Purification and biochemical characterization of ferulic acid esterase from *Aspergillus niger* CFR 1105

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IN
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by
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DECLARATION

I declare that the thesis entitled *"***Purification and biochemical characterization of ferulic acid esterase from *Aspergillus niger* CFR 1105***"*** submitted to the **UNIVERSITY OF MYSORE,** Mysore for the award of the degree of **DOCTOR OF PHILOSOPHY IN BIOCHEMISTRY** is the result of the work carried out by me under the guidance of **Dr. G. Muralikrishna**, Scientist-F, Department of Biochemistry and Nutrition, Central Food Technological Research Institute (CFTRI), Mysore–570020, India, during the period from April 2006 to October 2009. I further declare that the results are not submitted for the award of any other degree or fellowship.

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CERTIFICATE

This is to certify that the thesis entitled *"***PURIFICATION AND BIOCHEMICAL CHARACTERIZATION OF FERULIC ACID ESTERASE FROM *Aspergillus niger* CFR 1105***"*** submitted by **Ms. Hegde Shyamala Ganapati** for the award of the degree of **DOCTOR OF PHILOSOPHY** in **BIOCHEMISTRY** to the **UNIVERSITY OF MYSORE, Mysore** is the result of research work carried out by her in the Department of Biochemistry and Nutrition, CFTRI, Mysore – 570020, India, under my guidance during the period from April 2006 to October 2009. I further declare that the results presented in this thesis have not been submitted for the award of any other degree or fellowship.

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DEDICATED TO MY

PARENTS
&
TEACHERS

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Hegde Shyamala Ganapati

CONTENTS

LIST OF ABBREVIATIONS

Abbreviations	Expansions
α	Alpha
β	Beta
µ	micro
^{0}C	Degree centigrade
APS	Ammonium persulfate
BSA	Bovine serum albumin
DCC	Dicyclohexyl carbodiimide
DEAE	Diethyl amino ethyl
DMSO	Dimethyl sulphoxide
EDTA	Ethylene diamine tetra acetic acid
ESI	Electrospray ionization
FA	Ferulic acid
FAE	Ferulic acid esterase
GPC	Gel permeation chromatography
GSH	Reduced glutathione
g	Gram(s)
h	Hour
HPLC	High performance liquid chromatography
IR	Infrared
kDa	kilo Dalton
M	Molar

min	Minute
ml	Milliliter
MS	Mass spectroscopy
MUTMAC	4-Methylumbelliferyl *p*-trimethyl ammoniocinnamate chloride
nm	Nanometer
NMR	Nuclear magnetic resonance
4NPF	4-nitrophenyl ferulate
NSP	Non-starch polysaccharide
p	Para
p-NP	*p*-nitrophenol
PAGE	Polyacrylamide gel electrophoresis
PVPP	Polyvinylpolypyrrolidone
SDS	Sodium dodecyl sulphate
ssf	Solid state fermentation
smf	Submerged fermentation
Tris	Tris(hydroxymethyl) aminomethane
TEMED	N,N,N^{I},N^{I} –tetramethylethylenediamine
U	Unit(s)
UV	Ultra violet
w/w	Weight/weight
w/v	Weight/volume

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ABSTRACT

Ferulic acid esterase (FAE, EC 3.1.1.73), a biotechnologically important enzyme is responsible for the hydrolysis of the ester linkage present between 3-methoxy 4-hydroxy cinnamic acid (ferulic acid), and sugars and for their subsequent utilization as bioactive compounds. The major focus of the present work was to study ferulic acid esterase from a strain of *Aspergillus niger* (CFR 1105) with respect to (a) optimization of growth conditions of *Aspergillus niger* CFR 1105 for the enrichment of ferulic acid esterase, (b) its purification and partial characterization. A spectrophotometric substrate namely 4-nitrophenyl ferulate was synthesized by a single step dehydrative coupling of ferulic acid and 4-nitrophenol and characterized by HPLC, ESI-MS, ^{1}H and ^{13}C NMR for the assay of ferulic acid esterase. Ferulic acid esterase activity was detected in the culture filtrate of *A. niger* grown on wheat bran, whereas it was absent in the culture filtrates of *Aspergillus oryzae* and *Rhizopus arrhizus* grown on wheat bran. FAE activities produced by *A. niger* grown in both solid state (ssf) and submerged fermentation (smf) using wheat bran as a carbon source and also as an inducer of the enzyme were comparable, however specific activity of the enzyme in culture filtrate of smf was double than that of ssf and was constant upto 5 days and this condition was chosen for isolation and purification of ferulic acid esterase. Two isoenzymes, designated as FAE-1 and FAE-2 were isolated and purified to homogeneity by conventional protein purification methods from the 5^{th} day culture filtrate of *A. niger* grown in smf with a fold purification of 11 and 25 respectively. Molecular weights of FAE-1 and FAE-2 were found to be 50 kDa and 55 kDa respectively. FAE-1 and FAE-2 are glycoproteins with carbohydrate content of 23% and 30% respectively. FAE-1 and FAE-2 exhibited pH optima of 9 and 6 respectively and both were stable over pH range of 6 to 9. FAE-1showed temperature optimum of 40 ^{0}C whereas FAE-2 showed wider temperature optimum of 40-50 ^{0}C. They showed highest specific activity towards *p*-nitrophenyl ferulate followed by *p*-nitrophenyl acetate and *p*-nitrophenyl

butyrate. The thesis ends with summary and conclusions along with list of references.

Synopsis

Title of the thesis**: "Purification and biochemical characterization of ferulic acid esterase from *Aspergillus niger* CFR 1105"**

Candidate: **Shyamala Hegde**

Polysaccharides such as arabinoxylans, 1,3/1,4-β-D-glucans, arabinogalacto-proteins and lignocellulosic complexes are the major constituents of the cell walls of cereals as well as pulses. Phenolic acids and their derivatives such as ferulic acid (4-hydroxy-3-methoxycinnamic acid) and *p*-coumaric acid (4-hydroxycinnamic acid) are esterified to the 5^{I} –OH group of arabinose residues present as side chains of xylans. Complete degradation of the plant cell walls requires synergistic action between main chain degrading enzyme such as endo-β-(1,4)-xylanase and accessory enzyme such as ferulic acid esterase which remove various substituents such as ferulic acid. The removal of phenolic acid substituents is important not only with respect to the degradation of plant cell walls, but also for obtaining bioactive compounds such as ferulic acid, *p*-coumaric acids which have various biotechnological applications. Ferulic acid is the most abundant hydroxycinnamic acid present in the cell walls of cereals. Cereal brans, one of the major byproducts of the milling industries are renewable source of ferulic acid. Ferulic acid (4-hydroxy-3-methoxycinnamic acid), due to its structural similarity, is an ideal potential precursor for the biotechnological production of vanillin (4-hydroxy-3-methoxy benzaldehyde), a widely used flavour in the food industry. Biotechnologically produced vanillin can be an alternate approach towards high demand for vanillin in the market, in addition to the one

produced by synthetic process as well as the natural vanillin obtained from vanilla pods.

Ferulic acid esterases (FAE) (3.1.1.73), a subclass of the carboxylic ester hydrolases (EC 3.1.1.1) are responsible for the hydrolysis of the ester linkage present between hydroxycinnamic acids and sugars and their subsequent utilization as bioactive compounds. These ferulic acid esterases have a number of biotechnological applications such as isolation of bioactive compounds from agro wastes, pulp and paper industries, production of fuel ethanol, synthetic tool to synthesize novel esters. Due to biotechnological importance of ferulic acid esterases, these enzymes have been studied from a large number of microbial sources (fungal and bacterial sources). There is an increased research effort in finding newer sources of FAE with novel properties. Hence a detailed study is envisaged to optimize the growth conditions for improved levels of ferulic acid esterase from a strain of *Aspergillus niger* (CFR 1105), an industrially important microorganism grown on cereal brans.

The major focus of the present work was to study ferulic acid esterase from a strain of *Aspergillus niger* (CFR 1105) with the following objectives.

1. Optimization of growth conditions of *Aspergillus niger* CFR 1105 for the enrichment of ferulic acid esterase.

2. Purification and characterization of ferulic acid esterase isolated from *Aspergillus niger* CFR 1105 and study of its kinetic parameters.

An outline of the studies carried out towards this end forms the subject matter of this thesis and is presented chapterwise.

Chapter 1. General Introduction

This chapter begins with the general account on food processing and cell wall components of cereal brans, and bioactive compounds derived from them. This chapter provides an overview of the ferulic acid esterases from various microbial sources such as fungi and bacteria. The topics in this chapter include their biological significance (as endogenous) in plants, microbial production of ferulic acid esterases, their properties, classification and their biotechnological applications. A brief description of the growth conditions and substrates required by microorganisms to produce FAE is presented. This chapter also highlights the objectives and scope of the present investigation.

Chapter 2. Materials and Methods

Described here are various colorimetric and spectrophotometric methods such as estimations of total carbohydrate, reducing sugar, protein. A novel single step synthetic method is described pertaining to the synthesis of 4-nitrophenyl ferulate. Assay methods for amylase, xylanase, 1, 3 / 1, 4-β-D-glucanase and ferulic acid esterase are also provided.

Methods pertaining to the isolation of ferulic acid esterase, purification of this enzyme by ammonium sulphate fractionation, anion exchange chromatography on DEAE-cellulose, gel permeation chromatography on Sephacryl 100-HR are described in detail. The methods used to ascertain purity of ferulic acid esterase by PAGE, protein staining are also described.

This chapter also deals with various methods used for the characterization of ferulic acid esterase such as determination of molecular weight by GPC,

SDS-PAGE, ESI-MS. pH and temperature optima and their stability studies, various kinetic parameters such as determination of K_m and V_{max}, effect of metal ions, inhibitors are included.

Results and Discussion

Chapter 3. Synthesis of spectrophotometric substrate for the assay of ferulic acid esterases

A spectrophotometric substrate 4-nitrophenyl ferulate was synthesized by dehydrative coupling of ferulic acid and 4-nitrophenol using dicycohexylcarbodiimide as reagent. The synthesized product was characterized by HPLC, ESI-MS, and NMR. The method optimized, obviates the protection of phenolic group and its subsequent deprotection in the final step by the expensive enzymatic process employed in the published synthetic methodologies. Thus, this method affords facile access to 4NPF, accurately modeling the ester linkage of ferulic acid to polysaccharides found in cell walls of plants and serving as an appropriate substrate for the hydrolytic action of ferulic acid esterases. The 4NP released after enzyme action is measured spectrophotometrically at 410 nm and enzyme activities were calculated using 4NP standard graph.

Chapter 4. Selection of the fungal organism and growth substrate

Three fungal organisms namely *Aspergillus niger* CFR 1105, *Aspergillus oryzae* CFR 232 and *Rhizopus arrhizus* NCIM 997 were grown on brans of wheat, rice and ragi for 24, 48, 72 and 96 h time intervals, and the resultant moldy brans were evaluated for starch degrading and cell wall degrading enzymes. The activity of amylase, the most preponderant enzyme in all the moldy brans was several times higher than the pullulanase activity. Xylanase activity was 2-3 times higher than that of 1,3/1,4-β-D-glucanase in all moldy brans. *A. niger* CFR 1105 and *A. oryzae* CFR 232, were found to be better than *R. arrhizus* NCIM 997 with respect to the induction of starch degrading and cell wall degrading enzymes and can be utilized for the degradation of cell wall polymers of wheat, rice and ragi brans. Wheat bran was found to be a better substrate than rice bran and ragi bran for the induction and production of these enzymes. *A. niger* was found to produce more ferulic acid esterase than *A. oryzae* and *R. arrhizus* when grown on wheat bran.

As *Aspergillus niger* CFR 1105 was found to produce higher amounts of xylanase and ferulic acid esterase than *A. oryzae* and *R. arrhizus*, this organism was chosen for further detailed studies. It was inoculated into wheat and rice brans and grown for 24, 48, 72 and 96 h to understand the degradation pattern of non-starch polysaccharides and phenolic acid complexes. Native wheat bran polysaccharides mainly consisted of arabinose, xylose, galactose and glucose in % of 27:39:2:30 with traces of mannose whereas rice bran consisted of arabinose, xylose, galactose and glucose in % of 9:27:31:32. Both the cereal

bran arabinoxylans were degraded extensively by *A. niger* at 96 h, whereas the degradation of 1,3/1,4-β-D-glucans and cellulose was negligible. Bound phenolic acids of rice and wheat brans identified by HPLC were found to be mainly ferulic acid, coumaric acid, syringic acids in % of 93.6:6.3:0 (wheat) 34.7:55.8:9.5 (rice) and were drastically degraded/utilized at 96 h. The above results indicated preferential degradation of arabinoxylans of wheat bran by *A. niger* which can be exploited to obtain bioactive compounds such as ferulic acid.

Chapter 5. Purification and characterization of ferulic acid esterase from *A. niger* grown on wheat bran

A. niger CFR 1105 was grown on wheat bran for further isolation and purification and characterization of ferulic acid esterase. The effect of initial pH of the media, incubation temperature and autoclaving time of the substrate (wheat bran) were studied for FAE production. *A. niger* CFR 1105 was grown under solid state (ssf) and submerged fermentation (smf) using wheat bran as growth substrate and inducer of the enzyme. Ferulic acid esterase activities increased with incubation period and reached maximum on 5^{th} day both in ssf and smf. Though the ferulic acid esterase activity produced in ssf and smf were comparable on day 5, specific activity of the enzyme in submerged fermentation conditions, was nearly double than that of ssf. Therefore for purification of FAEs, the organism was grown under smf.

Ferulic acid esterase was purified to homogeneity from the culture filtrate of *A. niger* by ammonium sulfate precipitation, anion exchange chromatography and gel filtration chromatography. Two activity peaks, designated as FAE-1 and

FAE-2 were obtained from DEAE-cellulose chromatography indicating the presence of two isoenzymes. Gel filtration chromatography yielded FAE-1 and FAE-2 in 4.3% and 2.5% recovery. Both the isoenzymes showed almost similar kinetic properties and a single band on SDS-PAGE with slight difference in molecular weight. Both FAE-1 and FAE-2 were active and stable in alkaline pH. Both exhibited activity in the wide pH range of 5-10. FAE-1 showed optimum activity at pH 9 whereas FAE-2 exhibited optimum activity at pH 6, similar to many other fungal FAEs reported previously. However it still exhibited significant activity in the alkaline pH range, retaining about 60 % at pH 9. FAE-1 had a pH optimum at pH 9 and it showed about 70% activity at pH 6. Both FAE-1 and FAE-2 were stable at alkaline pH, retaining 52% and 48% of their original activity at pH 10 after 2 h of incubation. The carbohydrate content of FAE-1 and FAE-2 was found to be 23% and 30% respectively. The K_m and V_{max} for FAE-1 for hydrolysis of 4NPF were 2 mM and 98 U/ mg protein respectively. For FAE-2 K_m and V_{max} were found to be 3.3 mM and 158.7 U/ mg protein respectively.

The activities of FAE-1 and FAE-2 were enhanced by of Fe^{+2}, Ba^{+2}, Mg^{+2} whereas they were inhibited by Hg^{+2}, and Cd^{+2}. The ferulic acid esterase activities were inhibited in the presence of PMSF (phenyl methane sulphonylfluoride) and TLCK (Tosyl-L-lysine chloromethyl ketone) by about 40% indicating the possible presence of serine in the active site pocket. There was no significant effect on the enzyme activity in the presence of iodoacetamide and TPCK (Tosyl-L-phenylalanine chloromethyl ketone). Disulfide reducing agent

β-mercaptoethanol also did not have any effect on the enzyme activity indicating the absence of subunits in both FAEs.

Introduction

In the present study ferulic acid esterase (FAE, EC 3.1.1.73), a biotechnologically important enzyme has been studied from *Aspergillus niger* CFR 1105 grown on wheat bran both as growth medium and inducer of FAE. Utilization of cereal brans, abundantly available cheap byproduct of food processing industry for the production of value added products, enzymes including FAE are given below.

1.1 Food processing

To prevent food deterioration and prepare food for immediate or future use raw materials require processing, preservation and storage. Benefits of food processing include toxin removal, increasing shelf life, improving flavor and consistency, raising marketing and distribution task. In addition, it increases continuous availability of seasonal foods, enables transportation of delicate perishable foods across long distances and makes many kinds of foods safe to eat by removing microorganisms. Modern food processing also improves the quality of life for allergies, diabetics and also adds extra nutrients (Potter 1978).

Food processing mainly falls into two broad categories, primary and secondary processing. The primary processing of rice, wheat and pulses include mainly milling, polishing, cleaning etc. Secondary processing of cereals includes the production of ready to consume products such as bread, biscuits, cakes etc. Secondary processing of pulses includes the preparation of puffed pulses, papad and many breakfast and bakery products.

Byproducts from agriculture and food processing can become one of the most serious sources of pollution (Di Blasi et al., 1997). An increase in the number of industrial plants in turn increased the volume of by-products, which led to giving due consideration about the treatment and environmental friendly disposal of the processing by-products. Agricultural technology earlier only started to use the byproducts as fodder and for oil production from food processing industry. Food industry waste and by-products are substances that originated during processing and can be further utilized in other ways. If we could produce valuable products from food industry by-products through new scientific and technological methods, environmentally polluting by-products could be converted into products with a higher economic value along with the main products, such as cereal brans, pulse husks and fruit pulps etc.

1.1.1 Use of food processing byproducts as carbon source for growing useful microorganisms in the production of valuable chemicals and enzymes

Byproducts from the food processing industry as a whole can be used in a number of ways especially for biomass production. Microorganisms are grown on food processing by-products. Availability of by-products for this biomass production is useful in view of their use in foods and feeds, in the production of enzymes, single cell protein, amino acids, lipids, carbohydrates and organic acids. Agricultural and food processing waste can also be used for the production of various acids having a potential use in the food and chemical industries.

Many industrially important enzymes are produced from food processing waste by using microorganisms. Wheat bran has been studied extensively for

pectinase production by solid-state fermentation (Ghildyal et al., 1981). Lipase was produced from copra waste (coconut oil cake) and rice bran using *Candida rugosa* (Benzamin and Pandey, 1996). Production of xylanase has been reported on dried apple pomace (Bhalla and Joshi, 1993). Amylase has been produced from bean waste, banana waste wheat and maize bran using microorganisms. Cellulase has been produced from cabbage waste, apple pomace, wheat bran, sugar beet, rice straw, bagassae etc using microorganisms such as *Trichoderma harzianum*, *Aspergillus ustus*, *Tricho* spp, *Bortrytes* spp etc. (Hang and Woodams, 1987).

1.1.2 Isolation of value added products

The recycling of by-products and minimizing wastes are crucial aspects of the food processing strategy. There is an increasing consumer's appreciation of natural products as alternative to synthetic compounds in a variety of goods from food to personal care formulations. By-products of plant food processing found to be promising sources of biologically active compounds, which may be used because of their favorable nutraceutical properties. There are great variety of value added compounds in the by-products and wastes from biological origin. These products may be used as such or may be a starting material for the preparation of novel compounds. The products with desired characteristics of commercial interest include antioxidants, carbohydrates, dietary fibers, fat and oils, pigments, proteins and starches. Several secondary metabolites such as phenolic compounds, carotenoids and other bioactive compounds such as

enzymes and vitamins with vital biological activities can be isolated from food processing by-products.

1.1.3 Cereal processing by-products

Wheat and rice are the important cereal consumed worldwide. Finger millet (ragi) is an important staple food in the south part of Karnataka. Basic staples like rice, wheat and pulses reach the market after going through many primary mechanical processing operations that convert the grain into an edible material (Seetharamaiah and Prabhakar, 1986).

During processing of rice, bran (3-7%), husk (21-24%), broken rice and germ are the major by-products. Bran, which comprises the testa and pericarp, is known to be rich in number of components such as oil, vitamins, sugars and proteins. Wheat is an important staple food, germ (3%) and bran (15%) are by-products obtained during milling. Figure 1 shows the cross sectional view of a whole wheat grain.

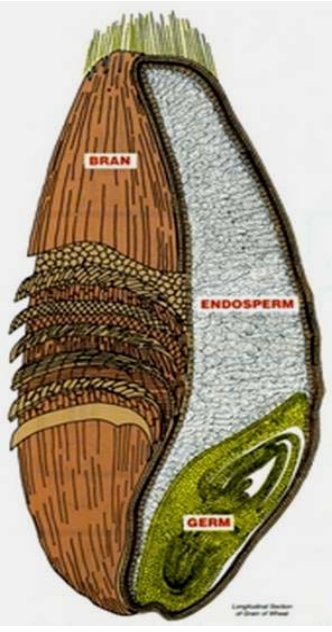


Figure 1. Cross section of wheat grain

The whole wheat grain is composed of the pericarp and the outermost tissues of the seed including the aleurone layer. It is considered as the protective skin for the rest of the kernel. The bran contains fiber, as well as important antioxidants and B vitamins. The next part of the wheat grain structure is the germ or it is also called the embryo from which a new plant may sprout. It is a good source of vitamin E, β-carotene, fats, valuable proteins, and enzymes such as lipase and esterase. Finally there is the endosperm, which makes up the bulk of the seed and feeds the germ and the rest of the seed. It is mostly made up of starchy carbohydrates and protein, but also contains small quantities of vitamins and minerals. Wheat and rice bran are found to be rich source of dietary fiber (Ge et al., 2002).

In the present study wheat bran has been used as a carbon source for the induction of FAE from *A. niger* CFR 1105. Its composition is given in the Table 1.

Table 1 Nutritional composition of cereal brans

Brans	Polysaccharides (%)	Lignin (%)	Protein (%)	Ash (%)	Moisture (%)
Wheat bran	66	12	6	4	12
Rice bran	62.4	12	9	2.7	15
Ragi bran	70	5	8.5	5	12

Source: Ring and Selvendran, 1980; McDonough et al., 1986.

1.1.4 Application of enzymes in food processing

Enzymes are biocatalysts. They have remarkable catalytic power, often far superior than synthetic catalysts. They are highly specific and accelerate specific chemical reactions under mild conditions of temperature and pH. Application of enzymes to alter the properties of foods is an area, which has attracted considerable interest, since enzymes are considered as natural products. Enzymes are also favored as they require milder conditions, have high specificity, required only in optimum quantities, and less likely to produce toxic products.

Thus, enzymes are becoming common use in many industries for improving the functional properties of food proteins (Pereria et al., 1999). They find great use in a large number of fields such as food, dairy, pharmaceutical, detergent, textile and cosmetic industries. Attempts made to produce enzymes by induction have been successful and played a very important role in many industries. Food industrial byproducts are the rich dietary source for the production of industrially important enzymes.

1.2 Cereal bran polysaccharides

Cereal brans contain polysaccharides such as cellulose, hemicelluloses, arabinoxylans, 1,3/1,4-β-D-glucans, glucomannans, pectins and arabinogalactans. These are non-digestible in the small intestine and included under the broad category of nonstarch polysaccharides which are nutritionally termed as dietary fibre components (Fincher and Stone, 1986; Izydorczyk, and Billiaderis, 1995).

Cellulose, a β-1,4 linked polymer of glucose forms the micro-fibrils of the cell wall. All other non-starch polysaccharides are very heterogeneous in their structure and form the “matrix” of the cell wall. The main nonstarch polysaccharides other than cellulose in cereals are 1,3/1,4-β-D-glucans and arabinoxylans, out of which arabinoxylans are the most preponderant (Smith and Hartley, 1983).

The rigidity and strength of the cell wall is related to the integrity of cellulose/hemicellulose network. During cell growth, wall expansion takes place which is dependent on the enzymatic modification of the hemicellulosic component (Pauly et al., 2001; McNeill et al., 1984).

1.2.1 Arabinoxylans

Arabinoxylans (AX) are heteropolysaccharides consisting predominantly of arabinose and xylose residues which are also referred to as pentosans. They consist of a β-1,4-linked xylan backbone, with side chains of α-L-arabinose (Wilde & Woo, 1977) or 4-O-methyl D-glucuronic acid (Brillouet & Joseleau, 1987) substituted either at O-2/O-3 (Figure 2). Based upon their solubility they can be classified as both water soluble and water insoluble arabinoxylans (alkali-extractable and referred as hemicelluloses). Polysaccharides are synthesized in the golgi complex which are exported as UDP sugar complexes and followed by their incorporation into the cell walls of plants (Liners et al., 1994).

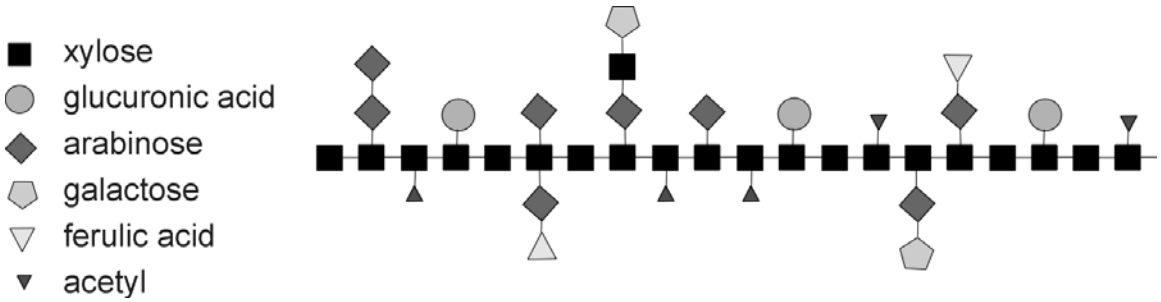


Figure 2. Schematic representation of cereal arabinoxylan

1.2.3 Minor constituents of cereal arabinoxylans

Esters

In cereals, acetic acid and cinnamic acid derivatives are present as minor constituents, ester-linked to the xylan back bone and to the arabinose side chain respectively. The acetyl groups are esterified at positions O-2 and O-3 of the xylopyranoid ring. They are known to modulate the properties of arabinoxylans (Jelaca and Hlynka, 1971). Cinnamic acid derivatives such as ferulic acid, *p*-coumaric acid and caffeic acid are ester-linked to the 5^{th} hydroxyl group of arabinose.

Functions of cinnamic acid esters

Cinnamic acid esters are biosynthesized through the shikimic acid pathway (Dixon and Paiva, 1995). They are present in many plants in significant quantities especially enriched in agro-industrial derived by-products, such as cereal brans and pulse husks (Table 2). They have been implicated in the regulation of cellular expansion and plant defense and they reduce the digestibility of cell walls both *invitro* and *invivo* by restricting accessibility to the cell wall degrading enzymes. Phenolic components of the plant cell wall,

especially *p*-coumaric acid and *p*-hydroxybenzaldehyde, inhibit the growth of rumen microorganisms. Phenolic acids derived from the plant cell wall have long been used as food preservatives to inhibit microbial growth. They are known to exhibit antiplatelet, antioxidant, antinflammatory, antimicrobial, antiviral, antiglycemic and oestrogenic activities, suggesting their potential role in the prevention of coronary heart diseases and cancer (Hertog et al., 1993, Jang et al., 1997).

Table 2 Ferulic acid content in some of the agro-industrial by-products (on w/w dry weight basis)

Agro-industrial by-products	Ferulic acid content (%)	Reference
Wheat bran	0.8	Ralet et al., 1990
Rice bran	0.9	Shibuya, 1984
Ragi bran	1.5	McDonough et al., 1986
Maize bran	3.0	Saulnier et al., 1995
Barley spent grain	0.3	Bartolome et al., 1997
Sugar beet pulp	0.8	Bonnin et al., 2001

Phenolic acids, both benzoic acid/cinnamic acid derivatives (Figure 3) are known to act as antioxidants not only because of their ability to donate hydrogen or electrons but also because of their stable radical intermediates, which prevent the oxidation of various food ingredients, particularly fatty acids and oils (Subba Rao and Muralikrishna, 2002). Out of the various bound cinnamic acids present in the cell wall, ferulic acid is the most preponderant in cereals. A detailed

description pertaining to its nature, chemistry, occurrence and biological role have been enumerated below.

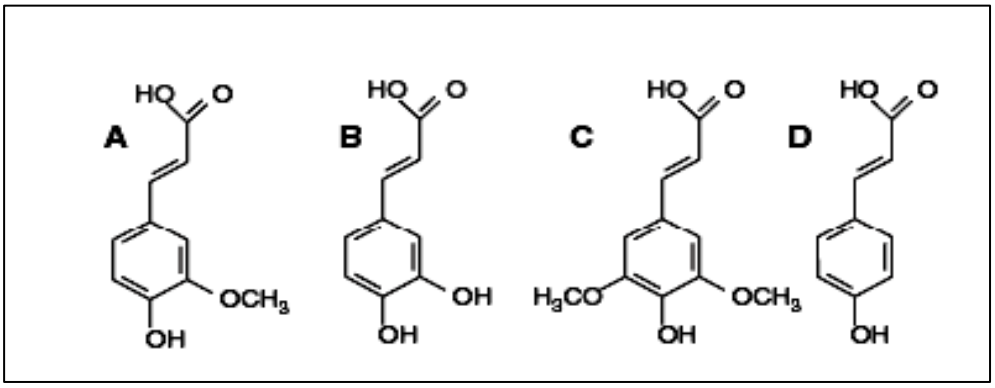


Figure 3. Structure of phenolic acids

A. Ferulic acid B. Caffeic acid C. Sinapic acid and D. *p*-coumaric acid

Nature, Chemistry and Biochemistry of ferulic acid

Ferulic acid (4-hydroxy-3-methoxycinnamic acid) is a phenylpropenoid derived from the cinnamic acid. It exists as two isomers: *cis* (a yellow oily liquid) and *trans* (crystalline). It is concentrated mainly in the aleurone layer (~ 75%) of the grain and comprises about 0.5% in wheat and 0.14% in barley grains.

Occurrence

Ferulic acid (FA) is generally esterified to 5^{th} hydroxyl group of α-L-arabinose side chains of arabinoxylans; at position O-2 of α-L-arabinose residues in arabinans; at position O-6 of β-galactose residues in pectic substances and galactans (Faulds and Williamson, 2003); and at position C-4 to α-D-xylopyranosyl residues in xyloglucans (Mueller-Harvey, 1986). The ferulic acid esterified to carbohydrates is found either unsubstituted or linked to another

esterified ferulic acid to form several types of diferuloyl bridges connecting two polysaccharide chains (Figure 4) (Iiyama et al., 1990; Ishii, 1997). Ferulic acid can also be involved in ether linkages with lignin components, thereby providing the covalent linkage between lignin and hemicellulose. These cross-links have a number of physical consequences, including influences on nutritional value and also on cell wall growth and mechanical strength (Benoit et al., 2006).

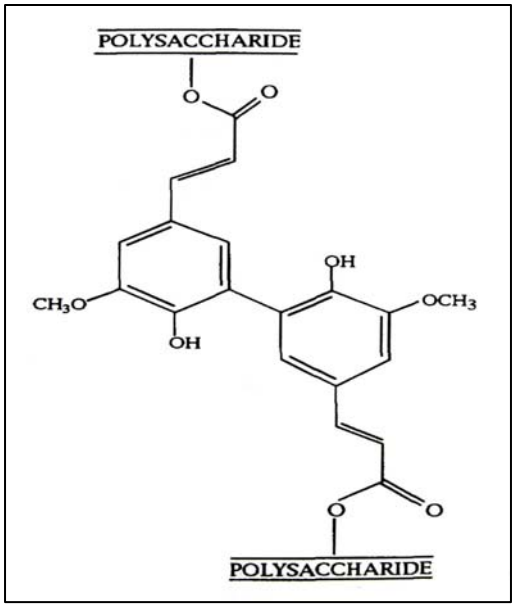


Figure 4. Structure of a diferulate cross linkage

Biological role *in vivo*

Feruic acid has many biological roles. (i) inhibition or prevention of cancers of the breast, colon, lung, stomach, and tongue; (ii) prevention of brain damage by Alzheimer's proteins; (iii) inhibiting prostate growth; (iv) strengthening of bone; (v) prevention of diabetes-induced free radical formation; (vi) expansion of pancreatic islets; (vii) reduction of elevated lipid, triglyceride, and blood glucose levels; (viii) lowering cholesterol production; (ix) prevention of hot

flashes; (x) prevention of free radical damage to cell membranes; (xi) protection of skin from aging effects of UV light; (xii) stimulation of the immune system and finally, (xiii) stimulation of retinal cell growth in degenerative retinal diseases (Fazary and Ju, 2007).

1.3 Arabinoxylan degrading enzymes

Enzymatic hydrolysis of cereal arabinoxylans requires the participation of several hydrolytic enzymes. These are classified into two groups based on the nature of the linkages that they cleave, primarily the carbohydrases and secondly the esterases, which act synergistically in order to increase digestibility of the plant cell wall. Many bacteria and fungi produce a wide range of hemicellulases as well as cinnamic acid esterases to degrade these cell wall polymers (De Vries and Visser, 2001).

The first group of enzymes hydrolases are involved in the hydrolysis of the glycosidic bonds of xylan. These include endoxylanase (EC 3.2.1.8), which randomly degrades the xylan backbone into xylooligosaccharides; β-xylosidase (EC 3.2.1.37), which cleaves the xylooligosaccharides to xylose from the nonreducing end (exo-cleavage); α-L-arabinofuranosidase (EC 3.2.1.55) which removes arabinose from the xylan backbone and α-D-glucuronidase (EC 3.2.1.1) removes 4-O-methylglucuronic acid substituents from the xylan backbone. The second group includes enzymes that cleave the ester linkages present in several biomolecules called esterases such as acetic acid esterase (EC 3.1.1.6) and cinnamic acid esterases (ferulic acid esterase, FAE, EC 3.1.1.73), which are listed in Table 3.

Table 3 Enzymes involved in the hydrolysis of arabinoxylans

Enzyme	Mode of action
Endo-xylanase	Hydrolyzes mainly interior β-1,4-xylose linkages of the xylan backbone releasing xylooligosaccharides
Exo-xylanase	Hydrolyzes mainly interior β-1,4-xylose linkages releasiong xylobiose
β-Xylosidase	Releases xylose from xylobiose and short chain xylooligosaccharides
α-Arabinofuranosidase	Hydrolyzes terminal nonreducing α-arabinofuranose from arabinoxylans
α –Glucuronidase	Releases glucuronic acid from glucuronoxylans
Acetylxylan esterase	Hydrolyzes acetylester bonds in acetyl xylans
Ferulic acid esterase	Hydrolyzes feruloylester bonds in xylans
p-Coumaric acid esterase	Hydrolyzes *p*-coumaryl ester bonds in xylans

Degradation of plant cell wall polysaccharides is of major importance in the food and feed, beverage, textile, paper and pulp industries, as well as in several other industrial production processes.

1.4. Esterases

Esterases (EC 3.1.1.x) are a group of hydrolytic enzymes catalyzing the cleavage and formation of ester bonds and are widely distributed in animals, plants and microorganisms. A considerable number of carboxyl esterases have been discovered and overexpressed (Bornscheuer, 2002). A number of

esterases were purified and characterized from animals, plants and microbes (Table 4). Generally most of the esterases act on a broad range of substrates.

Table 4 Sources of esterases

Microbial sources	Fungi:	*Aspergills* sp. (Faulds and Williamson, 1995), *Aureobasidium* sp. (Rumbold et al., 2003), *Talaromyces* sp. (Garcia Conesa et al., 2004), *Sporotrichum* sp. (Topakas et al., 2003a), *Penicillium* sp. (Donaghy and McKay, 1997).
	Yeast:	*Saccharomyces* sp. (Lomolino et al., 2003, *Candida* sp. (Ghosh et al., 1991), *Aureobasidium pullulans* (Rumbold et al., 2003).
	Bacteria:	*Streptomyces* sp. (Garcia et al., 1998), *Bacillus* sp. (Degrassi et al., 1998), *Pseudomonas* sp. (Kim et al., 2002).
Plant sources	Barley (Sancho et al., 2001; Humberstone and Briggs, 2002a and b), Finger millet (Upadhya et al., 1985; Madhavi Latha et al., 2007), Root nodules of *Medicago sativa* (Pringle and Dickstein, 2004), suspension of cultured rice cells (Tomoko et al., 2002)	
Animal sources	Mammalian liver, Brain, Serum, Dental composites (Finer et al., 2004)	

1.5. Ferulic acid esterases (FAE)

Out of several cinnamoyl esterases reported, ferulic acid esterases (FAEs) are widely studied from microbial sources. Ferulic acid esterases (FAEs; also known as ferulic acid esterases, cinnamoyl esterases and cinnamic acid hydrolases; EC 3.1.1.73) are a subclass of carboxylic acid esterases. Mode of action of esterases is described in Figure 5.

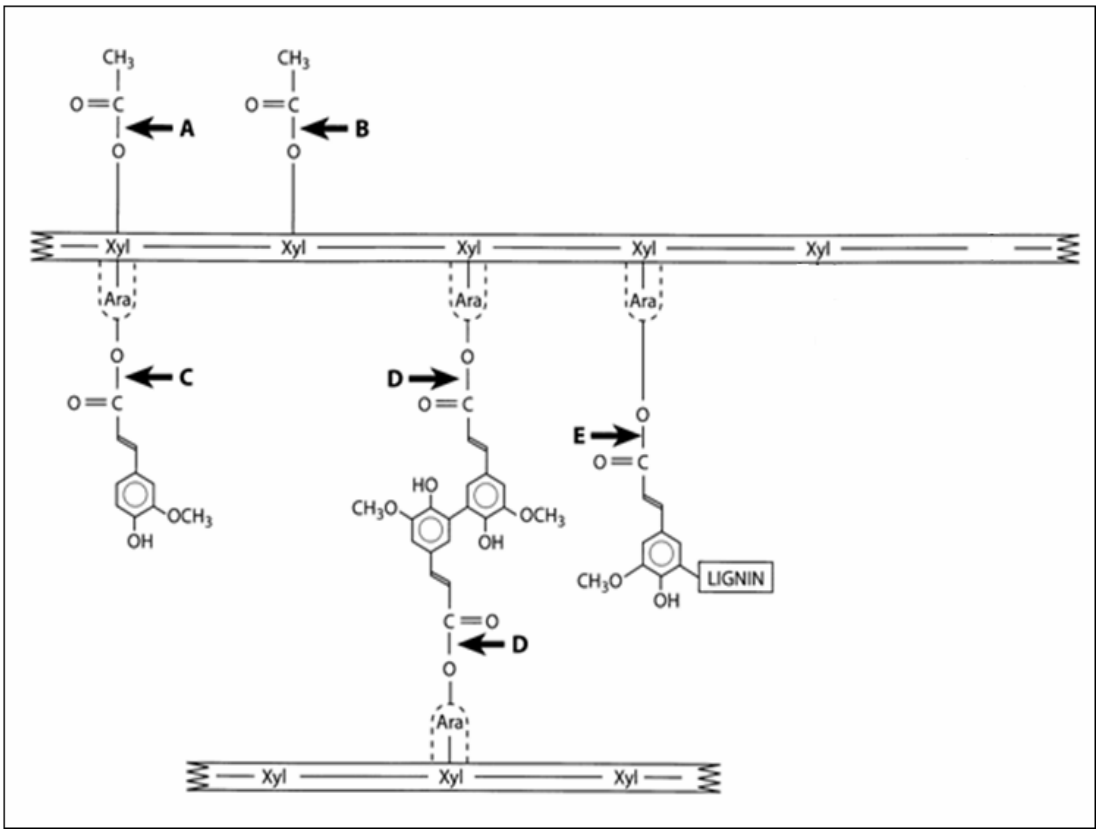


Figure 5. Mode of action of esterases; A, B –Acetyl xylan esterase; C, D, E –Ferulic acid esterase, (Source: Williamson et al., 1998)

1.5.1. Production

Microbial enzymes are often more useful than enzymes derived from plants or animals because of the great variety of catalytic activities available, the high yields possible, ease of genetic manipulation, regular supply due to absence of seasonal fluctuations and rapid growth of microorganisms on inexpensive media. Microbial enzymes are also more stable than their corresponding plant and animal enzymes and their production is more easy and convenient (Wiseman, 1995; Hasan et al., 2006). Hence the demand for industrial enzymes particularly of microbial origin is ever increasing owing to their applications in a wide variety of processes. They are produced in large quantity by a process called fermentation.

Fermentation can be defined as a process of breakdown of organic complex molecules into simpler ones, the process being catalyzed by microorganisms.

1.5.1.1. Solid state fermentation

Solid state fermentation (SSF) could be defined as any fermentation process allowing the growth of microorganisms on moist solid materials in the absence of free liquid. The solid material could be used as support (SSF) or as support and carbon energy. These fermentations have advantages of using simple media with cheaper, natural and abundantly available components such as agricultural waste materials, lower energy requirements, lower risk of contamination, nonrequirement of complex machinery and sophisticated control

system. However, industrial scale-up is much more difficult since heat removal in these systems is often a limiting step (Mitchell et al., 2000).

1.5.1.2. Submerged fermentation

Submerged fermentation (SMF) is carried out in the presence of free water. Though SSF is economically cheaper and simpler, it has some limitations such as the limited choice of microorganisms capable of growth under reduced moisture conditions, controlling and monitoring of parameters such as temperature, pH, humidity and air flow (Lonsane et al., 1985). In addition, the broth obtained from SSF is coloured probably due to black spores of *Aspergillus* strains and it is also mixed with a number of unwanted ingredients coming from solid substrates after extraction.

1.5.2. Classification of ferulic acid esterases

FAEs have been classified into four types based on their functionality (Table 5). The subclasses are A, B, C, and D depending on their (i) primary amino acid sequence identity (ii) substrate specificity towards hydroxycinnamic acid methyl esters and (iii) ability to release diferulic acids from model and complex substrates (iv) inducible plant cell wall materials.

Table 5 Classification of ferulic acid esterases

Parameter	Type A	Type B	Type C	Type D
	Aspergillus niger FaeA, TsFaeA, FoFaeA[a,c,d]	*Penicillium funiculosum* FaeB[e]	*A. niger* FaeB[b,c,d] *Talaromyces stipitatus* FaeC[f]	*Pen. Equi* EstA[g], *Pseudomonas fluorescens* XYLD[h]
Preferential induction medium	WB	SBP	SBP-WB	WB
Hydrolysis of methyl esters	MFA, MSA, MpCA	MFA, MpCA, MCA	MFA, MSA, MpCA, MCA	MFA, MSA, MpCA, MCA
Release of diferulic acid	Yes (5-5')	No	No	Yes (5-5')
Sequence similarity	Lipase	CE family 1 acetyl xylan esterase	Chlorogenate esterase, tannase	Xylanase

CE: carbohydrate esterase, MFA: Methyl ferulate, MSA: methyl sinapate, MCA: methyl caffeate, MpCA: methyl p-coumarate, CE: carbohydrate esterase, SBP: sugar beet pectin, WB: wheat bran.

[a] Faulds and Williamson, 1994; [b] Kroon et al., 1996; [c] Kroon et al., 1999; [d] Ralet et al., 1994; [e] Kroon et al., 2000; [f] Crepin et al., 2003; [g] Fillingham et al., 1999; [h] Ferreira et al., 1993.

Type A ferulic acid esterases tend to be induced during growth on cereal-derived substrates. These show preference for the phenolic moiety of the substrate containing methoxy substitutions, especially at meta-position(s), as occurs in ferulic acid and sinapinic acids. Type B FAEs shows complementary activity to type A esterases, showing preference to substrates containing one or

two hydroxyl substitutions as found in *p*-coumaric acid or caffeic acid (Kroon et al., 1997; Topakas et al., 2005). Type A and D FAEs in contrast to type B and C are also able to release low quantities of diFA. Type C and D FAEs exhibit broad specificity against synthetic hydroxycinnamic acids (ferulic, *p*-coumaric, caffeic and sinapinic acid) showing difference only in the ability to release 5-5' diFA (Crepin et al., 2004a; Crepin et al., 2004b; Vafiadi et al., 2006).

FAE releasing FA from wheat bran were first detected in cultures of *Streptomyces olivochromogenes* (MacKenzie et al., 1987). The members of the fungal genus *Aspergillus* are commonly used for the production of polysaccharide-degrading enzymes. This genus produces a wide spectrum of cell wall-degrading enzymes, allowing not only complete degradation of the polysaccharides but also tailored modifications by using specific enzymes purified from these fungi. Table 6 summarizes various FAEs produced under SSF and SMF conditions.

Table 6 Production of FAEs by microorganisms

Condition	Organism	Carbon source	Cultivation condition	Reference
SMF	*A. niger*	MFA	26 ^{0}C, 4 days	Koseki et al., 1998
	Au. pullulans	BX	30 ^{0}C, 60 h	Donaghy and McKay, 1995
	A. awamori	WB	30 ^{0}C, 3 days	Rumbold et al., 2003
	C. stercorarium	BX	65 ^{0}C	Donaghy et al., 2000
	F. oxysporum	CC	30 ^{0}C, 70 h	Topakas and Christakopoulos, 2004
	L. acidophilus	Glu + lac	37 ^{0}C, 60 h	Wang et al., 2004
	N. crassa	WB	30 ^{0}C, 3 days	Crepin et al., 2003
	O. PC-1g	CBG + S	39 ^{0}C, 5 days	Borneman et al., 1990
	P. funiculosum	SBP	25 ^{0}C, 6 days	Kroon et al., 2000
	St. avermitilis	DSWB	37 ^{0}C, 1-2 days	Garcia et al., 1998
	T. stipitatus	SBP	25 ^{0}C, 7 days	Garcia-Conesa et al., 2004
SSF	*A. niger*	WB, SBP	26 ^{0}C, 7 days	Donaghy and McKay, 1995
	P. brasilianum	BSG	30 ^{0}C, 196 h	Panagiotou et al., 2006
	P. expansum	WB	26 ^{0}C, 7 days	Donaghy and McKay, 1995
	P. pinophilum	OSX + WB	30 ^{0}C, 12 days	Castanares et al., 1992
	Sp. thermophile	WS	50 ^{0}C, 163 h	Topakas et al., 2003a

Abbreviations: SMF: Submerged fermentation, SSF: Solid state fermentation, WB: wheat bran, SBP: sugar beet pulp, WS: wheat straw, BSG: brewer's spent grain, Glu: glucose, Lac: lactose, DSWB: destarched wheat bran, CBG: coastal bermuda grass, S: sisal, BX: birchwood xylan, CC: corn cobs, MFA: methyl ferulate, OSX: oat spelt xylan.

A.: *Aspergillus, Au: Aureobasidium C.: Clostridium, F.: Fusarium, L.: Lactobacillus, N.: Neocallimastix*, O.: *Orpinomyces, P.: Penicillium, St.: Streptomyces, T.: Talaromyces, Sp.: Sporotrichum.*

1.5.3. Isolation of ferulic acid esterases

Esterases from plant sources and microbial source exist as either soluble (cytosolic) or membrane bound. Various buffers such as sodium acetate, sodium phosphate, Tris-HCl, MOPS buffer etc., are used to obtain the esterases in the free form while the extraction media containing PVPP, reduced glutathione and Triton X-100 dissolved in buffer have been used to isolate the membrane bound esterases (Humberstone and Briggs, 2000).

1.5.4. Purification of ferulic acid esterases

Fractional precipitation

The large volume of enzyme extracts can be concentrated by fractional precipitation using salts such as ammonium sulphate or organic solvents such as acetone or ethanol. This step has to be carried out at lower temperature to minimize the inactivation of the enzyme at high salt or organic solvent concentrations. Three types of FAEs were purified from mesophilic fungus *Talaromyces stipitatus* following ammonium sulphate precipitation as the first step of purification (Garcia-Conesa et al., 2004).

Chromatographic methods

Several chromatographic methods were used to purify esterases from cereals and microbes.

(a) Ion exchange chromatography

It is a form of adsorption chromatography in which proteins are separated on the basis of their net charge. Proteins display reversible electrostatic interactions with a charged stationary phase. An ion exchanger contains insoluble matrix containing charged groups

attached by covalent bonds. Choice of buffer pH, ionic strength plays an important role during ion exchange chromatography. DEAE-anion exchange resins have been used for the purification of most of the cereal and microbial esterases (Humberstone and Briggs, 2002a). DE-23 cellulose was used in the purification of esterases from Barley (Ward and Bamforth 2002). A ferulic acid esterase (FAE-II) was purified from *Fusarium oxysporum* by cation exchange chromatography using SP-Sepharose (Topakas et al., 2003b).

(b) Hydrophobic Interaction Chromatography

The hydrophobicity of proteins is exploited for purification of proteins in hydrophobic interaction chromatography. Three types of FAEs were purified by HIC using Butyl sepharose 4 from the culture supernatant of *Talaromyces stipitatus* (Garcia-Conesa et al., 2004). A ferulic acid esterase (FAE-III) was purified from *Aspergillus niger* by hydrophobic interaction chromatography using phenyl Sepharose column (Zhao & Sinnott, 2000).

(c) Size exclusion chromatography (SEC/GPC)

It is a technique in which the proteins are separated on the basis of their size. This technique is also applied to molecular weight determination of proteins. Sephacryl, Sephadex, sepharose etc. are the matrices used to purify esterases from cereals and microbes.

(d) Affinity chromatography

This exploits the specific, high affinity, noncovalent binding of a protein to another molecule, the ligand. A ligand 8-aminooctyl 5'-S-coniferyl-5'-deoxy-thio-α-L-arabinofuranoside has been synthesized, attached to cyanogen-bromide-activated

Sepharose and shown to be a selective affinity ligand for the ferulic acid esterase III of *Aspergillus niger* (Zhao and Sinnott, 2000).

(e) Chromatofocusing

This is an extension of ion-exchange chromatography, used for the separation of proteins/enzymes based on their isoelectric points (pI). It is carried out at very low ionic strength, using polymeric zwitterionic buffer species. It offers high resolution due to the separation based on difference in pI values varying even in the range of 0.05 units.

(f) HPLC

It is a non-destructive automated liquid chromatographic method with a capability for high-resolution separation of wide range of samples in a cumulative period. There is no laborious process for derivatization of samples and can be recovered after the chromatographic process. Separation is mainly by ion exchange or partition of compounds in a mobile phase. The compounds can be detected by either UV or refractive index detectors. Proteins are mostly purified by preparative reverse phase HPLC using C-18 column and are detected by UV detector both at 225 nm and 280 nm (Mahoney and Hermodson, 1980).

1.5.5 FAE assays

A number of FAE assay procedures have been developed based on the quantification of the liberated FA after the action of FAE. A variety of natural substrates such as feruloylated oligosaccharides (Fillingham et al., 1999; Blum et al., 2000) and de-starched wheat bran have been used to assay the enzymes. Synthetic phenolic acid methyl esters such as methyl esters of caffeate (MCA),

sinapate (MSA), ferulate (MFA), *p*-coumarate (MpCA), isoferulate, cinnamate, 3,4-dimethoxy cinnamate, vanillate, syringate, chlorogenic acid and feruloyl glycerol (Humberstone and Briggs, 2000) were also used as substrates to assay the esterases (Garcia-Conesa et al., 2004). Chromogenic substrates such as 4-Nitrophenyl 2-O-(E)-feruloyl-α-L-arabinofuranoside and 4-Nitrophenyl 5-O-(E)-feruloyl-α-L-arabinofuranoside with coupling of the action of the FAEs with α-L-arabinofuranosidase have been employed for differentiation of FAEs which differ in the affinity for 2-O-and 5-O-feruloylated α-L-Araf residues (Mastihubova et al., 2003) (Figure 6).

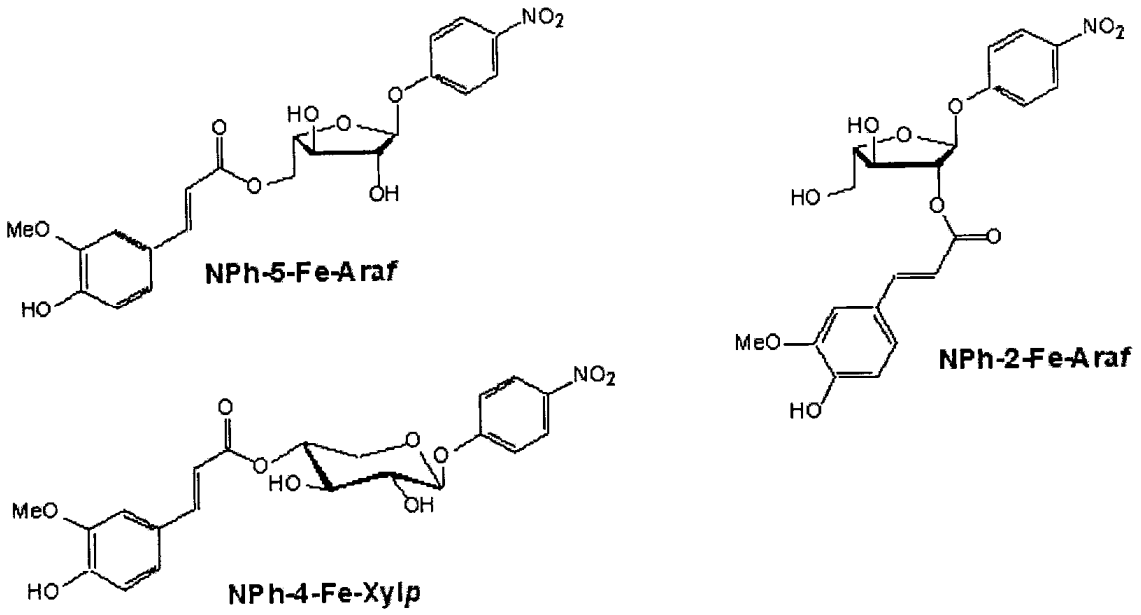


Figure 6. Structures of ferulic acid esterase substrates NPh-5-Fe-Araf, NPh-2-Fe-Araf, and NPh-4-Fe-Xylp.

Spectrophotometric analyses for cinnamoyl esterase activity described in the literature rely on the use of differences in spectral properties of free hydroxycinnamic

acid and its natural esters or their analogues. Such methods measure relatively low changes of absorbance and have not been adopted (Mastihuba et al., 2002).

These assay procedures require expensive equipments such as HPLC, GC, time consuming and are not suitable for rapid analysis of large numbers of samples in time, especially during isolation, purification and characterization of the enzyme. The methods wherein natural substrates are used require the isolation of natural substrates, which adds another laborious step (Topakas et al., 2007). Capillary zone electrophoresis and gas chromatography have also been applied to FAE assays using natural substrates, their analogs and hydroxycinnamic methyl esters, but these methods possess similar disadvantages.

A spectrophotometric assay using the substrate 4-nitrophenyl ferulate (4NPF) is based on the measurement of 4-nitrophenol (4NP) released upon enzyme action. This substrate was synthesized according to the chemoenzymatic procedure (Mastihubova et al., 2001), which involved four step reaction and the product yield was considerably low. An alternative method for the synthesis of 4NPF is reported and employed for the first time in the present study which is synthesized in a single step replacing the earlier four step procedure.

1.5.6 Purity criteria of esterases

Different methods are used to ascertain the homogeneity of enzymes which include gel electrophoresis (native and SDS-PAGE), activity staining, biological activity,

reverse phase HPLC using C_{18} column, capillary electrophoresis, N-terminal sequence etc. (Price, 1996).

Gel electrophoresis

This is carried out under native (based on charge) and denatured (based on the mass/size) conditions to determine the purity and molecular mass of the enzyme by comparing with the standard molecular weight protein markers. The activity staining of FAE was carried out by incubating the gel in an activity staining solution after electrophoresis. The activity staining of FAEs was carried out using MUTMAC (4-Methylumbelliferyl *p*-trimethyl ammoniocinnamate chloride), a fluorescent substrate dissolved in phosphate buffer (Ferreira et al., 1999). The esterase activity was determined by observing a visible or fluorescent band on the gel. The presence of carbohydrate in FAEs can be visualized by periodate sciff staining.

1.5.7. Different techniques used in the study of esterases to characterize the released products

Spectroscopic methods

Spectroscopic methods give more details about the structure and are often much simpler to carry out. Data obtained from these methods complement data obtained from chemical and enzymatic methods. Some of the most important spectroscopic methods are nuclear magnetic resonance spectroscopy (NMR), infra red (IR), Mass spectrometry (MS).

(a) Nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy is a rapid and nondestructive method for studying molecular structure including relative configuration, relative and absolute concentrations, and intermolecular interactions without modification or degradation. ^{13}C and ^{1}H NMR together give the molecular complexity and fine structure of a compound in solution and solid state. It can also be used to ascertain the purity of biomolecules such as proteins, peptides, polysaccharides etc. High resolution ^{13}C and ^{1}H NMR (gCOSY), NOESY, TOCSY, gHSOQC, HMBC) are nowadays used to elucidate the complete structure of a compound (Lavertu et al., 1991).

(b) Infrared spectroscopy

Infrared waves are absorbed by the vibrating chemical bonds in the polysaccharides giving characteristic IR spectra (vibrational) in the frequency range of 4000 to 400 cm^{-1}. The sample is prepared either as a pellet, by mixing with dry KBr, or as smear, by mixing with nujol (a paraffin oil). IR spectroscopy can be used for the detection of functional groups, configuration of sugar residues and to know the substitution pattern (Kacurakova et al., 1998).

(c) Mass spectrometry

Mass spectrometry is based on the principle that ions of different mass:charge (m/z) ratios are separated because they are differentially deflected by magnetic and electrostatic fields. Chemical ionization and electron ionization are the two important methods by which ionization can be achieved. In chemical ionization, molecular ions remain intact and spectra obtained are simple to interpret. On the other hand, electron ionization may result in complicated spectra because ions entering the analyzer may get fragmented by the high energy transferred from the bombarding electrons. Due to the

advent of mass spectrometric techniques such as matrix assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF-MS) and fast atom bombardment-mass spectrometry (FAB-MS), it is very easy to analyze samples without any derivatization. These methods have been used to determine the molecular mass and sequence of various biomolecules such as peptides, small proteins, glycolipids, oligosaccharides (Harrison and Cotter, 1990).

1.5.8 Physicochemical properties of FAEs

More than 30 FAEs have been purified and characterized from several microorganisms such as *Aspergillus niger, A. awamori, A. nidulans, A. oryzae, A. tubingensis, A. pullulans, Clostridium stercorarium, C. thermocellum, Fusarium oxysporum, F. proliferatum, Lactobacillus acidophilus, P. expansum, P. funiculosum, P. pinophilum, Talaromyces stipitatus*. The purified FAEs show significant variations in physical characteristics such as molecular weight, isoelectric point, optimal hydrolytic reaction conditions. Molecular weights range from 11 to 210 kDa. A cinnamoyl esterase *Aspergillus niger* CS 180 was a dimer and heavily glycosylated (Kroon et al., 1996). A ferulic acid esterase with 48% glycosylation from *Aureobasidium pullulans* was reported (Rumbold et al., 2003). Both monomeric and dimeric ferulic acid esterases were purified from *Aspergillus niger* CBS 120.49 (Faulds and Williamson, 1993). Glycosylated ferulic acid esterase (TsFaeC) was isolated from a mesophilic fungus *Talaromyces stipitatus* (Garcia-Conesa et al., 2004). Isoelectric points vary from 3 to 9.9. Microbial FAEs have a broad range of pH and temperature dependence. They exhibit optimal activities between pH 4-8 and temperature 30-65 ^{0}C.

Ferulic acid esterase from *Sporotrichum thermophile* showed a pH optimum of 8 and stable over a pH range of 6-8 for 1 h (Topakas, 2003a). The type B ferulic acid esterase from *Fusarium proliferatum* NRRL 26517 showed a pH optimum at 6.5-7.5 and it was stable over a broad pH range 5-9 (Shin and Chen., 2006). The high activity and stability in alkaline conditions of FAEs may be advantageous in biotechnological applications and especially in the treatment of alkaline pulp. Similar application of alkaline ferulic acid esterase was isolated from *Fusarium oxysporum* (Topakas and Christakopoulos, 2004), for which optimal FAE activity was observed at pH 7 and 50 ^{0}C with 68% and 55% activity at pH 8 and pH 9, respectively. The esterase was fully stable at pH 5-8 and upto 40 ^{0}C and retained 72% and 40% of its activity after 6 h at pH 9 and pH10, respectively. FAEs from different sources exhibited different temperature optima and is shown in Table 7.

The recombinant FAE (FAEB) from *Penicillium funiculosum* showed N-terminal amino acid sequence of ASLTQVNNFGDNPGSLQMYIYVPNKLASKP which has sequcence homology with acetyl xylan esterases from *Aspergillius* sp such as *A. awamori* (Koseki et al., 1997) and *A. niger* (De Graff et al., 1992).

Table 7 Temperature optimum of FAEs from different sources

Source	Temperature optimum	Reference
Cultured rice cells	45 ^{0}C	Tomoko et al., 2002
Ragi (*Eleusine coracana*)	45 ^{0}C	Madhavi Latha et al., 2007

Neocallimastix strain MC-2	40 ^{0}C	Borneman et al., 1992
Fusarium oxysporum	45 ^{0}C,	Topakas et al., 2003b
A. awamori	45 ^{0}C	McCrae et al., 1994
Fusarium proliferatum	50 ^{0}C	Shin and Chen, 2006
A. niger	50 ^{0}C	Kroon et al., 1996
Penicillium expansum	37 ^{0}C	Donaghy and McKay, 1997
A. niger	55-60 ^{0}C	De Vries et al., 1997
Au. pullulans,	60 ^{0}C	Rumbold et al., 2003
Sporotrichum thermophile	60 ^{0}C	Topakas et al., 2003a
Penicillium pinophilum	55 ^{0}C	Castanares et al., 1992
Clostridium stercorarium	65 ^{0}C	Donaghy et al., 2000

1.5.9 Mechanism of action

Catalytic center of FAE: The α/β hydrolase fold of FAE contains the classical Ser-His-Asp “catalytic triad” first observed in the structure of the serine protease chymotrypsin (Figure 7). The catalytic triad of FAE comprises residues Ser954, Asp1018, and His1058.

The mechanism for ester hydrolysis or formation is essentially the same for lipases and esterases and is composed of four steps: First, the substrate is bound to the active serine, yielding a tetrahedral intermediate stabilized by the catalytic His and Asp residues. Next, the alcohol is released and an acyl-enzyme complex is formed (Figure 8). Attack of a nucleophile (water in hydrolysis, alcohol or ester in (trans-) esterification) forms again a tetrahedral intermediate, which

after resolution yields the product (an acid or an ester) and free enzyme. Catalysis is performed via the formation and subsequent breakdown of a covalent acyl-enzyme intermediate via tetrahedral transition states sometimes known as the “tetrahedral intermediates.” (Source: Prates et al., 2001).

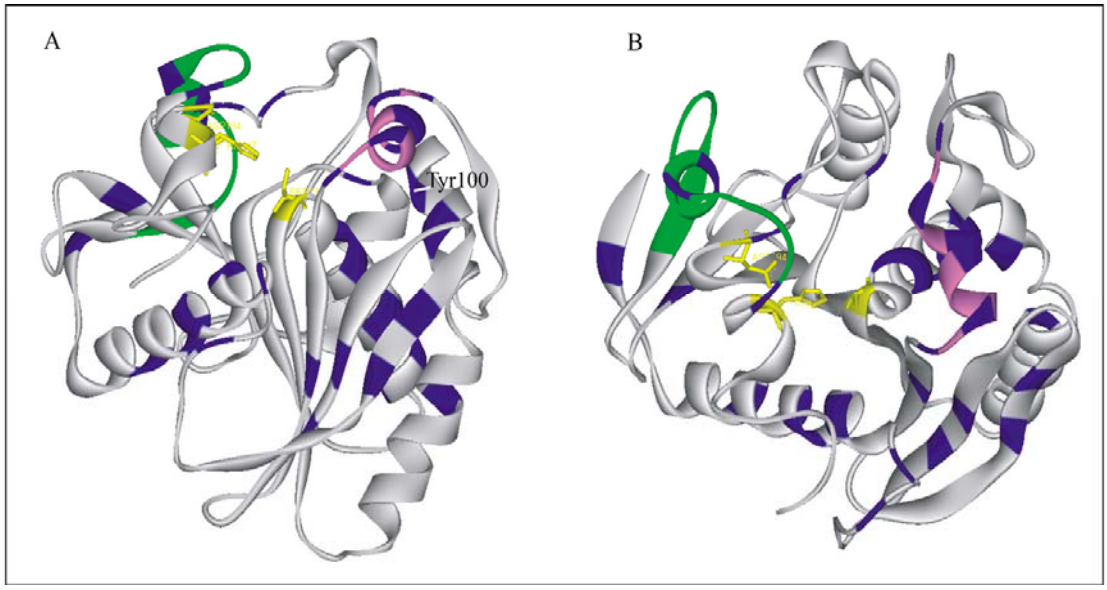


Figure 7. 3D structure of *A. niger* FAE (Source: Levasseur et al., 2006)

The catalytic triad is displayed in yellow. Two different orientations (A & B) are depicted.

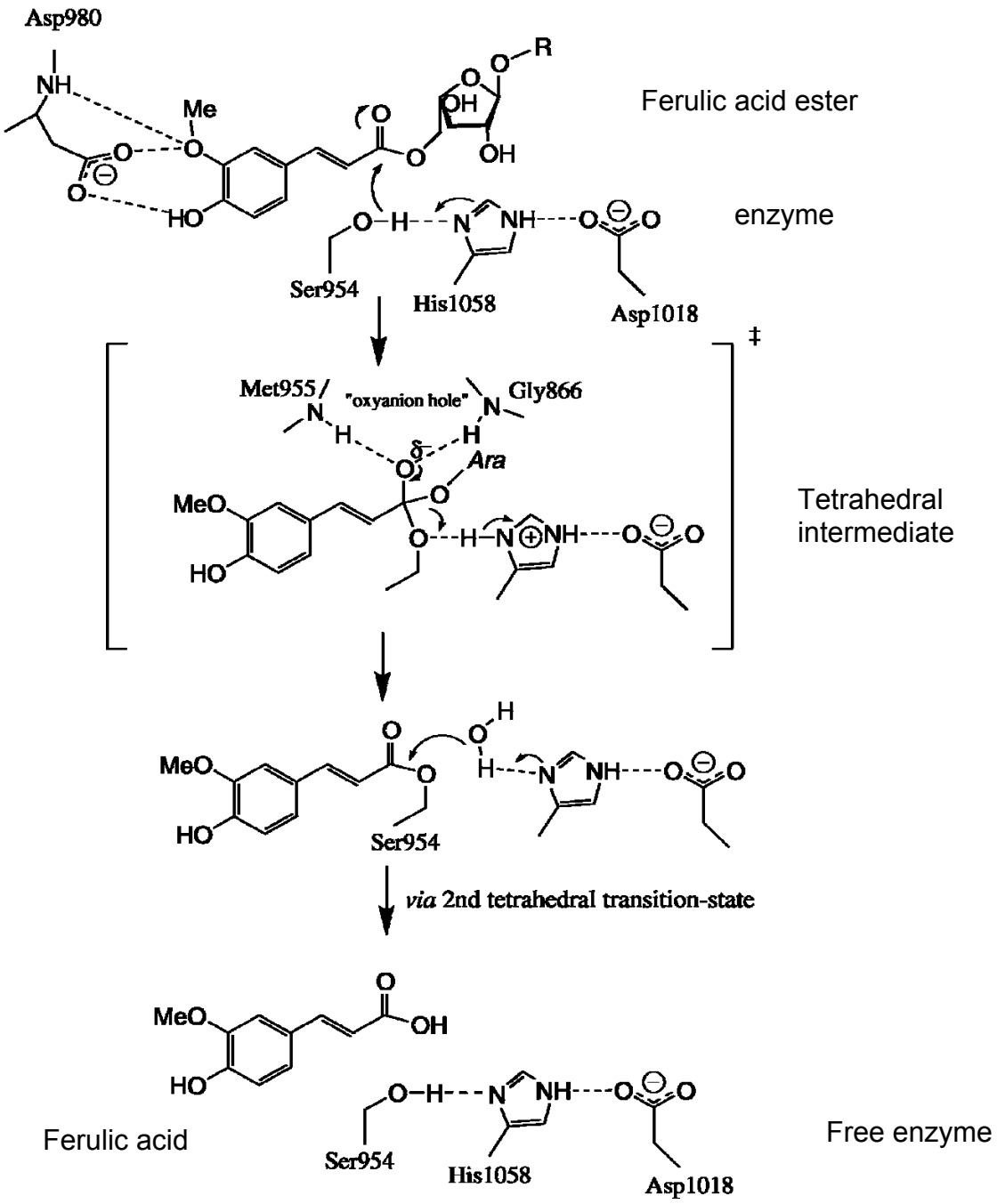


Figure 8. Catalytic mechanism of a ferulic acid esterase

(Source: Prates et al., 2001)

1.5.10. Difference between lipases and esterases

Esterases and lipases catalyze the hydrolysis of an ester bond resulting in the formation of an alcohol and carboxylic acid. Both belong to the family of serine hydrolases, share structural and functional characteristics including a catalytic triad containing serine, aspartate which form hydrogen bond with histidine and show sequence homology i.e. Gly-Xaa-Ser-Xaa-Gly.

Lipases can be distinguished from esterases by the phenomenon of interfacial activation, which was only observed for lipases. Esterases obey classical Michaelis-Menten kinetics, lipases need a minimum substrate concentration before high activity is observed. Furthermore, lipases prefer water-insoluble substrates, typically triglycerides composed of long-chain fatty acids, whereas esterases preferentially hydrolyze `simple' esters (e.g. ethyl acetate) and usually only triglycerides bearing fatty acids shorter than C6 (Table 8). Both enzymes have been shown to be stable and active in organic solvents, but this feature is more pronounced with lipases. Lipases show maximum activity at pH values around 8, whereas esterases are active in the pH range around 6.

Lipases can be distinguished from carboxyl esterases by their substrate specificity, using *p*-nitrophenyl palmitate (cleaved by lipases) vs. *p*-nitrophenyl butyrate (cleaved by esterases and sometimes also by lipases).

Table 8 Differences between lipases and carboxyl esterases

Property	Lipase	Esterase
Preferred substrates	Triglycerides (long chain), secondary alcohols	Simple esters, triglycerides (short-chain)
Interfacial activation	Yes	No
Substrate hydrophobicity	High	High to low
Enantioselectivity	Usually high	High to low to zero
Solvent stability	High	High to low

Source: Bornscheuer, 2002

1.5.11. Application of FAE in biotechnology

Release of FA: FAEs have potential application in obtaining FA from agro-industrial wastes, produced by milling, brewing and sugar industries. Ferulic acid can be used as an antioxidant toward peroxynitrite, low density lipoprotein *in vitro*. FA is used as feedstock for the biocatalytic conversion into other valuable molecules such as styrenes, polymers, epoxides alkylbenzenes, vanillic acid derivatives, protocatechuic acid-related catechols, guaiacol, catechol and vanillin (Falconnier et al., 1994).

Production of fuel ethanol: The utilization of hemicellulose sugars is essential for efficient and cost-effective conversion of lignocellulosic material to fuel ethanol (Lesage-Meessen et al., 1996).

Farming Industry: Pretreatment of low-value waste products of the milling industries such as wheat, maize and rice bran with enzymatic cocktails

containing FAEs can increase the digestibility and the calorific value (Gasson et al., 1998).

Use of FAEs as biosynthetic tools: Various hydroxycinnamic acids (ferulic acid, *p*-coumaric acid, caffeic acid, sinapinic acid) have widespread industrial potential by virtue of their antioxidant properties. The modification of these compounds to make them more lipophilic, via esterification with aliphatic alcohols, lipases and FAEs act as biosynthetic catalysts for enzymatic esterification. Enzymatic esterification offers an alternative to the poor selectivity of chemical synthesis. Esterification could take place by lipases only if the aromatic ring is not para-hydroxylated and the lateral chain is saturated. Therefore, enzymatic esterification of cinnamoyl substrates can be obtained by using only FAEs (Topakas et al., 2005).

FAEs benefit microorganisms : Some of the ester-linked substituents on plant cell wall polysaccharides retard or inhibit microbial infection. Microbial esterases increase the chances of successful infection of a plant tissue by removal of the methoxyl side chains. Microbial esterases also increase the rate at which main-chain degrading enzymes hydrolyze the polysaccharide backbone, by removing groups which prevent access of the substrate to the active site by steric hindrance.

FAEs as analytical aid: Esterases have potential as analytical aids in modern carbohydrate chemistry. In combination with other plant-cell-wall-degrading enzymes, the esterases will provide important tools in understanding the fine

structure and linkage patterns which exist in the plant cell wall (Panda and Gowrishankar, 2005; Williamson et al., 1998).

1.6. Recombinant FAEs

The esterase genes of a number of microbes were cloned and expressed in *Escherichia coli* for the large-scale production of recombinant esterases. Various microbial FAE genes such as *Aspergillus niger*, *Neurospora crassa*, *Cellvibrio japonicus*, *Butyrivibrio fibrisolvens* etc has been cloned and expressed in *E. coli*. The FAE (III) from *Aspergillus niger* was encoded by faeA gene (De Vries, 2003).

1.7. Scope of the present investigation

Polysaccharides such as arabinoxylans, 1,3/1,4-β-D-glucans, arabinogalacto-proteins and lignocellulosic complexes are the major constituents of cell walls of cereals as well as pulses. Phenolic acids and their derivatives such as ferulic acid (4-hydroxy-3-methoxycinnamic acid), *p*-coumaric acid (4-hydroxycinnamic acid) are esterified to the 5^{I} –OH group of arabinose residues present as side chains of xylans. Complete degradation of the plant cell walls requires synergistic action between main chain degrading enzyme such as endo-β-(1,4)-xylanase and accessory enzyme such as ferulic acid esterase which remove various substituents such as ferulic acid. The removal of phenolic acid substituents is important not only with respect to the degradation of plant cell walls, but also for obtaining bioactive compounds such as ferulic acid, *p*-coumaric acids which have various biotechnological applications. Ferulic acid is the most abundant hydroxycinnamic acid present in the cell walls of cereals. Cereal brans, one of the major byproducts of the milling industries are renewable source of ferulic acid. Ferulic acid (4-hydroxy-3-methoxycinnamic acid), due to its structural similarity, is an ideal potential precursor for the biotechnological production of vanillin (4-hydroxy-3-methoxy benzaldehyde), a widely used flavour in the food industry. Biotechnologically produced vanillin can be an alternate approach towards high demand for vanillin in the market, in addition to the one produced by synthetic process as well as the natural vanillin obtained from vanilla pods.

Ferulic acid esterases (FAE) (3.1.1.73), a subclass of the carboxylic ester hydrolases (EC 3.1.1.1) are responsible for the hydrolysis of the ester linkage present between hydroxycinnamic acids and sugars and their subsequent release for further utilization as bioactive compounds. These ferulic acid esterases have a number of biotechnological applications such as isolation of bioactive compounds from agro wastes, pulp and paper industries, production of fuel ethanol, synthetic tool to synthesize novel esters. Due to biotechnological importance of ferulic acid esterases, these enzymes have been studied from a large number of microbial sources (fungal and bacterial sources). There is an increased research effort in finding newer sources of FAE with novel properties. Hence a detailed study is envisaged to optimize the growth conditions for improved levels of ferulic acid esterase from a strain of *Aspergillus niger* (CFR 1105), an industrially important microorganism grown on cereal brans.

The major focus of the present work was to study ferulic acid esterase from a strain of *Aspergillus niger* (CFR 1105) with the following objectives.

3. Optimization of growth conditions of *Aspergillus niger* CFR 1105 for the enrichment of ferulic acid esterase.

4. Purification and characterization of ferulic acid esterase isolated from *Aspergillus niger* CFR 1105 and study of its kinetic parameters.

a. General

- All the results are average values of minimum of three experiments, unless otherwise mentioned.
- Reagent preparations and extractions were carried out using double glass-distilled water.
- Room temperature was ~ 25 ± 2 ^{0}C.
- Boiling temperature was ~ 95 ^{0}C, unless otherwise mentioned.
- Concentrations / evaporation of samples was carried out by using Buchi Rotavapor (RE111), Switzerland with a water bath temperature ranging from 30 to 40 ^{0}C.
- Colorimetric and spectrophotometric readings of test samples/solutions with appropriate controls were taken using Shimadzu double beam spectrophotometer (UV-160 A), Japan.
- Lyophilization was carried out using Virtis Freeze Mobile (12SL) at -60 ^{0}C, Germany.
- Centrifugation of samples was carried out either in Sigma (202 C), Hermle (Z 320 K) or Remi (RC 8) centrifuges, Bangalore or Hitachi Koki, Japan centrifuges.
- Autoclaving was done at ~ 121 ^{0}C, ~ 15 lbs for ~ 20 min.
- Dialysis against double distilled water or buffer was carried out at ~4 ^{0}C by using dialysis bags with a cut off range ~ 8kDa.

2.2. Materials

Cereal brans such as wheat and rice were obtained in bulk quantity from a local market. Ragi seeds were milled at the Department of Grain Science and technology, CFTRI to obtain the bran with a particle size of around 2 mm.

2.3. Chemicals

Chemicals used in the present investigation were obtained from various agencies/ companies as mentioned below.

2.3.1. Sigma Chemical Company, St. Louis, USA:

Lipase (*Candida rugosa*), Lipase (*Candida cylindracea*), Esterase (porcine liver), Larchwood xylan, Laminarin, p-nitrophenyl ferulate, *p*-nitrophenyl acetate, *p*-nitrophenyl butyrate, *p*-nitrophenyl β-D-galactopyranoside, *p*-nitrophenyl α-L-arabinofuranoside, *p*-nitrophenyl β-D-mannopyranoside, *p*-nitrophenyl β-D-xylopyranoside. Gel matrices and ion exchange resins: Sephacryl 100-HR, DEAE-cellulose (0.99 meq/g), Amberlite IR 120-P, $CDCl_3$, trifluoroacetic acid (TFA), ferulic acid, 4-nitrophenol, dicyclohexylcarbodiimide (DCC).

2.3.2. Other sources

Lipase (Zeus Biotech, India, *Aspergillus niger*), Hemicellulase (Biocon India, fungi), Cellulase (Novozyme, Viscozyme L), Protein molecular weight markers were obtained from Genei, Bangalore, India. Pectinase ((Fluka, *Aspergillus niger*),

Sugar standards (fucose, rhamnose, arabinose, xylose, mannose, galactose, glucose and inositol) were from ICN Pharmaceuticals Inc., Life Sciences group, Cleveland, USA. Sodium borohydride was from Aldrich

Chemical Company, Milwaukee, USA. Pullulan and soluble starch were procured from SISCO Research Laboratory, India.

Microbiological culture media ingredients were obtained from HiMedia, Mumbai, India. Microbiological filters (0.2 μ) were from Millipore, Bangalore, India.

All other chemicals used were of analytical grade and were obtained from E-Merck, BDH and SRL (India).

2.4. Instruments

Following instruments were used for various experiments:

Orbital shaking Incubator: Alpha scientific company, Bangalore, India.

GC-15A, equipped with FID, from Shimadzu, Japan.

HPLC–LC 10A, equipped with RI and UV-photo diode array detectors, from Shimadzu, Japan.

Ion exchange and gel filtration column chromatography fractions were collected using Gilson FC 203 B fraction collector. Gradient elution of ion exchange chromatography was carried out using Pharmacia gradient mixer Uppsala, Sweden.

Broviga mini slab gel electrophoresis unit, Balaji Scientific Services, Chennai, India.

UV-visible Spectrophotometer (UVH 60 A) from Shimadzu, Japan.

ESI-MS by Alliance, Waters 2695 mass spectrometer.

IR-Nicolet 5700.

NMR-Bruker AMX 500 MHz Spectrometer from Bruker Biospin, Germany.

2.5. Columns

2.5.1. GLC analysis

OV-225 (Φ 1/8" x 8 ft), 3% on Chromosorb W (80-100 mesh) was obtained from Pierce Chemical Company, Rockford, Illinois, USA.

2.5.2. HPLC Analysis

Shimpack C_{18} column (Φ 4.6 mm x 250 mm) was obtained from Shimadzu Corporation, Tokyo, Japan.

2.6. Methods

2.6.1. Distillation / purification of solvents

Phenol, acetic anhydride, pyridine, DMSO, chloroform, and dioxane were purified by distilling in all glass distillation apparatus following standard distillation protocols.

2.6.2. Activation / regeneration of diethylaminoethyl (DEAE)-cellulose

Anion exchanger was kept in water at room temperature for 24 h for swelling, followed by washing with distilled water to remove the fines. DEAE-cellulose was treated with HCl (0.5 N) for 30 min at room temperature with occasional stirring using a glass rod. The excess acid was washed with water till it comes to neutral pH and subsequently treated with NaOH (0.5 M) as mentioned above. DEAE-cellulose was then thoroughly washed with water and preserved in Tris-HCl buffer (20 mM, 7.2 pH) at 4 ^{0}C.

2.6.3. Fungal strain and maintenance

Microorganisms such as *Aspergillus niger* CFR 1105, *Aspergillus oryzae* CFR 232 and *Rhizopus arrhizus* NCIM 997 were obtained from the stock culture

collection of Food Microbiology Department, Central Food Technological Research Institute, Mysore, India. The cultures were maintained at 6 ^{0}C on malt extract agar, with regular subculturing at an interval of 30 days.

2.6.4. Preparation of Inoculum

An inoculum of the various fungal cultures used in the experiment was prepared by harvesting a culture (5-day-old slants) of organisms with 20 ml of sterile distilled water. Spore counts (per milliliter) of *A. niger* CFR 1105, *A. oryzae* CFR 22 and *R. arrhizus* NCIM 997 were maintained at 1x10^8 cfu/ml.

2.6.5. Inoculation and culture conditions

2.6.5.1. Solid state fermentation (ssf)

Brans of wheat, rice and ragi in quantities of 10 g each, were taken in 500 ml Erlenmeyer conical flasks and to this distilled water (10 ml) was added and mixed well and autoclaved for 30 min, followedf by inoculation with 1 ml of fungal spore suspension. The water and inoculum used contributed 60% of the moisture level. The flasks containing inoculum were incubated at 30 ^{0}C for 24, 48, 72 and 96 h (Lonsane et al., 1985).

2.6.5.2. Submerged fermentation (smf)

Submerged fermentation (smf) was carried out in 500 ml Erlenmeyer flasks, with basal medium (100 ml), containing diammonium tartrate 1.842 g as the nitrogen source, yeast extract 0.5 g, KH_2PO_4 0.2 g, $CaCl_2.2H_2O$ 0.0132 g and $MgSO_4.7H_2O$ 0.5 g per litre. Wheat bran (10 g dry weight per 100 ml) were used as the natural carbon source and 2.5 g L^{-1} of maltose as starter. Cultures were inoculated with 1 ml spore suspension of *A. niger* CFR 1105 (1 x 10^8 spores/ml),

prepared by harvesting the spores from a 5-day old malt extract agar grown slant with sterile water and incubated at 30 ^{0}C, 100 rpm, up to a period of 7 days. A separate set of flasks with medium were used. The submerged fermentation broth of *A. niger* grown on wheat bran was filtered through muslin cloth and the culture filtrate was assayed for ferulic acid esterase activity (Asther et al, 2002).

2.6.6. Enzyme assays

Moldy brans of wheat, rice and ragi (2 g) were extracted with sodium acetate buffer (50 mM and pH 4.8) for 2 h, at 4 ^{0}C, centrifuged and supernatants were dialyzed with sodium acetate buffer (50 mM and pH 4.8) for 8 h at 4 ^{0}C. The supernatant was assayed for starch degrading enzymes such as amylase (EC 3.2.1.1 and EC 3.2.1.2) and pullulanase (EC 3.2.1.41) and cell wall degrading enzymes such as xylanase (EC 3.2.1.8) and 1,3/1,4-β-D-glucanase (EC 3.2.1.4) and ferulic acid esterase (EC 3.1.1.73). The enzyme activity was measured by estimating reducing sugar by the dinitrosalicylic acid method. The following substrates and conditions were employed for assaying the previously mentioned enzymes.

2.6.6.1. Amylase

Soluble starch (1%) in 50 mM sodium acetate buffer of pH 4.8 was incubated for 30 min with appropriately diluted enzyme at 45 ^{0}C. One unit of activity was defined as the amount of enzyme required to liberate 1 µmol of reducing sugar per minute under the assay conditions (Bernfeld et al., 1955).

2.6.6.2. Pullulanase

Pullulan (0.25%) in 100 mM sodium acetate buffer of pH 4.8 was incubated for 120 min with appropriately diluted enzyme at 35 ^{0}C. One unit of activity was defined as the amount of enzyme required to liberate 1 µmol of reducing sugar (maltotriose) per minute under the assay conditions (Norman, 1982).

2.6.6.3. Xylanase

Larchwood xylan (0.1%) in 100 mM sodium acetate buffer of pH 4.8 was incubated for 60 min with appropriately diluted enzyme at 50 ^{0}C. One unit of activity was defined as the amount of enzyme required to liberate 1 µmol of reducing sugar (xylose) per minute under the assay conditions (Cleemput et al., 1997).

2.6.6.4. 1,3 /1,4-β-D-Glucanase

Laminarin (0.1%) in 50 mM sodium acetate buffer of pH 4.8 was incubated for 240 min with appropriately diluted enzyme at 38 ^{0}C. One unit of activity was defined as the amount of enzyme required to liberate 1 µmol of reducing sugar (glucose) per minute under the assay conditions (Fink et al., 1988).

2.6.6.5. Ferulic acid esterase

2.6.6.5.1. HPLC method

One gram quantity of uninoculated and *A. niger* grown wheat and rice bran samples of varying incubation periods were extracted with extraction medium (6.6 ml), containing Tris (hydroxymethyl) aminomethane hydrochloride, 50 mM, pH 8.0, reduced glutathione, 25mM, Triton-X-100, 1% (wt/vol),

polyvinylpolypyrrolidone, 0.2 g for 2 h at 4 ^{0}C, centrifuged, dialyzed against tris buffer (50 mM, 8.0 pH). Ferulic acid esterase activities were assayed using ethyl ferulate as the substrate, incubating at 37 ^{0}C for 1 h (Donaghy et al., 1999). Reaction was stopped by the addition of methanol. Ferulic acid released was analyzed and quantified by HPLC using C-18 column (4.6 x 250 mm) with an eluant mixture consisting of water:acetic acid:methanol (isocratic, 70:5:25). Enzyme activity was defined as the increase in ferulic acid concentration per hour (mmol/h) (Humberstone and Briggs, 2000).

2.6.6.5.2. Spectrophotometric method

Synthesis of 4-nitrophenyl ferulate, spectrophotometric substrate

All the solvents and reagents employed in the synthesis were of analytical reagent grade. All solvents were purified and dried before use.

To ferulic acid (1 g; 5.15 mmol, m.p. 168-170 ^{0}C) dissolved in dry dioxane (10 ml), 4-nitrophenol (0.716 g; 5.15 mmol, m.p. 112-114 ^{0}C), dissolved in dioxane was added dropwise. The reaction mixture was made basic by addition of 1 ml pyridine and 1ml triethylamine. After stirring for 10 min, solid dicyclohexylcarbodiimide (1.18 g; 5.7 mmol) was added in portions of 200 mg at intervals of 3 min under nitrogen atmosphere. The reaction mixture was stirred under nitrogen atmosphere at 25 ^{0}C and monitored on TLC. The precipitate of dicyclohexyl urea (DCU) started appearing in the reaction mixture after 15 min. The progression of the reaction was assessed by the complete disappearance of the reactants by TLC on silica gel using 10% methanol in chloroform as the developing solvent. R_f values of ferulic acid, 4-nitrophenol and 4-nitrophenyl

ferulate were 0.39, 0.56 and 0.72 respectively. After completion of the reaction (8 h), the reaction mixture was filtered to separate DCU and filtrate was evaporated under reduced pressure. The product was purified by column chromatography using silica gel (100-200 mesh size), with chloroform as the solvent. Fractions, containing 4NPF (R_f, 0.72 on TLC) were pooled and evaporated. The final product was analyzed by HPLC on reverse phase C-18 column using methanol: water: TFA 70:30:0.1 with a flow rate of 1 ml/min at 340 nm. The absorbance measurements were recorded from 200 to 500 nm on a UV-visible spectrophotometer (UVH 60A).

Fourier transform infrared spectroscopy: FTIR spectra of 4-NPF was obtained from discs containing 2 mg of dry samples and 10 mg potassium bromide (KBr) ground together under dry conditions. The spectra were recorded using infrared spectrophotometer (Nicolet 5700) from 4000 cm^{-1} to 400 cm^{-1} (Kacurakova et al., 1998).

Nuclear magnetic resonance spectroscopy: ^{1}H and ^{13}C NMR spectra for the compounds were recorded on a Bruker Avance 500 MHz spectrometer using deuterated solvent, $CDCl_3$. Coupling constants (J values) are given in Hz. Mass spectral analysis of the synthesized compound was carried out using MS (Waters Q-Tof Ultima) in the ESI negative mode (Hoffmann et al., 1991; Hoffmann et al., 1992).

Assay of ferulic acid esterase: Dialyzed culture filtrate of *A. niger* grown on wheat bran was assayed for ferulic acid esterase activity, essentially following the procedure reported earlier (Mastihuba et al., 2002). The substrate solution

was prepared by mixing 9 vol of 0.1 M potassium phosphate buffer solution, pH 6.5, with 1 vol of 11 mM 4NPF in DMSO containing 2.5% Triton X-100, followed by immediate vortexing. The assay mixture comprising 1 vol of appropriately diluted enzyme and 10 vol of the substrate was incubated at 37 ^{0}C for 1 h. The final concentration of substrate in the assay mixture was 1 mM. The substrate blanks were also run to avoid the possible absorption by the substrate. The 4NP released after the enzyme action is measured spectrophotometrically at 410 nm and enzyme activities were calculated using 4-nitrophenol standard graph (R^2=0.999). Enzyme activity is expressed as µmole of 4NP released/h/mg protein.

Ferulic acid esterase activities, which are present as contaminants in few related commercially available enzyme preparations (lipase, esterase, pectinase, cellulase) were assayed under the conditions described above.

2.6.7. Colorimetric estimations

2.6.7.1. Total carbohydrate estimation To the sample (0.5 ml) containing carbohydrate, phenol (0.3 ml, 5%) followed by concentrated H_2SO_4 (1.8 ml) were added successively and mixed thoroughly. The absorbance was read at 480 nm after 20 min incubation at room temperature (Figure 9). Sugar content was determined by referring to the standard graph, prepared by using either D-glucose or D-xylose (10-50 µg/0.5 ml) (Dubois et al., 1956).

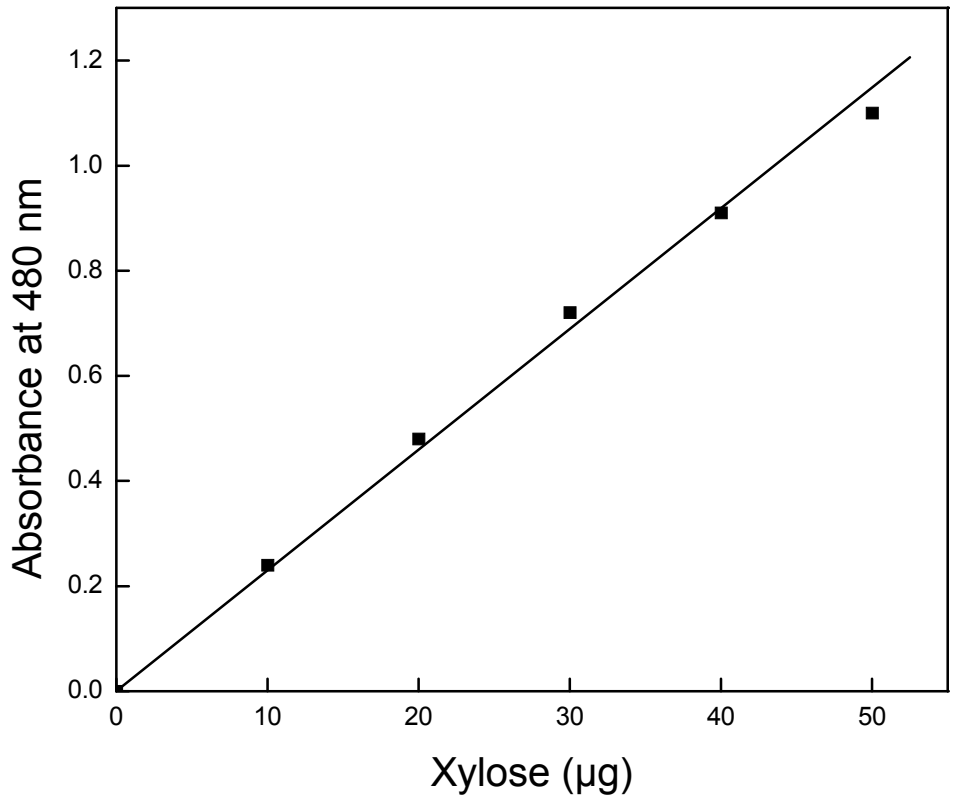


Figure 9. Standard graph for estimation of total sugar

2.6.7.2. Reducing sugar estimation by dinitrosalicylic acid method

Preparation of DNS reagent: Dinitrosalicylic acid (1 g) was dissolved in a solution containing sodium potassium tartrate (30 g) and 0.4 N NaOH and the content was made upto 100 ml with water. The reagent was filtered through a whatman No. 1 filter paper and stored in a brown bottle at 4 ^{0}C (Figure 10).

To the sample (1 ml) in a test tube, DNS reagent (1 ml) was added and incubated in a boiling water bath for 10 min. The content was cooled and diluted with water (2 ml) and the absorbance was read at 550 nm. Reducing sugar

content was determined by referring to the standard curve prepared by using D-glucose (0.1-1.0 mg/ml) (Miller, 1959).

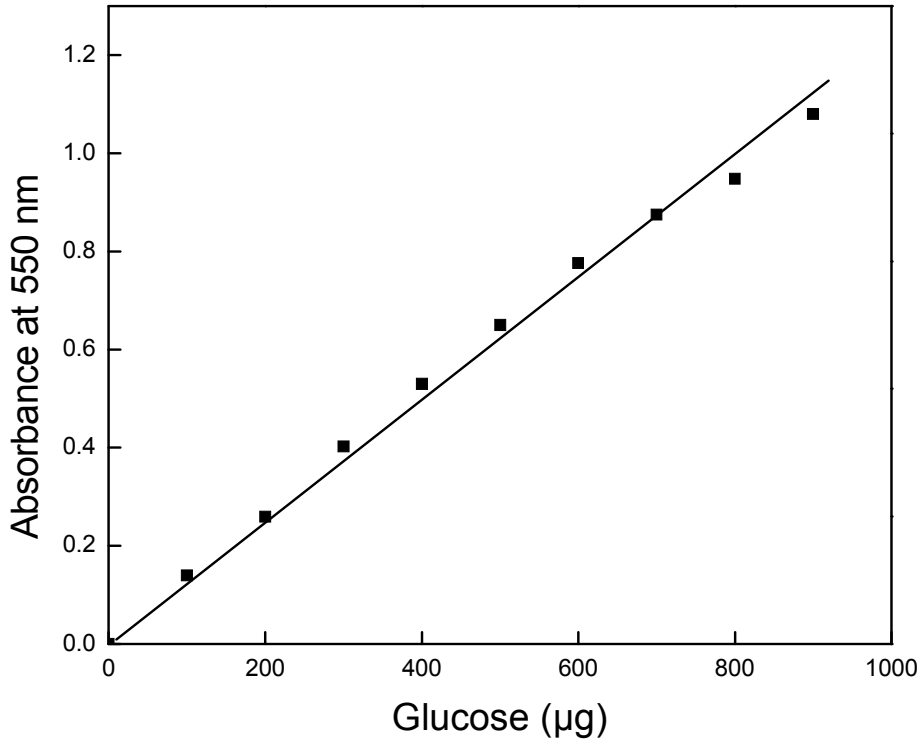


Figure 10. Standard graph for estimation of reducing sugar

2.6.7.3. Protein estimation

Bradford method

Preparation of Bradford reagent: Coomassie brilliant blue G-250 (10 mg) was dissolved in ethanol (5 ml, 95%) and o-phosphoric acid (10 ml, 85%). The solution was made upto 100 ml with water and filtered through Whatman No. 1 filter paper. To the sample (0.1 ml), Bradford reagent (1 ml) was added and mixed well. The absorbance was read at 595 nm. Protein content was determined by referring to the standard graph (Figure 11) prepared by using BSA (2-10 µg/0.1 ml) (Bradford, 1976).

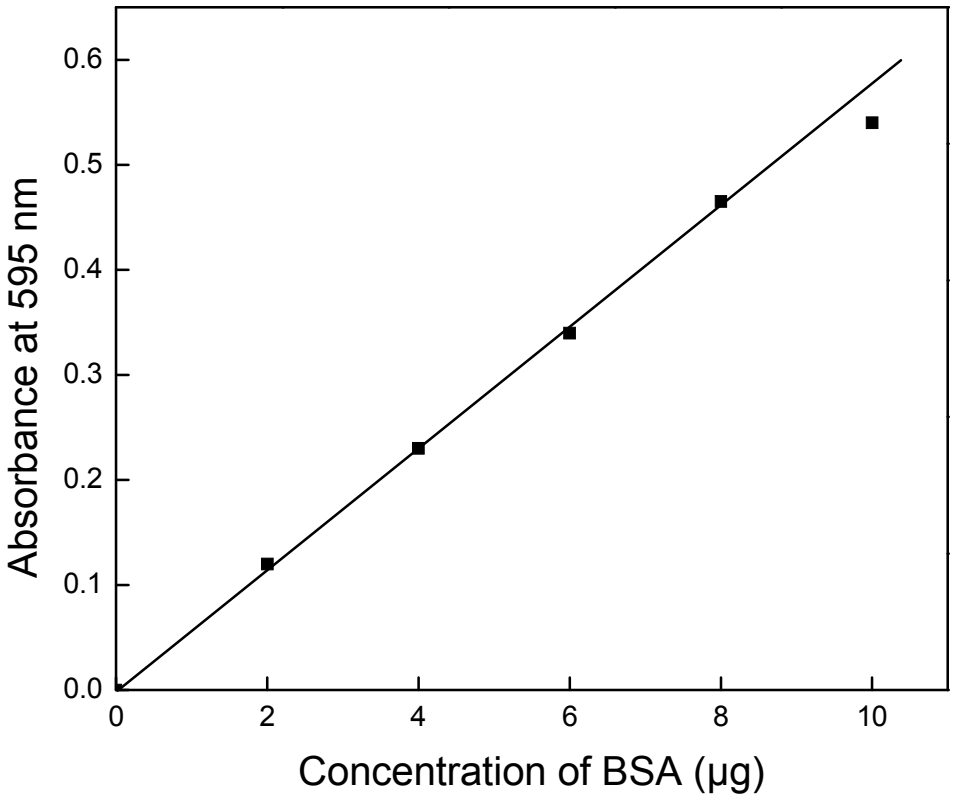


Figure 11. Standard graph for estimation of protein

Spectrophotometric method The presence of protein/peptide bonds in column fractions was monitored by taking absorbance at 280/220 nm respectively (Peterson, 1983).

2.6.7.4. *p*-nitrophenol estimation

Stock solution of *p*-nitrophenol (4NP) was prepared by dissolving in potassium phosphate buffer (6.5 pH). 4NP was taken in a concentration range (2.5-12.5 µg) was taken in a test tube and diluted to 1.0 ml with the above buffer. The absorbance was read at 410 nm taking appropriate blank (buffer). The standard graph of 4NP was used to quantify 4NP released from 4NPF for the assay of FAE (Figure 12).

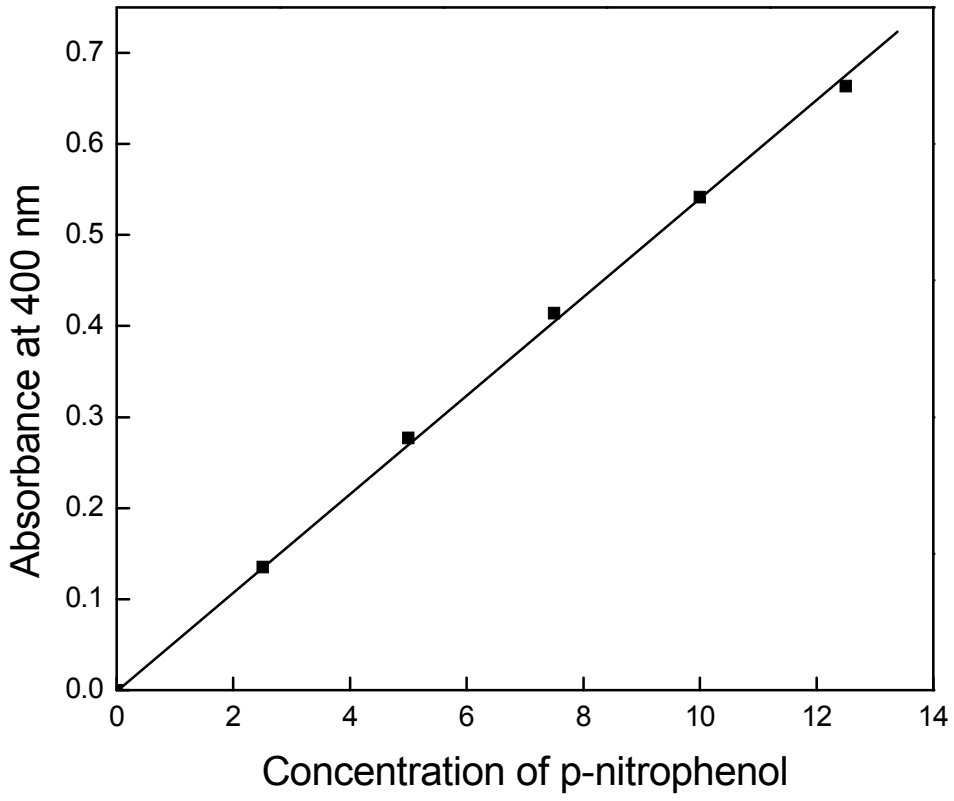


Figure 12. Standard graph for estimation of *p*-nitrophenol

2.6.8. Characterization of non-starch polysaccharides (NSP)

Hydrolysis of polysaccharides: Twenty milligram quantities of uninoculated and *A. niger* grown wheat and rice bran samples of varying incubation periods were suspended in water (0.5 ml) and solubilized with concentrated sulphuric acid (0.6 ml) at ice cold temperature, after which the concentration of sulphuric acid was brought down to 8% by the addition of water. The above mixture was refluxed in a water bath for 10–12 h; neutralized with barium carbonate, filtered, concentrated, deionized with Amberlite (IR 120-H^+) and reduced with sodium borohydride (Selvendran and O'Neill, 1987). Excess sodium borohydride was removed by adding acetic acid (3%). To the resultant glycitol, acetic anhydride and pyridine were added and kept in boiling water bath for 2 h. After acetylation excess reagents were removed by co-distillation with water and toluene. Alditol acetates were extracted with chloroform, filtered through glass wool and dried using nitrogen stream (Sawardekar et al., 1965). The component sugars were separated and identified on a 3% OV-225 (1/8" x 6") column using a Shimadzu 14-B gas liquid chromatograph equipped with flame ionization detector. The parameters used in GC analysis were 200 ^{0}C column temperature and 250 ^{0}C injector and detector port temperatures. Nitrogen (40 ml/min) was used as carrier gas. A sugar mixture consisting of rhamnose, fucose, arabinose, xylose, mannose, galactose and glucose was used as reference and inositol as internal standard (Sawardekar et al., 1965).

2.6.9. Isolation and characterization of bound phenolic acids: Bound phenolics were extracted according to the method of Nordkvist et al (1984). One

gram quantity of uninoculated and *A. niger* grown wheat and rice bran samples of varying incubation periods were defatted with chloroform and methanol (3 x 50 ml) in the ratio of 2:1. Sugars and free phenolics were removed with 70% alcohol (3 x 50 ml). The samples were dried sequentially with 80%, 90%, absolute alcohol and finally with diethyl ether. The dried samples were extracted with 1 M NaOH (2 x 100 ml, 2 h each) containing 0.5% sodium borohydride under nitrogen atmosphere and the clear supernatants were collected upon centrifugation and processed as described earlier (Subba Rao and Muralikrishna, 2001) and analyzed on a C-18 HPLC column (4.6 x 250 mm) using a diode array detector (operating at 280 and 320 nm) and a solvent system of water: acetic acid: methanol (isocratic, 80:5:15). For identification and quantification of phenolic acids present in the samples, standards such as protocatechuic acid, syringic acid, gentisic acid, vanillic acid, caffeic acid, ferulic acid and coumaric acids were used.

2.6.10. Isolation and characterization of free phenolic acids: One gram quantity of uninoculated and *A. niger* grown wheat and rice bran samples of varying incubation periods were defatted as mentioned in the case of bound phenolic acids and extracted with 70% ethanol (3 x 50 ml, 2 h each); and the supernatants obtained by centrifugation were concentrated, and their pH was adjusted in the range of 2–3 with 4 N HCl. Phenolic acids were separated by ethyl acetate phase separation (3 x 50 ml), and analyzed by HPLC as mentioned in the case of bound phenolic acids (Subba Rao and Muralikrishna, 2002).

2.6.11. Purification and characterization of ferulic acid esterase: The 5th day culture filtrate of *A. niger* CFR 1105 grown on wheat bran under submerged state fermentation was used as the source of ferulic acid esterase for further purification.

2.6.11.1. Fractionation by ammonium sulphate: The crude culture filtrate of *A. niger* grown on wheat bran was precipitated with 0-35, 35-60, 60-80% ammonium sulphate. Each addition of $(NH_4)_2SO_4$ was followed by a 4 h equilibration at 4 ^{0}C and centrifugation at 10,000 g at 4 ^{0}C. The 35-60% precipitate dissolved in 4 ml of 20 mM Tris-HCl buffer (pH 7.2) and dialyzed extensively with the same buffer.

2.6.11.2. Chromatographic methods

Ion exchange chromatography: Ion exchange chromatography was carried out on DEAE-cellulose column (Humberstone and Briggs, 2002a).

Purification of ferulic acid esterase: Dialyzed ferulic acid esterase enriched (ASF 35-60%) fraction (4.5 ml) was loaded onto DEAE-cellulose column (3 cm x 18 cm), that was equilibrated with 20 mM Tris-HCl buffer (pH 7.2) at a flow rate of 0.5 ml/min. The column was washed with the equilibrating buffer to remove unbound proteins. The ferulic acid esterase was eluted with a linear gradient of 0-0.5 M NaCl in the same buffer, at a flow rate of 0.3 ml/min. Fractions (3 ml each) having ferulic acid esterase activity were pooled and the major ferulic acid esterase peaks were further purified by gel filtration chromatography on a Sephacryl 100-HR column (1 cm x 110 cm), which was equilibrated with 20 mM Tris-HCl buffer (pH 7.2) and fractions (1.5 ml) were

collected and monitored for protein (280 nm) and FAE activity. Protein was quantified according to the Bradford method using bovine serum albumin (BSA) as standard. The active fractions were pooled, lyophilized and used for further studies.

2.6.11.3. Polyacrylamide Gel Electrophoresis (PAGE)

Vertical slab gel electrophoresis was carried out at room temperature (Laemmli, 1970).

Preparation of reagents for PAGE/SDS-PAGE

a) *Monomer*: Acrylamide (29.2 g) and Bis acrylamide (0.8 g) were dissolved in water (50 ml) and final volume was made to 100 ml, filtered and stored in a brown bottle at 4 ^{0}C.

b) *Separating gel buffer***:** Tris (18.5 g) was dissolved in water, pH was adjusted to 8.8 with 6 N HCl and made upto 100 ml and stored at 4 ^{0}C.

c) *Stacking gel buffer***:** Tris (3 g) was dissolved in water, pH was adjusted to 6.8 with 6 N HCl and made upto 50 ml and stored at 4 ^{0}C.

d) *Sodium dodecyl sulphate (SDS):* 10 g was dissolved in 100 ml water.

e) *Ammonium persulphate (APS):* 100 mg was dissolved in 1 ml of water.

f) *Tank buffer:* Tris (0.3 g) and glycine (1.44 g) and SDS (0.1 g) were dissolved in water and made upto 100 ml.

g) *Cocktail buffer:* Mixed 2.5 ml of solution (c), 2 ml solution (d), glycerol (2 ml), β-mercaptoethanol (1 ml) and bromophenol blue (0.01 %, 0.5 ml) and stored at 4 ^{0}C.

Staining reagents

a) Coomassie staining

a.1) *Staining reagent*: Coomassie brilliant blue R-250 (100 mg) was dissolved in methanol: acetic acid: water (100 ml, 12.5:7.5:30, v/v). The reagent was filtered and stored at room temperature.

a.2) *Destaining solution*: Methanol: acetic acid: water (12.5: 7.5: 30 v/v).

b) Silver staining

b.1) *Fixing*: Acetic acid (10 ml), methanol (30 ml) and formaldehyde (75 µl) were mixed and made upto 100 ml with water.

b.2) *Ethanol* (50 %)

b.3) *Pretreatment solution*: Sodium thiosulphate (200 mg) in 100 ml water.

b.4) *Impregnating solution*: Silver nitrate (200 mg) dissolved in water, added formaldehyde (25 µl) and made upto 100 ml.

b.5) *Developing solution*: Sodium carbonate (6 g) was dissolved in water containing sodium thiosulphate (1 mg), formaldehyde (25 µl) and made upto 100 ml.

b.6) *Stopping solution*: Acetic acid (7 ml) and methanol (10 ml) were mixed and made upto 100 ml.

Running gel (10 %) was prepared by mixing solution ‘a’ (2 ml), solution ‘b’ (1.5 ml) and water (2.4 ml). Solution ‘d’ (60 µl), TEMED (10 µl) and solution ‘e’ (60 µl) were added to this, mixed well and poured between the assembled glass plates with the edges sealed with 2 % agar. Gels were allowed to polymerize at room temperature for 2 h.

Stacking gel (4 %) was prepared by mixing solution ‘a’ (0.35 ml) and ‘c’ (0.5 ml) with water (1.17 ml). To this mixture was added solution‘d’ (20 µl), TEMED (10 µl) and solution ‘e’ (20 µl) and poured above the polymerized running gel. The polymerization was facilitated at room temperature for 2 h.

Protein samples (20 µg) were prepared by dissolving in solution ‘g’ (10 µl). This was heated in a boiling water bath for 3 min. Cooled samples were loaded (25 µl) on to the wells immersed in solution ‘f’ and were run at a constant voltage (50 V) for 3 h or until the tracking dye was just above the lower end of the gel.

2.6.11.4. Native PAGE

PAGE (10 %) under native conditions was carried out to evaluate the purity of ferulic acid esterases. The electrophoresis was carried out as described above without SDS and β-mercaptoethanol. Duplicate samples (25 µl) were loaded on the wells and run for 3 h at a constant voltage (50 V). The gels were stained for protein.

2.6.11.5. SDS-PAGE

SDS-PAGE was performed using 10% separation gel and 4% stacking gel, in presence of 0.1% sodium dodecyl sulfate. Samples were prepared in Tris-HCl buffer containing 1% SDS, 5% mercaptoethanol, 10% glycerol and boiled for 5 min. The gel was run at a constant voltage of 50 V. Following the run, the proteins were stained with 0.1% Coomassie brilliant blue for 5 h and destained by a mixture of 10 % acetic acid and 50% methanol in water.

Gel staining

After electrophoresis the gels were stained for protein by any one of the following methods.

a) Coomassie blue staining: After electrophoresis, gel was kept in solution (a.1) for 5 h sustained by solution (a.2).
b) Silver staining: Gel after electrophoresis was mixed in solution (b.1) for 45 min. followed by washing with solution (b.2) twice for 30 min each. Subsequently the gel was placed in solution (b.3) for 1 min. with continuous shaking. After washing thoroughly with water, the gel was kept in solution (b.4) for 20 min with shaking. The protein bands were visualized by treating with solution (b.5) for 10-15 min or till the bands appear. Staining was arrested by placing the gel in solution (b.6) and stored in 6% acetic acid (Wray et al., 1981).

2.6.11.6. Glycoprotein staining and estimation of carbohydrate content

Glycoprotein nature of purified enzymes was determined by performing periodic Acid-Schiff staining (Zacharius and Zell, 1969), after the SDS-PAGE. The carbohydrate content in purified ferulic acid esterase was estimated by phenol-sulfuric acid method using glucose as standard (Dubois et al, 1956).

2.6.11.7. Molecular weight determination by SDS-PAGE

The molecular weight of the purified ferulic acid esterase was determined by using a mixture containing following protein standards: lysozyme (14.3 kDa), soyabean trypsin inhibitor (20.1 kDa), carbonic anhydrase (29 kDa), ovalbumin (43 kDa), BSA (66 kDa) and phosphorylase b (97.4 kDa). Molecular weight was determined using the standard graph shown in Figure 13.

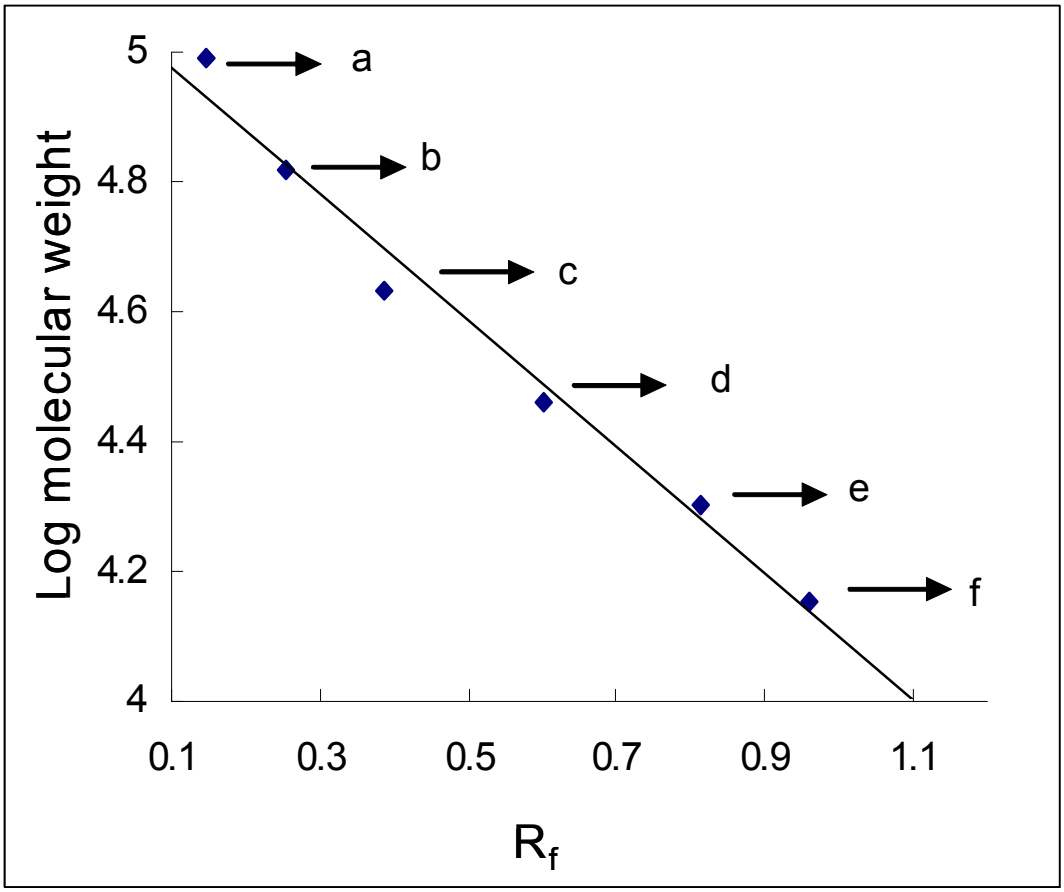


Figure 13. Standard graph for determination molecular weight by SDS-PAGE, protein molecular weight standard consisting of (a) phosphorylase-b (97.4 kDa); (b) bovine serum albumin (66 kDa); (c) ovalbumin (43 kDa); (d) carbonic anhydrase (29 kDa); (e) soyabean trypsin inhibitor (20.1 kDa); (f) lysozyme (14.3 kDa)

2.6.11.8. Molecular weight determination by gel filtration chromatography

In order to determine the molecular weight of purified FAE-1 and FAE-2 by using Sephacryl 100-HR, a calibration run was made. The void volume (V_0) was determined using blue dextran (2000 kDa). The log molecular weight of each standard was plotted against its V_e/V_0 (V_e-elution volume of particular standard as

shown in Figure 14. Following molecular weight standards were used for the column calibration: cytochrome c (12.4 kDa), carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa), alcohol dehydrogenase (150 kDa) (Whitaker, 1963).

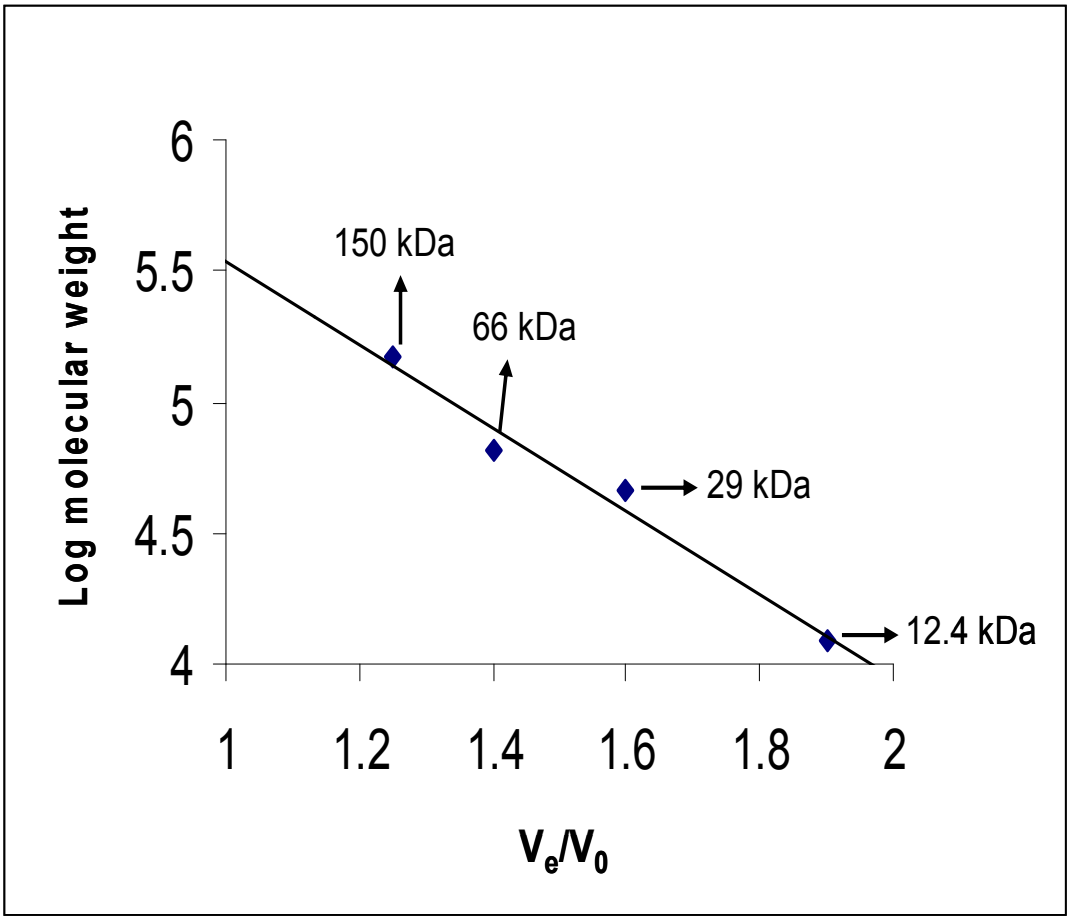


Figure 14. Standard graph for determination of molecular weight using Sephacryl 100-HR gel filtration chromatography, protein molecular weight standard: cytochrome c (12.4 kDa), carbonic anhydrase (29 kDa), BSA (66 kDa) and alcohol dehydrogenase (150 kDa).

2.6.11.9. ESI-MS of FAE

Mass spectra of the purified enzymes was measured by Alliance, Waters 2695 mass spectrometer in positive mode electro spray ionization. Capillary voltage was 3.5 kV, core voltage 100 V, source temperature 120 ^{0}C, disolvation temperature 300 ^{0}C, core gas (Argon) 50 lt h^{-1} and disolvation gas (nitrogen) 500 lt h^{-1} (Stapels et al., 2004).

2.6.12. Effect of pH

Determination of optimum pH: The optimum pH of purified FAEs was determined by assaying the activity at pH values ranging from pH 3.5-6.0 (50 mM, sodium acetate buffer), pH 6.0-8.0 (50 mM, sodium phosphate buffer) and pH 8-10 (50 mM, Tris-HCl buffer). The enzyme assay was carried out as described earlier. The maximum activity was taken as 100% and the relative activity was plotted against different pH values (Nirmala and Muralikrishna, 2003a).

Determination of pH Stability: To analyze the pH stability of purified FAEs, the enzyme samples were preincubated in pH 3.5-6.0 (50 mM, sodium acetate), pH 6.0-8.0 (50 mM, sodium phosphate) and pH 8-9.5 (50 mM, Tris-HCl), at different time intervals (15 min, 30 min, 45 min, 1 h, 2 h), and the residual activities were calculated. The original activity was taken as 100%, and relative activities were plotted against different time intervals (Nirmala and Muralikrishna, 2003b).

2.6.13. Effect of temperature

Determination of temperature optima: The purified FAEs were incubated with 4NPF (1 mM) in their respective pH optimum (50 mM, pH 9 and 6 respectively) in a temperature range of 30-65 ^{0}C, with an interval of 5 ^{0}C. The optimum activity was taken as 100%, and relative activities were plotted against different temperatures (Nirmala and Muralikrishna, 2003a).

Determination of thermal stability: The thermal stabilities of the purified FAEs were determined by preincubating the enzymes at temperatures of 30 to 65 ^{0}C, with an interval of 5 ^{0}C, for 30 minutes and performing the assay. The original activity was taken as 100%, and relative activities were plotted against different temperatures (Nirmala and Muralikrishna, 2003b).

2.6.14. Effect of substrate concentration

Purified FAE-1 and FAE-2 were incubated with different concentrations of 4NPF (0.1mM to 1mM) at their respective pH and temperature optima and their activities were measured. Initial velocities (V) were calculated for all substrate concentrations, and K_m and V_{max} values were calculated from a double reciprocal plot (Lineweaver and Burk, 1934).

2.6.15. Determination of substrate specificity

The activities of purified FAEs were determined by using 1mM *p*-nitrophenyl derivatives of different sugars and fatty acids as substrate. The following substrates were used: *p*-nitrophenyl ferulate, *p*-nitrophenyl acetate, *p*-nitrophenyl butyrate, *p*-nitrophenyl β-D-galactopyranoside, *p*-nitrophenyl α-L-

arabinofuranoside, *p*-nitrophenyl β-D-mannopyranoside, *p*-nitrophenyl β-D-xylopyranoside.

2.6.16. Effect of metal ions: Purified FAEs were pre-incubated with salts of metal ions such as Fe^{+2}, Ba^{+2}, Mg^{+2}, Hg^{+2} and Cd^{+2} at 5 mM concentration and their residual activities were measured. The enzyme activities without metal ions were taken as 100% and the relative activities determined in the presence of metal ions were calculated (Nirmala and Muralikrishna, 2003a).

2.6.17. Effect of group specific reagents: Purified FAEs were pre-incubated with PMSF and TLCK in sodium acetate buffer (50 mM, pH 6) at 40 ^{0}C and the residual activities were assayed. The enzyme activities without these group specific reagents were taken as 100%, and relative activities were calculated.

Ferulic acid esterases (FAEs), which hydrolyze ferulic acid esters, are important enzymes under both physiological and pathological conditions in plants (Williamson et al., 1998) and also have several industrial applications (Topakas et al., 2007, Panda and Gowrishankar 2005). Few methods have been reported for measuring this enzyme activity, but most of them are based on high-performance liquid chromatography (HPLC) techniques (Mastihuba et al., 2002; McCallum et al., 1991). Mastihuba and coworkers reported a spectrophotometric method for easy, quick, and routine assay using 4-nitrophenyl ferulate (4NPF) (Mastihuba et al., 2002). As 4NPF is not commercially available, they synthesized it by a chemoenzymatic procedure (Mastihubova et al., 2001). This method, involving four synthetic steps, also uses expensive lipase enzyme.

3.1. Synthesis of 4NPF by single step

We attempted to synthesize 4NPF by dehydrative coupling of ferulic acid and 4NP using dicyclohexylcarbodiimide (DCC) as reagent in a reaction as shown in Figure 15. Conditions of synthesis are described in detail under materials and methods (page 46).

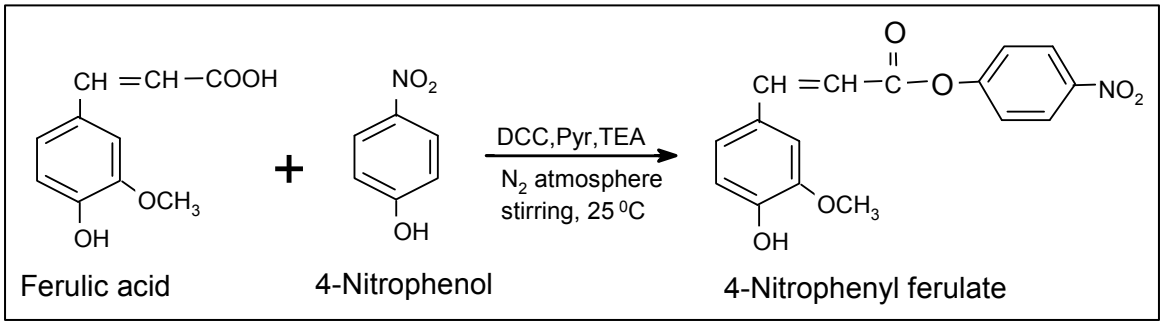


Figure 15. Scheme for the synthesis of 4-nitrophenyl ferulate

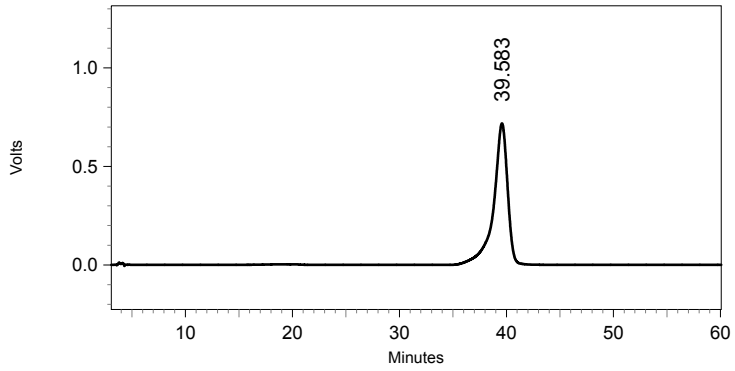
3.2. Characterization of 4NPF

The final product (50% yield) showed a melting point of 176–178 ^{0}C and a single peak on HPLC (reverse phase, C-18 column, methanol/water/ trifluoroacetic acid [TFA] 70:30:0.1, flow rate 1 ml/min) at 340 nm (Figure. 16). The ester formed was characterized by FT-IR. The compound showed –C=O stretching frequency at 1726 cm^{-1} indicating an ester bond, supported by -C(C=O)-O stretching frequency 1214 cm^{-1} of acetate. The N=O stretching at 1590 cm^{-1} indicated covalent linkage of ferulic acid to 4-NP (Figure. 17). The mass spectrometry (MS) analysis of the product [electrospray ionization (ESI)–MS] in the negative ion mode exhibited molecular mass ion at 314.3, corresponding to the molecular mass of 4NPF (Figure 18).

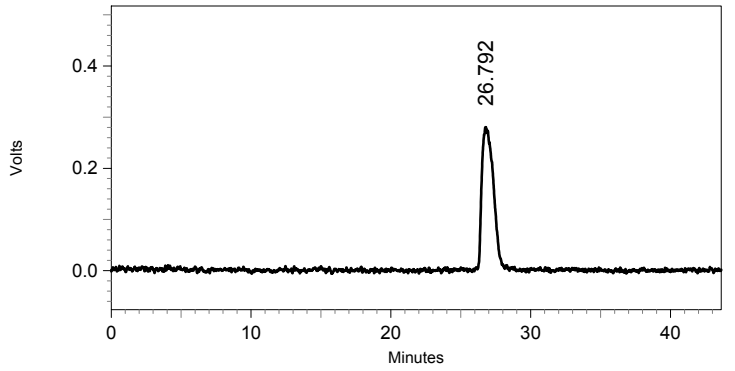
4NPF was further characterized by ^{1}H and ^{13}C nuclear magnetic resonance (NMR) using $CDCl_3$ (Table 9). The ^{1}H NMR of 4NPF showed signals at 3.94, 5.93, 6.45, 7.82, 6.95, 7.07, and 7.15 from ferulic acid and showed signals at 7.35 and 8.28 from 4NP (Figure 19). The absence of 9.57 (br, ^{1}H) in the spectra of 4NPF, which represents the –COOH proton of ferulic acid, confirmed the coupling of ferulic acid and 4NP. Similarly, whereas the carbonyl in ferulic acid appeared at 168.13, the product showed the signal at 164.30, corresponding to the ester carbonyl moiety. The ^{13}C spectrum of the product also showed methoxyl carbon signal at 55.71 as in ferulic acid (Figure 20). In addition to the two olefinic and six aromatic carbon signals from ferulic acid, the spectra contained an additional four signals for four aromatic carbons from the nitrophenol moiety.

Dehydrative coupling is a chemical reaction often employed in synthetic chemistry for the synthesis of esters. Such a strategy was adopted previously for the synthesis of *p*-nitrophenyl ester of piperic acid; however, this does not contain the phenolic functional group (Mishra et al., 2005a, Mishra et al., 2005b). In the current study, we were able to synthesize 4NPF by reaction of ferulic acid with 4NP, where the reaction conditions employed led to the ester preparation in good yield with little intermolecular interaction of the phenolic group with carboxyl moiety in ferulic acid. Accordingly, this methodology obviates the protection of phenolic group (Hatfield et al., 1991) and its subsequent deprotection in the final step by the expensive enzymatic process employed in the previous synthetic methodologies (Mastihuba et al., 2002). Also, the latter reaction, being slow and partial, necessitates the chromatographic separation of product from the starting material. Thus, the current method affords facile access to 4NPF, accurately modeling the ester linkage of ferulic acid to polysaccharides found in cell walls of plants and serving as an appropriate substrate for the hydrolytic action of ferulic acid esterases.

(a)



(b)



(c)

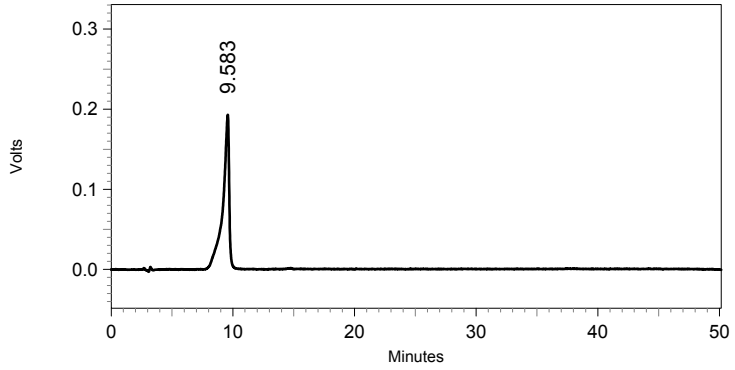


Figure 16. HPLC profiles of (a) ferulic acid, (b) 4-nitrophenol and (c) 4-nitrophenylferulate

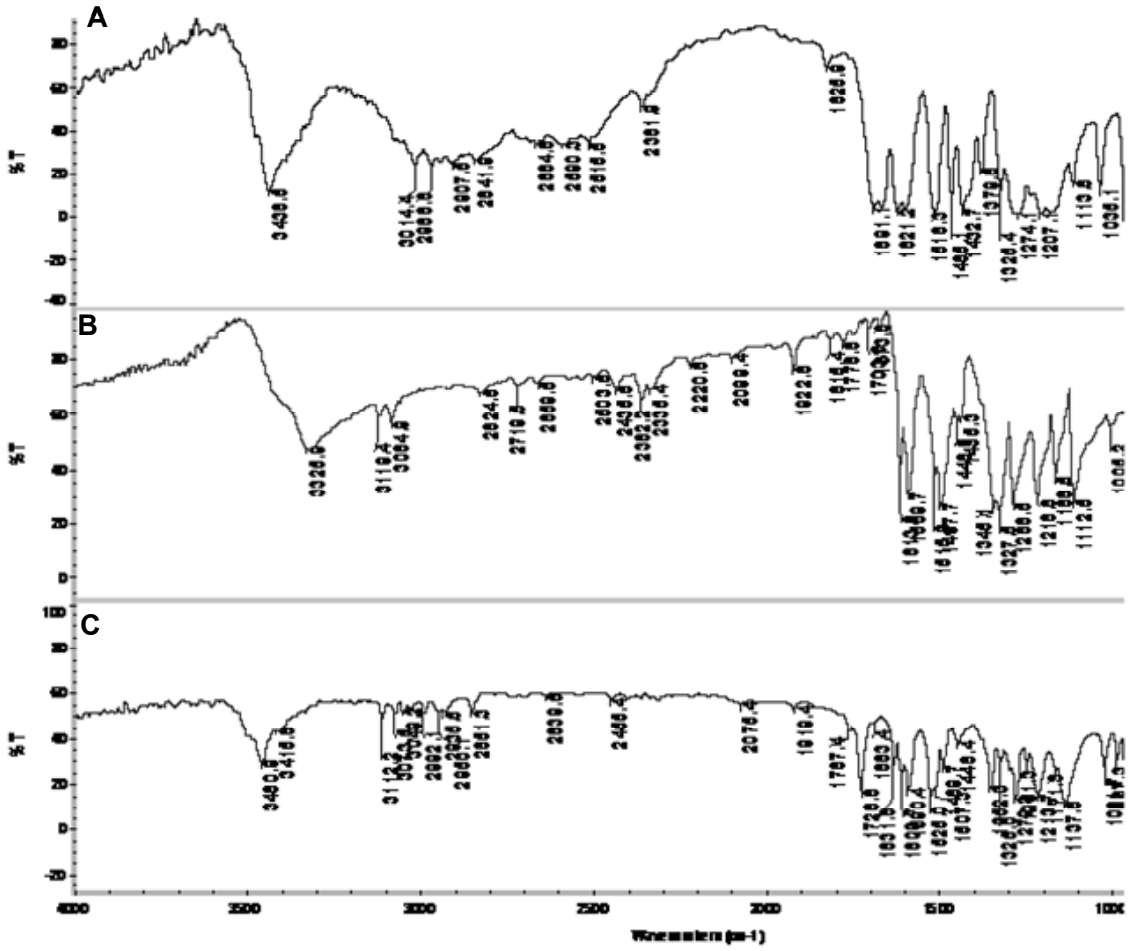


Figure 17. IR spectra of (A) Ferulic acid, (B) 4-nitrophenol and (C) 4-nitrophenyl ferulate

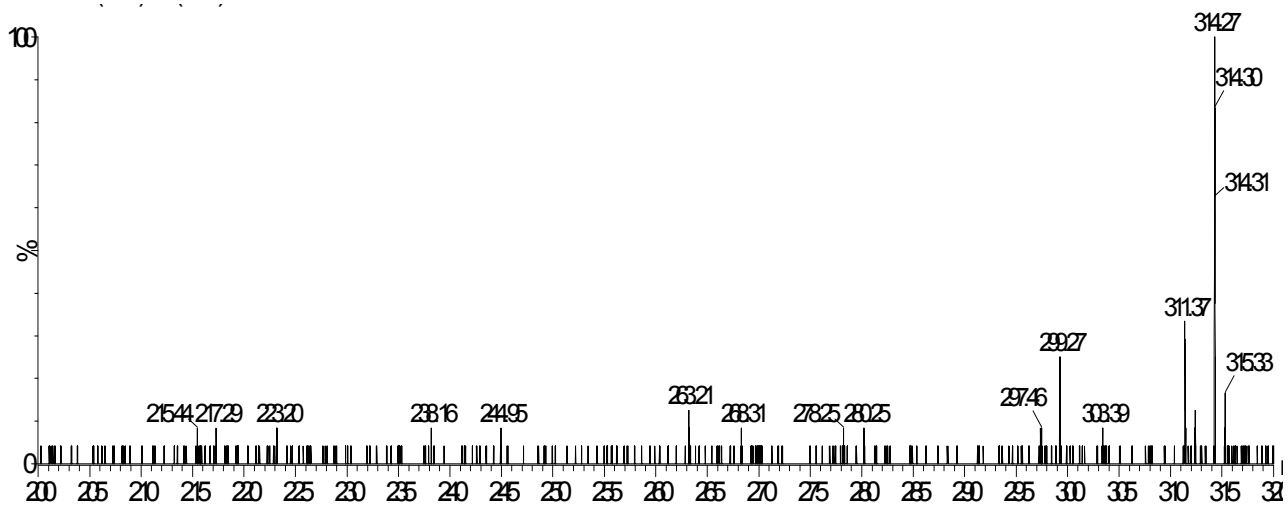


Figure 18. ESI-MS of 4-nitrophenyl ferulate

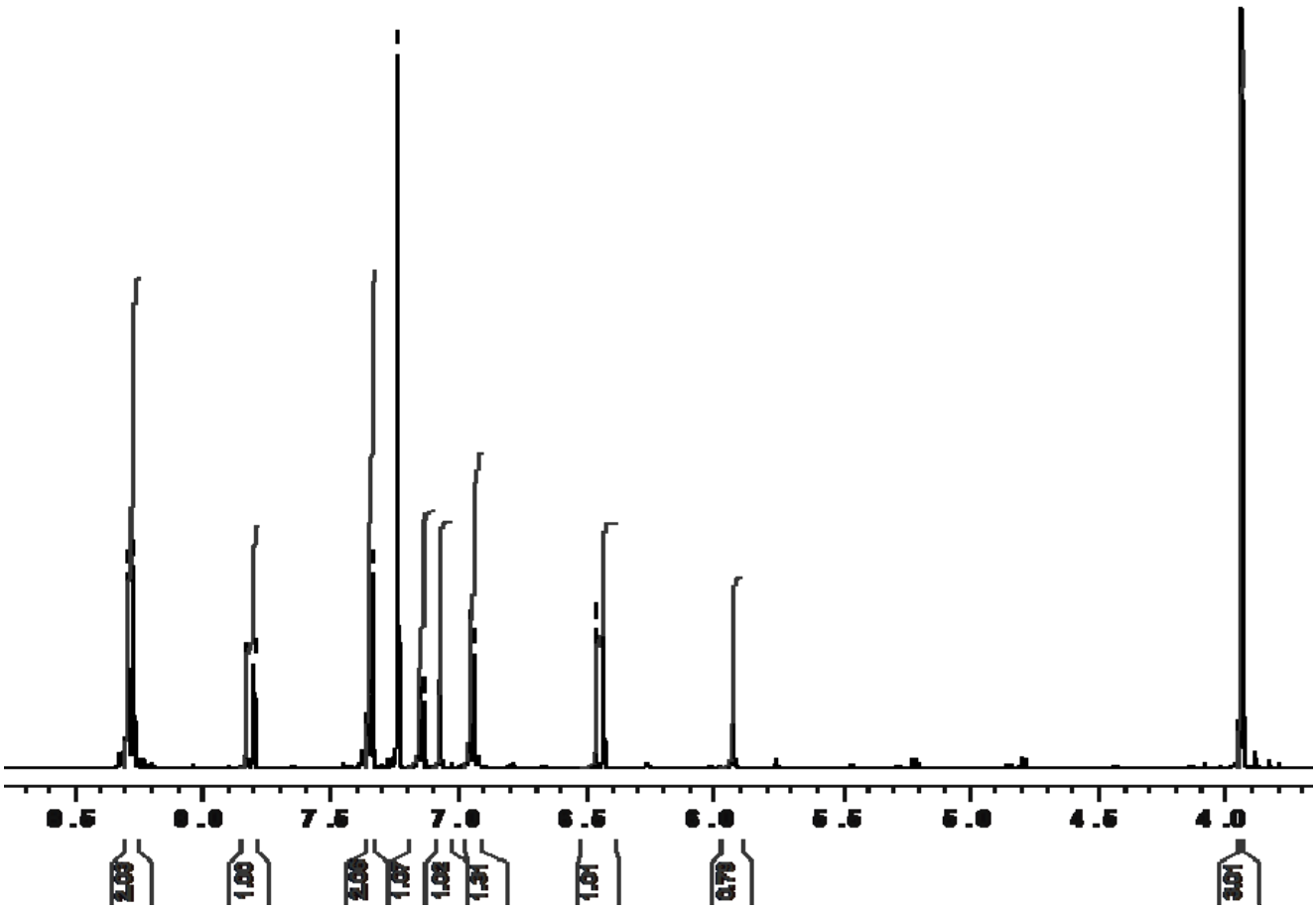


Figure 19. ^{1}H NMR of 4-nitrophenyl ferulate

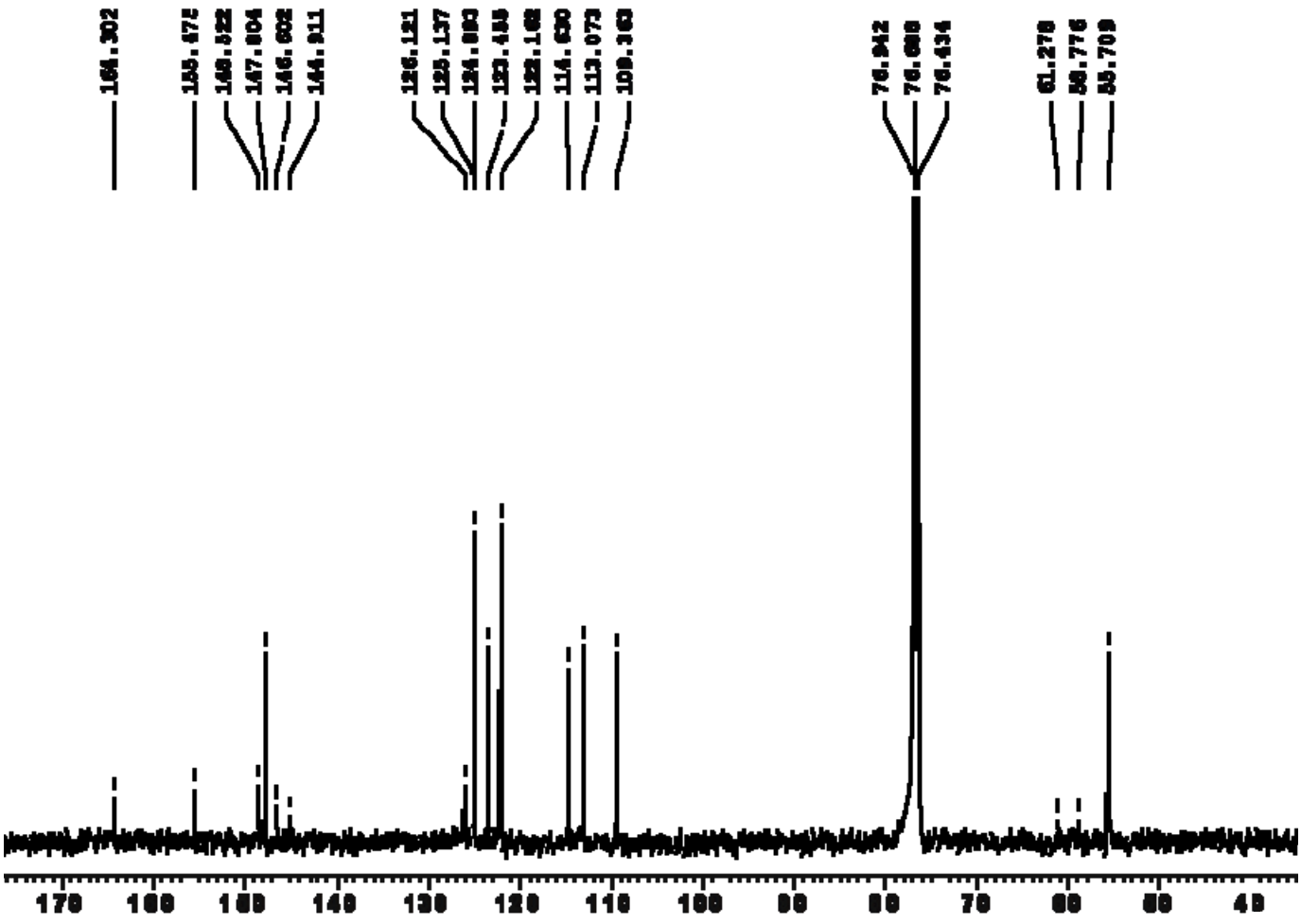


Figure 20. ^{13}C NMR of 4-nitrophenyl ferulate

Table 9. ^{1}H and ^{13}C spectral data of 4-nitrophenyl ferulate

^{1}H	3.94 (s, 3H); 5.93 (s, 1H); 6.45 (d, 1H, J = 16 Hz); 6.95 (dd, 1H, J = 8 Hz); 7.07 (d, 1H, J = 1.5 Hz); 7.15 (dd, 1H, J = 1.5 Hz, 8 Hz); 7.35 (m, 2H); 7.82 (d, 1H, J = 16 Hz); 8.28 (m, 2H).
^{13}C	55.71, 109.36, 113.07, 114.63, 122.16, 123.46, 124.89, 126.12, 144.911, 146.60, 147.80, 148.52, 155.48, 164.30

Coupling constants (J values) are given in hertz (Hz)

3.3. Assay of FAE using 4NPF as a substrate

Dialyzed culture filtrate *A. niger* was assayed for ferulic acid esterase (described in materials and methods), under conditions reported earlier (Mastihuba et al., 2002). Culture filtrate showed significant ferulic acid esterase activity. Ferulic acid esters are used often to study the substrate specificity of different esterases and also to determine ferulic acid esterase activity present in other related enzyme preparations as contaminant. Ferulic acid esterase activity of few commercially available enzymes was assayed (Table 10).

Table 10. Ferulic acid esterase activities using 4NPF as a substrate

Enzyme & Source	Activity (µmoles of 4-NP released/h/mg protein)
Ferulic acid esterase (*Aspergillus niger* CFR1105)	9.0
Lipase (Zeus Biotech, India, *Aspergillus niger*)	2.7
Lipase (Sigma, *Candida rugosa*)	19.25
Lipase (Sigma, *Candida cylindracea*)	36.72
Esterase (Sigma, Porcine liver)	1.18
Pectinase ((Fluka, *Aspergillus niger*)	0.36
Cellulase (Novozyme, Viscozyme L)	0.07
Hemicellulase (Biocon India, fungi)	5.9

Lipase from different sources showed ferulic acid esterase activities to different extent. Lipase from *Candida cylindracea* showed maximum activity and lipase from *Aspergillus niger* showed less activity. Content of ferulic acid esterase activity in hemicellulase is significant, whereas it is negligible in pectinase and cellulase preparations.

This simple, single-step method for the synthesis of 4NPF for assay of ferulic acid esterases may find extensive use by researchers for their in-house use and also may increase the prospects of its commercialization. The method described here may find further application for the synthesis of 4-nitrophenyl esters of 4-coumaric acid, caffeic acid, and the like for spectrophotometric assay of 4-coumaroyl esterase, caffeoyl esterase, and other related enzymes given that the spectrophotometric substrates for these enzymes are not commercially available.

Cereal and millet brans, by-products of milling industry in India, are available in plenty. They are mainly used as animal feed. They are ideal and cheap sources for obtaining dietary fiber compounds such as arabinoxylans, 1,3/1,4-β-D-glucans, cellulose, lignin and an array of phenolic acids. Among the phenolic acids, ferulic acid (3-methoxy-4-hydroxycinnamic acid) is the most abundant in cereal and millet brans, and is ester-linked to the 5'-OH group of arabinoxylans (Ishii, 1997). It comprises about 0.8% (w/w) dry matter of wheat bran (Hartley and Jones, 1977), 0.9% (w/w) dry matter of rice bran (Shibuya, 1984) and 1.5% (w/w) dry matter of ragi bran (McDonough et al., 1986). Only a few microbes secrete ferulic acid esterase to cleave ferulic acid from the cell wall polymers (Faulds and Williamson, 1995). Efficient enzymatic removal of ferulic acid from cereal brans requires the synergistic interaction between an esterase and cell wall degrading enzymes such as xylanase and 1,3/1,4-β-glucanase (Bartolome et al., 1995).

4.1. Selection of the fungal organism and growth substrate

Among the microorganisms, fungi are known to produce a wide spectrum of hydrolytic enzymes, used both in solid-state as well as in submerged fermentations (Maria et al., 1989). In order to choose the best organism for the degradation of ferulic acid containing cell wall polymers of cereal and millet brans. Three fungal organisms, namely *Aspergillus niger* CFR 1105, *A. oryzae* CFR 232, and *Rhizopus arrhizus* NCIM 997 were selected since they are devoid of mycotoxins and were evaluated for starch degrading and cell wall degrading enzymes.

4.2. Starch degrading enzymes

4.2.1. Amylase

Amylase activity was maximum in the extracts of *A. niger* CFR 1105 grown on wheat, rice and ragi brans (Figure. 21). A three fold increase was observed with respect to *A. niger* CFR 1105 grown on wheat bran at 96 h compared to 24 h. However, a 15-fold increase was observed with respect to 96-h extract of rice bran. Wheat bran extract showed a two fold increase in amylase activity at 48, 72 and 96 h of incubations compared to the rice bran extract. Amylase activity was maximum at 48 h in the moldy extracts of ragi and is higher than 24, 72 and 96 h extracts.

The 96 h extract of *A. oryzae* CFR 232 grown on wheat bran showed maximum amylase activity (Figure 22). However the amylase activity of *A. oryzae* CFR 232 was maximum at 48 h of incubation whereas in the moldy ragi bran extracts showed maximum activity at 96 h. Considerable amount of α-amylase activity was reported from *A. oryzae* (McKelvy and Lee, 1969).

Amylase activity was considerably less in *R. arrhizus* NCIM 997 extracts of moldy wheat, rice and ragi brans compared to *A. niger* CFR 1105 and *A. oryzae* CFR 232 (Figure 23), grown on wheat, rice and ragi brans. Moldy extract of *A. oryzae* grown on rice flour (4%) as a substrate showed an amylase activity of 30.2 U/ml (Michelena and Castillo, 1984).

Figure 21

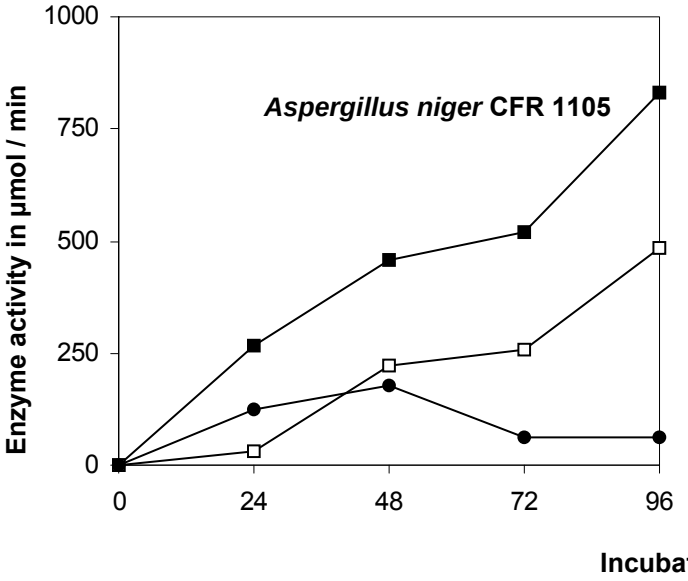
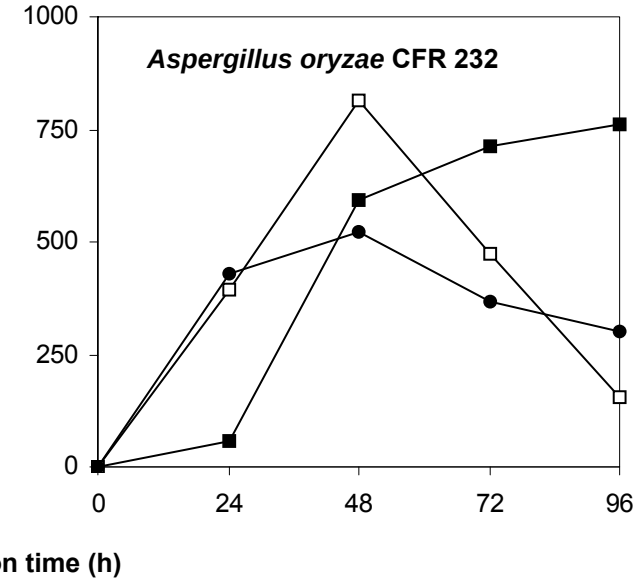


Figure 22



Incubation time (h)

Figure 23

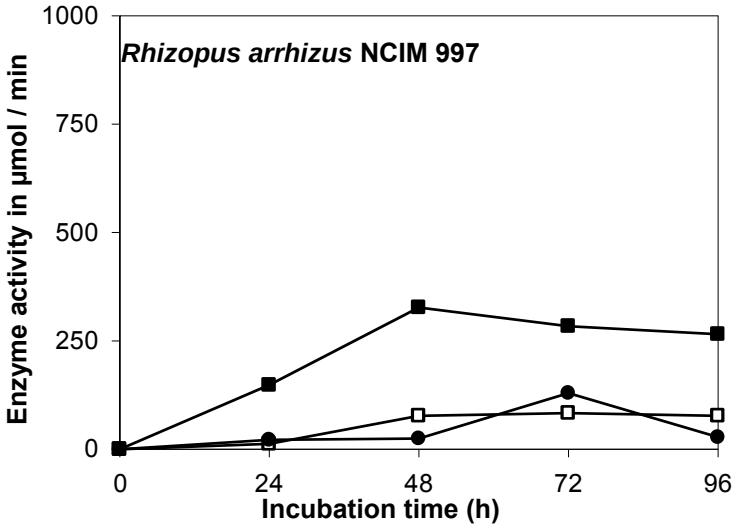


Figure 21. **Amylase activity in the extracts of moldy brans of (*Aspergillus niger)* '■', wheat; '□', rice and '●', ragi**

Figure 22. **Amylase activity in the extracts of moldy brans (*Aspergillus oryzae*) of '■', wheat; '□', rice and '●', ragi**

Figure 23. **Amylase activity in the extracts of moldy brans (*Rhizopus arrhizus* NCIM 997) of '■', wheat; '□', rice and '●', ragi**

4.2.2. Pullulanase

Pullulanase activity was maximum in 72-h extracts of moldy wheat and rice brans grown with *A. niger* CFR 1105 and *A. oryzae* CFR 232 (Figure 24 & 25). However, in the extracts of moldy ragi bran, pullulanse activity was maximum at 72 and 96 h. The extracts of *A. oryzae* CFR 232 showed a substantial increase in pullulanase activity when grown on ragi bran as compared with the other two fungal organisms.

In case of *R. arrhizus* NCIM 997, the maximum pullulanase activity was observed in 48-h extracts, unlike the amylase activity, which was maximum on wheat bran extracts (Figure 26). Pullulanase activity was found to be more in extracts of moldy rice bran grown with *A. niger* CFR 1105. However, the extracts of *A. oryzae* CFR 232 showed maximum activity when grown on ragi, rather than on wheat and rice brans. The low level of pullulanase activity was perhaps due to the extensive degradation of starch by amylase, wherein resultant oligosaccharides produced are relatively poor substrates for pullulanase, compared to the undegraded starch and amylopectin. Generally, the activities of pullulanase will be always several times lower than that of amylase.

Figure 24

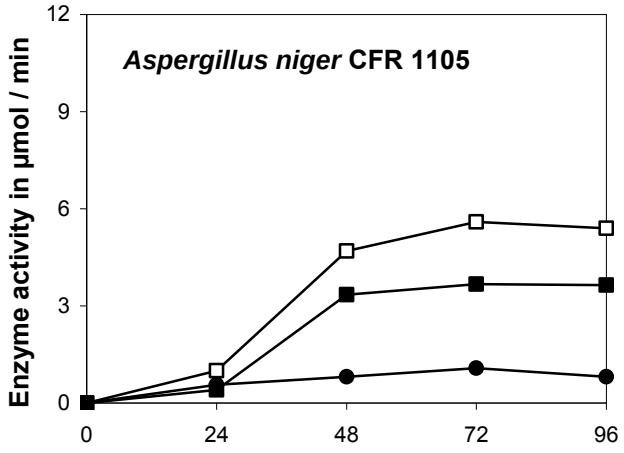
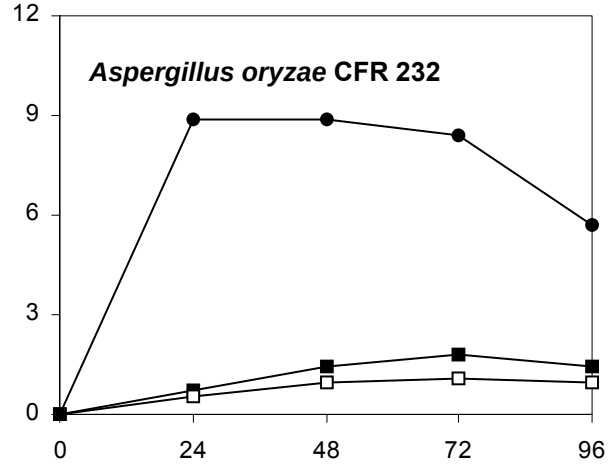


Figure 25



Incubation time (h)

Figure 26

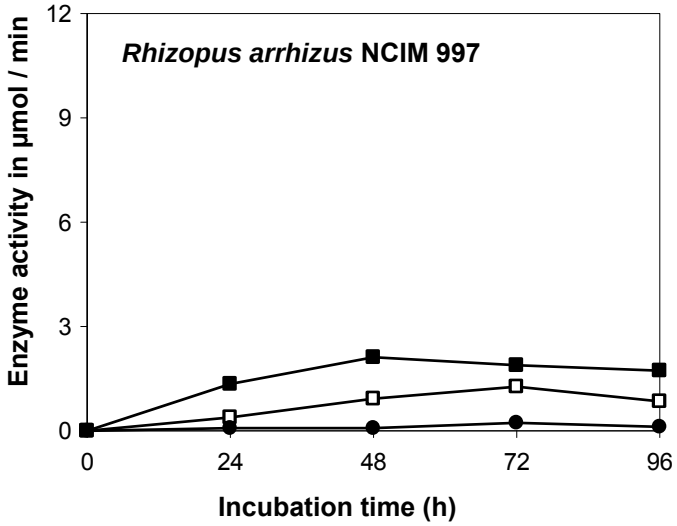


Figure 24. Pullulanase activity in the extracts of moldy brans (*Aspergillus niger*) of '■', wheat; '□', rice and '●', ragi

Figure 25. Pullulanase activity in the extracts of moldy brans (*Aspergillus oryzae*) of '■', wheat; '□', rice and '●', ragi

Figure 26. Pullulanase activity in the extracts of moldy brans (*Rhizopus arrhizus*) of '■', wheat; '□', rice and '●', ragi

4.3. Cell wall degrading enzymes

4.3.1. Xylanase

An increase in xylanase activity was observed with an increase in time in all the three fungal organisms and was maximum activity was observed in 96-h extracts (Figure 27, 28 and 29).

However, *R. arrhizus* NCIM 997 showed very low activity when grown on ragi bran and the activity was maximum at 24 h (Figure 29). Xylanase activity was maximum in *A. oryzae* CFR 232 (Figure 28), followed by *A. niger* CFR 1105 (Figure 27) and *R. arrhizus* NCIM 997. About 11-fold increase was observed in the xylanase activity in *A. oryzae* CFR 232 grown on wheat bran compared with *A. niger* CFR 1105 and *R. arrhizus* NCIM 997. Of the three substrates, wheat bran was the best substrate for xylanase induction compared with the brans of ragi and rice. Rice bran was the poorest inducer among the three brans tested. Xylanase activity is a prerequisite for the induction of ferulic acid esterase, which can cleave feruloyl–xylooligosaccharides more efficiently than feruloyl arabinoxylans. Couri et al. (2000) obtained high levels of xylanase activity (30.62 U/ml) of *A. niger* grown on wheat bran in solid-state fermentation. High xylanase activities from *A. ustus* using wheat bran (615.26 U/g) and rice straws (740.0 U/g) were reported (Shamala and Shreekantiah, 1986). The xylanase activity (8.76 U/g) from *Rhizopus* spp. grown on malted barley was around 9 U/g (Noots et al., 2001).

Figure 27

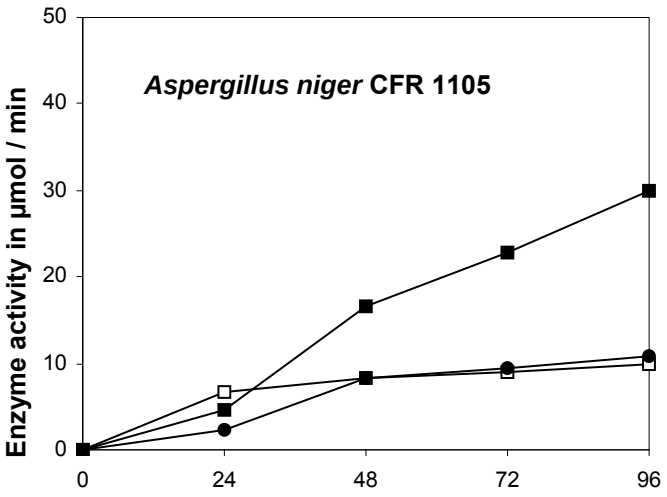
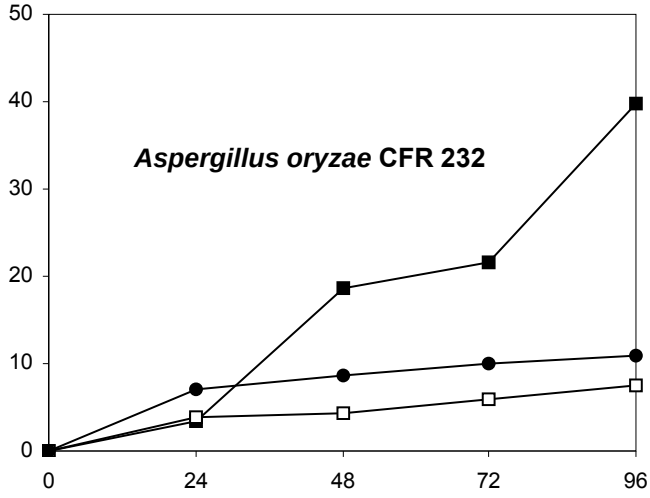


Figure 28



Incubation time (h)

Figure 29

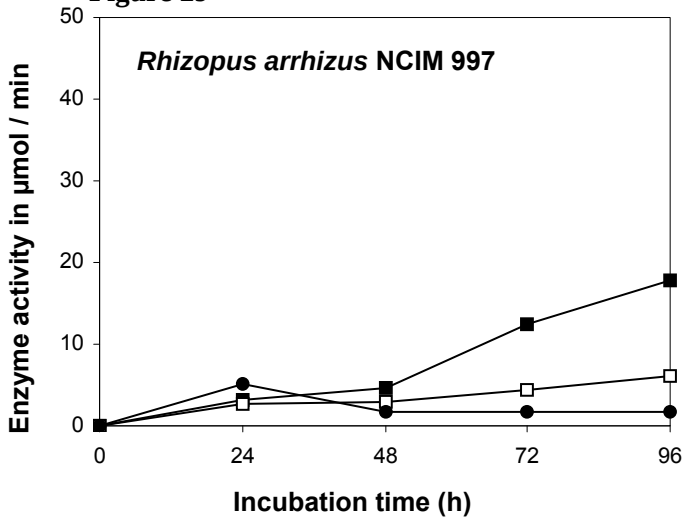


Figure 27. Xylanase activity in the extracts of moldy brans (*Aspergillus niger*) of '■', wheat; '□', rice and '●', ragi

Figure 28. Xylanase activity in the extracts of moldy brans (*Aspergillus oryzae*) of '■', wheat; '□', rice and '●', ragi

Figure 29. Xylanase activity in the extracts of moldy brans (*Rhizopus arrhizus*) of '■', wheat; '□', rice and '●', ragi

4.3.2. 1,3/1,4-β-D-Glucanase

1,3/1,4-β-D-Glucanase activity was maximum at 96 h with respect to *A. niger* CFR 1105 grown on brans of wheat, rice and ragi (Figure. 30). The extract of *A. oryzae* CFR 232 showed the maximum 1,3/1,4-β-D-glucanase activity when grown on ragi and wheat bran (Figure. 31). The extract of *R. arrhizus* NCIM 997 produced almost the same enzyme levels when grown on all three brans (Figure. 32).

However *A. niger* CFR 1105 and *A. oryzae* CFR 232 utilized wheat bran more efficiently than *R. arrhizus* NCIM 997 with respect to 1,3/1,4-β-D-glucanase. This is also due to the presence of higher amount of β-D-glucan present in wheat bran than rice bran. A high 1,3/1,4-β-D-glucanase activity (114.3 U/g) of *Rhizopus* spp. grown on malted barley was reported (Noots et al., 2001). In contrast very low 1,3/1,4-β-D-glucanase activity (1.18 U/ml) was detected from *R. arrhizus* QM 1032 (Clark et al., 1978).

The studies clearly indicated *A. niger* CFR 1105 and *A. oryzae* CFR 232 were able to produce higher levels of cell wall degrading enzymes compared with *R. arrhizus* NCIM 997 at 96-h incubation time. Wheat bran was found to be a better substrate than rice and ragi brans for all three fungi used in this study with respect to both starch degrading and cell wall degrading enzymes. Amylase activity was several fold higher than the activities of pullulanase, xylanase and 1,3/1,4-β-D-glucanase. Among the cell wall degrading enzymes, xylanase was more predominant than 1,3/1,4-β-D-glucanase.

Figure 30

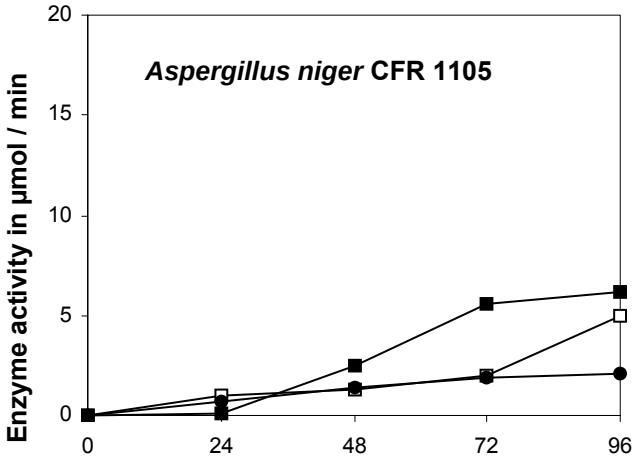
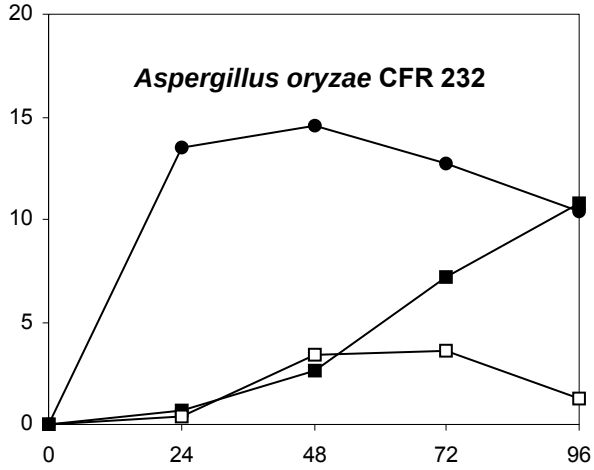


Figure 31



Incubation time (h)

Figure 32

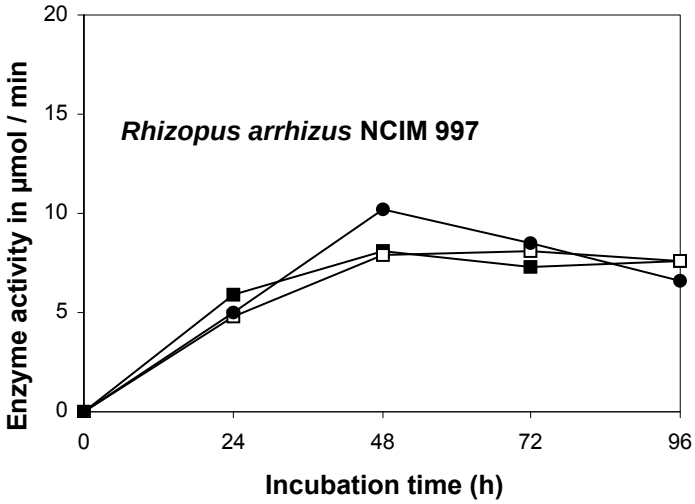


Figure 30. 1, 3/1, 4-β-D-glucanase activity in the extracts of moldy brans of (*Aspergillus niger*) '■', wheat; '□', rice and '●', ragi

Figure 31. 1, 3/1, 4-β-D-glucanase activity in the extracts of moldy brans of (*Aspergillus oryzae*) '■', wheat; '□', rice and '●', ragi

Figure 32. 1, 3/1, 4-β-D-glucanase activity in the extracts of moldy brans of (*Rhizopus arrhizus*) '■', wheat; '□', rice and '●', ragi

4.4. Degradation of non-starch polysaccharides

The major sugars identified in the non-starch polysaccharides (NSP) in native and experimental samples of wheat and rice brans were arabinose, xylose, galactose and glucose. Mannose and rhamnose are present as minor constituents. Native wheat bran polysaccharides mainly consisted of arabinose (27%), xylose (39%), galactose (2%) and glucose (30%) with traces of mannose. As against the native wheat bran, in the experimental samples, there was 9- and 19-fold decrease in the arabinose and xylose contents, respectively, in a 96 h growth period. Similarly a marginal decrease was observed in the rhamnose/fucose content (Table 11).

The substantial decrease in the arabinose and xylose content indicates the degradation of arabinoxylan backbone of the wheat bran polysaccharide. This is in good agreement with the various levels of cell wall degrading enzymes, wherein, xylanase activity is found to be higher, indicating extensive degradation of the xylan backbone. Native rice bran consisted of arabinose (9%), xylose 27 (%), galactose (30%) and glucose (32%). More than 4-fold decrease was observed in the arabinose content in the experimental sample of rice bran, whereas xylose was degraded by 13-fold. These results indicated extensive degradation of arabinoxylans rather than hexosans during the growth of the fungus, which can be correlated with higher activity of xylanase. There was a significant amount of glucose in the native bran as well as in all experimental samples, which might have originated from cellulose (Bonnin et al., 2002), β-D glucan (Brillouet et al., 1982) and associated starch. In the above

experiments, the mass utilized at different time intervals of *A. niger* are as follows: wheat bran: 24 h – 1%; 48 h – 6.3%; 72 h – 17%; 96 h – 17% and rice bran : 24 h – 6.3%; 48 h – 7.3%; 72 h – 11.2%; 96 h – 25.8%. The % of arabinose and xylose remaining in the 96 h experimental sample compared to the control is as follows: wheat bran: arabinose – 11%; xylose – 5%. Rice bran: arabinose – 22%; xylose – 12%.

Table 11. Comparison of degradation of non-starch polysaccharides of *Aspergillus niger* grown on wheat and rice brans

Sugars	Wheat bran					Rice bran				
	Native	24 h	48 h	72 h	96 h	Native	24 h	48 h	72 h	96 h
Rha/Fuc	0.65	6.0	1.0	3.4	ND	1.2	1.0	1.1	4.6	3.7
Ara	26.6	8.2	7.1	4.4	3.0	8.6	6.5	7.0	3.5	1.9
Xyl	38.7	10.6	5.0	3.1	2.0	27.1	9.5	11.5	4.0	2.2
Man	1.2	ND	1.9	3.0	2.4	ND	2.3	7.0	7.4	1.0
Gal	2.6	ND	8.9	14.4	7.8	30.3	35.1	34.5	3.2	7.5
Glc	30.4	75.2	76.1	72.7	84.8	31.8	45.5	38.8	77.2	84.9

Sugar composition (%)

ND, Not detectable

Rha, rhamnose; Fuc, fucose; Ara, arabinose; Xyl, xylose; Man, mannose; Gal, Galactose; Glc, glucose.

4.5. Changes in bound phenolic acids

Ferulic acid and coumaric acid are the major bound phenolic acids present in the native wheat bran. However, rice bran, in addition to the above phenolic acids, consisted of substantial amounts of syringic acid. Ferulic acid decreased by 8- and 4-fold in the experimental samples of wheat and rice brans respectively at 96 h. A 7-fold decrease in the content of bound coumaric acid was observed in the experimental sample of wheat bran at 96 h. A 2-fold decrease was observed with respect to coumaric acid in the experimental sample of rice bran at 24 h. Thereafter the degradation was not substantial. This may be perhaps due to the inter-conversion of liberated ferulic acid to coumaric acid at 72 and 96 h. Decrease in bound syringic acid was substantially less than ferulic and coumaric acids. Degradation of these bound phenolic acids clearly indicates the induction of various phenolic acid esterase(s) (Table 12).

Table 12. Degradation of bound phenolic acids in the moldy wheat and rice brans (µg/ g sample)

Incubation time (h)	Wheat bran					Rice bran				
	Native	24	48	72	96	Native	24	48	72	96
Ferulic acid	911.3	489.0	208.6	129.5	116.3	716.5	239.7	133.2	153.9	139.7
ρ-Coumaric acid	61.3	55.4	25.6	23.0	8.1	1150.0	609.5	484.0	511.3	532.0
Syringic acid	ND	ND	ND	ND	ND	195.8	179.9	158.0	155.8	149.3

ND, Not detectable

Degradation of bound ferulic acid can be correlated with maximum activity of ferulic acid esterase in the 72 h experimental sample of wheat bran (Figure 33). The activity of ferulic acid esterase in the moldy brans of rice and ragi could not be detected. Further studies with respect to changes in free phenolic acids were restricted to wheat and rice moldy brans.

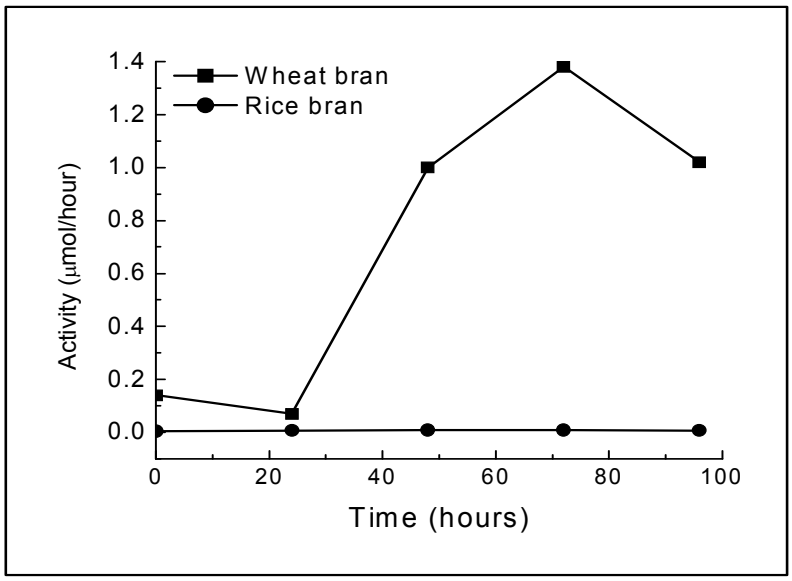


Figure 33. Activity of ferulic acid esterase in the moldy wheat and rice brans of *A. niger*

4.6. Changes in free phenolic acids

Wheat bran consisted of both benzoic acid and cinnamic acid derivatives. Gallic acid, protocatechuic acid and vanillic acids were present in the ratio of 2.2:3:1. Cinnamic acid derivatives such as coumaric acid and ferulic acids are present in 1:3 ratios. Gallic acid and protocatechuic acids are completely degraded in 96 h experimental sample. Coumaric acid is almost completely

degraded at 96 h, whereas the decrease in ferulic acid content at 96 h was negligible. A direct correlation with the decrease in the bound ferulic acid with a concomitant increase in the free phenolic acids could not be correlated from these results. This may be perhaps due to the oxidation or interconversion of benzoic acid as well as cinnamic acid derivatives (Figure 34(A)). Rice bran exclusively consisted of benzoic acid derivatives such as gallic acid, protocatechuic acid and vanillic acid. Cinnamic acid derivatives are completely absent. Gallic acid and protocatechuic acids are completely degraded after 24 h of incubation of *A. niger* grown on rice bran. At 96 h, a 3-fold increase was observed with respect to vanillic acid. Gallic acid and protocatechuic acid are completely disappeared in the experimental samples of rice bran (Figure 34(B)).

Thus it can be concluded from the above results that arabinoxylan degrading enzymes (pentosanases) are induced, which is prerequisite for the induction of esterase to cleave ferulic acid from the cell walls. Increase in esterase activity was directly influenced by the rate of polysaccharide degradation, which also dependent on the activity of the xylanase (Faulds et al., 1997).

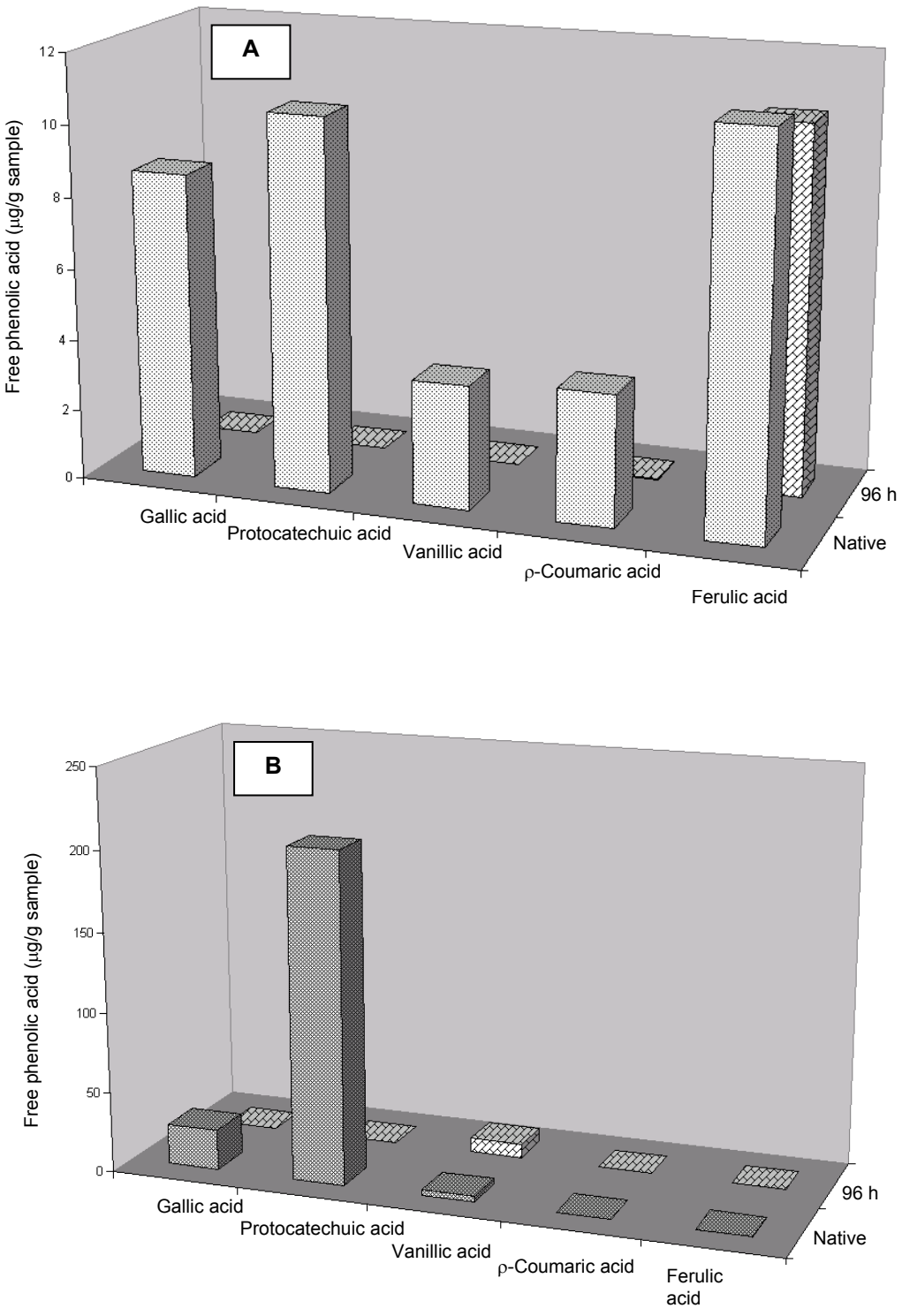


Figure 34. Free phenolic acids in wheat (A) and rice (B) brans grown with *A. niger* CFR 1105 for 96 h

From the above studies, it was clear that there is a preferential degradation of arabinoxylans rather than hexosans by *A. niger*. Substantial decrease in bound ferulic acid was also noticed in experimental samples of wheat and rice brans, indicating the possible induction of ferulic acid esterase. The amount of free ferulic acid was comparatively very low in rice bran compared to wheat bran. All the free phenolic acids were degraded in the 96 h experimental sample.

These studies also showed that wheat bran was found to be a better substrate for the growth of *A. niger* with respect to the induction of arabinoxylan degrading enzymes as well as phenolic acid esterases compared to rice bran. The degradation of cereal bran polysaccharide-phenolic acid complexes can be exploited to obtain bioactive compounds such as ferulic acid as well as xylooligosaccharides for various end uses.

5.1. Standardization of growth conditions of *A. niger* for production of ferulic acid esterase

Ferulic acid is the most abundant hydroxycinnamic acid present in the cell walls of cereals. Cereal brans, one of the major byproducts of the milling industries are good source of ferulic acid. Ferulic acid comprises about 0.5-1% w/w dry matter of wheat bran (Hartley and Jones 1977). These cinnamic acid derivatives/phenolic acids/hydroxycinnamic acids associated with plant hemicelluloses play an important role in cell wall integrity and protection of plant tissues against digestion by plant invading microorganisms. However, under situations when agroindustrial wastes are one of the major environmental pollutants, their degradation is also an important factor. These phenolic acids impede the breakdown of agroindustrial wastes. Therefore, the removal of phenolic acid substituents is important not only for the degradation of agroindustrial wastes, but also for obtaining bioactive compounds such as ferulic acid, *p*-coumaric acids which have various biotechnological applications.

Ferulic acid esterases (FAE) (3.1.1.73), a subclass of the carboxylic ester hydrolases are responsible for the hydrolysis of the esters of hydroxycinnamic acids (Benoit et al. 2006). These ferulic acid esterases have a number of biotechnological applications such as isolation of bioactive compounds from agro wastes, pulp and paper industries, production of fuel ethanol, synthetic tool to synthesize novel esters. Due to biotechnological importance of ferulic acid esterases, this enzyme has been studied from a large number of fungal and

bacterial sources (Topakas et al. 2007). There is an increased research effort in finding newer sources of FAE with novel properties.

As discussed earlier, a strain of *A. niger* (CFR 1105) was able to degrade wheat bran efficiently and also produced cell wall/starch degrading enzymes namely, ferulic acid esterase, xylanase, 1,3/1,4-β-D glucanase, pullulanase, and amylase. We further studied the ferulic acid esterase of this organism grown under solid state fermentation (ssf) and submerged fermentation (smf) condition and purification and partial characterization of enzyme.

5.2. Enzyme production

Aspergillus niger CFR 1105 was grown on wheat bran (carbon source and inducer) in both solid state (ssf) and submerged fermentation (smf) for 7 days (Asther et al. 2002). Under ssf conditions ferulic acid esterases were produced after one day of incubation (Figure 35) and activity was maximum after 4 days (32.5 U/g dry weight of wheat bran). The enzyme activity per mg protein (specific activity) was maximum on 2nd day (12.8 U/mg protein, Figure 35b). The specific activity of ferulic acid esterase decreased gradually after 2nd day, though the total activity increased upto 5 days which may be due to the increased production of non-FAE proteins.

In smf at 24 h, a very low FAE activity (0.9 U/mg protein) was detected (Figure 35b). After 24 h, the secretion of FAE increased and continued to increase progressively up to 5 days and reached the maximum (31.5 U/g dry weight of wheat bran) on 5^{th} day (Figure. 35a). The specific activity reached maximum (11.7 U/mg protein) on 3^{rd} day and it was almost stable upto 5 days,

thereafter drastic decrease was noticed. The total activity increased significantly from 2nd day of incubation and reached maximum on 5th day. Incubation for longer periods (7 days) showed decrease in the enzyme titer. Though the ferulic acid esterase activity produced in ssf and smf were comparable on 5th day, specific activity in submerged fermentation conditions was nearly double than that of ssf. Therefore for purification of enzymes, the organism grown under smf condition was used. FAE activity was maximum at 30 ^{o}C at pH 6 of the media when wheat bran was autoclaved for 60 min (Figure 36).

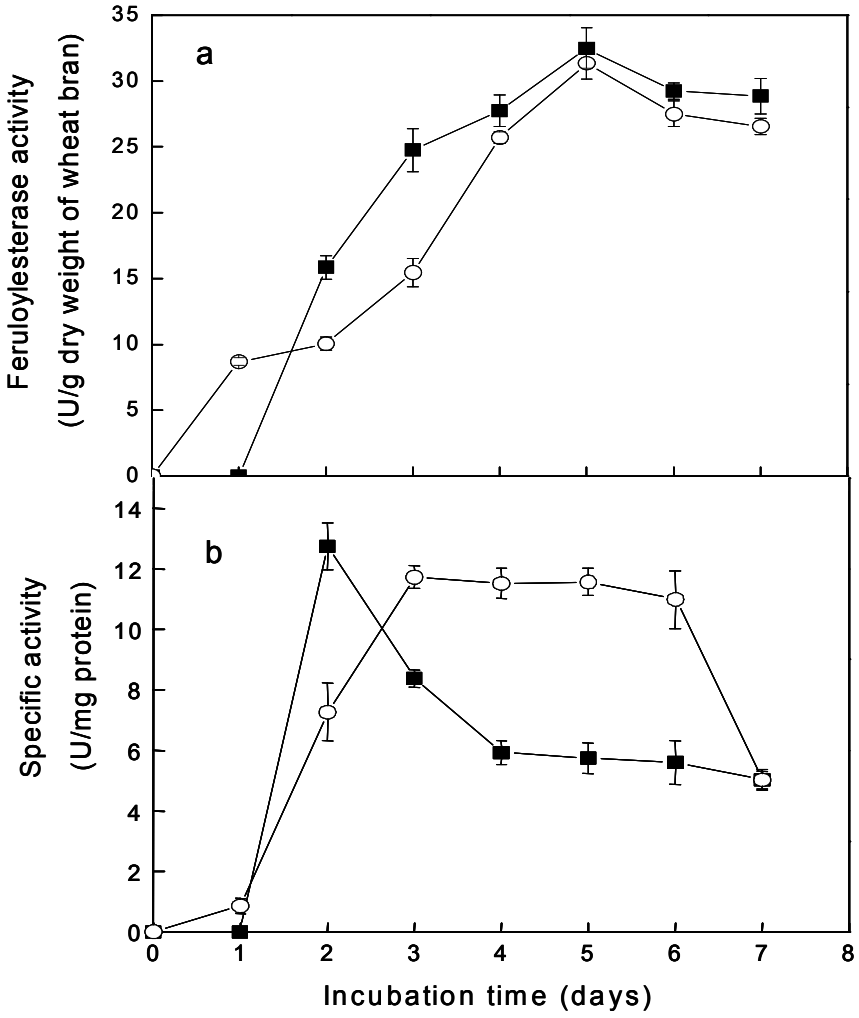


Figure 35. Ferulic acid esterase activity of *A. niger* grown on wheat bran in solid state (-■-) and submerged (-O-) fermentations (a) Total activity (b) Specific activity

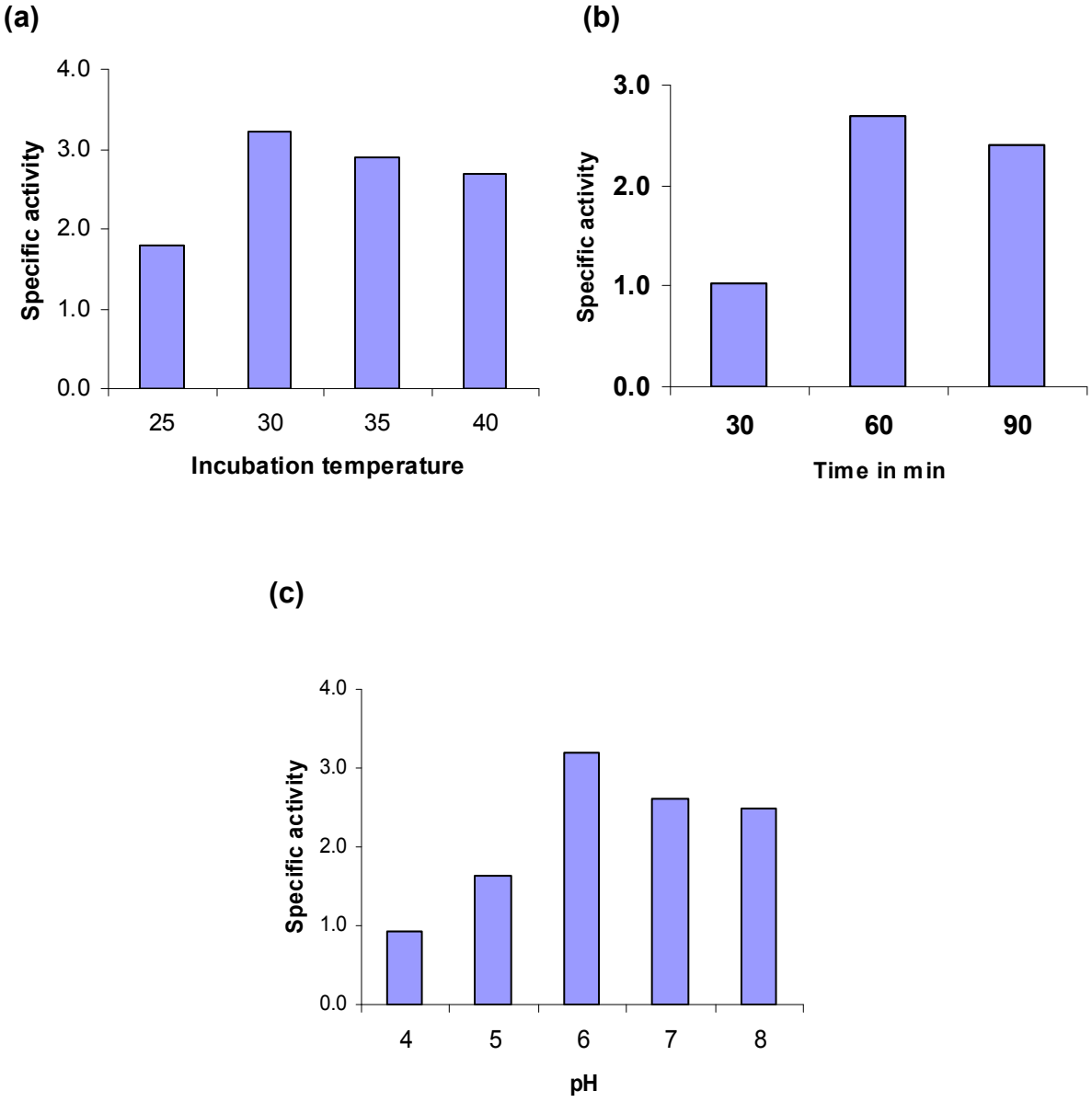


Figure 36. Optimization of fermentative conditions (a) Effect of temperature (b) Effect of autoclaving time of wheat bran (c) Effect of pH

(Average values of three independent experiments were taken)

5.3. Purification and characterization of ferulic acid esterase

The 5th day culture filtrate of *Aspergillus niger* CFR 1105 grown on wheat bran under submerged fermentation condition was subjected for ammonium sulphate precipitation, which yielded three fractions i.e. 0-35%, 35-60% and 60-80%. About 70% of activity was present in 35-60% saturated ammonium sulphate fraction, which was loaded onto DEAE-cellulose ion exchange column (Figure 37). The bound proteins were eluted with linear gradient of NaCl (from 0 to 0.5 M) which yielded two major FAE activity peaks designated as FAE-1 and FAE-2, eluted from the column at 0.3 M and 0.35 M NaCl concentrations with a recovery of 10% and 8.4% respectively (Table 13).

The FAE-1 and FAE-2 were further purified on Sephacryl 100-HR separately (Figure 38 a & b). Procedures followed for purification of FAE from different strains of *A. niger* in earlier reports resulted in purification of FAE, active in neutral pH (pH 5-6) whereas the procedure reported in the present communication resulted another isoenzyme of FAE having pH optimum in alkaline range (pH 9) in addition to neutral enzyme. The summary of purification is given in Table 13.

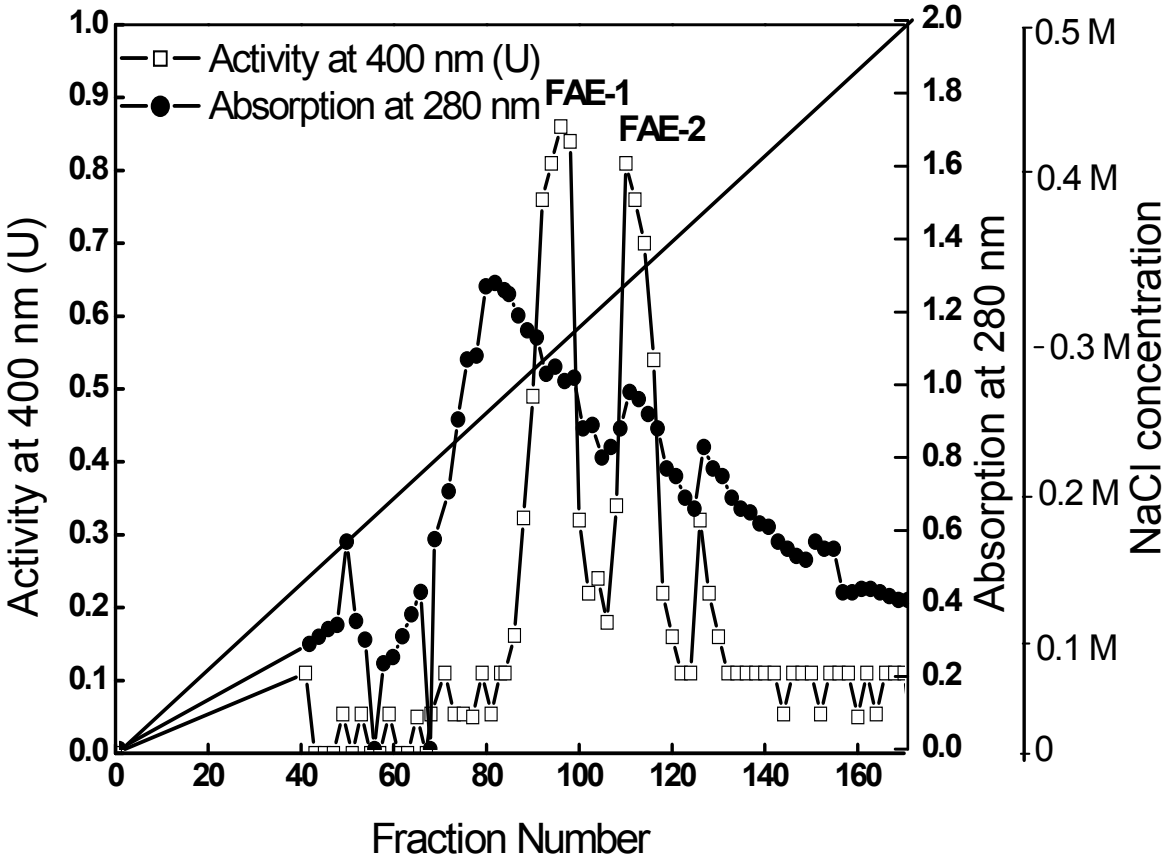


Figure 37. Elution profile of FAE (35-60% ammonium sulphate fraction) on DEAE-cellulose column

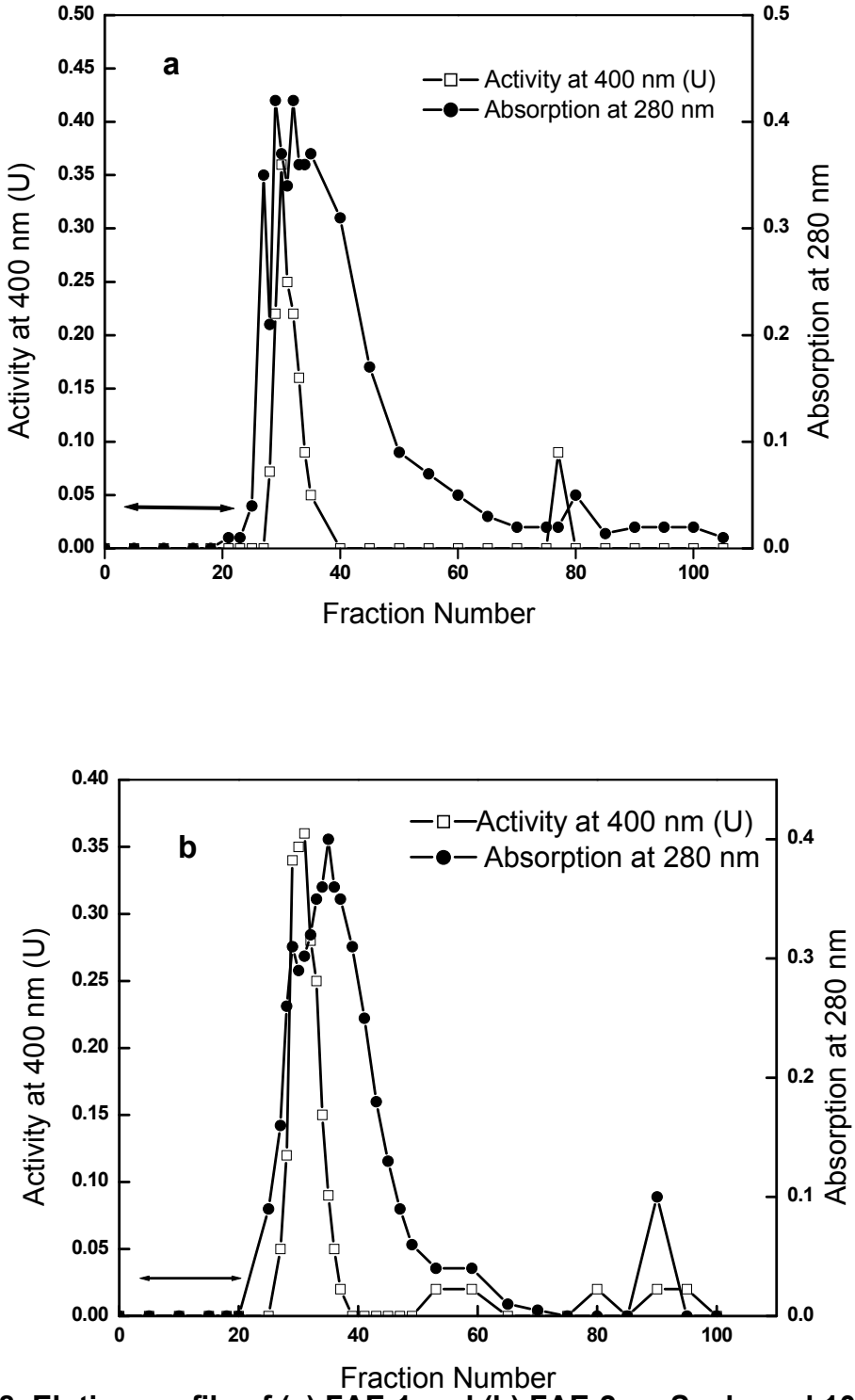


Figure 38. Elution profile of (a) FAE-1 and (b) FAE-2 on Sephacryl 100-HR column

Table 13. Purification of ferulic acid esterases

Purification step		Total activity (U)	Total protein (mg)	Specific activity (U/mg protein)	Fold purification	Recovery (%)
Crude		323.5	100	3.23	1	100
35-60% $(NH_4)_2SO_4$ Fraction		215.7	18	11.9	3.7	66.7
DEAE-cellulose	FAE-1	32.2	1.5	21.5	6.7	10
	FAE-2	27.1	0.9	30.1	9.3	8.4
Sephacryl 100-HR	FAE-1	14	0.39	35.9	11.11	4.3
	FAE-2	8	0.1	80	24.8	2.5

5.4. Characterization of FAEs

Electrophoresis of the purified proteins under native conditions showed single band (Figure 39). Coomassie staining after SDS-PAGE of the lyophilized sample showed single band corresponding to the molecular weight of approximately 50 kDa and 55 kDa (Figure 41) respectively. An identical molecular mass was obtained by GPC (Figure 40) indicating the monomeric nature of the purified FAE-1 and FAE-2. Periodic acid-schiff staining of the gel indicated the presence of glycans in the protein. The carbohydrate content of the FAE-1 and FAE-2 estimated by the phenol-sulfuric acid method was found to be 23% and 30% respectively. Ferulic acid esterases from different microbial

sources showed significant variations in molecular weights ranging from 11 kDa to 210 kDa (Topakas et al. 2007). Even the ferulic acid esterases from different *Aspergillus* species varied from 29 kDa to 132 kDa (Topakas et al. 2007). Enzymes with 50 kDa and 55 kDa were not reported from *Aspergillus* species so far. A cinnamoyl esterase from *Aspergillus niger* CS 180 was reported to be a dimer and heavily glycosylated (Kroon et al. 1996). A ferulic acid esterase with 48% glycosylation from *Aureobasidium pullulans* was reported by Rumbold et al. (2003). Both monomeric and dimeric ferulic acid esterases were purified from *Aspergillus* niger CBS 120.49 (Faulds and Williamson, 1993).

(a)

(A) FAE-1
(B) FAE-2

(b)

(A) FAE-1
(B) FAE-2

(c)

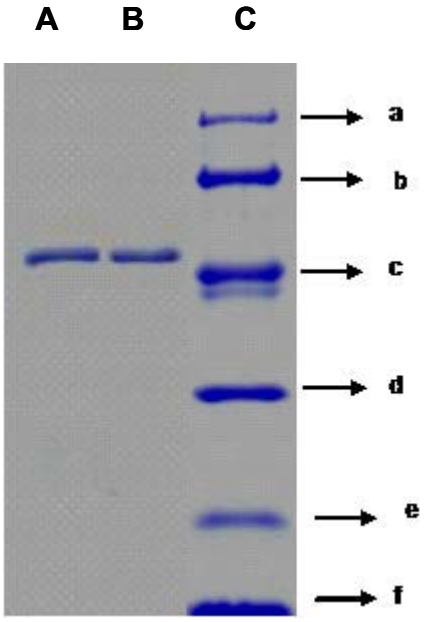


Figure 39. PAGE of purified FAE-1 (lane A), FAE-2 (lane B) and molecular weight standards (lane C)

(d) Coomassie staining of native PAGE

(e) Periodic acid-schiff (PAS) staining of SDS- PAGE

(f) Coomassie staining of SDS-PAGE

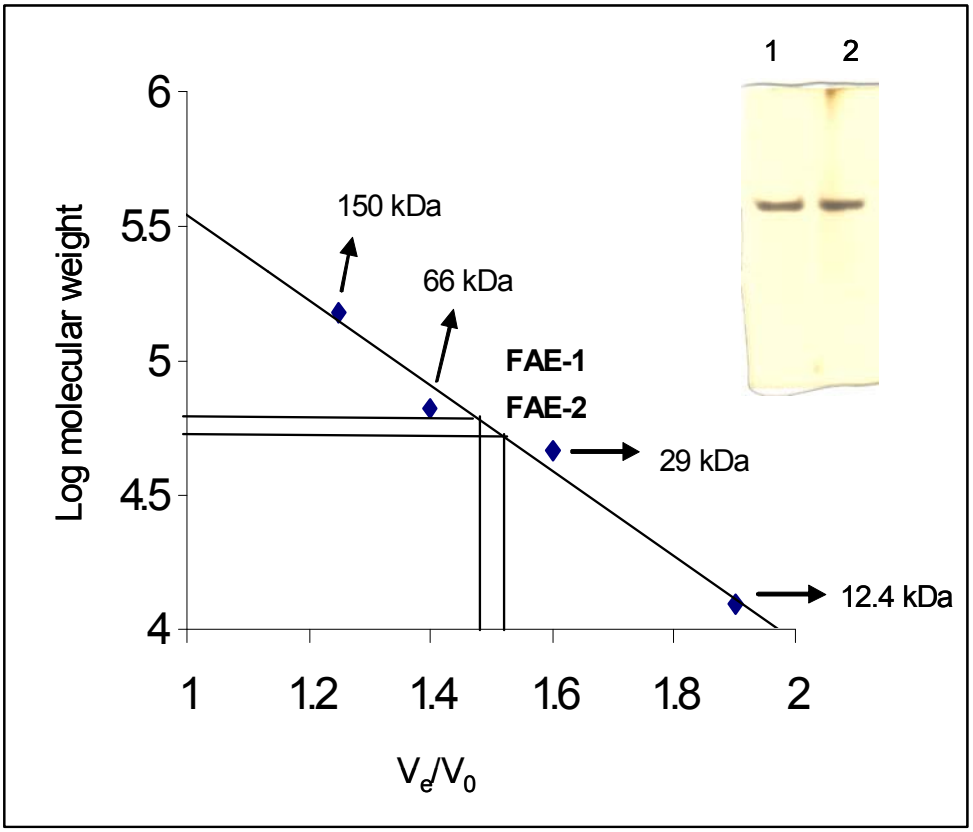


Figure 40. Determination of molecular mass of purified FAE-1 and FAE-2 on Sephacryl 100-HR. Protein molecular weight standard; cytochrome c (12.4 kDa), carbonic anhydrase (29 kDa), BSA (66 kDa) and alcohol dehydrogenase (150 kDa). Inset, silver staining of gel filtration peaks of FAE-1 (lane 1) and FAE-2 (lane 2).

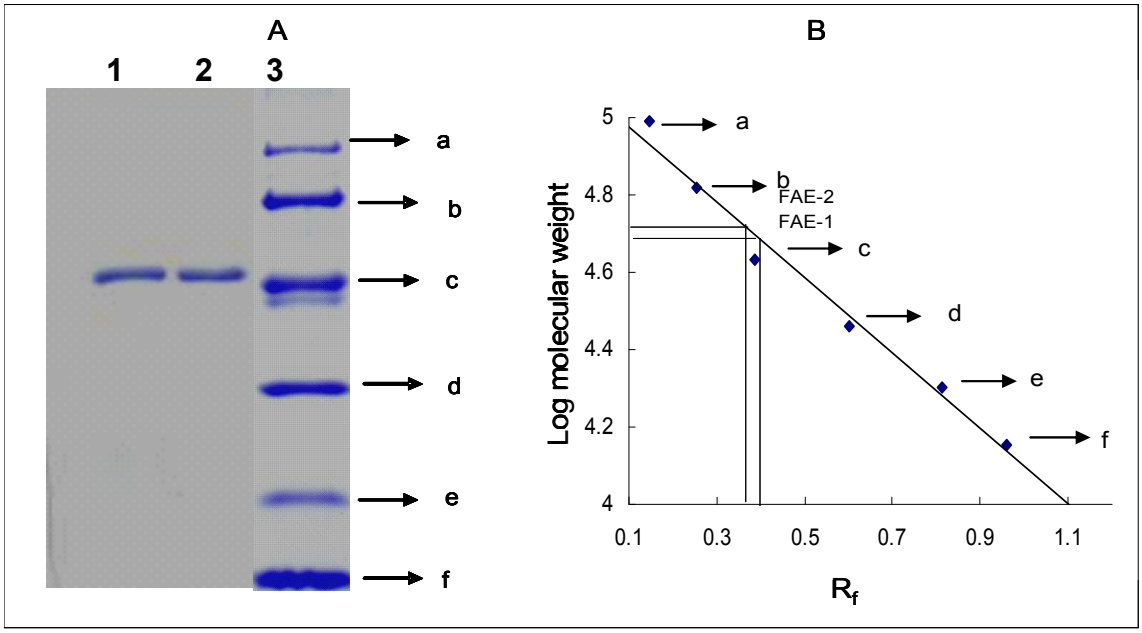


Figure 41. Molecular weight determination of FAEs by SDS-PAGE

(A) Lane 1: FAE-1; lane 2: FAE-2; Lane 3: protein molecular weight standard consisting of (a), phosphorylase-b (97.4 kDa); (b), bovine serum albumin (66 kDa); (c), ovalbumin (43 kDa); (d), carbonic anhydrase (29 kDa); (e), soyabean trypsin inhibitor (20.1 kDa); (f), lysozyme (14.3 kDa)

(B) Plot of R_f versus log molecular weight of FAE-1, FAE-2 and standard proteins

5.5. pH optima and pH stability

Effect of pH on FAE activity and its stability were studied in the pH range of 4-10. As shown in Figure. 42, both FAE-1 and FAE-2 exhibited activity in the wide pH range (pH 5-10). FAE-1 showed optimum activity at pH 9 whereas FAE-2 similar to many other fungal FAEs reported, had pH optimum at pH 6.0, but still exhibited significant activity in the alkaline pH, retaining about 60% at pH 9.0. FAE-1 had pH optimum at pH 9.0 and it showed about 70% activity at pH 6.0 (Figure 42). Thus both FAE-1 and FAE-2 were active in alkaline pH. To investigate enzyme stability with changes in pH, both FAE-1 and FAE-2 were incubated in 50 mM buffer of different pH at 30 ^{0}C for 2 h and residual activities were measured under standard assay conditions. Both FAE-1 and FAE-2 were quite stable at alkaline pH retaining 52% and 48% of their original activity at pH 10 after 2 h of incubation. Ferulic acid esterase from *Fusarium oxysporum* F3 showed a pH optimum at 6-7 and retained 55% of its activity at pH 10 after 6 h (Topakas and Christakopoulos 2004). Ferulic acid esterase from *Sporotrichum thermophile* showed a pH optimum of 8 and was stable over a pH range of 6-8 for 1 h (Topakas et al. 2003a). The ferulic acid esterase from *Fusarium proliferatum* NRRL 26517 showed a pH optimum at 6.5-7.5 and it was stable over a broad pH range 5-9 (Shin and Chen, 2006). The high activity and stability in alkaline conditions of FAEs reported in the present study may be advantageous in biotechnological applications and especially in the treatment of alkaline pulp.

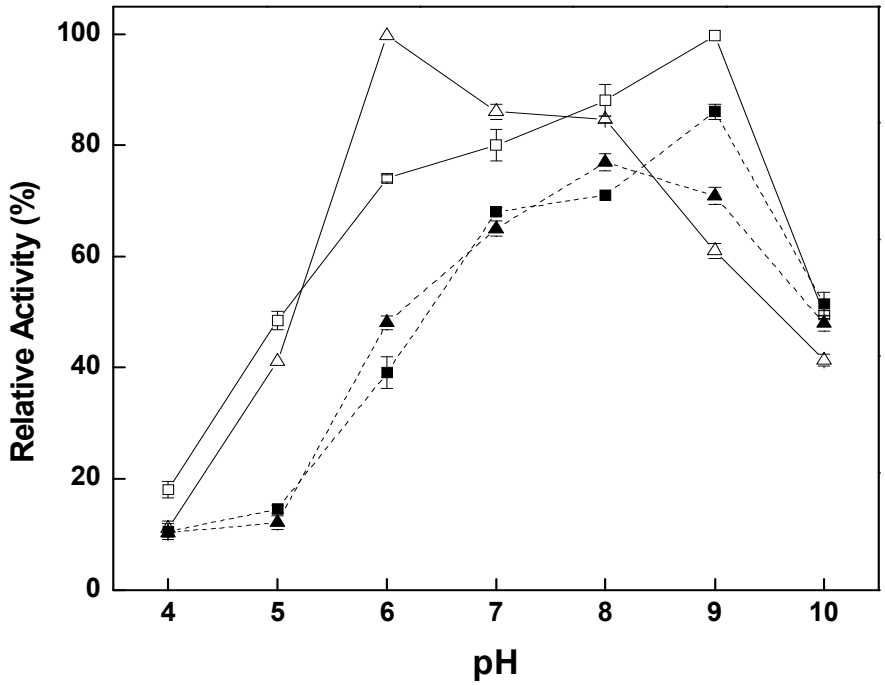


Figure 42. Effect of pH on activity (—) and stability (---) of FAE-1 ((-□- / -■-) and FAE-2 (-∆- / -▲-)

5.6. Temperature optima and thermal stability

The temperature optimum of FAE-1 was found to be 40 ^{0}C which is similar to the ones reported from other mesophilic fungal organisms. FAE-2 showed wider temperature optimum of 40-50 ^{0}C (Figure 43) which is similar to FAEs of *A. niger* (Kroon et al. 1996), *A. awamori* (McCrae et al. 1994), *Neocallimastix strain* MC-2 (Borneman et al. 1992), *Fusarium oxysporum* (Topakas et al. 2003b), *Fusarium proliferatum* (Shin and Chen 2006), slightly higher than ferulic acid esterase from *Penicillium expansum* (Donaghy and McKay 1997) and lower than the one reported for another strain of *A. niger* (De Vries et al. 1997). Both FAEs in the present study were stable over temperature of 30-40 ^{0}C and ~60% of the

activities were retained when the enzymes were preincubated at 50 ^{0}C for 30 min. Thermal stabilities of FAEs in the present study were comparable with the FAE of *Neocallimastix* strain MC-2 (Borneman et al. 1992). However higher temperature stabilities were reported for *A. awamori* (Koseki et al. 1998), *Fusarium oxysporum* (Topakas et al. 2003b), *Fusarium proliferatum* (Shin and Chen, 2006) and *Sporotrichum thermophile* (Topakas et al. 2003a).

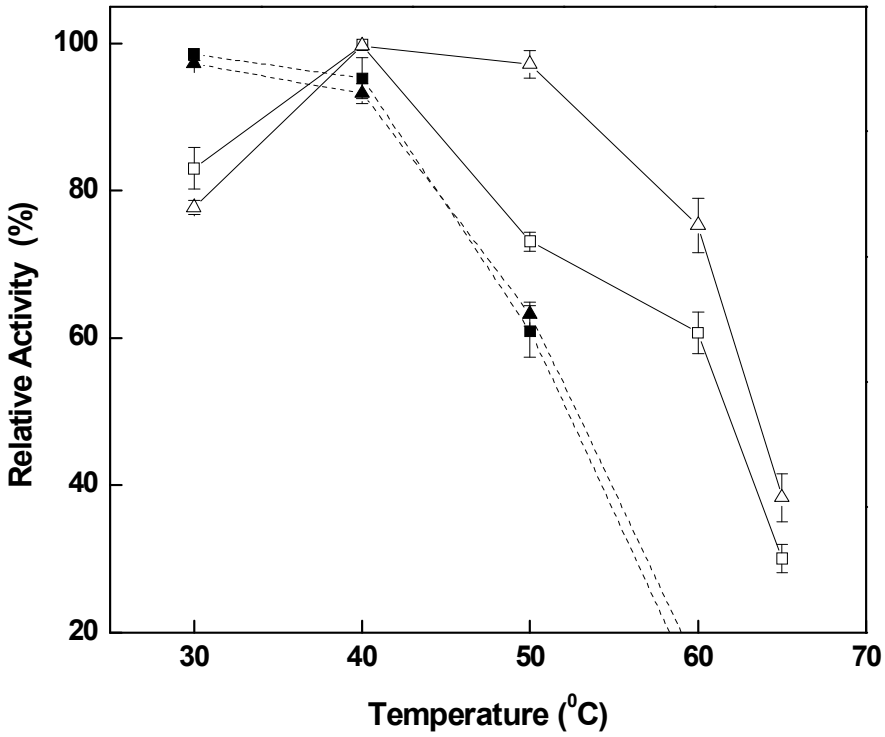


Figure 43. Effect of temperature on activity (—) and stability (---) of FAE-1 (-□- / -■-) and FAE-2 (-△- / -▲-)

5.7. Effect of Substrate concentrations

The effect of different substrate concentrations on the initial velocity was determined, and the kinetic constants, K_m and V_{max} were calculated from the double reciprocal plots (Lineweaver and Burk, 1934) (Figure 44). The K_m and V_{max} for FAE-1 for hydrolysis of 4NPF were 2 mM and 98 U/mg protein respectively. For FAE-2, K_m and V_{max} were found to be 3.3 mM and 158.7 U/mg protein respectively. V_{max} for FAE-2 was 62% more than that of FAE-1 indicating that FAE-2 is more active than FAE-1. This was also reflected in the specific activity of the purified enzymes (Table 13). These two isoenzymes have a slight difference in their carbohydrate content. Therefore difference in catalytic potential of the enzyme may be due to the difference in carbohydrate content. It is well established that in glycoenzymes, carbohydrates have a great influence on structure, activity and stability of the enzyme (Rademacher et al., 1988).

Purified FAE-1 and FAE-2 showed highest activity towards *p*-nitrophenyl ferulate followed by *p*-nitrophenyl acetate. Broad specificities of esterases towards *p*-NP esters ranging from acetate to caproate, triacetin, tripropionin, tributyrin and triolein were reported by Ferrer et al. (2005). The two ferulic acid esterases identified in *A. niger* (FAEA and FAEB) are both involved in the degradation of xylan and pectin, but have different preferences for these polysaccharides (De Vries et al., 2002).

FAE-2 has slightly more molecular weight and enzyme activity than FAE-1 and both these enzymes showed almost similar properties, perhaps these two enzymes might have originated from the same source (proenzymes) or FAE-1

might have been derived from FAE-2. Proteolytic modification of FAE-III to FAE-II was suggested by Faulds and Williamson (1994) due to their similarities in their substrate specificities.

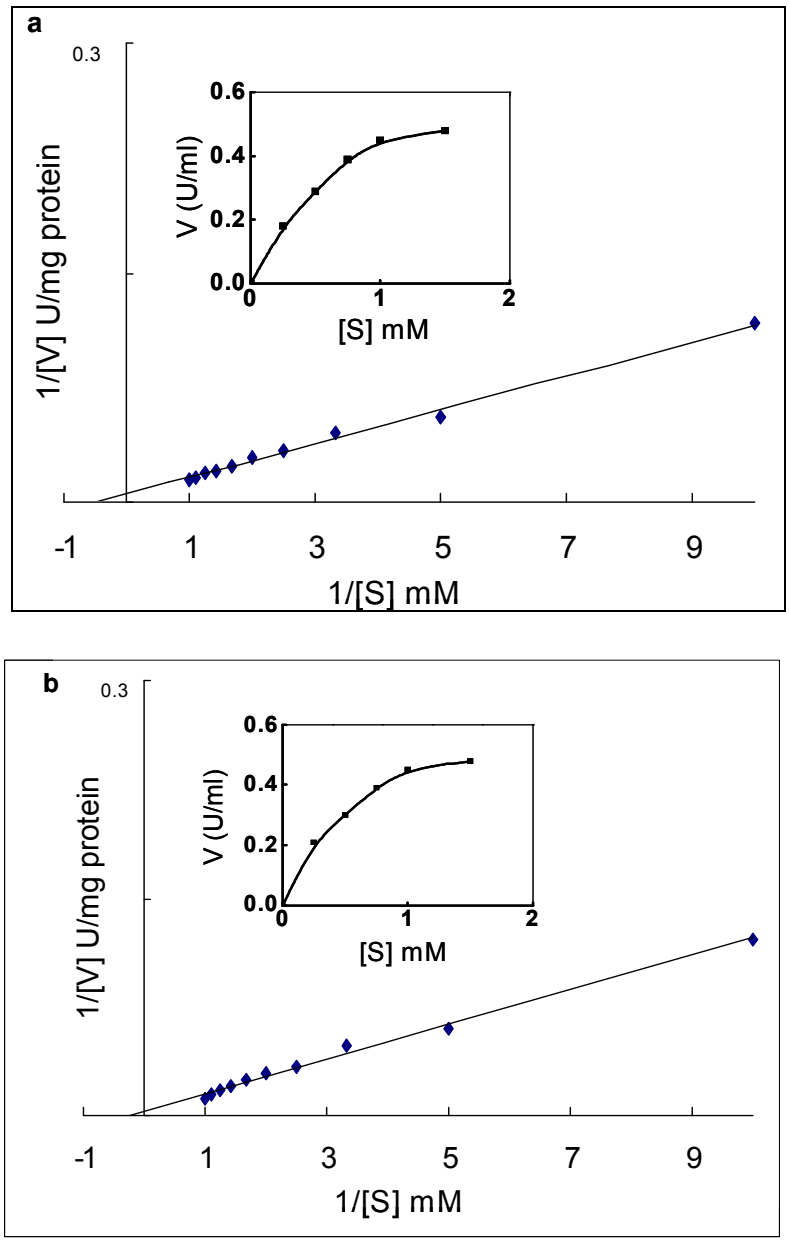


Figure 44. Determination of K_m and V_{max} of (a) FAE-1 and (b) FAE-2 by Lineweaver- Burk double reciprocal plots. Corresponding Michaelis-Menten graph of initial velocity versus substrate (4NPF) concentration are shown as insets

5.8. Substrate specificities of FAE-1 and FAE-2

Few *p*-nitrophenyl derivatives of sugars and fatty acids were used as substrate (1mM) to evaluate the substrate specificities of purified FAE (Table 14).

Table 14. Substrate specificities of FAE-1 and FAE-2

Substrates	Specific activity (U/mg protein)	
	FAE-1	FAE-2
p-nitrophenyl ferulate	9.0 ± 0.01	11.2 ± 0.02
p-nitrophenyl acetate	6.2 ± 0.01	6.1 ± 0.03
p-nitrophenyl butyrate	1.0 ± 0.01	1.9 ± 0.02
p-nitrophenyl β-D-galactopyranoside	1.0 ± 0.02	1.2 ± 0.01
p-nitrophenyl α-L-arabinofuranoside	1.5 ± 0.03	0.7 ± 0.01
p-nitrophenyl β-D-mannopyranoside	1.0 ± 0.03	0.7 ± 0.02
p-nitrophenyl β-D-xylopyranoside	1.5 ± 0.02	0.7 ± 0.01

5.9. Effect of metal ions on the activities of FAE-1 and FAE-2

A range of metal ions such as Fe^{+2}, Ba^{+2}, Mg^{+2}, Hg^{+2} and Cd^{+2} at 5 mM concentration were tested for the ferulic acid esterase activation/inhibition effect and the results are given in Table 15.

Table 15. Effect of metal ions on the activities of FAE-1 and FAE-2

Metal ions	Relative Activity (%)	
	FAE-1	FAE-2
Control	100	100
Fe^{+2}	190	135
Ba^{+2}	160	123
Mg^{+2}	160	140
Hg^{+2}	43	55
Cd^{+2}	51	22

The activities of FAE-1 and FAE-2 were enhanced by of Fe^{+2}, Ba^{+2}, Mg^{+2} whereas they were inhibited by Hg^{+2}, and Cd^{+2} (Table 15).

5.10. Effect of group specific reagents

Ferulic acid esterase activities were determined in the presence of group specific reagents such as PMSF, TLCK, TPCK at 50 mM concentration. The ferulic acid esterase activities were inhibited in the presence of PMSF (phenyl methane sulphonylfluoride) and TLCK (Tosyl-L-lysine chloromethyl ketone) by about 40% at 50 mM concentration indicating the possible presence of serine in the active site pocket (Table 16).

Table 16. Effect of group specific reagents on ferulic acid esterase activity

Group specific reagents	Relative activity (%)	
	FAE-1	FAE-2
Control	100	100
PMSF	63	66
TLCK	59	64
TPCK	98	100
β-mercaptoethanol	97	100

There was no significant effect on the enzyme activity in the presence of iodoacetamide and TPCK (Tosyl-L-phenylalanine chloromethyl ketone) at 50 mM concentration. Disulfide reducing agent β-mercaptoethanol also did not have any effect on the enzyme activity.

Summary

The results obtained from the Ph.D. thesis entitled “Purification and biochemical characterization of ferulic acid esterase from *Aspergillus niger* CFR 1105” are summarized as follows.

- Spectrophotometric substrate 4-nitrophenyl ferulate for the assay of feruloyl esterase, was synthesized by a single step dehydrative coupling of ferulic acid and 4-nitrophenol and characterized by HPLC, ESI-MS, ^{1}H and ^{13}C NMR.
- *A. niger* CFR 1105, *A. oryzae* CFR 232 and *R. arrhizus* NCIM 997 were evaluated for the induction of various starch degrading / cell wall degrading enzymes grown on wheat, rice and ragi brans. Amylase activity was several folds higher than the activities of pullulanase, xylanase and 1,3/1,4-β-D-glucanase in the moldy wheat and rice brans. Among the cell wall degrading enzymes xylanase was more predominant than 1,3/1,4-β-D-glucanase.
- *A. niger* CFR 1105 and *A. oryzae* CFR 232 were able to produce higher levels of xylanase and 1,3/1,4-β-D-glucanase compared with *R. arrhizus* NCIM 997 at 96 h incubation time.
- Ferulic acid esterase (FAE) activity was detected in the culture filtrates of *A. niger* grown on wheat bran, whereas it was not present in the culture filtrates of *A. oryzae* and *R. arrhizus*. Hence *A. niger* CFR 1105 was chosen to study the polysaccharide degradation of rice bran and wheat bran.

- Wheat bran was found to be a better substrate than rice and ragi brans for all the three fungi used in this study with respect to the levels of starch degrading and cell wall degrading enzymes in all the moldy extracts.
- Native wheat bran polysaccharides consisted of arabinose (27%), xylose (39%), galactose (2%) and glucose (30%) with traces of mannose. There was 9- and 19-fold decrease in the arabinose and xylose content respectively in *A. niger* grown on wheat bran, in a 96 h growth period. Native rice bran consisted of arabinose (9%), xylose (27%), galactose (30%) and glucose (32%). There was about 4- and 13-fold decrease in the arabinose and xylose content respectively, in the *A. niger* grown rice bran. These results (decrease in the arabinose and xylose content of experimental samples of wheat and rice brans) indicated extensive degradation of arabinoxylans rather than hexosans during the growth of *A. niger*.
- Wheat bran consisted of ferulic acid and coumaric acids as the major bound phenolic acids, whereas rice bran consisted of syringic acid in addition to ferulic and coumaric acids. Ferulic acid decreased by 8- and 4-fold in the experimental samples of wheat and rice brans respectively at 96 h. Coumaric acid decreased by 7- and 2-fold in the experimental sample of wheat bran and rice bran at 96 and 24 h respectively. Decrease in the bound syringic acid was substantially less than ferulic and coumaric acids. Gallic, protocatechuic and vanillic acids were present in the ratio of

2.2:3:1. Rice bran exclusively consisted of benzoic acid derivatives such as gallic, protocatechuic and vanillic acids as free phenolic acids.

- Feruloyl esterase activities produced by *A. niger* grown in both solid state (ssf) and submerged fermentation (smf) using wheat bran as carbon source and inducer of the enzyme were comparable, however specific activity of the enzyme in culture filtrate of smf was nearly double than that of ssf and was constant upto 5 days. Hence this condition was chosen for isolation and purification of ferulic acid esterase.
- Two isoenzymes of feruloyl esterases designated as FAE-1 and FAE-2 were isolated and purified to homogeneity by conventional protein purification methods from the 5^{th} day culture filtrate of *A. niger* grown in smf with a fold purification of ~11 and 25 respectively.
- Electrophoresis of FAE-1 and FAE-2 under native as well as denatured conditions showed single band indicating them to be monomeric proteins.
- FAE-1 and FAE-2 were found to be glycoproteins by periodic acid schiff (PAS) staining and their carbohydrate contents were found to be 23% and 30% respectively.
- Molecular weights of FAE-1 and FAE-2 were found to be 50 kDa and 55 kDa respectively as determined by gel filtration.
- FAE-1 and FAE-2 exhibited pH optima of 9 and 6 respectively and both were stable over pH range of 6-9. FAE-1 showed temperature optimum of 40 ^{0}C whereas FAE-2 showed wider temperature optimum of 40-50 ^{0}C.

Both FAEs were stable over temperature of 30-40 ^{0}C and ~60% of the activities were retained at 50 ^{0}C.

- The K_m and V_{max} for FAE-1 for hydrolysis of 4NPF were 2 mM and 98 U/mg protein respectively. For FAE-2, K_m and V_{max} were found to be 3.3 mM and 158.7 U/mg protein respectively. FAE-2 was more active than FAE-1 as indicated by its higher V_{max}.
- Purified FAE-1 and FAE-2 showed highest specific activity towards *p*-nitrophenyl ferulate followed by *p*-nitrophenyl acetate and *p*-nitrophenyl butyrate.
- Metal ions such as Fe^{+2}, Ba^{+2}, Mg^{+2} enhanced the enzyme activity to different extent whereas Hg^{+2} and Cd^{+2} reduced the FAE activities.
- Around 40% of the feruloyl esterase activities were decreased in the presence of PMSF (Phenyl methane sulphonyl fluoride) and TLCK (Tosyl-L-lysine chloromethyl ketone) indicating the possible presence of serine in the active site pocket. Enzyme activity was not affected in the presence of iodoacetamide and TPCK (Tosyl-L-phenylalanine chloromethyl ketone) and β-mercaptoethanol.

Conclusions

In the global market, there has been an increasing demand for “natural products”, such as vanillin. In this direction, the present study was attempted to evaluate fungal organisms as a source of starch degrading and cell wall degrading enzymes which have a role to play in obtaining ferulic acid from brans of cereal and millet in order to convert it to vanillin since it has structural similarity with ferulic acid. *A. niger* CFR 1105 was found to be the best compared to *A. oryzae* CFR 232 and *R. arrhizus* NCIM 997 with respect to the induction of starch degrading and cell wall degrading enzymes including ferulic acid esterase.

A spectrophotometric substrate 4-nitrophenyl ferulate (4NPF) is synthesized by a single step for the assay of feruloyl esterases by dehydrative coupling of ferulic acid and 4-nitrophenol. This is the first report on the synthesis of 4NPF by a single step and may find extensive use by researchers and also may increase the prospects of its commercialization. The method described here may find further application for the synthesis of 4-nitrophenyl esters of 4-coumaric acid, caffeic acid, and the like for spectrophotometric assay of 4-coumaroyl esterase, caffeoyl esterase, and other related enzymes given that the spectrophotometric substrates for these enzymes are not commercially available.

Feruloyl esterases (FAEs) from a strain of *Aspergillus niger* (CFR 1105) grown in solid state (ssf) and submerged fermentations (smf) using wheat bran both as carbon source and inducer of the enzyme were studied. Two isoenzymes of ferulic esterases were isolated and purified and characterized from 5^{th} day culture filtrate of *A. niger* CFR 1105 grown under smf condition. The characteristic feature of these two enzymes is that they showed different pH optima. FAE-1 and FAE-2 exhibited pH optima of 9 and 6 respectively and both were stable over pH range of 6-9.

Thus, in this study we showed that FAE is produced from *A. niger* growing exclusively on wheat bran. Therefore this approach may not only help to produce FAE, an industrially important enzyme, but also to degrade cereal brans which is very difficult to degrade and cause pollution in the environment.

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List of Publications

1. **Shyamala Hegde** and Muralikrishna, G., 2009. Isolation and partial characterization of alkaline feruloyl esterases from *Aspergillus niger* CFR 1105 grown on wheat bran. World J Microbiol Biotechnol 25:1963–1969.

2. **Shyamala Hegde**, Srinivas, P. and Muralikrishna, G., 2009. Single step synthesis of 4-nitrophenyl ferulate for spectrophotometric assay of feruloyl esterases. Anal Biochem. 387, 128-129.