxidative damage (Balasundram et al. 2006; Yu 2008). Rooibos plant material is known for poor extraction of the plant phenolics and a previous study showed that treatment of rooibos plant material with hydrolytic enzymes enhanced the release some of these phytochemicals (Pengilly et al. 2008). However, enzyme treatment in an aerobic environment causes oxidation of some of the phytochemicals, most notably the dihydrochalcones, aspalathin and nothofagin, with a concomitant decrease in TPC and TAC, as reported by Coetzee et al. (2013). When compared to *A. niger* D15[pGTP], the *A. niger* D15[*AtfaeA*] cocktail significantly improved the ferulic acid content of both green and fermented rooibos SS. Furthermore, the *A. niger* D15[*AtfaeA*] cocktail overcame the loss in TAC and some individual antioxidants displayed by the *A. niger* D15[pGTP] cocktail for both plant materials, suggesting that the *At*FAEA released additional polyphenols through the hydrolysis of important linkages.

The *A. niger* D15[pGTP] cocktail increased the SS content of the fermented rooibos infusion by 17%, accompanied by a 10% decrease in TPC, which suggests a release of mostly non-phenolic compounds without antioxidant properties. Although the *A. niger* D15[*AtfaeA*] cocktail slightly increased the SS and TP content of the fermented rooibos infusion (7% and 6%, respectively), it significantly increased the levels of ferulic acid (53%), isoquercitrin (33%) and luteolin-7-glc (150%) in the SS. Analysis of the infusions of a large number of fermented rooibos samples, similarly prepared as for the present study, indicated that the ferulic acid content of the SS could vary between undetectable and 0.18% (Joubert et al 2012). Whilst the ferulic acid content of untreated fermented rooibos SS was within this range, treatment with *A. niger* D15[*AtfaeA*] increased this level to 0.23%. Significant increases in isoquercitrin (67%) and luteolin-7-glc (100%) were also observed with the *A. niger* D15[pGTP] cocktail, suggesting that the increase in these two antioxidants are associated with the *A. niger* D15 strain and not the presence of *At*FAEA.

Both the *A. niger* D15[pGTP] and *A. niger* D15[*AtfaeA*] cocktails reduced the TAC and TP content of the green rooibos SS, but the *A. niger* D15[*AtfaeA*] cocktail overcame the loss in aspalathin (unique to rooibos) and nothofagin observed with the *A. niger* D15[pGTP] cocktail, and displayed a 250% increase in ferulic acid relative to the control treatment. The study therefore demonstrated that the *A. niger* D15[*AtfaeA*] cocktail can add value to rooibos

infusions in terms of ferulic acid, isoquercitrin and luteolin-7-glucoside content, but the treatment process itself is detrimental to the total antioxidant content of the green rooibos SS. It would be interesting to evaluate the effect of a purified *At*FAEA in the absence of other lignocellulosic hydrolases (as found in crude enzyme cocktails).

# 5. Conclusions

The *A. niger* D15[*AtfaeA*] cocktail was superior to the *A. niger* D15[pGTP] cocktail at extracting phytochemicals from all four substrates investigated, i.e. triticale and maize bran, green and fermented rooibos. Particularly encouraging was the high levels of ferulic acid extracted from maize bran (531 mg/l) and triticale bran (177 mg/l), as well as *p*-coumaric acid (77 mg/l) from triticale bran. Since triticale bran and maize bran routinely used as animal feed, treatment of maize and triticale bran with FAEA can ensure a higher ferulic acid content, and thus a higher nutritional value. As a natural extracted product, ferulic acid can be converted to other industrially significant molecules such as vanillin, which is widely used in the food, pharmaceutical and cosmetic industry. Ferulic acid can also be converted to other valuable molecules such as polymers, epoxides, alkylbenzenes, protocatechoic acids that have industrial significance (Huang et al. 2011).

Ferulic acid is ubiquitous to the plant kingdom, but its availability and utilization has been limited by the high cost associated with its synthesis. The use of enzyme cocktails such as the one developed in this study could result in the development of cost-effective processes, which could allow the large-scale extraction of ferulic acid. This would allow industry to increase their use of natural ferulic acid as an additive in food, cosmetic and medical products, as well as animal feed. This study confirmed that an extracellular FAEA isolated from *A. niger* D15[*AtfaeA*] is effective for the release of ferulic acid (and selected other phytochemicals) from different plant substrates. This could be of significant value to industries in search of fine chemicals (such as ferulic and *p*-coumaric acid) or strategies to increase the antioxidant content (i.e. enrichment) of food/ fodder products.

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## 7. Declaration

ENZ performed the experimental work, analysed the data and drafted the manuscript. EJ, DdB and GD assisted with plant treatments and analyses of the polyphenolics. KR, WHVZ and MVB participated in the design of the study and revision of the manuscript. All authors read and approved the final manuscript.

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# CHAPTER 5

# Summary and General Conclusions

## 1. Summary

An abundance of agro-industrial waste is generated during agricultural practices that could serve as a renewable source for the production of lignocellulosic bioethanol as well as valuable plant-derived phytochemicals. However, most of the phytochemicals, such as ferulic acid, are tightly bound to the hemicellulosic backbone and are therefore not readily bioavailable. The current demand for natural phenolic compounds suggests a growing market for eco-friendly methods – such as microbial hydrolysis - for the extraction of bioactive phytochemicals on a commercial scale. Enzyme-assisted hydrolysis of the lignocellulosic plant components will also improve the digestibility of animal feed and enhance the hydrolysis of cellulose to fermentable sugars, a requirement for cost-effective biofuel production.

Since ferulic acid esterases (FAEs) are able to release ferulic acid and possibly other phenolic compounds from plant material, the aim of this research was to identify a fungal ferulic acid esterase, over-express the relevant gene in *Aspergillus niger* and evaluate the potential of the recombinant strain for commercial-scale production. The main results can be summarised as follow:

### 1.1 Screening, identification and evaluation of FAE producers

The production of FAE, involved in the release of ferulic acid side groups from xylan, was investigated in strains of *Aspergillus tubingensis, Aspergillus carneus, A. niger* and *Rhizopus oryzae* (Chapter 2). The *A. tubingensis* T8.4 strain showed the highest activity on triticale bran as sole carbon source, producing a type A ferulic acid esterase active against methyl *p*-coumarate, methyl ferulate and methyl sinapate. The native *A. tubingensis* ferulic acid esterase gene was subject to glucose-inhibition as well as substrate-induction in the presence

of maize bran. Furthermore, the data suggested a combined action of endoglucanase, endoxylanase and ferulic acid esterase for the utilization of maize bran.

## 1.2 Cloning and expression of a FAE-encoding gene in *A. niger*

The *A. niger* D15#26 strain, which has reduced protease activity and does not acidify the media (thus promoting high-level expression of recombinant enzymes), was used as host for the expression of a genomic copy of the *A. tubingensis faeA* gene under control of the *A. niger gpdA* promoter (Chapter 2). The *A. niger* D15[*AtfaeA*] strain produced 13.5 U/ml FAEA after 5 days on autoclaved maize bran as sole carbon source, which was 3-fold higher than for the *A. tubingensis* donor strain. The recombinant *At*FAEA extracted 50% of the available ferulic acid from non-pretreated maize bran, indicating its potential application for the release of ferulic acid from lignocellulosic plant material.

The recombinant *At*FAEA was purified 7-fold with anion-exchange chromatography and its identity confirmed with peptide mass fingerprinting (Chapter 3). The observed physical properties of the recombinant *At*FAEA were mostly similar to that of the native enzyme; the enzyme was optimally active at pH 6 and 50$^{o}$C, stable at pH 3 to 7 and 30$^{o}$C to 60$^{o}$C, with a $K_m$ of 0.43 mM, $K_{cat}$ of 0.48/min and $K_{cat}/K_m$ of 1.1/ min.mM. These properties suggested that the *At*FAEA would be suitable for the food, pulp and paper, and animal feed industries where ferulic acid and other phenolic compounds could be released from the lignocellulosic backbone and become available for absorption in the gastro-intestinal tract.

## 1.3 Optimise expression of recombinant FAE

Culturing *A. niger* D15[*AtfaeA*] in a bioreactor (Chapter 3) significantly improved *A*tFAEA production, with fed-batch fermentation being more effective with a 2-fold higher FAE activity recorded with the fed-batch fermentation relative to batch fermentation. The fed-batch conditions resulted in a higher biomass yield, volumetric productivity and volumetric activity than batch fermentation, suggesting that it would be the preferred cultivation strategy for larger-scale production of *At*FAEA.

### 1.4 Evaluate potential value-addition through treatment of different plant substrates with recombinant FAE

In Chapter 4, the evaluation of *At*FAEA for its ability to improve the release of phenolic compounds from maize bran, triticale bran and rooibos (an antioxidant-rich herbal tea) is described. A crude preparation of the *A. niger* D15[*AtfaeA*] extracellular enzyme cocktail extracted 531 mg/l and 177 mg/l ferulic acid from maize bran and triticale bran, respectively, as well as 77 mg/l *p*-coumaric acid from triticale bran. This confirmed that *At*FAEA can increase the ferulic acid content and thus the nutritional value of maize and triticale bran, which are both routinely used as animal feed. The cocktail was also able to extract 0.2 g ferulic acid/100 g hot water soluble solids from *Aspalathus linearis* (rooibos) leaves and stems, indicating the potential of *At*FAEA for the enrichment of extracts prepared from plant substrates known for their antioxidant and/or neutraceutical value. In parallel to improving the nutritional value of products, *At*FAEA can also be used for the extraction of natural ferulic acid and other phenolic compounds that have significance in the pharmaceutical industry.

## 2. General Conclusions

The alkaline extraction of phenolic acids from lignocellulosic material is a simple and inexpensive method, but by-product formation compromises the subsequent purification steps. The use of enzymes is a more natural way of extracting phenolic compounds and eliminates the production of by-products associated with alkaline extraction. Current knowledge of lignocellulosic hydrolysis indicates that significant enzyme-assisted hydrolysis can mainly be achieved after significant pretreatment. In this study, the *At*FAEA enzyme cocktail released 50% of the esterified ferulic acid in untreated maize bran, suggesting that efficient pretreatment of this highly recalcitrant substrate could yield significantly higher levels of ferulic acid - and possibly other phenolic acids as well. Note that FAEA only catalyses the release of ester bound ferulic acid, whereas total ferulic acid extraction (i.e. both ester and ether bound ferulic acid) is achieved through alkaline extraction.

Various features associated with the *Aspergillus* species render them favourable strains for exploring their industrial application as recombinant strains, including their GRAS status (Generally Regarded As Safe), their ability to produce a large range of enzymes as well as

their suitability for large-scale enzyme production. However, Aspergilli also produce proteases that pose a challenge for the industrial production of native or recombinant enzymes in high quantities and purification thereof to homogeneity.

In this study, over-expression of the *A. tubingensis fae*A gene in *A. niger* D15 proved effective as the reduced protease activity of the *A. niger* D15 strain enabled the accumulation of an enzyme cocktail with FAEA representing a significant portion of the total enzymes secreted by *A. niger* D15[*AtfaeA*]. The FAE activity compares favourably to FAE activities reported from other microorganisms; relatively high levels of FAEA would reduce the cost of enzyme preparation and purification. The constitutive *gpdA* promoter allows for high expression levels and without the need for an inducer, the production process is simplified and allows for bulk production of the recombinant enzyme. The enzyme cocktail also allowed easy downstream processing of the *At*FAEA as was evident by the one-step purification required to purify the enzyme, which would further benefit large-scale industrial production of *At*FAEA.

Fed-batch fermentation proved to be a successful strategy for increasing the production of *At*FAEA. However, these results were preliminary and further optimization of the fed-batch fermentation process is required to unlock the full potential of *At*FAEA production in *A. niger*. The levels of activity can be further increased through strain development, for example obtaining higher copy numbers of the *AtfaeaA* gene through retransformation of *A. niger* D15[*fae*A]. In this regard, the study provides a basis for industrial microbiologists and process engineers to design a scale-up strategy for the optimisation of *At*FAEA production.

Attempts to add value to biomass through enzyme treatment remains a challenge to bioengineers. The complicated structure of biomass requires an array of enzymes and optimal conditions to exploit the synergy among the different enzymes. A purified enzyme, such as *At*FAEA, is not able to completely hydrolyse a complex substrate; a mixture or enzyme cocktail is required to attack the various chemical linkages and ensure complete hydrolysis of the lignocellulose. We were able to show that the *A. niger* D15[*AtfaeA*] cocktail was able to extract additional phenolic compounds from maize bran, triticale and rooibos tea. Since maize bran and triticale are used mostly as animal feed, this study suggests that the addition of the *At*FAEA enzyme cocktail has the potential to enrich animal feed. Although not investigated in this study, the native *A. niger* cellulases in the *A. niger* D15[*AtfaeA*] cocktail will also contribute towards the microbial hydrolysis of these feedstocks and thus improve ruminal degradation and nutrient absorption.

It is noted that additional cellulolytic activities may not be beneficial to all plant substrates, as the presence of native *A. niger* hydrolases also contribute to the extraction of non-polyphenolics, which was particularly evident in the rooibos extracts evaluated in this study. This suggested that alternative cocktails should be developed for rooibos to ensure that predominantly polyphenols are extracted.

There are numerous challenges currently associated with industrial enzyme production. This study attempted to address some of these challenges, such as limited production levels of the enzyme and the costs associated with enzyme production and purification. This was done by engineering a fungal strain of *A. niger* D15 to produce an enzyme cocktail that contained mainly *At*FAEA, allowing the easy purification of the enzyme. The study showed potential towards the successful extraction of phytochemicals from various plant substrates, but further research needs to focus on the extraction efficiency of bioactive compounds. We expect that the findings reported here will pave the way for the development of a new enzyme-based process for the production of an enzyme cocktail that is mainly focused on the extraction of phenolic compounds, whether it is for substrate enrichment or value addition to biomass as part of the biorefinery concept. Furthermore, this process could create natural commercial products that will more likely be embraced by consumers.