Biorefining of wheat bran for the production of feruloyl esterase, ferulic acid and their applications

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By

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CERTIFICATE

This is to certify that the work incorporated in this Ph.D. thesis entitled "**Biorefining of wheat bran for the production of feruloyl esterase and ferulic acid and their applications**" submitted by **Mr. Nishant Gopalan** to Academy of scientific and Innovative Research (AcSIR) in fulfillment of the requirements for the award of the Degree of, **Doctor of Philosophy In Biological Sciences,** embodies original research work under my guidance. We further certify that this work has not been submitted to any other University or Institution in part or full for the award of any degree or diploma. Research material obtained from other sources has been duly acknowledged in the thesis. Any text, illustration, table etc., used in the thesis from other sources, have been duly cited and acknowledged.

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-Nishant Gopalan

Dedicated To

My Teachers & My Mother

Contents

List of Abbreviations

%	Percent
µ	micron
µg	microgram
µm	micrometre
µM	micromolar
µL	microlitre
°C	Degree Celsius
ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
BSA	Bovine Serum Albumin
CCD	Central Composite Design
cm	centimetre
CSIR	Council for Scientific and Industrial Research
G	gram
g/L	gram per litre
gds	Gram dry substrate
h	hour
HCA	Hydroxycinnamic acid
HPLC	High Performance Liquid Chromatography
kDa	kilodalton
K_m	Michaelis constant
L	litre
M	Molar
mg	milligram
min	minute
mL	millilitre

mM	millimolar
NIIST	National Institute for Interdisciplinary Science and Technology
nm	nanometre
PAGE	Polyacrylamide Gel Electrophoresis
PBD	Plackett - Burman Design
PDA	Potato Dextrose Agar
Rpm	Rotations per minute
SDS	Sodium Dodecyl Sulphate
sec	seconds
SEM	Scanning Electron Microscopy
sp.	Species
SSF	Solid State Fermentation
U	Units
U/gds	Units per dry gram substrate
U/mL	Units per millilitre
UV	Ultraviolet
v/v	Volume per volume
V_{max}	Maximum velocity
w/v	Weight per volume
DSWB	Destarched Wheat Bran
DMSO	Dimethyl sulfoxide
o-	ortho
p-	para
TGA	Thermogravimetric analysis
DTG	Differential thermogravimetric analysis
TS	Tensile strength
MPa	Mega Pascal
FAE	Ferulic acid esterase/Feruloyl esterase

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Chapter I

Introduction and Review of literature

1.1 Introduction

With the looming fuel crisis, cutting edge research is focused on fuel production from lignocellulosic biomass. Second generation bioethanol is one of the major thrust areas. The technology involves the breakdown of lignocellulosic materials into component sugars that can be then converted into ethanol via the fermentative route. The breakdown of lignocellulosic biomass requires the use of various carbohydrate degrading enzymes like cellulases and hemicellulases. Feruloyl esterases (FAEs) are hemicellulase accessory enzymes that do not release sugars directly, however, make it better available for carbohydrate binding enzymes. The process currently is not economical enough to replace automobile fuels (Menon and Rao 2012). To bring down the cost of fuel ethanol, subsidiary processes or by-products of high-value must be spun out, more akin to a petroleum refinery, and hence the process is claimed to be biorefining.

Producing copious amounts of the enzyme feruloyl esterase is the primary requirement of this study with a focus on a cheap process; it has thus been decided to use agro-residual substrate wheat bran for the process of production of the enzyme. The enzyme feruloyl esterase can have its own applications which will further add value to the whole process. Understanding the biochemical properties of feruloyl esterase enzyme once it has been produced helps us in zeroing in on conditions which might be most useful to store the enzyme and to use it to its full potential. Therefore it is very essential to study the biochemical characteristics ot the enzyme. Finally it becomes essential to test the enzyme in processes similar to those in the industries to extract the maximal possible ferulic acid out from agro-residual substrates, so as to test the enzymes feasibility for industrial use. The wide economic gap in biofuel generation cost and final sale price is a problem that threatens the viability of the future generations. Ferulic

acid is a low-volume, high-value products that can help bridge this economic gap. A compound that has use as ingredient in the high profit sector of cosmeceuticals and even in the pharmaceutical sector with neuroprotective action and anti glycemic activities as further discussed in section 1.2, currently it has a value close to 80USD/Kg (Zauba_Ferulic_Acid_import_data 2017). Products with ferulic acid are in their infancy and therefore the current price is definitely subject to sharp increases as the demand for these products goes up. HCAs are ubiquitously present in plant material; however, the distribution of these compounds is varied. By-products of first generation ethanolic fermentations as a fuel (maize) or as a drink (wheat/barley) can be used as a source of ferulic acid. Removing ferulic acid and other HCAs from the animal fodder meant for monogastric animals and ruminants increases the digestibility and hence the nutrient quality of the feed. The extracted biomass may, therefore, be used as forage feedstock (Harholt, Bach et al. 2010).

Therefore it is absolutely necessary that processes like production of feruloyl esterase and ferulic acid from agro-residual biomass like wheat bran be studied thoroughly.

1.2 Objectives

1. To study amount of alkali releasable ferulic acid in various agro-residual biomass and assess if there is a relation between ferulic acid content of biomass and feruloyl esterase production.

2. To study the major parameters influencing the production feruloyl esterase in solid state fermentation using *Aspergillus niger* ATCC 13497 and to maximize the production of the enzyme through the understanding of the process.

3. To study the biochemical characteristics of the enzyme feruloyl esterase produced.

4. To study the enzyme feruloyl esterase as to find the conditions for maximal extraction of ferulic acid from destarched wheat bran and to test the parameters in a packed column reactor.

5. To study applications of feruloyl esterase enzyme and ferulic acid for esterification of ferulic acid with arabinose, and understand the influence of ferulic acid in chitosan dextran composite films.

1.3 Ferulic acid

Ferulic acid is a derivative of cinnamic acid, it is trans-4-Hydroxy-3-methoxycinnamic acid, with CAS # 537-98-4. It is a phenolic acid found abundantly in nature esterified to plant cell wall components (Smith and Hartley 1983, Mathew and Abraham 2004, Zhao and Moghadasian 2008). The phenolic acid was first discovered in 1866 by a pair of researchers from the Vienna Science academy, viz. Halsiwetz and Barth (Hlasiwetz and Barth 1866). The pair found the compound in the form of a yellow precipitate in the alcoholic extract from the plant *Ferula foetida*. Dutt (1925) later chemically synthesized ferulic acid from malonic acid and the crystallographic structure of ferulic acid was solved by (Nethaji, Pattabhi et al. 1988). Ferulic acid is a phenolic or hydroxycinnamic acid found abundantly in nature as a component of the plant cell walls. Ferulic acid has a molecular weight of 194.186 g/mol, with a chemical formula $C_{10}H_{10}O_4$(Pubchem_CID_445858). Naturally the compound can dissociate two hydrogen ions , the first one from the carboxylic acid to form the carboxylate ion, this is followed by the donation of hydrogen from the hydroxyl group from the 4’ carbon to form a phenoxy moiety. The phenoxy moiety thus formed is stabilized by the resonant structure of the ring. Due to the apparent stability of this phenoxy moiety, ferulic acid is one of the best antioxidant molecules. Ferulic acid is found in nature in two forms the trans and the cis form of which the trans form is much more abundant due to a more favorable entropy state of the hydrogen atoms across the double bond. The trans form is a solid at room temperature and forms

long needle shaped crystals, and an average particle size of 61 µm, while cis-ferulic acid is present as a yellowish liquid at room temperature. Trans Ferulic acid has a melting temperature between 168-171^{o}C, and a vapor pressure of 2.69X10^{-6} mm Hg at 25^{o}C. Trans ferulic acid has a solubility of 5.97 x 10^{3} mg/L in water at 25^{o}C). The pKa for the hydroxyl group of the carboxylic acid group is 4.58. The partition coefficient for trans ferulic acid in n-octanol and water system is 0.3753 at 25^{o}C, when the pH is adjusted to 3 indicating that trans ferulic acid is quite hydrophilic (Sohn and Oh 2003). Ferulic acid also is a UV-B blocking agent as it absorbs ultraviolet light near 320 nm. The absorbance maximum of ferulic acid is 322 nm. Ferulic acid is the active pharmaceutical component in traditional Chinese medicines made from *Angelica Sinensis,* and has many properties including neuroprotective, anti ageing, skin whitening (α Tocopherylferulate), and that of being hypoglycemic (Funaska, Komot et al. 2000, Ohnishi, Matuo et al. 2004, Cheng, Su et al. 2008, Barone, Calabrese et al. 2009, Koh 2012, Mancuso and Santangelo 2014, Ojha, Javed et al. 2015). This hydroxycinnamic acid is one of the most abundant phenolic acids found in the plant cell wall. Ferulic acid in plants is produced through the products of the shikimate pathway, with amino acids phenylalanine and tyrosine acting as precursors(Weaver and Herrmann 1997). These amino acids are deaminated respectively by ammonia lyase enzymes specific for phenyl alanine and tyrosine, which leads to the formation of cinnamic acid, and p-coumaric acid respectively. Ferulic acid is a derivative of p-coumaric acid which undergoes serial hydroxylation and methylation to finally give the product. Most of the hydroxycinnamic acids are obtained through this route by modification of the original parent molecule of cinnamic acid, these include sinapic acid, ferulic acid, caffeic acid and p-coumaric acid(Boudet, Lapierre et al. 1995). These molecules are essential for the development of the plant as these molecules are precursors for lignin, which forms the first line of defense for plant cells. The corresponding acids are activated by ligation with Co enzyme A and then transferred to a lignin superstructure. A portion of the acid derivatives ligated to Co enzyme A are also diverted to the flvanoid pathway. The lignin is therefore composed of alcohol derivatives of these hydroxycinnamic acids (ether bonds)

and the free acids esterified may also be esterified to the lignin and arabinan content which account for the phenolic component of the cell walls in plant.(Hu, Kawaoka et al. 1998).Varying percentages of ferulic acid are found as diferrulates, oligoferulates or dehydrodimers of ferulic acid, depending on the amount of cross linking in the heteropolymer (Hatfield, Ralph et al. 1999). The antioxidant activity of ferulic acid is known to stem from the potential of the molecule to donate hydrogen from the hydroxyl group present with the phenolic ring. The substitution of the hydroxyl group in the ortho position to a methoxy group further increases the probability of donation of the hydrogen from the hydroxyl group in the para position as it reduces the hydrogen bond strength between the ortho and para groups(de Heer, Korth et al. 1999, Zhang, Ge et al. 1999).It was found that the antioxidative potential of coniferyl alcohol was higher than that of ferulic acid (Nenadis, Zhang et al. 2003), which would explain the use of coniferyl alcohol as a monolignol instead of ferulic acid as it would easily oxidatively cross link to form the lingnin macromolecular complex. Ferulic acid has been found in plant cell walls in different forms including the free form, dimer, trimer and the oligomer form(Barberousse, Roiseux et al. 2008). Ferulic acid as a precursor for monolignol formation is converted to sinapic acid with an additional methoxy group, giving syringyl lignin for higher plants including many dicots.

C type lignin Obtained from p-coumaric acid (p-hydroxyphenyl) is a hallmark of monocots, while G lignin obtained from ferulic acid with coniferyl alcohol moieties is found abundantly in gymnosperms. Both S (obtained from sinapic acid with syringylalcohol) and G lignins are found in angiosperms. Lignin is known to incorporate other non conventional molecules like C (obtained from caffeic acid) lignin and 5H lignin apart from a wide variety of molecules including ferulate monomers, other hydroxycinnamic acids, hydroxycinnamaldehydes etc. The ferulate ornaments normally function as linkers to other polymers of the plant cell wall viz. hemicelluloses or cellulose(Zhao 2016). Ferulic acid can therefore be safely assumed to be a vital part of the plant metabolome.

1.4 Presence of ferulic acid in Planta

1.4.1 Ferulic acid in dicots

Ferulic acid in dicots are found normally in the pectic polymer, linked to various sugar moieties, while other plants which posses the ability to carry out cell wall suberization have a large percentage of the hydrophobic extractable components to be ferulic acid esters of omegahydroxy fatty alcohols(Riley and Kolattukudy 1975, Marques, Pereira et al. 1994). Suberized cells are found in plants on the outer surfaces, where there is a need of a barrier for water. Suberin also functions as a protectant from pathogen attacks, and excessive heating (Schönherr 1982, Kolattukudy 2001, Thomas, Fang et al. 2007). These are complex polymers are composed of polyphenols, polysaccharides, amines and crosslinked aliphatic fatty acids and aliphatic fatty alcohols. A large part of the hexane extractive from suberin accounts for feruloyl esters of aliphatic acid (Marques, Pereira et al. 1994) .While the poylphenols are essentially guaicyl lignin type polymers that use ferulic acid as precursor. Most of the studies on suberinhas been carried out using the potato healing wound tissue model. The complex polymer consists of crosslinks made by diferulates linked to tyramine and octopamine which are in turn linked to the aliphatic main chains(Negrel, Pollet et al. 1996). Different dehydro dimers corresponding to the 5-5’ and 8-5’ diferulates are found in the suberin structure of the potato endoderm(Graça 2010). Suberins and related polymers are found in dicots, monocots and even in gymnosperms. Ferulic acid esters were found in the bark of hardwood trees (dicots) *Erythrina variegata, E. stricta,* and *E. blake(Kolattukudy and Espelie 1989).* Recently an unusual ester of alpha hydroxyl fatty acid with ferulic acid was discovered and isolated from the suberized tissue of *Eucalytus globulus* , The said compound was very hydrophobic and would cause problems during the pulping of the wood from the said trees(Freire, Silvestre et al. 2002).

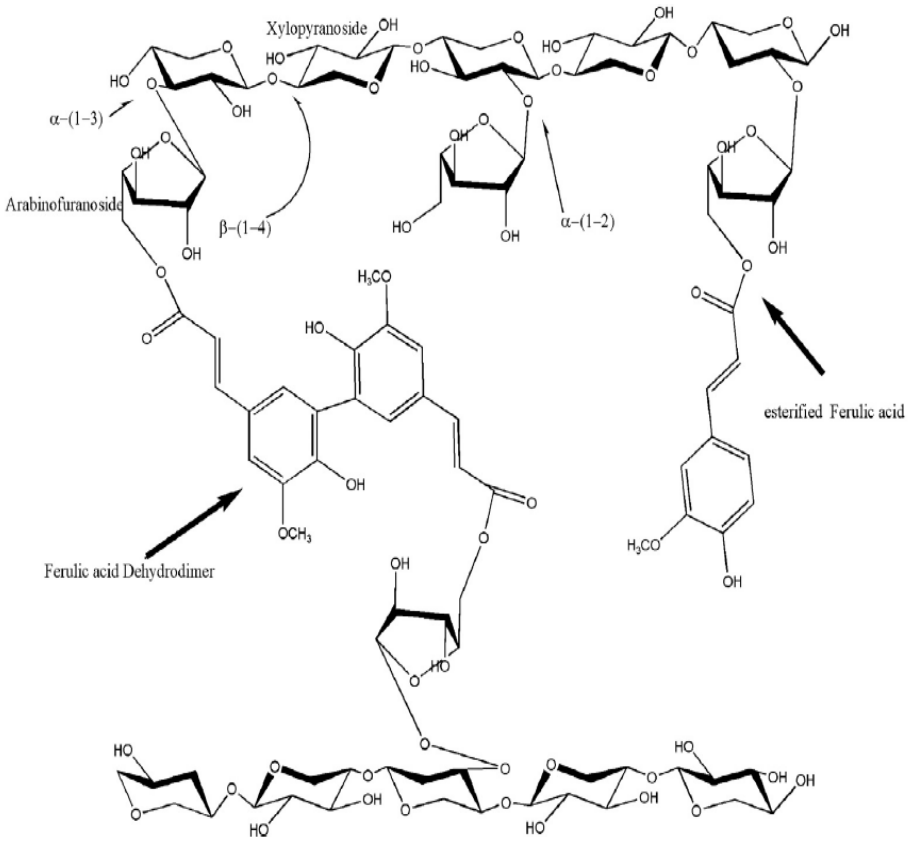
In dicots like sugar beet and spinach, the ferulic acid moiety is linked to the O-6 of the galactose residue in pectin. About 0.14% w/w is in the form of diferulates, while majority of it is monomeric esterified ferulic acid in sugar beet(Saulnier and Thibault 1999). Apple marc and coffee pulp have been shown to be a valuable source of chlorogenic acid, caffeic acid, *p*-coumaric acid. Coffee pulp has significant amounts of chlorogenic acids, (~2600 mg/Kg) while apple cider processing facilities have chlorogenic acid about 0.5mg/g(Benoit, Navarro et al. 2006, Torres-Mancera, Baqueiro-Peña et al. 2013). Classically sugar beet pulp has been used for the production of FAEs and is also used as a substrate for the enzymatic release of free ferulic acid. Sugar beet pulp can have as much as 6000 mg/Kg of total alkaline extractable ferulic acid (Benoit, Navarro et al. 2006). The phenolic acid is found in other dicots like carrots, cabbage, tomatoes, sweet potato, turnips, Japanese rasin tree among others(Cho, Moon et al. 2000, Mattila, Hellström et al. 2006, Mattila and Hellström 2007). Ferulic acid found in orange (*Citrus sinensis*) is partitioned mostly in the peel of the fruit, with contents as much as 178mg/Kg while the peel of grape fruit (*Citrus paradisi*) has 155 mg/Kg, another member of the citrus family, *Citrus poonensis*has higher ferulic acid content in the peel, in the range 1000-1500 mg/Kg(Peleg, Naim et al. 1991, Xu, Ye et al. 2008). The grain of amaranth (*Amaranthus caudatus*) has 602 mg/Kg of trans-ferulic acid and 203 mg/Kg of cis-ferulic acid (Bunzel, Ralph et al. 2005). Pectins are composed of smooth regions which are essentially linear polymeric structures of galacturonic acid linked by α-(1-4) linkages; these linear polymers are interrupted by rhamnose residues generally by α-(1-2) linkage as a branching point(Mohnen 2008). The rhamnoglacturanones in turn bear side chains in the form of arabinans and glactans. The main linear polymer composed of glactunronic acid may be acetylated or methylated at regular intervals. Apart from above mentioned rhamnoglacturanones, other type of complex rhamnoglacturanones (type II) also exist, where branching happens due to a glycosidic link between D-apiose and glacaturanone(Mohnen 2008). Typically smooth regions consist of majority of pectins followed by type I rhamnogalacturanones with decorations of arabinans and galactans. Ferulic acid is

exclusively linked to main chain arabinans via 2-O- ester linkages and to galactans via the 6-O- ester linkages (Guillon, Thibault et al. 1989). Total esterified ferulic acid in pectins are almost equally distributed as 2-O linked arabinose ester and 6-O linked galactose ester(Ralet, Faulds et al. 1994). The function of the ferulic acid decorations is normally because cross linking of the ferulate moieties between the side chains. Various different diferulate moieties have been reported from sugar beet pectin including 5-5, 8-O-4, 8-5 and 8-8 dehydrodiferulic acids (Micard, Grabber et al. 1997, Oosterveld, Grabber et al. 1997).

1.4.2 Ferulic acid in monocots

Ferulic acid, is an abundant phenolic acid in monocots, especially in poales, with increased distribution of the phenolic acid in the bran of the grains. Ferulic acid functions as a cross linker for the hemicellulosic part of the plants (Figure 1.1) (Hatfield, Ralph et al. 1999, Kroon and Williamson 1999, Buanafina 2009). Ferulic acid and related phenolic acids are responsible for the blue green fluorescence found in grains, when excited with a UV source of wavelength 354 nm(Harris and Hartley 1976). Ferulic acid serves as a link between lignin polyphenols and heicellulose (Figure 1.2). The amount of feruloylation increases with the increase in the arabinose substitution along the xylan backbone. Peroxidase activity increases as the cell ages and the cell wall becomes set.

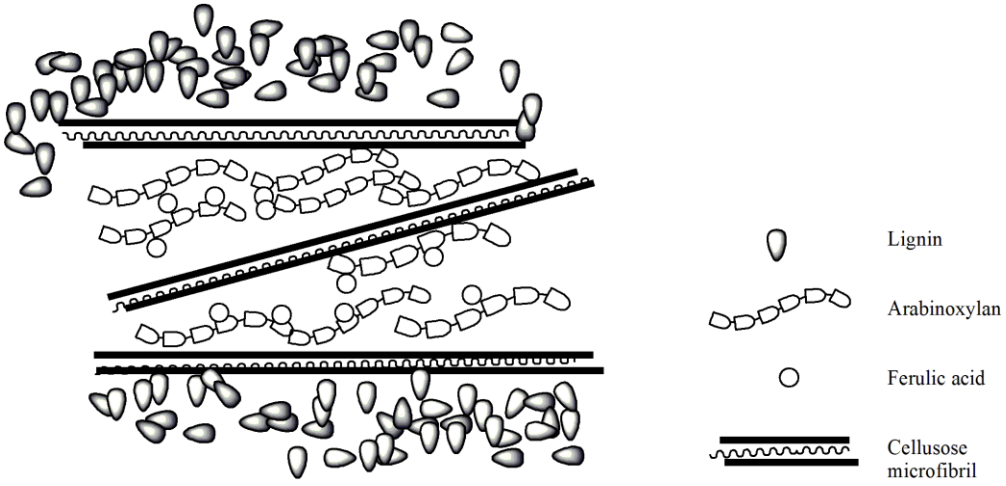
Figure 1.1 typical ferulic acid linkages in monocots within hemicelluloses.



Many cereal brans have very high concentrations of ferulic acid and related HCAs and are used as a starting material for enzymatic extraction of ferulic and allied HCAs. Grasses like switchgrass, sugarcane bagasse, wheat straw, barley straw (Qi, Wang et al. 2011), have been known to be used for the enzymatic release of hydroxycinnamates. Among bran substrates, maize/corn bran has the highest amount of esterified ferulic acid content (30000 mg/Kg), while de starched wheat bran can have compositions ranging from 4000 – 6000 mg/Kg (Faulds, Mandalari et al. 2004, Topakas, Stamatis et al. 2004, Benoit, Navarro et al. 2006). De starched wheat bran is even used as a substrate to measure the enzyme activity of FAE. Apart from wheat bran other synthetic substrates like methyl and ethyl ferulate, methyl sinapate, methyl *p*-coumarate, methyl caffeate are also used as substrates for analysing the FAE activity (Couteau, McCartney et al. 2001). Steam exploded wheat straw is a source of *p*-

coumaric acid, with 2130 mg/Kg and ferulic acid at 1350 mg/Kg (Benoit, Navarro et al. 2006, Wu, Abokitse et al. 2012). Sugarcane bagasse also contains *p*-coumaric acid and ferulic acid (Xu, Sun et al. 2005). Rice bran and triticale bran both have been used as substrates for the enzymatic release of ferulic acid. Rice bran is known to have ferulic acids about 900 mg/Kg, and triticale bran has 2200 mg/Kg esterified ferulic acids (Mathew and Abraham 2004, Wu, Abokitse et al. 2012). Brewers spent grain is also a valuable source as a substrate for the enzymatic release of ferulic acids, along with oat hulls (Crepin, Connerton et al. 2004, Yu, McKinnon et al. 2004). All of the above-mentioned sources are by-products, with very little economic value. Many of these can be used as feedstock for lignocellulosic bioethanol. Generating high-value phenolic acids through maximal enzymatic recovery is therefore of priority.

Figure 1.2 Positioning of Ferulic acid in lignocelluloosic biomass



1.4.3 Ferulic acid in gymnosperms

Unlike the angiospersm where ferulic acid is majorly found esterified to cell walls of only certain as part of the complex structure of suberinsgroups of plants within the monocots (commelinid clade) and dicots (core caryophylales), all the extant gymnosperm genera have ferulic acid esterified to the primary cell wall (Carnachan and Harris 2000). Just like their angiosperm relatives, ferulic

acid is also found esterified to long chain fatty acids and fatty alcohols in gymnosperms (Lotfy, Javelle et al. 1995). Trans-ferulic acid is present in the range of 88-1,561 mg/Kg of cell wall preparation of gymnosperms (Carnachan and Harris 2000).

1.5 Feruloyl esterase

Feruloyl esterases (EC#3.1.1.73) is a group of versatile enzymes that can be used as xylanase accessory enzymes to increase the efficiency of hydrolysis of lignocellulosics or can also be used as a synthetic catalyst to produce various esters of ferulic acid and the ferulic acid released from different sources can be converted by controlled bio-oxidation to flavor compounds vanillin and vinyl guiacol (Faulds and Williamson 1995, Bonnin, Saulnier et al. 2002, de Vries, vanKUYK et al. 2002, Laszlo, Compton et al. 2006a, Mastihubová, Mastihuba et al. 2006, Mathew, Abraham et al. 2007). Cinnamoyl ester hydrolase, ferulic acid esterase and hydroxycinnamoly esterase are alternative names to the said enzyme. Feruloyl esterases aid in the release if ferulic acid from various lignocellulosic feedstocks without the use of alkali, hence the process can be categorized as a green process. Traditionally, ferluloyl esterases are assayed by detecting the ferulic acid released from a methyl/ethyl ester by HPLC or by the colorimetric detecting 4-nitrophenol by the action of the enzyme on 4-nitrophenol ferulate as the substrate (Mastihuba, Kremnický et al. 2002, Damásio, Braga et al. 2013a, Esteban-Torres, Reverón et al. 2013, Chyba, Mastihuba et al. 2014).

1.5.1 Classification and Sources of FAE

FAEs are a subclass of the carboxylic acid esterases that are able to hydrolyse the ester bond between HCAs and sugars present in the plant cell walls. FAEs were classified into 4 groups (type-A, B, C and D) by Crepin et al. (2004) based on substrate utilization data and supported by primary sequence identity. Type A FAEs show preference for the phenolic moiety of the substrate that contain methoxy substitutions, especially at meta-position, as occurs in ferulic and

sinapinic acids. Type B FAEs shows preference to substrates containing one or two hydroxyl substitutions as found in p-coumaric or caffeic acid. Type A and D FAEs are able to release low quantities of diferulates. Type C and D FAEs exhibit broad specificity against synthetic HCA esters (methyl sinapate and methyl p-coumarate and methyl caffeate) showing difference only in the ability to release diferulates. Type A FAEs are active only on substrates containing FA ester linked to the O-5 and not on substrates containing ferulic acid ester linked to the O-2 linkages of L arabinofuranose. Type B FAEs are active on substrates containing FA ester linked to both O-5 and O-2 of L arabinofuranose. Type C and D FAEs are able to hydrolyze both linkages (Crepin, Faulds et al. 2004, Topakas, Vafiadi et al. 2007). The empirical classification system of four groups has been further expanded and improved (Benoit, Danchin et al. 2008, Udatha, Kouskoumvekaki et al. 2011), however, it continues being widely used in scientific papers.

Extensive study by Udatha et al. (2011) has led to the classification of FAEs into 12 distinct families. The improved distinction was possible through the use of descriptor based classification and machine learning algorithms. This classification system does not contradict but expands the knowledge in the area and allows a systematic understanding of the mode of action of FAE. Dilokpimol, Mäkelä et al. (2017) have carried out multiple sequence alignment and phylogenetic analysis of all available feruloyl esterase protein sequences and constructed a phylogenetic tree based on the amino acid sequences. The analysis revealed the presence of thirteen superfamilies of the enzyme.

Extensive research for finding FAEs that release greater amounts of alkali releasable HCAs has led to a wide variety of organisms and enzymes. Aspergilli have been found to have FAEs that are effective in releasing HCAs in various species. Anaerobic fungi and bacteria have also been employed for mining FAEs. These include fungi like Piromycesequii, Anaeromycesmucronatus, Neocallimastixspp, Orpinomyces, Clostridium stercorarium, Clostridium thermocellum, etc. (Borneman, Hartley et al. 1990, Borneman, Ljungdahl et al. 1992, Fillingham, Kroon et al. 1999, Blum, Kataeva et al. 2000, Donaghy,

Bronnenmeier et al. 2000, Qi, Wang et al. 2011). FAEs are extensively prevalent in ascomycta and recently a study based on the information provided by FAE sequences in Ascomycota found the enzyme sequences phylogenetically important (Olivares-Hernández, Sunner et al. 2010).

1.5.2 Production of feruloyl esterase

FAE is produced by filamentous fungi or bacteria in submerged fermentation and solid state fermentation. Culture media usually contain soluble ferulic acid esters (methyl or ethyl ferulate) or agro industrial waste rich in bound ferulic acid (e.g. wheat bran, oat spelt xylan, sugar beet pulp) as the carbon source and enzyme inducer. Under these conditions, titres of very low activity have been obtained (usually less than 1 U/mL of culture medium) (Faulds and Williamson 1994, Bonnina, Brunel et al. 2001, Shin and Chen 2006, Mukherjee, Singh et al. 2007).

To enhance the production of feruloyl esterase, several researchers have evaluated the use of solid-state fermentation. Solid-state fermentation has several advantages over submerged fermentation for the production of certain enzymes. These advantages include higher enzyme titres and higher productivity, low level of catabolic repression and increased stability of the excreted enzymes. Furthermore, solid-state fermentation allows the use of agro-industrial wastes as and support carbon source. Asther, Haon et al. (2002)compared the production of FAE by *Aspergillus niger* I-1472 in solid-state fermentation and submerged culture conditions using sugar beet pulp as support and carbon source. Maximal activity was reached around 4 d of incubation. Activities against methyl-sinapate and methyl-ferulate did not significantly differ in liquid and solid fermentation. However, when methyl caffeate and methyl *p*-coumarate were used as substrates, activities were significantly higher in solid-state fermentation. These observations indicated the induction of at least two esterases with different substrate specificity. Hegde and Muralikrishna (2009) studied the production of FAE by *Aspergillus niger* CFR 1105 grown in solid-state and submerged fermentation, using wheat bran as a carbon source and inducer of the enzyme were studied.

Maximum activity titers were similar in solid-state and submerged fermentations (32.5 and 31.5 U/g dry wheat bran, respectively), but the highest FAE activity was reached a day earlier (4 days) in solid-state fermentation than submerged fermentation.

1.5.3 Molecular approaches for enhanced production of feruloyl esterase

Record, Asther et al. (2003) constructed a vector containing the cDNA encoding *Aspergillus niger* FAE A with its signal peptide and used it to transform a protease deficient *Aspergillus niger* strain. Using glucose as carbon source (60 g/L) the transformed strain produced 20.6 nkat/mL of FAE. This enzyme was easily purified and used for wheat straw pulp bleaching. Levasseur, Benoit et al. (2004)inserted the *fae*B gene from *Aspergillus niger* BRFM13 into an expression vector under the control of the *gpd* promoter and expressed in a protease deficient *Aspergillus niger* strain. This homologous overproduction system allowed reaching a FAE activity of 18 nkat/mL, 16-fold higher as compared to the production with non-transformed *Aspergillus niger* strain induced by sugar beet pulp.

There have been few reports of FAEs from different sources being cloned heterologously or otherwise and used for characterizing the property of the enzyme with respect to enzymatic release capacity of the same from different agro by-products. Levasseur, Saloheimo et al. (2006) constructed a chimeric enzyme, consisting of the *AnFaeA* and *Trichoderma reesei* swollenin. It was expressed in *Trichoderma reesie* Rut30 and CL847 strains. The pure form of the enzyme was not as effective as the complete extracellular component of the chimeric protein expressed in CL847 strain, which could release 45% of total alkali releasable ferulic acids from de starched wheat bran. FAEZ a part of a xylanase enzyme in *Clostridium thermocellum* was expressed in *E. coli* and used to release ferulic acid from defatted jojoba meal, with about 1.8 U of the enzyme, 76% of the total ferulic acid was released by 24 hours (Laszlo, Compton et al. 2006b).*Thermoanaerobacter tengcongensis TtFaeA* was expressed in *E.coli* and used to release ferulic acid from triticale bran. The enzyme was thermostable

up to 80^{o}C and released 30% of ferulic acid using approx 0.8 U/g as enzyme loading and solid loading of 5% wt/vol. The reaction mixture was initially treated with 10 U of xylanase initially at 37 ^{o}C for 16 hours, followed by the action of the FAE at 55^{o}C for 5 hours (Abokitse, Wu et al. 2010). In a study by Wong, Chan et al. (2011), cloned and expressed *AnfaeA* gene from *Aspergillus niger* into *Saccharomyces cerevisiae*and the extracellular fraction of the yeast in YMB medium was able to release about 3.0µg /100mg of ferulic acid from switch grass (total releasable ferulic acid 2 mg/g). A landmark publication for FAEs from bacterial origin was published, with the bacterial enzymes being able to release high amounts of ferulic acid and *p*-coumaric acid from triticale bran, de starched wheat bran and even condensate of steam exploded wheat straw. The *Sorangium cellulosum* FAEs *ScFae1* and *ScFae2* were cloned into *E.coli* cells and were purified. The maximal release of ferulic acid from triticale bran of 96% was observed, when 0.45 U/ml of the enzyme *ScFae2* was incubated with 5.3U/ml of xylanase to triticale bran suspension of 5%, within 3h of incubation at 37^{o}C with shaking at 200rpm (Wu, Abokitse et al. 2012).

1.6 Application of FAEs for the release of HCAs

There is considerable interest in the academic and industrial sects in the search for new sources of FAEs and applications, therefore. The interest is evident through the number of patent applications and patents granted in the last 15 years (Table 1.1). Most of these patents are related to FAEs from microbial sources, the polypeptide sequence of the same, polynucleotides encoding the polypeptides, nucleic acid constructs, vectors, and host cells comprising the polynucleotides as well as methods of producing and using the polypeptides. Patented applications include the production of ferulic acid and other HCAs, its use as an additive in food and feed. The use of FAE as an accessory enzyme in the hydrolysis of lignocellulosic materials for the production of oligosaccharides and biofuels has been patented as well. Among the companies that have patented on FAEs include Novozymes AS and its subsidiary Novozymes Inc. On the academic side,

researchers from several universities and research institutes in UK, Canada, China and France have filed patent applications on FAEs

Table 1.1 Recent patents related to feruloyl esterase

Patent number	Applicant	Title
WO200212472	Novozymes AS [DK] Novo Nordisk AS [DK]	Stereoselective esterase from *Aspergillus oryzae*
WO2004009804	Biocatalysts Ltd [GB] University of Nottingham [GB] Institute of Food Research [GB]	Feruloyl esterase and uses thereof
EP1913136	InstitutNational de la RechercheAgronomique [FR] InstitutFrançais du Pétrole [FR]	Fused protein containing plant cell wall-destroying enzymes and use thereof
US2010047320	McGill University [CA]	Oral polymeric membrane feruloyl esterase producing bacteria formulation
WO2008116319	McGill University [CA]	Bioproduction of ferulic acid and uses thereof
GB2324302	Biotechnology and Biological Sciences Research Council [GB] University of Newcastle [GB]	Phenolic acid esterase
US2010256353	Biocatalysts Ltd [GB]	Use of type C and D feruloylesterases in the manufacture of biofuels

RU2358756	OooNpkFermtek [RU]	Method of producing enzymatic agent for degradation of hemicellulose heteropolysaccharides of herbal cytoderm and enzymatic agent
US8034995	Novozymes AS [DK]	Polypeptides having feruloyl esterase activity and polynucleotides encoding same
CN101228921	China Agricultural University [CN]	Compound feruloyl esterase additive for feed and using method thereof
US8058513	NovozymesInc [US]	Polypeptides having feruloyl esterase activity and polynucleotides encoding same
US8609933	NovozymesInc [US]	Polypeptides having feruloyl esterase activity and polynucleotides encoding same
US8637292	Novozymes AS [DK]	Polypeptides having esterase activity and nucleic acids encoding the same
CN101492700	Jianshe Wang [CN]	Intensive processing method for stalk articles or agricultural castoff
CN102002508	Jilin University [CN]	Feruloyl esterase, code gene and application thereof
CN102190586	Chengdu Institute of Biology [CN] Chinese Academy of Sciences [CN]	Two new compounds for enzyme activity assays of feruloyl esterase, and preparation methods thereof
CN102220299	Chengdu Institute of Biology [CN] Chinese Academy of Sciences [CN]	Feruloyl esterase A mutant and purpose thereof

CN102286442	Zhejiang University [CN]	Method for producing feruloyl esterase by fermentation of *Aspergillus fumigates*
CN102605021	Tianjin Modern Vocational Technology College [CN]	Method for preparing xylo-oligosaccharide syrup and powdered sugar by using complex enzyme
CN102676468	Shandong Agricultural University [CN]	Feruloyl esterase PCFAE2 from Phytophthoracapsici coding gene and application
CN102703402	Shandong Agricultural University [CN]	Feruloyl esterase PCFAE1 from Phytophthoracapsici, coding gene and application thereof
CN102703403	Jiangnan University [CN]	Cloning of feruloyl esterase gene (Fae-A) and preparation of recombinant enzyme
CN102796670	Zhejiang University [CN]	*Aspergillus niger* strain and application thereof
WO2014020141	Dupont Nutrition Biosciences APS [DK]	Feed additive composition
CN102864180	Huaqiao University [CN]	Method for simultaneously preparing ferulic acid, xylo-oligosaccharides and ethanol from spent grains
CN102796673	Yellow River Delta Chambroad Research Institute of Chemical Industry Co. Ltd [CN] Shandong Jingbo Holdings Co., Ltd. [CN]	Feruloyl esterase production strain and method for producing feruloyl esterase by using same
CN102888388	Zhejiang University [CN]	Method for producing feruloyl esterase by solid fermentation
CN102894228	Huaqiao University [CN]	Broiler chicken feed

CN102978180	Jiangnan University [CN]	Clone and expression of *Aspergillus oryzae*feruloyl esterase gene (FaeA)
CN103061181	Yellow River Delta Chambroad Research Institute of Chemical Industry Co. Ltd [CN]	Complex enzyme preparation and technique for preparing dissolving pulp by using same
CN103074314	Zhejiang University [CN]	Method for producing feruloyl esterase through *Aspergillus niger* fermentation
WO2014135063	Novozymes AS [DK]	Milling process
CN103320496	Northwest A & F University [CN]	Method for detecting activity of feruloyl esterase of anaerobic fungi
CN103789357	Qilu University of Technology [CN]	Method for producing 4-vinyl guaiacol by fermenting *Bacillus circulans*
CN103642850	Jiangnan University [CN]	Determination method of ferulic acid antioxidant activity in vitro
CN106753945A	Jiangnan University [CN]	A method for improving the flavor characteristics of wheat beer
CN106434711A	Jiangnan University [CN]	Method for improving enzyme activity of feruloyl esterase
CN106263010A	Hubei tobacco industry private limited [CN]	Method for improving sensory quality of reconstituted tobaccos through biotransformation

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Enzymatic release of HCAs and its efficiency are directly related to the type of substrate that is employed and to an extent the type of enzyme that has

been used to carry out the process. Pre-treatment of the substrate also influences the efficiency of the process, along with the parameters that are used for the release of the HCAs, like temperature, time, agitation, presence or absence of co-solvents like TWEEN and DMSO. Ferulic acid is the primary HCA that is targeted by the enzymatic treatment. Some of these processes have been summarized in Table 1.2

Table 1.2. Enzymatic extraction of HCAs from plant material

Biomass	**Enzyme**	**Phenolic acid**	**Efficiency**	**Reference**
Sugar beet pulp	*A. niger* crude extract	Ferulic acid	70%	Bonnin, Saulnier et al. (2002)
Autoclaved maize bran	*A. niger* crude extract	Ferulic acid	95.5%	Bonnin, Saulnier et al. (2002)
Wheat bran	*P. funiculosum* FAE B + *Trichoderma viride*xylanase	Ferulic acid	98%	Kroon, Williamson et al. (2000)
Wheat bran	*Talaromyces stipitatus* FAE C	Ferulic acid	1.5%	Garcia-Conesa, Crepin et al. (2004)
Sugar beet pulp	*Talaromyces stipitatus* FAE C	Ferulic acid	5.8%	Garcia-Conesa, Crepin et al. (2004)
Sugar beet pulp	*Talaromyces stipitatus* FAE C + *A. niger*endo-arabinanase and α-L-arabino-furanosidase	Ferulic acid	20%	Garcia-Conesa, Crepin et al. (2004)
De starched wheat bran	*Sporotrichum thermophile* FAE	Ferulic acid	0.7%	Topakas, Stamatis et al. (2004)

De starched wheat bran	*Sporotrichum thermophile* FAE + xylanase	Ferulic acid	33%	Topakas, Stamatis et al. (2004)
Wheat bran	*Humicola insolens* commercial enzyme	Ferulic acid	83%	Faulds, Mandalari et al. (2004)
		5,5′ diferulic acid	61%	
		8-O-4′ diferulic acid	44%	
		8,5′ diferulic acid	69%	
		p-coumaric acid	88%	
Brewer's spent grain	*Humicola insolens* commercial enzyme	Ferulic acid	76%	Faulds, Mandalari et al. (2004)
		5,5′ diferulic acid	71%	
		8-O-4′ diferulic acid	73%	
		8,5′ diferulic acid	0%	
		p-coumaric acid	40%	
Oat hulls	Food grade *A. niger*feruloyl esterase and *Trichoderma* Xylanase	Ferulic acid	50%	Yu, McKinnon et al. (2004)
		p-Coumaric acid	4.8%	
Corn bran	*Fusariumproliferatum* FAE B + xylanase	Ferulic acid	32%	Shin and Chen (2006)
Corn bran	*Neosartorya spinosa* crude enzyme	Ferulic acid	100%	Shin, McClendon

				et al. (2006)
Coffee pulp	*A. niger* FAE B	Caffeic acid *p*-Coumaric acid	100% 73%	Benoit, Navarro et al. (2006)
Apple marc	*A. niger* FAE B	Caffeic acid *p*-Coumaric acid	83% 34%	Benoit, Navarro et al. (2006)
Steam-exploded wheat straw	*A. niger* FAE B	*p*-Coumaric acid Ferulic acid	16% 58%	Benoit, Navarro et al. (2006)
Autoclaved maize bran	*A. niger* FAE A	Ferulic acid	40%	Benoit, Navarro et al. (2006)
Autoclaved maize bran	*A. niger* FAE B	Ferulic acid	8%	Benoit, Navarro et al. (2006)
Sugar beet pulp	*A. niger* FAE A	Ferulic acid	0%	Benoit, Navarro et al. (2006)
Sugar beet pulp	*A. niger* FAE B	Ferulic acid	8%	Benoit, Navarro et al. (2006)
Sugar beet pulp	*Streptomyces tendae* crude extract	Ferulic acid	10%	Ferreira, Diez et al. (2007)
Maize bran	*Neocalimastix* sp. crude enzyme	Ferulic acid *p*-Coumaric acid	8% 69%	Yang, Yue et al. (2009)
Maize bran	*Anaeromyces* sp. crude extract	Ferulic acid *p*-Coumaric acid	2% 53%	Yang, Yue et al. (2009)
Wheat bran	*Neocalimastix* sp. crude enzyme	Ferulic acid *p*-Coumaric	36%	Yang, Yue et al.

		acid	1%	(2009)
Wheat bran	*Anaeromyces* sp. crude extract	Ferulic acid	16%	Yang, Yue et al. (2009)
		p-Coumaric acid	1%	
Brewer's spent grain	*Fusariumoxysporum* FAE C	Ferulic acid	40%	Xiros, Moukouli et al. (2009)
Coffee pulp	*Rhizopus pusillus* + comercial pectinase	Chlorogenic acid	54%	Torres-Mancera, Cordova-López et al. (2011)
		Caffeic acid	7.2%	
		Ferulic acid	25%	
Wheat straw	*Xylariapolymorpha* glycoside hydrolase	Ferulic acid	14.5%	Nghi, Bittner et al. (2012)
		p-Coumaric acid	10.1%	
Brewer's spent grain	*Lactobacillus acidophilus*FAE	Ferulic acid	2.1%	Szwajgier, Waśko et al. (2010)
		p-Coumaric acid	2.9%	
		Sinapic acid	2.8%	
		Caffeic acid	3.7%	
Wheat arabinoxylan	*A. clavatus* FAE	Ferulic acid	200%	Damásio, Braga et al. (2013b)
		p-Coumaric acid	85%	
Sugar cane bagasse	*A. clavatus* FAE	Ferulic acid	37%	Damásio, Braga et al. (2013b)
		p-Coumaric acid	7%	
Wheat bran	*Thermobifida fusca* crude extract	Ferulic acid	15%	Huang, Chen et al. (2013)
De starched wheat bran	*A. usamii* FAE + xylanase	Ferulic acid	35%	Gong, Yin et al. (2013)

Coffee pulp	Commercial pectinase + crude extracts from *R. pusillus, A. tamarii* and *Trametes* sp.	Chlorogenic acid Caffeic acid	68.3% 38.2%	Torres-Mancera, Baqueiro-Peña et al. (2013)

Sugar beet pulp, a waste product obtained after extracting water soluble sugars from sugar beet has been historically used as a medium for the growth of microbes possessing FAE and its induction. Sugar beet pulp/pectin contains about 6-8 mg/g ferulic acid. Bonnin, Saulnier et al. (2002) carried out enzymatic treatment of sugar beet pulp using *Aspergillus niger* crude extracellular fraction grown on sugar beet pulp and maltose. The treatment yielded 70% efficiency on sugar beet pulp. The reaction was carried out at 1% solid loading (solid to liquid wt/vol) and 1% enzyme loading (wt/wt), and was carried out for 24 hours at 40^{o}C. This crude enzyme preparation was also tested on maize bran and autoclaved fraction of maize bran. Maize bran as such was recalcitrant to the enzyme, however, after autoclave pre-treatment, 95.5% of bound ferulic acid was released from the reaction mixture. FAE type B that is more active on *p*-coumaric and caffeic acid compared to ferulic and sinapic acid are expressed when fungi are grown on sugar beet pulp, however, the high activity towards release of ferulic acid in the above work may be attributed to other non-specific esterases (Crepin, Faulds et al. 2004).

Kroon, Williamson et al. (2000)were able to release 98% (3.3mg/g) of total releasable ferulic acid from wheat bran using *Penicillium funiculosum* FAE, at 1 U/g enzyme loading and 200 U/g xylanase loading, when the reaction was carried out for 24 hours. *Neurospora crassa* FAE of type D was first reported by Crepin, Connerton et al. (2004), the authors followed similarity approach where the genome sequence was scanned for putative FAE sequences, followed by which the gene was cloned and expressed in *Pichia pastoris.Talaromyces stipitatus* a type C FAE (TsFaeC) machinery was evaluated for the efficiency of the release of ferulic acid from wheat bran and sugar beet pulp. TsFaeC alone was

able to release only 1.5% and 5.8% from wheat bran and sugar beet pulp respectively, with the reaction being carried out at 37 ^{o}C, with about 3.3 U/g (substrate) and a solid substrate loading of 3% wt/vol of enzyme for 24 hours. When exogenous xylanases were added, 66% efficiency was achieved for wheat bran extraction of ferulic acid (Garcia-Conesa, Crepin et al. 2004).

Sporotrichum thermophile FAE was used to release ferulic acid from de-starched wheat bran, and the maximal release was observed in 33% of total alkali releasable ferulic acid, this was possible, with 0.4 U/g enzyme loading with 500U/g xylanase within one hour of reaction time (Topakas, Stamatis et al. 2004).

Faulds, Mandalari et al. (2004) conducted studies on HCAs release using commercial enzymes derived from *Humicola insolens*, Ultraflo L. With a solid loading of 1%, and 1 unit FAE equivalent of Ultraflo L, 24 hour reaction with shaking (100 rpm) at 50 ^{o}C yielded 50% efficiency for *p*-coumaric acid from brewers spent grain, however, ferulic acid recovery was only about 17% for both wheat bran and brewers spent grain. The vast difference in alkali extractable ferulic acid/HCAs and enzymatically released is due to the different types of feruloyl moieties that are present in substrates. Alkaline hydrolysis will release the HCAs irrespective of the type of the bonds; however, enzymatic reactions are very specific to the types of bond and the accessibility of the macromolecular enzyme to the substrate matrix. Earlier study gave the understanding that Ultraflo L was able to release all three ferulate dimers and 65% of total releasable ferulic acid within 3 hours, while the reaction was carried out at 37 ^{o}C with 50 U/g enzyme loading (Faulds, Sancho et al. 2002).

Yu, McKinnon et al. (2004) used oat hulls as a substrate for the enzymatic release of *p*-coumaric acid, however, *Aspergillus* FAE of a commercial, feed grade was used and was unable to release sufficient amounts of *p*-coumaric acid, however, maximal release of ferulic acid at 50% was observed when the FAE was coupled with xylanase. *Fusarium proliferatum* NRRL 26517 was used to produce a type B FAE (FpFaeB), the crude enzyme when supplemented with xylanase at 345 U/ml, along with 100 U/ml of endogenous xylanase, released 32% of ferulic

acid from corn bran. The enzyme loading was 5 U/g of FpFaeB and solid loading of 2% w/v. The reaction was carried out at 45^{o}C for 24 hours with shaking at 200 rpm (Shin and Chen 2006). The same group isolated a fungus *Neosartorya spinosa*, the extracellular enzyme component from the organism could lead to complete recovery of alkali releasable ferulic acid from corn bran, when the solid loading was 2% and the enzyme loading was 10 U/g FAE and 23 U/g xylanase activity, with the reaction held at 45^{o}C for 24 hours. The enzyme not only released the total ferulic acid, but also 97.3 % of reducing sugars were released (Shin, McClendon et al. 2006).

Benoit, Navarro et al. (2006) used type A (AnFaeA) and type B (AnFaeB) *A. niger* FAE and studied the release of HCAs from coffee pulp, apple marc, corn bran and sugar beet pulp. AnFaeB performed better compared to AnFaeA. AnFaeB could release 100% of caffeic acid, 73% of *p*-coumaric acid (0.08 mg/g released) from coffee pulp and 40% of ferulic acid 83% caffeic acid and 34% *p*-coumaric acid from apple marc. A significant release of 58% ferulic acid from steam exploded wheat straw was observed while only 16% of *p*-coumaric acid was released 40% of ferulic acid from maize bran was released (AnFaeA gave maximal activity) and 25% of *p*-coumaric acid from the same substrate. Sugar beet pulp was almost recalcitrant, with only 8% of the total releasable ferulic acid being released by AnFaeB.

Hegde, Kavitha et al. (2006) did a study using *Aspergillus niger* CFR 1105, where instead of the released phenolic acid, the content of bound phenolic acids was characterized. De starched wheat bran and rice bran was used as substrate for fermentation by the 4th day; 86% of ferulic acid from wheat bran and 78% of ferulic acid from rice bran was reduced. The study was focused on the degradation of cereal brans; hence the fate of the released HCAs was unknown.

Ferreira, Diez et al. (2007) published a study with *Streptomyces tendae* being able to release maximum of 10% of total releasable ferulic acid from sugar beet pulp using a 3 day old culture supernatant from the organism grown on sugar beet pulp. *Neocalimastix* sp. and *Anaeromyces sp.* FAE crude preparation were

tested for release of ferulic acid from Chinese wild ryegrass, wheat bran, maize bran, cornstalk, wheat straw, however, the enzyme mixtures of *Neocalimastix* and *Anaeromyces* were able to release significant amounts of about 69% and 53% *p*-coumaric acid respectively from maize bran. While the only significant amount of release that was observed was that of 36% ferulic acid from wheat bran using the *Neocalimastix* enzyme mixture (Yang, Yue et al. 2009).

While studying *Fusarium oxysporum* FAE type C (FoFaeC) it was found that with 0.32 U/g of FoFaeC and 400 U/g xylanase, and solid loading of 7.5%, the reaction gave 40% of total releasable ferulic acid from brewer's spent grain within three hours, when the reaction was carried out at 50^{o}C with 15000 rpm (Xiros, Moukouli et al. 2009).

Work focusing on coffee pulp was neglected after 2006 as far as enzymatic extraction was concerned till Torres-Mancera, Cordova-López et al. (2011), published work on the release of HCAs from coffee pulp, using enzymes produced by *Rhizopus pusillus* using two different inducers (coffee pulp and olive oil) and using commercial pectinases. The work resulted in the release of a maximum of 54% of total releasable chlorogenic acid from the coffee pulp, amounting to 1.29 mg/g of chlorogenic acid yield. Maximal caffeic acid was released using the same combination, as 7.2% of releasable caffeic acid, corresponding to 0.04 mg/g and 25% of ferulic acid corresponding to 0.02 mg/g (Torres-Mancera, Cordova-López et al. 2011).

Xylaria polymorpha expressed an enzyme with rhamnosidase activity coupled with FAE activity and the enzyme could release 14.5% of ferulic acid and 10.1% of *p*-coumaric acid from native wheat straw within 2 hours, incubated at 37% with a solid loading of 1% and enzyme at 0.05 µM (Nghi, Bittner et al. 2012).

Earlier FAE from *Lactobacillus acidophilus* K1 was used in the pure form to release HCAs from brewers spent grain, which resulted in the release of ferulic,

p-coumaric, caffeic acid in the range of 2-6% of the total alkali releasable amounts (Szwajgier, Waśko et al. 2010).

Recent studies on *Aspergillus clavatus* FAE indicated that the enzyme was capable of releasing 85% of *p*-coumaric acid (~2.5mg/g) from wheat arabinoxylan within 15 hours at 30^{o}C, while it could release twice the amount of ferulic acid from wheat arabinoxylan (4mg/g) compared to mild alkali treated wheat arabinoxylan. The enzymatic extract was not as active on sugar cane bagasse, releasing on 0.5 mg/g of each ferulic acid and *p*-coumaric acid, accounting for 37% and 7% efficiency respectively (Damásio, Braga et al. 2013b).

Huang, Chen et al. (2013)were able to release 15% of total releasable ferulic acid from wheat bran using *Thermobifida fusca* a thermophilic actinomycete from the fermentation medium. *Aspergillus usamii* FAE was studied for the release of ferulic acid from de starched wheat bran and the study resulted in the release of 35% maximal release, in the presence of 56 U of the recombinant FAE and about 300 units of recombinant xylanase from the same organism after 10 hrs of incubation at 40 ^{o}C (Gong, Yin et al. 2013).

A study on the growth of koji mould *Rhizopus oryzae* on the rice bran showed that the content of ferulic acid in the bran when unfermented was about 33 mg/g, while after fermentation for 120 hrs (in solid-state), the bran content of ferulic acid was increased to 765 mg/g, indicating an enrichment of ferulic acid, due to degradation of the rest of the components in rice bran (Schmidt, Gonçalves et al. 2014).

The most recent study on extraction of chlorogenic acid and caffeic acid from the coffee pulp gave maximal efficiency of 68% release of chlorogenic acid (1.6 mg/g) and 38% efficiency for caffeic acid (0.715 mg/g) using a combination of four enzymes from the following sources, commercial pectinase, *R. pusillus*, *A. tamarii*and *Trametes sp*. at 4 U/g enzyme loading and 10% solid loading (Torres-Mancera, Baqueiro-Peña et al. 2013).

1.7 Potential applications of feruloyl esterase and ferulic acid

Primarily feruloyl esterases have been used as xylanase accessory enzymes. But lately they have been used for a wide variety of other purposes. Feruloyl esterase has been used for synthetic purposes for preparation of various esters of ferulic acid, which include esters with glycerol, with lipids and even alkyl esters(Tsuchiyama, Sakamoto et al. 2007). It becomes necessary to produce these esters because the bioavailability of these active ingredients increases with the increase in solubility of the compounds in lipids (Moussou and Danaoux 2004, Vafiadi, Topakas et al. 2008). Using feruloyl esterase for such processes become necessary because of feruloyl esterase having higher specificity for such reactions (Zeuner, Riisager et al. 2011) and in general because using chemical methods involve strong acids and high temperatures (Li, Shi et al. 2009), which may lead to oxidation of ferulic acid hence its loss. Feruloyl esterase have been used indirectly for different applications like improving the silage quality of barley for feed uses by employing feruloyl esterase producing strains of *lactobacilli* (Jin, Duniere et al. 2015). Feruloyl esterase has found use during malting of wheat for producing malts which are rich in ferulic acid which in turn will give rise to 4-vinyl guiacol after alcoholic fermentation, a food flavor used in the manufacture of clove beers (Lu Jian 2017). Feruloyl esterase expressing bacteria have also been used for the production of functional foods (Guglielmetti, De Noni et al. 2008), while other probiotic formulations with feruloyl esterase expressing microbes have led to reduction of symptoms for non-alcoholic fatty liver disease (Bhathena, Martoni et al. 2013).

Ferulic acid on the other hand is a molecule with multiple biological effects. Ohsaki, Shirakawa et al. (2008) found out that ferulic acid had ameliorating effects on hypertensive rats. Most of the biological functions of ferulic acid is due to its antioxidative function (Graf 1992). Ferulic acid has even been tested as a replacement for propyl gallate in guar gum formulations for prevention of thermal decomposition (Hill and Gray 1999). Sodium ferulate has

been shown to have anti-thrombosis and hence activity against atherosclerosis (Xu, Xu et al. 1984).

1.8 Conclusion

Thus it is quite clear that even with a large amount of prior research feruloyl esterase and ferulic acid still have explored horizons when it comes to its applications. Patent search also indicates newer patents for multitudes of applications which is only an indicator that feruloyl esterase and ferulic acid both have immense potential for fresh research.

1.9 Organization of the thesis

The thesis is divided into eight chapters. Chapter 1 deals with the introduction of the research work, its history and its need. Chapter 2 has general material and methods. Chapter 3 deals with the statistical optimization of parameters governing feruloyl esterase production. Chapter 4 works on purification of the enzyme in question. Chapter 5 works on characterizing the enzyme biochemically. Chapter 6 deals with finding the optimal conditions for extracting maximal ferulic acid from destarched wheat bran. Chapter 7 incorporates application studies featuring feruloyl esterase and ferulic acid. Finally Chapter 8 summarizes the results and makes final conclusions that have culminated into this thesis.

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Chapter II

General Materials and Methods

2.1 Materials

List of major instruments used during the progress of this work can be seen in Table 2.1, while a list of major chemicals, kits and other material used for this work is listed in Table 2.1

Table 2.1 List of Chemicals and vendors

Chemicals or materials	**Suppliers**
Ammonium chloride, Ammonium nitrate	SD fine chem.Ltd. India
Acetone,Potassium dihydrogen phosphate, Ammonium nitrate, Magnesium sulphate heptahydrate, Potassium acetate, Calcium chloride dehydrate, Zinc sulphate heptahydrate, Boric acid, Copper sulphate pentahydrate, Manganese sulphate monohydrate, Sodium molybdate dehydrate, Methanol (AR), Sulphuric acid (concentrated), Concentrated Hydrochloric acid, Glacial acetic acid, Ethyl acetate.	SiscoResearchLaboratories, Pvt.ltd. India
Trisodium Citrate, Glucose, Arabinose, Xylose. Brad fords reagent, Sodium citrate dehydrate, Citric acid monohydrate, Ferulic acid, Ethyl ferulate, Caffeic acid, p-Coumaric acid, Bovine serum albumin, Pluronic 123, Poly ethylene glycol (PEG), Ammonium persulphate, Tris Buffer, Sodium Dodecyl Sulphate (SDS)	Sigma- Aldrich, USA
Methanol (HPLC grade), Sodium nitrate, Ammonium sulphate, bicihoninic acid protein estimation kit, DMSO (Emplura), Sinapic acid, Phosphoric acid (HPLC grade), F254 Silica gel plates 20x20 cm, F254 preparatory silica	Merck, India

gel plates 20x20 cm, Tetraethyl orthosilicate, 3(Amino propyl) triethoxysilane, glutarladehyde	
Potato Dextrose Agar, Agar-Agar, Dipotassium Phosphate, Potassium Chloride, Potassium Nitrate, Sodium Chloride, Sodium phosphate monobasic monohydrate, Disodium hydrogen phosphate: anhydrous	HI media, India

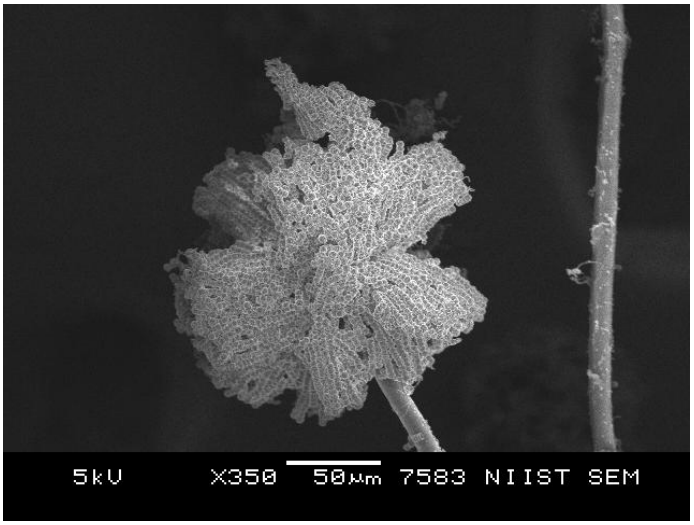
2.2 Biological Methods

2.2.1 Microorganism-growth and maintenance

*Aspergillus niger ATCC 13497*obtained from the TUB culture collection (www.tub-collection.com) was used throughout the study. The fungus was maintained on potato dextrose agar (PDA)(Composition provided in Annexure I(b)) plates at 28 °C. *Aspergillus niger* ATCC 13497 or the Armbruster C-14 strain which is a mutant form of *Aspergillus niger* is known to be a potent producer of alpha amylase. Aspergillus niger has septate, hyaline (clear) hyphae. Conidiophores are long with spherical vesicles at the apex. Aspergillus niger is biserate. The conidia are globose with dark brown to black color when matured

The scanning electron micrograph of the fungal conidiophore with a conidial head can be seen in Figure 2.1. A spore inoculum was prepared by dislodging spores from completely sporulated PDA plates with 0.1% Tween 80 in sterile de-ionized water.

Figure 2.1 Scanning electron micrograph of *Aspergillus niger* ATCC 13497



Spore suspensions obtained by the above method were diluted appropriately and were enumerated using a neubauer counting chamber (Sigma Aldrich USA). The average number of spores counted from four different 1mm x 1mm chambers (with a volume of 10^{-4}mL per chamber) is used to estimate the spore count in the sample using the formula in Equation 2.1

Equation 2.1 Equation for calculating spore suspension concentration

Spore concentration (spores/mL) = (Average number spores in 1mmx1mm chamber * dilution factor *10^4)

Unless otherwise specified, 1 ml of spore suspension used for inoculating one flask contains 10^6spores/ml.

2.2.2 Solid State Fermentation and enzyme extraction

Solid state fermentation (SSF) was carried out with 3g destarched wheat branin 250 mL conical flasks. Inoculum size was fixed at 10^6 spores/3g dry substrate from slants of *Aspergilus niger*ATCC 13497. The destarched wheat bran was then wet with the appropriate quantity of Vogel's minimal medium in order to achieve desired initial moisture and unless specified, it was maintained at 60 % (w/w). The flask after inoculation was incubated at 30^{o}C (static condition).After a specified time the enzyme was extracted by adding 10ml de-ionized water/g dry substrate, followed by mixing the mixture in a shaker incubator set to 200 rpm for one hour. Solids were separated and the liquid portion with the enzyme was obtained by squeezing the mixture through a cheese cloth followed by centrifugation at 1000 rpm for 10 minutes at 4^{o}C.

2.2.3 Destarching of wheat bran

Feed quality wheat bran was obtained from Arya Shalai local market in Thiruvananthapuram, Kerala and subjected to pretreatment to remove starch attached to the wheat bran, using a modified protocol from Mukherjee, Singh et al. (2007). 10 % w/v suspension of wheat-bran in 0.3% w/v potassium acetate was heated to 100^{o}C with continuous mixing. This was followed by separation of the solids from the slurry and washing the obtained solids multiple times with distilled water at 100^{o}C, followed by multiple washes with distilled water at room temperature. The solids thus obtained were then oven dried to obtain the final destarched wheat bran. After oven drying, if the wheat bran particles do form large clumps, then additional washes with water at 100^{o}C are carried out, after which the solids were oven dried.

Vogel's minimal medium was used to wet the pretreated substrate, without the addition of biotin. The composition of the minimal medium is shown in Annexure I(a).

2.3 Analytical Methods

2.3.1Feruloyl esterase standard assay

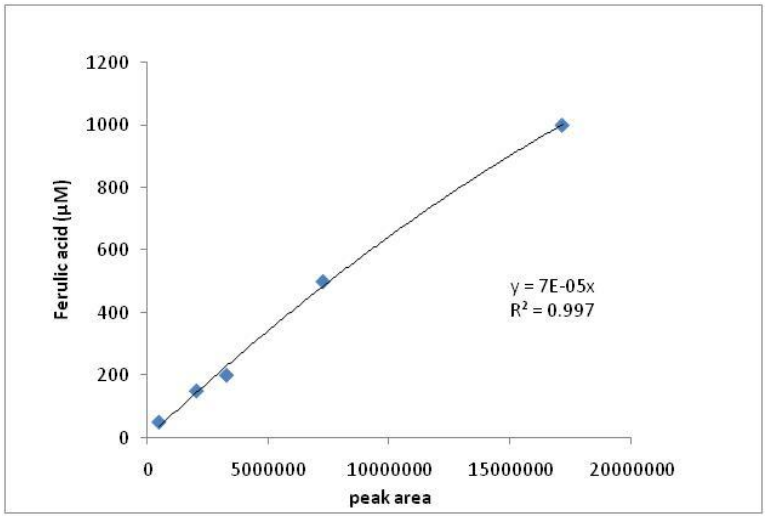
The feruloyl esterase assay conducted was a modified method from (Topakas, Stamatis et al. 2003). 750 µL of 5mM ethyl ferulate dissolved in 100mM MOPS buffer with 15% DMSO.250 µL of test enzyme solution appropriately diluted is added to the substrate and incubated at 37^{o}C for 15 minutes. The mixture was then steeped in a boiling water bath for 15 minutes to deactivate the enzyme. The samples were then filtered through 0.22 µm filters and analyzed by HPLC to detect the amount of released ferulic acid.

One unit of feruloyl esterase activity is defined as the amount of enzyme required to release one µM of ferulic acid in one minute under the above conditions (Topakas, Stamatis et al. 2003).

2.3.2High Performance Liquid Chromatography for ferulic acid detection

Shimadzu Prominence modular HPLC system equipped with a diode array detector SPD20A was used to separate the enzyme assay reaction mixture. A phenomenex Gemini c18 column (5µm, 110 Å, 150mm x 4.6mm) was eluted with an isocratic mixture of (65:35) Methanol: Water (0.01% phosphoric acid). The column oven was set at 37^{o}C. The elution was monitored at 320 nm, using a Shimadzu SPD M20A photodiode array detector. 10 µL of the reaction mixture appropriately diluted (if necessary) was injected into column for analysis. Fig 2.2 shows a standard curve plotted with peak area on X axis and concentration of ferulic acid on the Y axis.

Figure 2.2 Standard curve, peak area vs. concentration of ferulic acid



2.3.3Moisture analysis

A&D MX 50 moisture analyzer (Japan) was used to calculate the moisture content of destarched wheat bran prior to inoculation and use as substrate for release of ferulic acid so as to estimate the amount of dry biomass present in the substrate. The moisture analyzer would read out the percent moisture present in the biomass by heating the biomass to 105^{o}C till the mass of the biomass no longer decreased and remained constant.

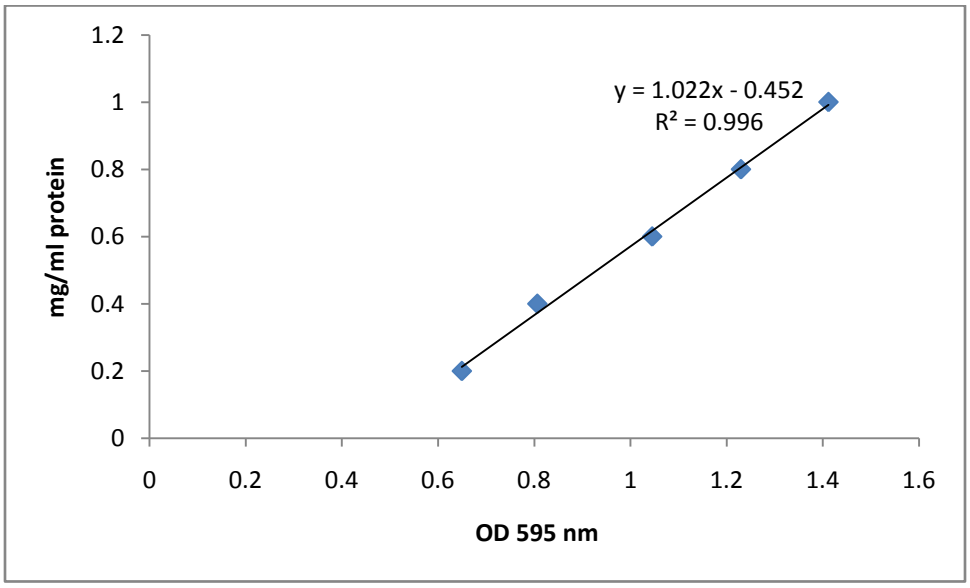
2.3.4Protein Estimation

2.3.4a Bradford's protein estimation method

Bradford protein assay (Bradford 1976) is a simple and accurate procedure for determining the concentration of protein in solution. Standard Bovine Serum Albumin was prepared on a concentration of 1mg/mL. Graded volumes of standard protein (0.2- 1mL) was pipetted out into a series of micro-centrifuge tubes and made to 1 mL by adding distilled water. 5µl of the above solution was added on to a well of micro titre plate.150 µL of Brad ford reagent was added and incubated for 5 minutes in dark .Absorbance was read at 595 nm on a micro plate reader. Then a linear graph was plotted on excel with concentration on X axis and Optical density on Y- axis with a regression value of 0.998. Solutions of unknown protein concentrations are

then assayed in a similar way and the regression equation obtained by using BSA is used to estimate the protein content in the solution using the standard curve as seen in Figure 2.3.

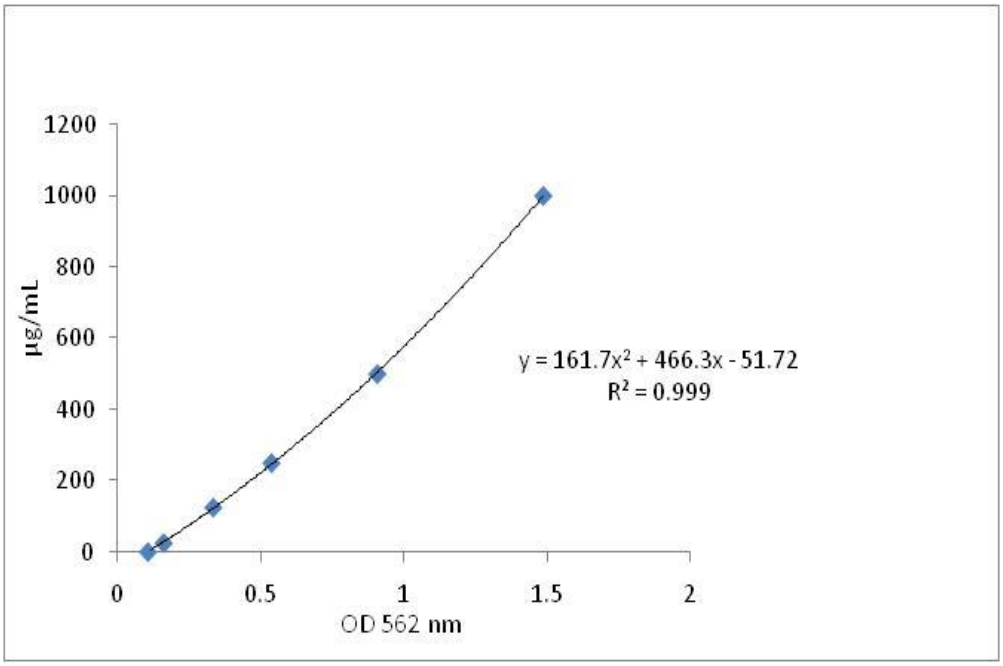
Figure 2.3 Bradfords assay standard curve



2.3.4b Bicinchoninic acid protein estimation method

The BCA protein assay kit (Smith, Krohn et al. 1985) was obtained from Merck, India. The kit was used as per the protocol advised by the supplier. Like brad fords assay, dilutions of BSA were prepared using the standard BSA solution (2 mg/mL) provided in the kit, with concentrations ranging from 1000 µg/mL to 25 µg/mL . Fresh BSA reagent was prepared by mixing the provided BCA solution to the 4% cupric sulphate solution in the ratio 50:1. 25 µL of the protein solutions are then mixed with 200 µL of the freshly prepared BCA, cupric sulphate mixture and incubated at 37^{o}C for 30 minutes. The microplate was then used for recording the absorbance of the solutions at 562 nm. Solutions of unknown protein concentrations are then assayed in a similar way and the regression equation (Figure 2.4) obtained by using BSA was used to estimate the protein content in the solution.

Figure 2.4BCA assay standard curve



2.3.5 SDS Polyacrylamide gel electrophoresis

The method was carried out according to Laemmli (1970). The polyacrylamide gel is cast between 10cm x x10 cm glass plates provided with Bio-Rad protean mini electrophoresis unit (Bio-Rad, USA). A separating gel solution (5mL) with 12% acrylamide in 375mM Tris HCL pH 8.8 with 0.1% SDS is poured with 0.1% freshly prepared ammonium persulphate and 2 µL of TEMED.A small layer of n-butanol is poured slowly over this layer to prevent oxidation of the end groups. This is followed by decanting the n-butanol layer and pouring the stacking gel with 5% acrylamide and Tris HCL 60 mM with pH 6.8, 0.1% SDS and 0.1 % ammonium persulphate with 2µL of TEMED. A comb for forming wells is placed before the gel sets. The buffer tank is filled with Tris glycine buffer pH 8.3 and 0.1% SDS. Samples are loaded on to the wells along with loading dye and run at 120V till the dye front reaches the bottom.

2.4 Summary

This chapter discusses the general materials and methods. All standard protocols were adopted in this work. Specific protocols will be discussed within the respective working chapters

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Chapter III

Production of feruloyl esterase under solid state fermentation

3.1 Introduction

Feruloyl esterases are enzymes that have the capacity to hydrolyze an ester bond between a hydroxycinnamic acid and a sugar moiety. The enzymes are naturally produced by saprophytic microorganisms that thrive on dead and decaying plant and wood material, and by symbiotic micro-organisms associated with animals that utilize plant material as their primary source of nutrition. Ferulic acid as discussed earlier is found as a decoration in arabinoxylans and in pectic structures in plant material. These decorations make the plant material recalcitrant towards pathogens that attack the plant cell wall structures as well as making the plant material difficult as a forage source (Akin 1982). The same can be said about the use of different lignocellulosic biomass for biofuel applications, as similar enzymes as that of the fungal pathogens are use for deconstruction of the lignocellulosic material(Anderson and Akin 2008, Raffrenato, Fievisohn et al. 2017). Feruloyl esterase enzyme group is very broad and has evolved from different families of enzymes including tannases, choline-esterases, acetyl xylan esterases, lipases(Benoit, Danchin et al. 2008). They have a wide substrate specificities and hence were classified originally into four classes according to their substrate specificities(Crepin, Faulds et al. 2004). With the growing importance of sustainable processes, it has become vitally important to find viable source of income from side processes in the biofuel industry. Feruloyl esterase is an an enzyme that can add value to such bioprocesses, by improving the digestibility of lignocellulosics and also by providing valuable byproducts like ferulic acid and other hydroxycinnamic acids like caffeic acid, p-coumaric acid (Koseki, Fushinobu et al. 2009, Damásio, Braga et al. 2013). Alternatively, feruloyl esterase enzyme can also be used for improving the quality of food so as to give nutraceutical value(Govindaswamy 2015). Production of feruloyl esterase through fermentative methods is therefore highly relevant and understanding and developing methods for enhanced production of the said enzyme is also a vital requirement to technological viability.

Agricultural by products are on the rise globally due to increased world population and the subsequent increase in production of food and non food crops. In an economy that is largely drive by fossil fuels, finding alternative means for fuel and chemicals has become binding. Grain crops and grasses form a large portion of agricultural produce and hence the byproducts from these produce also pose a problem. Wheat bran is an agricultural byproduct that has been used traditionally as an animal feed. It accounts for about 25 % of the total grain weight, and if it is assumed that all of the wheat produced in the world is going to be milled for human consumption, A gargantuan 150 million tons of wheat bran will be produced per annum(Prückler, Siebenhandl-Ehn et al. 2014). Using this by product solely for animal feed is not the solution, in fact utilizing wheat bran as a raw material for bio-refining is what is necessary. The bran is chemically non-uniform with starch, protein, cellulose and arabinoxylan components being the major constituents (Carré and Brillouet 1986, Maes and Delcour 2002, Sun, Liu et al. 2008).

The current study focuses on using agricultural residues as raw material for solid state fermentation for the production of feruloyl esterase.

3.2 Materials and Methods

All major chemicals used in the chapter are listed in table 2.1.

3.2.1 Raw material

To screen the ferulic acid content , initially four agro residues like wheat bran, wheat straw, chili stalk and sorghum stalk were selected for this study. Destarched wheat bran is prepared as per the protocol mentioned in section 2.2.3 and was also included for screening Wheat bran was purchased from Arya Shalai local market in Trivandrum and processed according to method mentioned in section 2.1.3. wheat straw was also purchased from the local market while chili stalk was obtained from Virudhunagar, Tamil Nadu, India. Sorghum stalk was received as a gift from Directorate Sorghum Research Institute, Hyderabad, India. All the raw materials were dried and milled using a knife mill so as to reduce the particle size (<10mm). The milled particles were then mixed thoroughly and stored in water tight containers and stored at room temperature till further use.

3.2.2Alkali releasable ferulic acid from different substrates

Alkali extractable ferulic acid was assayed by a modified method from (Faulds and Williamson 1995)by treating 1 g dry substrate(destarched wheat bran, wheat straw, sorghum stalk and chili stalk) with 1 N NaOH at 25^{o}C for 12 hours. The resulting slurry was then acidified using 5 N HCl till the pH dropped to 3.5. The acidified slurry was then extracted with three volumes of ethyl acetate. The extracted ethyl acetate volume was reduced by rotary evaporation (R210, Buchi, Switzerland), and the liquid residue was then dissolved in 10 mL of dry HPLC grade methanol (Sigma Aldrich, USA) and analyzed through HPLC.

3.2.3Enzyme recovery from fermented substrates

SSF was carried out as per the method mentioned in general materials and methods section 2.2.1, with the difference being that the solid substrates used were sorghum stalk, destarched wheat bran, wheat straw individually at 3 g levels, as well as combinations of these substrates in 1:1ratio at 3 g levels in 250 mL flasks. All the substrate filled flasks were sterilized and the solid matter was moistened with 2 ml of Vogel's minimal medium and the initial moisture was adjusted to 60 % using sterile distilled water. All the flasks were incubated (static condition) at 30^{o}C for 72 h i.e. only after initiation of sporulation.

After the incubation period, the fermented material from each flask was extracted by adding 10ml de-ionized water/g (dry substrate), followed by mixing the fermented matter in a shaker incubator set to 200 rpm for one hour. Solids were separated and the liquid portion with the enzyme was obtained by squeezing the mixture through a cheese cloth followed by centrifugation. The obtained enzyme extracts were then subjected to the feruloyl esterase assay followed by quantification of ferulic acid by HPLC as per prescribed methods ingeneral materials and methods section 2.2.2.

3.2.4 Growth of *Aspergillus niger* ATCC 13497 on destarched wheat bran

The growth of *Aspergillus niger* ATCC 13497on destarched wheat bran under SSF was analyzed using an indigenous CO_2 sensor (using an electrochemical CO_2 sensor MQ135). The growth conditions for the analysis were similar to the method mentioned in section 2.1.1 with initial moisture of 60%. The flask containing the sensor was incubated at 30^oC and the relative CO_2 content was plotted as mV analog signal. The micrograph of the fungal hyphae attaching and growing on destarched wheat bran particles was also recorded using an optical microscope at 100x and 200x magnification.

3.2.5 Determination of significant parameters affecting feruloyl esterase production through SSF

A Taguchi design for understanding the significant parameters affecting the process of production of feruloyl esterase using destarched wheat bran was designed using Minitab 17 trial (Minitab USA). The design chosen was a mixed design with parameters sodium nitrate (additional nitrogen source), initial moisture, initial pH and innoculum size at four levels, while particle size was assessed at two different levels, as per Table 3.1.

Table 3.1: Parameters and levels for Taguchi design

Factors	Levels
sodium nitrate	5-20 (mg/g dry substrate)
initial moisture	55-85 (percent moisture wt/wt)
initial pH	4-9
innoculum size	1 -30 ($x10^6$ spores/ 3 g dry substrate)
particle size	fine, coarse

The pH of the medium was adjusted by empirically calculating the amount of acid (0.5 N HCL) or base (0.5 N NaOH) required to attain the set pH and deducting the volume added in acid or base from the volume of sterile distilled water to make

up for the moisture set point. The software is used to generate a main effects plot and the factors are then ranked according to the influence that the factor has on the response.

3.2.6 Design of experiment using Central Composite Design to optimize the levels of the significant parameters for production of feruloyl esterase and tray fermentation

The parameters from the Taguchi design with the highest ranks were initial moisture and initial pH, and therefore, a central composite design with the following range of parameters was designed using Design Expert 7 Trial (Stat Ease USA).Table 3.2gives the details of the chosen parameters.

Table 3.2: Parameters and levels for central composite design

Parameter	**Coded levels**		
	-1	0	+1
Initial moisture (B)	62.0%	64.5%	67.0%
Initial pH (A)	3.0	4.0`	5.0

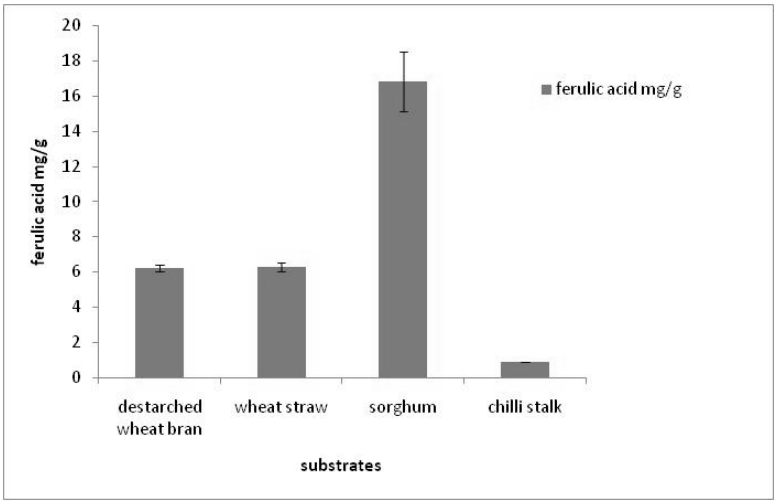
The central composite design was made so that the four parameters were tested at three different levels, viz. +1, 0, -1. Since there were two parameters at three levels, the design had a total of 14 runs. The statistical analysis of the experiment was carried out by means of analysis of variance to the obtained results using Design Expert 7.0 trial (Stat-Ease USA). The significance of the parameters was tested at 5 % level of significance. Tray fermentation with the optimized conditioned was performed with 50g of destarched wheat bran.

3.3 Results and Discussion

3.3.1 Alkali releasable ferulic acid from different substrates

Among the tested substrates, sorghum stalk was found to have the highest amount of alkali releasable ferulic acid with 16.81 ± 1.68 mg/g (ferulic acid mg/g dry substrate). Destarched wheat bran and wheat straw both had similar content of alkali releasable ferulic acid of 6.21 and 6.26 mg/g (standard deviation less than 5%). The amount of ferulic acid in destarched wheat bran is in agreement with previous reports(Faulds and Williamson 1995), however, wheat straw is reported to contain about 4.8 mg/g ferulic acid (Pan, Bolton et al. 1998). However it has been reported that ferulic acid content in grasses increase during certain kinds of stress (Wakabayashi, Hoson et al. 1997), which may account for the increased yield of ferulic acid in case of wheat straw. The summary of the results can be seen in Figure 3.1. Chili stalk had negligible amounts of alkali releasable ferulic acid compared to the other substrates. It was therefore decided to test destarched wheat bran, wheat straw and sorghum stalk for the production of feruloyl esterase individually as well as in 1:1 combinations.

Figure 3.1: Alkali releasable ferulic acid from different agro-residual biomass



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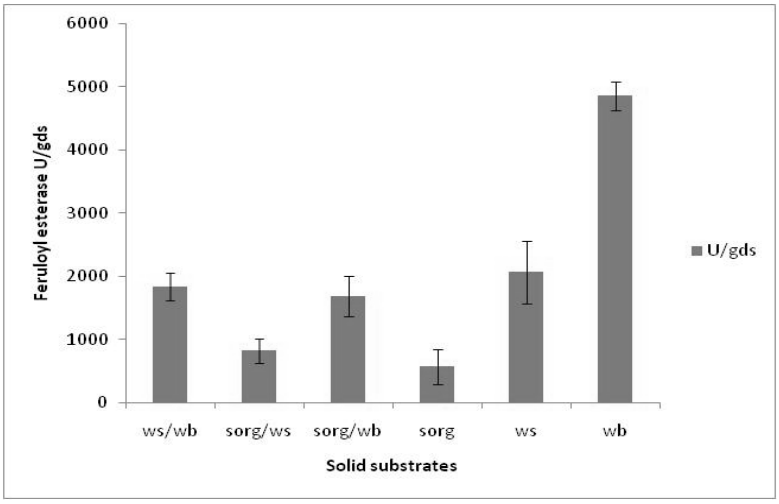
3.3.2 Enzyme production using different agro-residual substrates

Since destarched wheat bran, wheat straw and sorghum were observed to have higher amounts of alkali releasable ferulic acid, it was decided that these substrates be tested for production of feruloyl esterase by solid state fermentation. When these substrates were tested for production of feruloyl esterase using *Aspergillus niger* ATCC 13497, it was observed that there was no co-relation between the amount of esterified ferulic acid in the substrate to the actual amount enzyme that was produced by the organism when grown in solid state using the said substrate (Figure 3.2). It has been reported previously that presence of the phenolic acid may not necessarily be required for the induction of feruloyl esterase (Faulds and Williamson 1994, Brezillon, Kroon et al. 1996, Bonnin, Saulnier et al. 2002). In the current case, destarched wheat bran as a substrate for feruloyl esterase production gave the highest enzyme activity for feruloyl esterase with levels up to 4850 U/gds, and surprisingly the least feruloyl esterase activity was observed for sorghum stalk with 567.6 U/gds. None of the combinations were as efficient as destarched wheat bran for the production of feruloyl esterase as observed from

Figure 3.2: Production of feruloyl esterase using different agro-residual substrates by solid state fermentation.



Legend **ws/wb**= wheat straw: destarched wheat bran (1:1), **sorg/ws** = sorghum stalk : wheat straw (1:1), **sorg/wb** =sorghum stalk : destarched wheat bran (1:1), **sorg** = sorghum stalk, **ws** = wheat straw, **wb** = destarched wheat bran

3.3.3 Growth of *Aspergillus niger* ATCC 13497 on destarched wheat bran

The CO_2 production for the SSF process with the said conditions in section 3.2.4 peaked between 46 to 48h of the fermentation and reduced to base level by 72h which is also the time by which the substrate is covered with mycelia that are completely sporulated with black conidia, as can be observed in Figure 3.3. The micrographs of the destarched wheat bran with the fungus growing can be seen from Figure 3.4.

Figure 3.3 CO_2 evolution and growth analysis of *Aspergillus niger* ATCC 13497 in SSF.

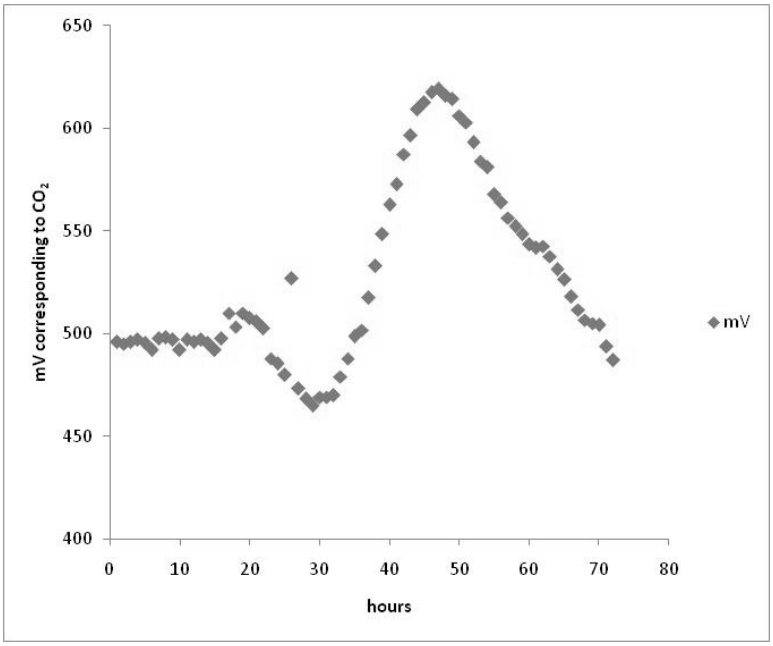


Figure 3.4 Optical micrograph of *Aspergillus niger* ATCC 13497 on destarched wheat bran

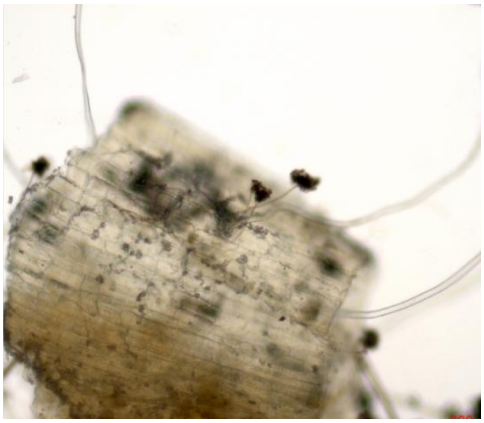


Figure 3.4 a 100 x magnification

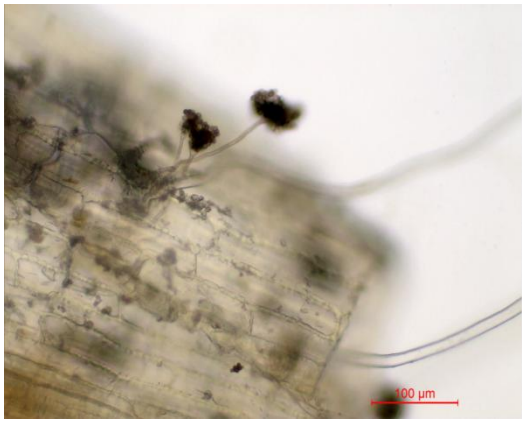


Figure 3.4 b 200 x magnification

3.3.3 Determination of significant parameters affecting the production of feruloyl esterase

Run number six gave the highest enzyme production from all the 16 runs of taguchi design as seen in Table 3.3. According to the p-values of the different factors, only initial moisture was a significant factor that determined the production of feruloyl esterase using destarched wheat bran as the substrate for solid state fermentation. The ANOVA for the factors of taguchi design is given in Table 3.4. The taguchi model thus obtained had an R^2 value of 0.98 and an adjusted R^2 value of 0.88.

Table 3.3: Response table for Taguchi design.

Run number	sodium nitrate	initial moisture	initial pH	innoculum size(x 10^6)	particle size	Response
1	5	55	4	1	fine	830.9
2	5	65	6	5	fine	838.4
3	5	75	7	15	coarse	40.13
4	5	85	9	30	coarse	47.51
5	10	55	6	15	coarse	512
6	10	65	4	30	coarse	**994.4**
7	10	75	9	1	fine	51.91
8	10	85	7	5	fine	39.44
9	20	55	7	30	fine	834
10	20	65	9	15	fine	503.6
11	20	75	4	5	coarse	583.4
12	20	85	6	1	coarse	31.49
13	40	55	9	5	coarse	245.5
14	40	65	7	1	coarse	589.3
15	40	75	6	30	fine	438.3
16	40	85	4	15	fine	254.9

Table 3.4: ANOVA table for variables tested using Taguchi design.

Terms	Sum of Squares	Degrees of freedom	Mean	F value	p-value probablity> F	
Sodium nitrate	26690	3	8897	0.69	0.636	
Initial moisture	1031886	3	343962	26.85	0.036	Significant
Initial pH	426658	3	142219	11.1	0.084	
Inoculum size	141781	3	47260	3.69	0.221	
Particle size	34945	1	34945	2.73	0.24	
Residual Error	25622	2	12811			
Total	1687581					

Although factors other than initial moisture did not turn out to be significant, other factor were ranked based on the difference between the highest mean response generated by the factor and the lowest mean response generated by the factor. According to this ranking system initial moisture was definitely at the top and it was closely followed by initial pH, as observed in Table 3.5.

Table 3.5: Response table for means from factors studied in the taguchi design.

Level	sodium nitrate	initial moisture	initial pH	innoculum size	particle size
1	439.24	605.61	665.91	375.92	380.47
2	399.42	731.42	455.03	426.69	473.93
3	488.13	278.44	375.73	327.65	
4	382	93.34	212.12	578.54	
Delta	106.13	638.08	453.79	250.89	93.47
Rank	4	**1**	**2**	3	5

3.3.4 Design of experiment using Central Composite Design to optimize the levels of the significant parameters for production of feruloyl esterase and tray fermentation

As per the taguchi design, the two factors with the highest ranks were chosen for optimization with central composite design, and the result obtained is listed in the following table (Table 3.6). Run number two gave the highest feruloyl esterase production and also the highest specific activity at 11984.1 U/gds and 274.87 U/mg (units/mg protein). The ANOVA for the data generated can be seen in Table 3.7and as can be observed that the model was significant at 5% level of significance. Usually central composite designs are used to understand the interaction between two different factors among a multitude of factors. In this case however among the two tested factors the only factor that came up to be significant is the initial pH of the solid medium being used as a substrate for feruloyl esterase production

Table 3.6 Feruloyl esterase as a response to the paramaters used in central composite design.

Run	A:pH	B:initial moisture	Response Feruloyl esterase (U/gds)	Specific activity U/mg
1	4	64.5	4899.7	117.78
2	5	67	**11984.1**	**274.87**
3	4	64.5	8172.6	171.83
4	3	62	755.1	14.383
5	3	67	0	0
6	4	64.5	8707.07	102.5
7	5	62	11200.3	247.25
8	4	60.96	3853.6	83.05
9	2.6	64.5	0	0
10	4	68.04	7855	207.26
11	4	64.5	7513.6	183.71
12	4	64.5	7520	193.32
13	5.4	64.5	11885.2	255.05
14	4	64.5	7577.1	189.9

The results of the same can be seen through the ANOVA table (Table 3.7) and the consequent regression equation that was generated using the statistical software

given in Equation 3.1.The equation has R^2 = 0.9180, Adjusted R^2= 0.9016 and a predicted R^2= 0.8214 which are in reasonable agreement with each other and hence under the given set of controlled conditions it can be said that the model will be unable to predict only about ten percent of variations.

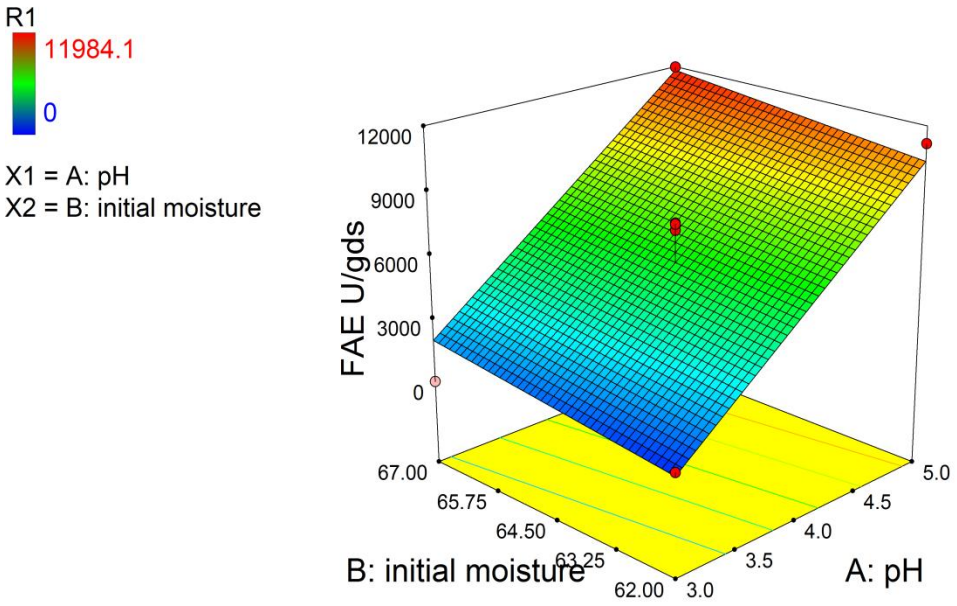
Table 3.7 ANOVA for model obtained using central composite design

Analysis of variance table	**Sum of Squares**	**df**	**Mean Squares**	**F Value**	**P Value**	
Source						
Block	2183560	1	2183560			
Model	1.96E+08	2	98245645	55.96593821	< 0.0001	significant
A-pH	1.92E+08	1	1.92E+08	109.6284808	< 0.0001	
B-initial moisture	4043506	1	4043506	2.303395578	0.1601	
Residual	17554543	10	1755454			
Lack of Fit	13396629	6	2232772	2.14797314	0.2397	not significant
Pure Error	4157913	4	1039478			
Cor Total	2.16E+08	13				

Equation 3.1 Model equation for predicting the production of feruloyl esterase using solid state fermentation.

FAE U/gds = -31755.34 + 4904.69 pH + 284.38* initial moisture*

Figure 3.5 Response surface for feruloyl esterase production.



The maximum activity that was recorded was 11984.1 U/gds which is 2.47 times the unoptimized enzyme levels. The interaction plot between initial pH and moisture can be observed in Figure 3.5. The figure shows a plane surface because of the lack of interaction between the two factors and the linear model used to describe the process. This is the maximum reported activity for feruloyl esterase activity as of yet with Kumar, Kamle et al. (2011) reporting 1162 U/gds at the

seventh day of fermentation (Productivity of 6.91U/gds/hour) and Singh, Nigam et al. (2014) reporting a maximum of 172 U/mg protein, specific activity on the eighth day of fermentation, both studies using maize bran as the substrate. Reports prior to 2010 report lesser activity as well as lesser productivity compared to the current report (Topakas, Vafiadi et al. 2007, Gopalan, Rodríguez-Duran et al. 2015). The current report gives the highest activity on the third day of fermentation, which corresponds to a productivity of 166.4 U/gds/hour. Optimization of process pH(of solid medium) for solid state fermentation is rarely reported(Deschamps, Giuliano et al. 1985, Battaglino, Huergo et al. 1991, Xia and Cen 1999, Senthilkumar, Ashokkumar et al. 2005), However, papers reporting pH optimum for production of feruloyl esterase through SSF are absent; making this as the first report optimizing the pH of the SSF medium for feruloyl esterase production. While papers on corresponding reports for FAE production in SMF have displayed similar pH optima (Hegde and Muralikrishna 2009, Singh, Nigam et al. 2014).

The process was scaled up using 50g destarched wheat bran and the tray fermentation recorded 10957 ±984U/gm which is similar to the optimized value. The growth of mycelia on the second day can be seen in Figure 3.6

Figure 3.6 Tray fermentation of destarched wheat bran

3.4 Summary

In conclusion, a linear equation for predicting the feruloyl esterase production using the factors initial pH and initial moisture had been established so as to produce significant amounts of the enzyme. It was also noted that the total alkali releasable ferulic acid content in a substrate may not be the driving force behind the expression of the enzyme feruloyl esterase. High productivity of 166.4 U/gds/h (11984.1 U/gds in 72h) was recorded with optimum conditions of pH 5 of the solid medium (destarched what bran) and the initial moisture set as 67%, and similar results were obtained at tray level fermentation (50g).

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Chapter IV

Purification of feruloyl esterase from *Aspergillus niger* ATCC 13497

4.1 Introduction

Feruloyl esterase as an enzyme was first detected as an extracellular enzyme activity component of the cellulolytic and xylanolytic systems of *Schizophyllum commune*(MacKenzie and Bilous 1988), and later the first report of a purified feruloyl esterase, from *Streptomyces olivochromogenes*(Faulds and Williamson 1991). Subsequent to this,Faulds and Williamson (1994) again described the occurrence of feruloyl esterase activity and characterized the enzyme from *Aspergillus niger*. This was followed by a plethora of work being carried out for identification of feruloyl esterase enzymatic activity in a variety of microbes as already discussed in the introduction (sections 1.3 and 1.4)of this thesis. A recent finding even extended the presence of a type C feruloyl esterase from *Aspergillus niger* (Dilokpimol, Mäkelä et al. 2017).

The objective of this particular chapter is to purify and characterize the feruloyl esterase activity from *Aspergillus niger* ATCC 13497.

4.2 Materials and methods

Procuring details of all chemicals used for this work were listed in Table 2.1

4.2.1Ammonium sulphate precipitation and fractionation of crude enzyme solution

Ammonium sulphate precipitation was done according toEnglard and Seifter (1990). Ammonium sulphate required to precipitate feruloyl esterase was optimized by its addition, at varying levels of concentrations (30, 60, and 90% saturation), to the crude extracts. To precipitate the protein, ammonium sulphate was slowly added initially at 30 % saturation to the crude extract while keeping in ice with gentle stirring. Precipitated protein was collected by centrifugation at 10000 rpm for 15 min at 4ºC. The precipitate was re-suspended in minimum volumes of 100mM MOPS buffer (pH 6.5) and stored at 4^{o}C. To the supernatant, ammonium sulphate required for next level of saturation, was added and the

procedure mentioned above was repeated. The process was repeated once more so as to obtain fraction from the 90% saturation of ammonium sulphate.

4.2.2Hydrophobic interaction chromatography (HIC) for purification of feruloyl esterase

Hydrophobic interaction chromatography was used to further fractionate the partially purified feruloyl esterase enzyme mixture. The said method involves binding of proteins with a hydrophobic moiety immobilized on to a support matrix, in this case, phenyl sepharose6 Fast flow (GE,USA) was utilized. Hydrophobic interaction is only possible once the surface charges of proteinsare neutralized. The surface charges are neutralized by suspending the protein solution and the matrix in high salt concentrations of ammonium sulphate (2M), Once all the proteins are bound , the salt concentration of the equilibration buffer is reduced stepwise which leads to regaining of surface charge of different proteins. Proteins that are highly polar elute first and the non polar proteins follow after further reduction of salt concentration. This method had been used previously by (Donaghy, Bronnenmeier et al. 2000).

Bio-Rad Low pressure chromatography system with inline conductivity and UV (280 nm) absorbance detector was used for this method. Phenyl-Sepharose matrix was packed in a glass column (10 cm x 2.5 cm). The column was equilibrated with ammonium sulphate solution of high molar strength (2M). After the column was equilibrated, 1.0 ml of the partially purified feruloyl esterase was loaded on to the column matrix. The column was then washed with 5–10 column volumes of 2M Ammonium sulphate solution or until the UV baseline and conductivity are stable, that is, when all unbound material has washed through the column. The bound proteins are then eluted with up to 5 column volumes of elution buffer at a salt concentration lower than that in the start buffer. Repeat lowering the salt content at each step until the target protein(s) has been eluted. i.e, 2M, 1.5M, 1M, 0.5M, 0.25M, deionised waterata flow rate of 1.0 mL/min. The fractions with high feruloyl esterase activity were pooled and

then dialyzed using a 14 kDa cutoff cellulose acetate membrane (Sigma Aldrich, USA) against 10 mM MOPS buffer (pH 6.5). The whole process was carried out at 4^{o}C so as to reduce the chances of protein precipitation. The dialyzed protein was then stored till further use at 4^{o}C.

Specific activity is a way to measure how much of a measured protein there is with all of the other contaminating proteins. Specific activity was calculated by dividing the enzyme units with protein content and was expressed as U/mg protein. The total protein content was measured initially by Bradford's method as described in section 2.2.6a. After column chromatography, the protein concentration dropped below the accurate quantification range of the Bradford's method and hence Bichoninic acid based BCA protein estimation was used to quantify the protein content as per method described in section 2.2.6b.Fold purification of the enzyme was calculated by dividing the specific activity of each fraction by the specific activity found in the crude enzyme. This number changes depending on the protein. Percentage yield is calculated by dividing the total enzyme activity at the end of the process with the initial total enzyme activity.

4.2.3Molecular size determination of feruloyl esterase

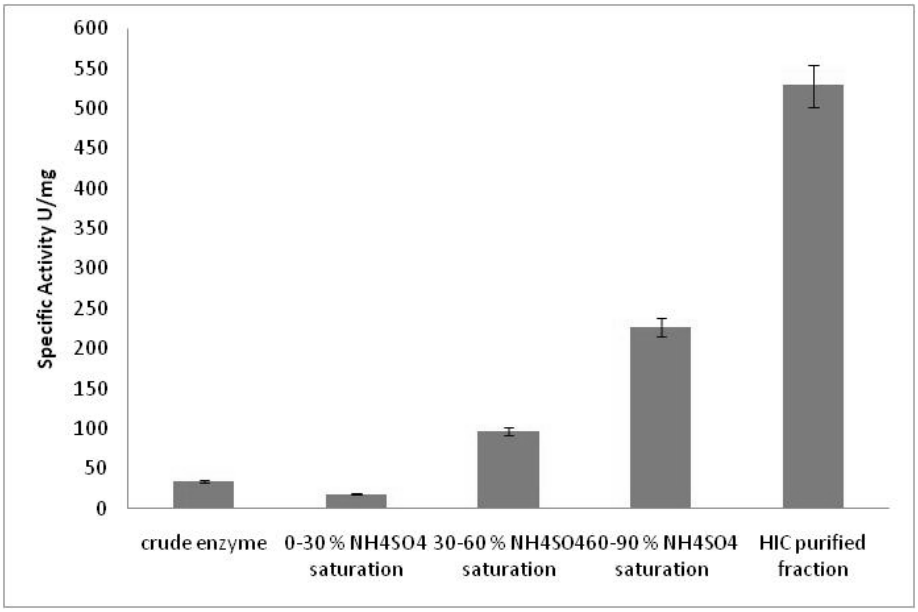
Discontinuous SDS -PAGE (12% polyacrylamide separating) (non reducing) according to Laemmli (1970)was carried out using the dialyzed protein samples as per section 2.3.5 in general materials and methods. Pre stained molecular size markers for PAGE (Bio-Rad USA) were used to compare the size of the separated peptide(s). ChemiDoc^{TM} (Bio-Rad USA) was used to capture the image of the gel, and the provided visualization software was also used to calculate the molecular weight of the separated bands.

4.3 Results and Discussion

4.3.1 Ammonium sulphate precipitation and fractionation of crude enzyme solution

The ammonium sulfate fraction at 60-90 % saturation had highest activity and comparatively higher specific activity.Feruloyl esterase from *Aspergillus niger* and other *Aspergilli* have been reported to be in the range of 30-36 kDa(Faulds and Williamson 1994, McCrae, Leith et al. 1994, Koseki, Furuse et al. 1998, Benoit, Asther et al. 2006) . Since we expect our target protein to be in the same size range it is natural that the 60-90% saturation fraction gave the highest specific activity for feruloyl esterase as smaller proteins are normally precipitated at relatively higher saturation percentages of ammonium sulphate. The graph depicting the increase in specific activity with subsequent steps of enzyme purification can be observed in Figure 4.1

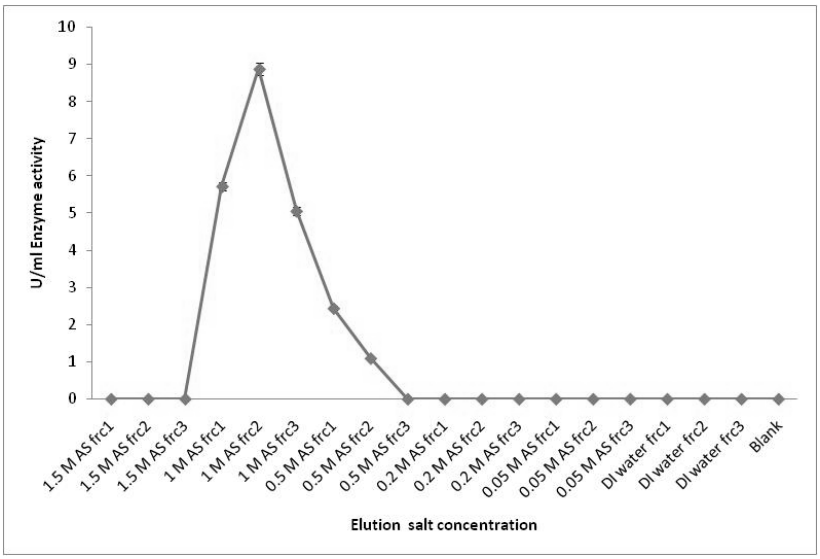
Figure 4.1: Increase in specific activity with subsequent purification of protein.



4.3.2 Hydrophobic interaction chromatography for purification of feruloyl esterase

Hydrophobic interaction chromatography fractions with high protein concentrations when subjected to the feruloyl esterase enzyme assay, it was evident that the fraction that was eluted when the Ammonium sulphate concentration was reduced to 1M gave the highest activity for feruloyl esterase, as can be seen from Figure 4.2. The fraction displayed a 15.67 fold purification (Table 4.1)

Figure 4.2: Feruloyl esterase activity of HIC fractions.



Legend **AS frc**- Ammonium sulphate fraction; **DI water** De-Ionized water

Table 4.1 Stepwise purification of feruloyl esterase

	Residual activity (U/ml)	**Fold purification**	**Specific activity (U/mg protein)**
Crude extract	100	1	33.73
60-90% ammonium sulphate saturation	36.73	6.72	226.66
Hydrophobic interaction chromatography-fraction	8.88	15.67	528.67

4.3.3 Molecular size determination of feruloyl esterase

The SDS PAGE profile of the protein separated can be seen in Figure 4.3. The molecular weight of the prominent band was calculated as 35kDa (Figure 4.4). AnFaeA was isolated and purified by (Faulds and Williamson 1994), and the protein described has a mass of 36 kDa, *Aspergillus awamori* feruloyl esterase A AwFaeA also has similar mass of 35 kDa (Kanauchi, Watanabe et al. 2008).

Figure 4.3: SDS PAGE (12%) analysis of purified feruloyl esterase.

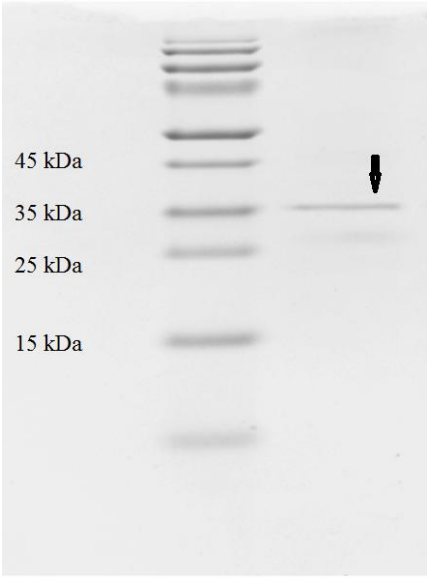
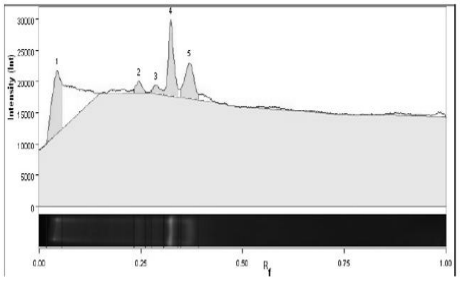


Figure 4.4: Report for protein size estimation of prominent band in SDS PAGE.



Band No.	Band Label	Mol. Wt. (KDa)	Relative Front	Volume (Int)	Abs. Quant.	Rel. Quant.	Band %	Lane %
1		170.0	0.046	34,332,318	N/A	N/A	39.4	22.9
2		46.0	0.244	4,495,521	N/A	N/A	5.2	3.0
3		39.1	0.286	4,039,506	N/A	N/A	4.6	2.7
4		34.9	0.323	24,993,621	N/A	N/A	28.7	16.7
5		28.9	0.369	19,356,837	N/A	N/A	22.2	12.9

4.4 Summary

From *A. niger* ATCC 13497 a feruloyl esterase of mass 35 kDa was isolated and purified from the crude extract obtained from solid state fermentation by fractionation and purification using ammonium sulphate precipitation and hydrophobic interaction chromatography.

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Chapter V

Characterization of feruloyl esterase from *Aspergillus niger* ATCC 13497

5.1 Introduction

Feruloyl esterase enzyme has uses in pulp bleaching, nutracuticals, pharmaceuticals and can also serve as an accessory enzyme for the deconstruction of complex lignocelluloses (Record, Asther et al. 2003, Benoit, Danchin et al. 2008, Govindaswamy 2015). Understanding the characteristics of the enzyme would only assist us to use the enzyme better for various applications that the enzyme maybe useful for. Understanding the conditions where the enzyme is the most active or conditions where the enzyme is the most specific would be advantageous for controlling the process parameters so as to control the quality of the final product, which is the deciding factor for the economics of the final product. Factors like the temperature stability and the temperature optimum of the enzyme will help determine the final concentrations of the enzyme for commercial preparations of the enzyme for example a thermostable enzyme will perform more efficiently and will have longer shelf life at higher temperatures compared to mesophilic temperatures making it more amenable for industrial process (Niehaus, Bertoldo et al. 1999). The optimal pH and the pH stability of the enzyme will determine the end application of the enzyme; an alkaline stable enzyme will have better use in the pulp and paper industry because of primary use of bases for pulping (Horikoshi 1996). An acidic or neutral pH stable enzyme maybe used more for food and feed applications where one would expect the enzyme to be active even after going through the acidic pH of the stomach. Feruloyl esterases are xylanase accessory enzymes (Braga, da Silva Delabona et al. 2014) and therefore, applications involving xylanase including feed improvement, lignocellulosic hydrolysis and food texture improvement would benefit because of the action of feruloyl esterase (Polizeli, Rizzatti et al. 2005). Understanding the type of enzyme for feruloyl esterase would determine the kind of product or the homogeneity of the product that one could expect from a reaction mixture, as feruloyl esterases may be promiscuous and hence might have side activities which may or may not be favorable for an industrial process(Yao, Chen et al. 2013).

The current chapter deals with understanding the optimal conditions for the enzyme for the hydrolysis of the ester bond and the kinetics for the same.

5.2 Materials and Methods

The details of all major chemicals used in the chapter are listed in table 2.1.The purified enzyme solution obtained through HIC was subjected to characterization studies. Unless specified, the assay mentioned in 2.2.2 was used to determine the enzyme activity at different conditions

5.2.1 pH optimum

The purified enzyme solution obtained through HIC (Hydrophobic Interaction Chromatography) was subjected to the enzyme assay using buffers in the acidic range, the neutral range and the basic range. Appropriate blanks were made for each of the buffers tested. Acetate buffer was used for acidic pH, phosphate buffer was used at pH close to 7 and glycine NaOH buffer was used for basic ranges. The reactions were carried out with 50mM concentration of each of the buffers at 37^{o}C for 15 min, followed by stopping the reaction by incubating the reaction mixture in boiling water bath for 20 min.100 U enzyme at pH 6.5 was used for assay.

5.2.2 Temperature optimum

The purified enzyme obtained through HIC was subjected to the enzyme assay at different temperatures using a gradient temperature profile on a Bio-RadMyCycler thermal cycler, with temperatures ranging from $30^{-}90^{o}$C in two batches. The reaction was carried out in 0.2 ml thin walled micro-tubes, and the reaction was stopped as per the enzyme assay conditions mentioned in general materials and methods 2.2.2 .100 U enzyme at pH 6.5 was used for assay.

5.2.3 Temperature stability of enzyme

The purified enzyme obtained through HIC was used to assess the temperature stability of the enzyme. Prior to the assay the purified enzyme

without the assay buffer was incubated at different temperatures from 50-90^{o}C for 20 min and was then subjected to the enzyme assay under defined conditions as mentioned earlier. 100 U enzyme at pH 6.5 was used for assay.

5.2.4 Effect of metal ions

Purified enzyme was used to assess the effect of metal ions on the activity of the enzyme. The enzyme assay was carried out in the presence of 5 mM of chloride salts of the metals $Co+^{2}$, Mn^{+2}, Ca^{+2}, Mg^{+2}, Fe^{+3},Ni^{+2},Cu^{+2}, Zn+2 and Ba^{+2} and the reaction carried out under defined assay conditions.100 U enzyme at pH 6.5 was used for assay.

5.2.5 Effect of Inhibitors

Purified enzyme was used to assess the effect inhibitors including detergents on the activity of the enzyme. The enzyme assay was carried out in the presence of 5 mM of SDS, Tween 80 (5mM) and guanidine hydrochloride 5 (mM). 160 U enzyme at pH 6.5 was used for assay.

5.2.6 Effect of solvents

Purified enzyme was used to assess the effect of different alcohols on theactivity of the enzyme. The enzyme assay was carried out in the presence of the solvents methanol, ethanol, propanol, butanol, octanol and polyethylene glycol (PEG at 5mM) at 10% v/v. 160 U enzyme at pH 6.5 was used for assay.

5.2.7 Determination of kinetic parameters of enzyme

Purified Enzyme of concentration 0.16 mg/ml was used for the Kinetic studies. (1:4 dilution). Substrate concentration ranging from 0.2 mM to 7.5 mM was used to probe the fixed enzyme concentration (40µg/mL) for the rate of product formation using a fixed time interval of 15 minutes. The velocities thus estimated and the product concentrations used were then used to fit the Michaelis-Menten function for single active site using the software package Simfit (Holzhütter and Colosimo 1990). The fitted equation was assessed by chi squared

test and the R^2 value. The fit is assumed to be good if the probability value of the chi squared term being less than weighted sum of squares (WSSQ) is <0.05 (for 5% significance) or p(chi squared ≥WSSQ) is greater than 0.95.

5.2.8 Preparation of methyl esters and feruloyl esterase typing

Methyl esters of various hydroxycinnamic acids were prepared by dissolving 10 mg of the said hydroxycinnamic acid in excess of methanol, followed by addition of 10 mol% of sulfuric acid as catalyst. The mixture was sealed and stirred at a constant temperature of 30^{o}C for 12 h. The reaction mixture was then applied onto silica gel 60 F 254 preparative TLC plates(Merck) as a single large band and was resolved to obtain a band of the methyl ester, which was scrapped off the glass TLC plate and extracted 3 times in 100% methanol, followed by evaporation of methanol to obtain relatively pure methyl esters of different hydroxycinnamic acids.

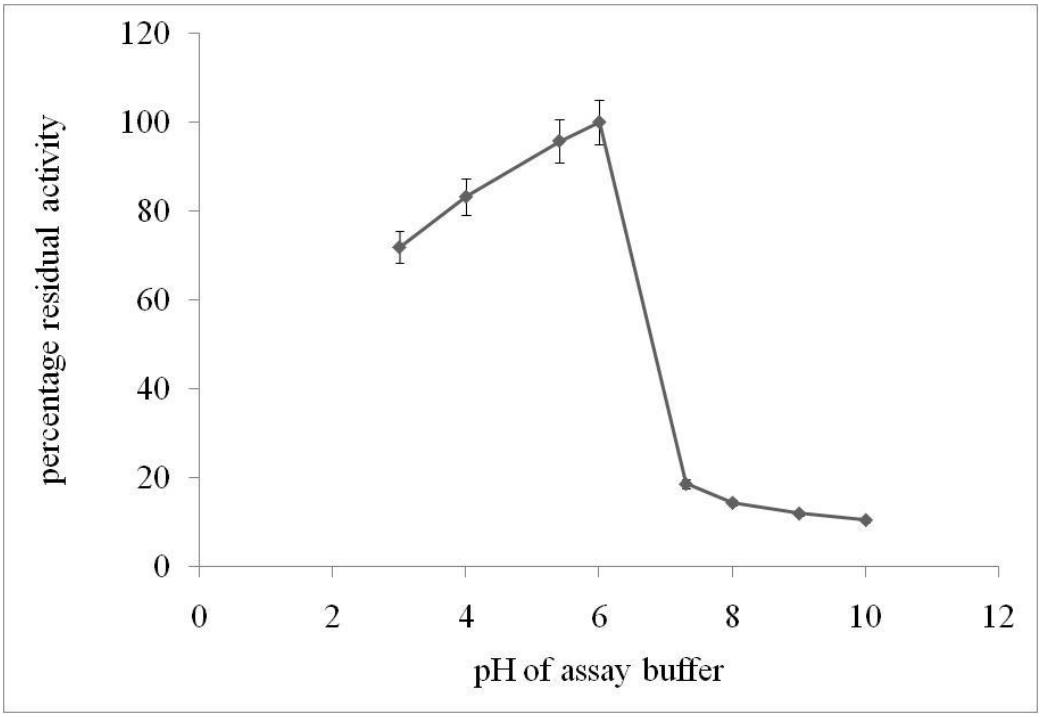
The feruloyl esterase was classified according to the action of the enzyme on synthesized methyl esters of cinnamic acid, ferulic acid, caffeic acid. Approximately one mM solution of the individual methyl esters were prepared in the enzyme assay buffer separately and were subjected to the enzyme assay. This was followed by detection of the respective hydroxycinnamic acid by HPLC (qualitative).

5.3 Results and Discussion

5.3.1 pH optimum

The purified enzyme fraction had maximal activity at pH 6 and was also active comparatively in the acidic pH range. The purified enzyme did not show any significant residual activity in the alkaline rangeas seen in Figure 5.1. The pH optimum is similar to that of feruloyl esterase produced by *Aspergillus niger* using sugar beet pulp as a growth substrate (Kroon and Williamson 1996) .

Figure5.1: Enzyme activity of the purified enzyme at different pH values

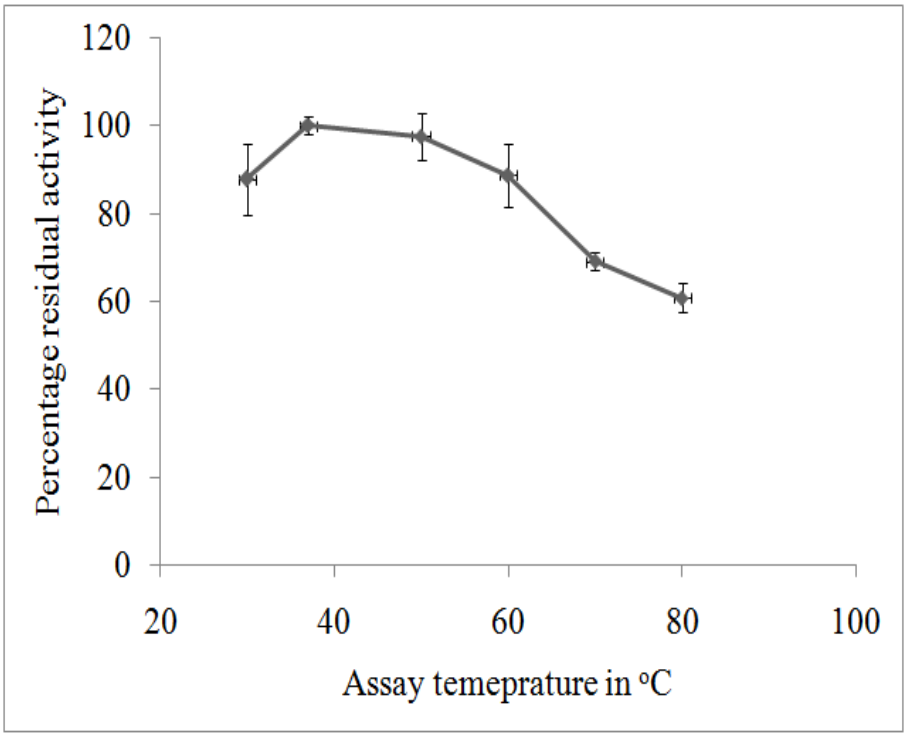


5.3.2 Temperature optimum

The purified enzyme displayed maximal activity at 37^{o}C, and the enzyme still retained 60% activity at 80^{o}C. The temperature profile of the enzyme is evident from Figure 5.2. Enzyme activity was above 80% of the activity at 37^{o}C for temperatures 30^{o}C, 50^{o}C, and 60^{o}C.The enzyme displayed similar temperature and pH profiles to AwFaeA and AtFaeA rather than AnFaeA described earlier (Faulds and Williamson 1994, De Vries, Michelsen et al. 1997, Kanauchi, Watanabe et al. 2008).Increasing the temperature of the reaction certainly increases the rate of reaction but with enzyme catalysed reactions the rate will increase till the time the enzyme is stable(Wasserman 1984). For industrial uses it is essential the reaction to be as fast as possible so as to achieve high productivity. It is therefore very essential to understand the temperature optimum, of the enzyme.Zhang and Wu

(2011)found that the wild type AnFaeA when expressed in *Pichia pastoris*, displays a similar pH optimum of 6.4 and even a temperature optimum of 40^{o}C.

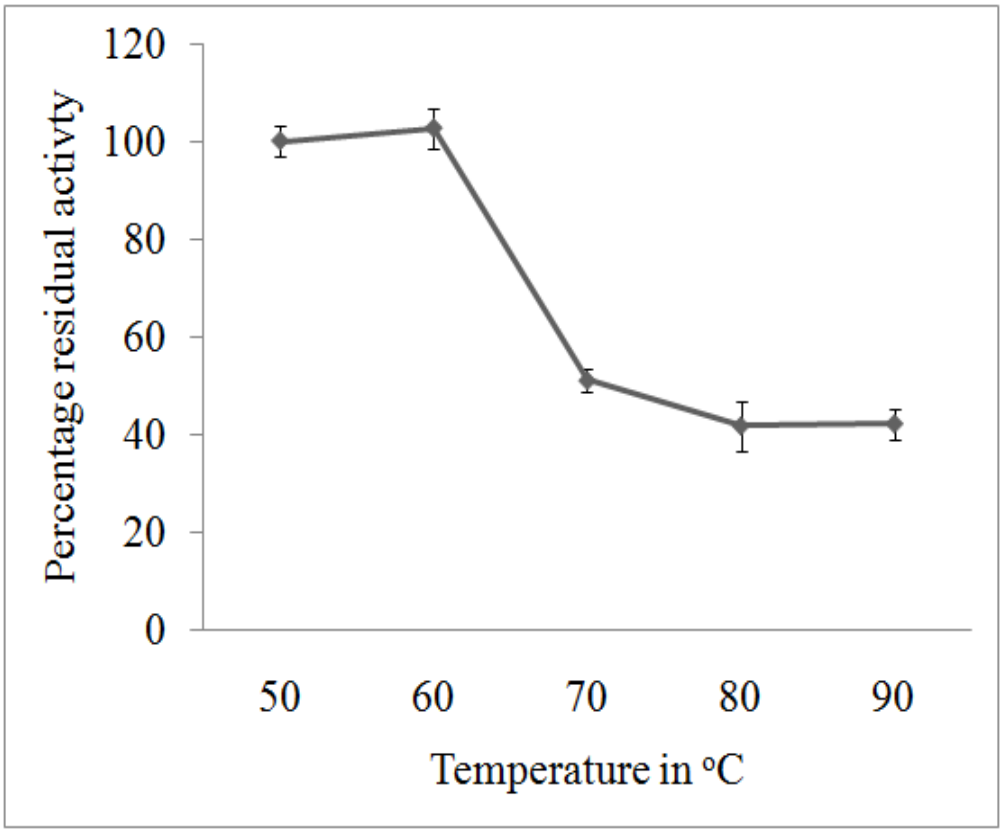
Figure5.2: Temperature optimum of purified enzyme.



5.3.3 Temperature stability of enzyme

The purified enzyme displayed about 100% activity for temperatures 50^{o}C and 60^{o}C, however, after incubation at 70^{o}C the activity dropped to 50%, followed by drop to 41% at temperature 80^{o}C, as observed in Figure 5.3. It is essential to understand the temperature at which the enzyme activity is maximal so as to understand the best conditions for the enzyme. The temperature stability of the enzyme is a trait that is sought out in the industrial arena. Using enzymes that are stable at higher temperatures give better reaction rates, which in turn lead to lesser amount of enzyme required for the process, also higher temperature reaction mixtures are less likely to be contaminated with microorganisms(Zamost, Nielsen et al. 1991).

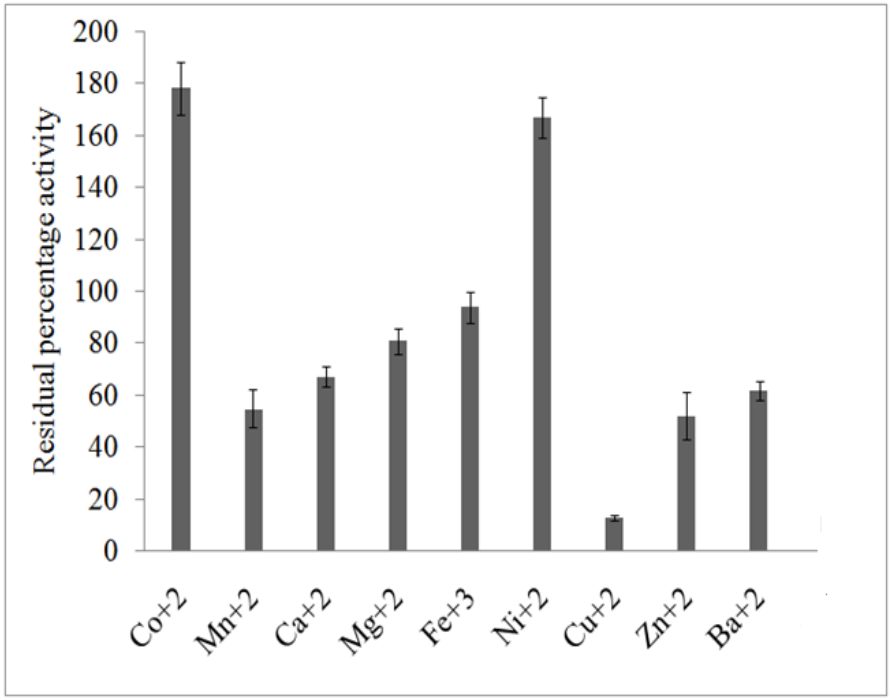
Figure 5.3: Temperature stability of purified enzyme.



5.3.4 Effect of metal ions

Metal ions Co^{+2} and Ni^{+2} (5mM) enhanced the activity of the enzyme to 178% and 166% respectively, while Ba^{+2}, Zn^{+2}, Mg^{+2} and Ca^{+2} reduced the activity to below 80%. Fe^{+3} marginally reduced the activity of the enzyme, while Cu^{+2} reduced the activity below 15%, as can be observed in Figure.5.4. Copper has been reported as a known inhibitor for feruloyl esterase (Donaghy, Bronnenmeier et al. 2000, Wang, Geng et al. 2004, Sang, Li et al. 2010). Ni+2 was earlier found to be slightly enhancing the activity of a feruloyl esterase expressed from a cotton soil metagenomic library (Yao, Chen et al. 2013). The inhibitory action of Zn^{+2} and Ba^{+2} has been recorded for FAE from *Aspergillus awamori* (McCrae, Leith et al. 1994).

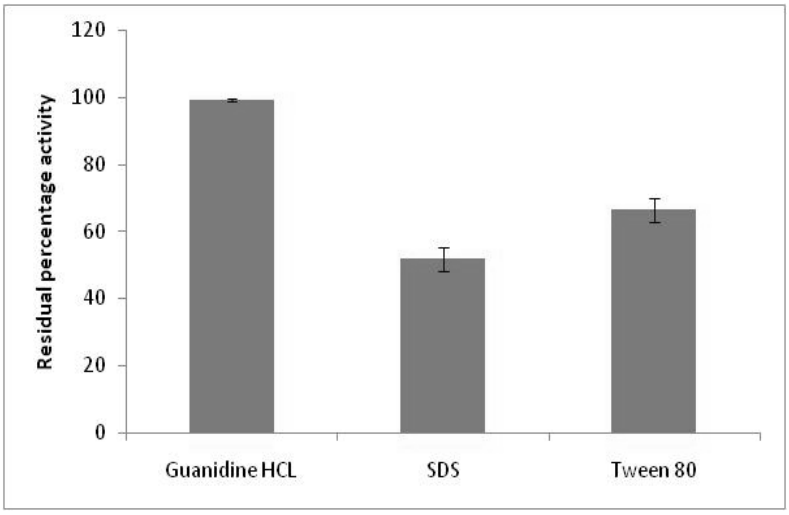
Figure5.4: Effect of metal ions on purified enzyme.



5.3.5 Effect of Inhibitors on enzyme

Inhibitors reduced the activity of the enzyme with inhibition in the order SDS>Tween 80 >Guanidine hydrochloride. Guanidine hydrochloride did not inhibit feruloyl esterase at 5mM level as seen in Figure 5.5.

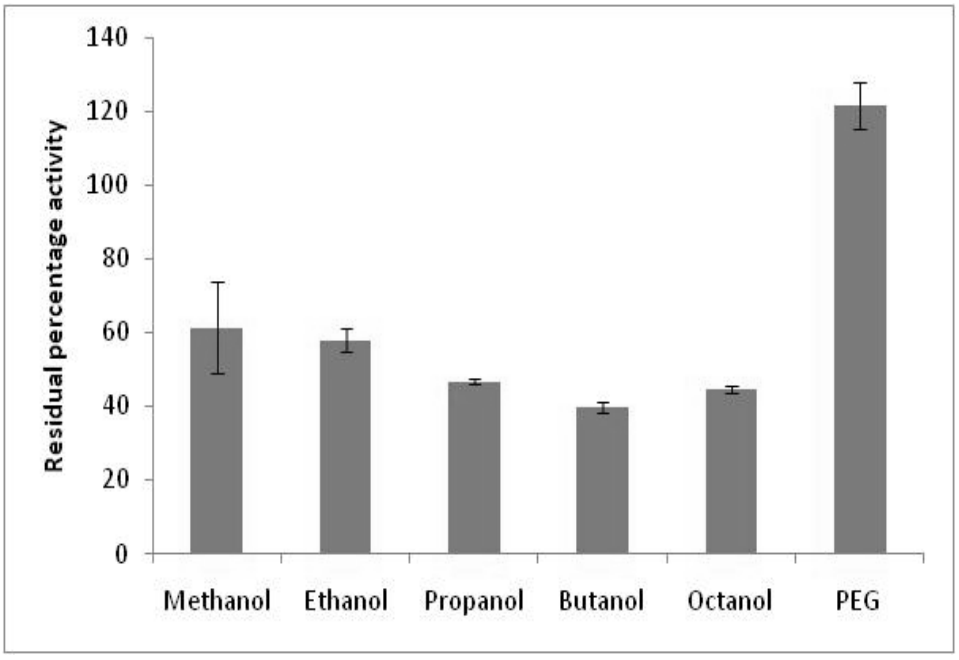
Figure5.5: Effect of inhibitors/detergents on feruloyl esterase.



5.3.6 Effect of solvents

All solvents inhibited the activity of feruloyl esterase enzyme and the extent of inhibition increased with the increase in the carbon chain length of the alcohol being used. PEG or poly ethylene glycol seemed to enhance the activity of feruloyl esterase as see in Figure 5.6. It has been noted in earlier studies that cellulolytic enzymes were stabilized by the addition of PEG to the reaction mixture (Ouyang, Dong et al. 2010). Reports of PEG enhancing feruloyl esterase have not been noticed. Esterases are enzymes which are used for biotransformation reactions in non-aqueous medium(Torres and Castro 2004, Petkar, Lali et al. 2006). It therefore becomes important to study the behavior of the enzyme in question in the presence of solvents, as we have to understand whether the enzyme can be used for esterification reaction. According to Figure 5.6, there is enzyme activity loss with the presence of alcohol solvents but it does not lead to complete inhibition indicating that the enzyme may still act as a catalyst in non aqueous solvents. Enhancement of activity in presence of PEG maybe due to biphasic reaction enhancement that happens in presence of PEG(Chen, Spear et al. 2005)

Figure 5.6: Effect of solvents on feruloyl esterase.



5.3.7 Determination of kinetic parameters of enzyme

The response of enzyme velocity vs. concentration of the substrate can be visualized through Figure 5.7. The same data was used as input for the curve fitting software Simfit, and the following output was obtained as seen in Table 5.1. The diagnostic statistic P(chi-sq. >= WSSQ) = 0.9998, and theR^2 = 0.9820, which helps us conclude that the fit of the curve is good and therefore the subsequent Km and Vmax calculations are also accurate within the given intervals. Since the protein content of the reaction is 40µg, the specific velocity V_{max}= 275.5 µM/min/mg and the K_m= 1.5258 mM. The values fall well within the range of reported values for the action of AnFaeA on ethyl ferulate in the presence of DMSO (Garcia-Conesa, Kroon et al. 1999)

Figure 5.7: Substrate concentration and corresponding velocity for fixed enzyme amount

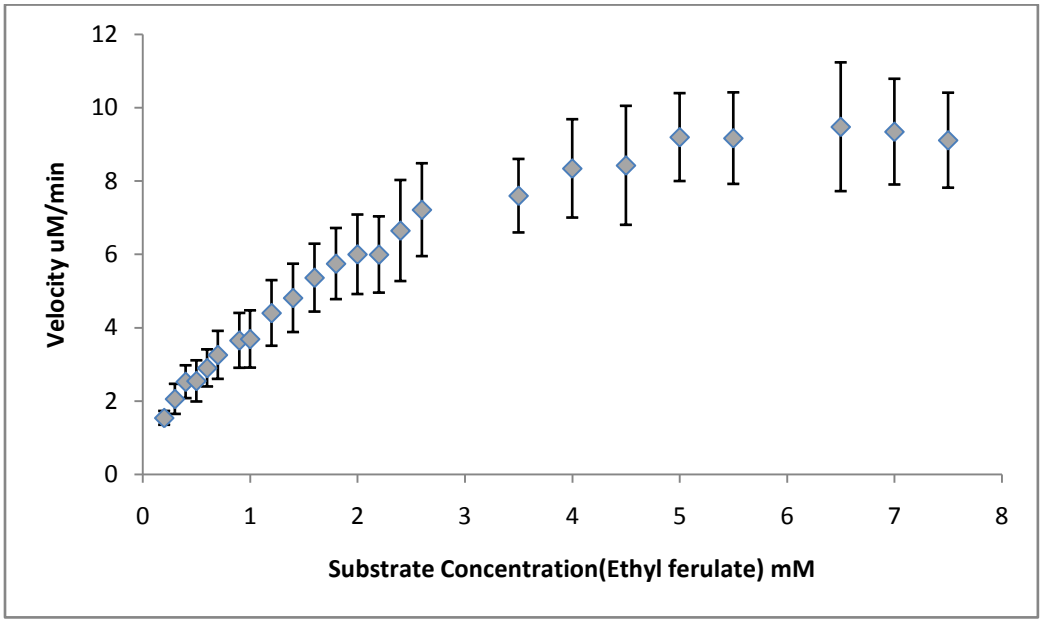


Table 5.1. Calculated K_m(µM**)** and V_{max} (µM/min) using Simplot software.

No	Parameter	Value	Std. Error	Lower 95% confidence Interval	Upper 95% confidence Interval	p
1	Vmax(1)(µM/min**)**	1.10E+01	4.45E-01	1.01E+01	1.19E+01	0.0
2	Km(1)(mM)	1.53E+00	1.23E-01	1.27E+00	1.78E+00	0.0

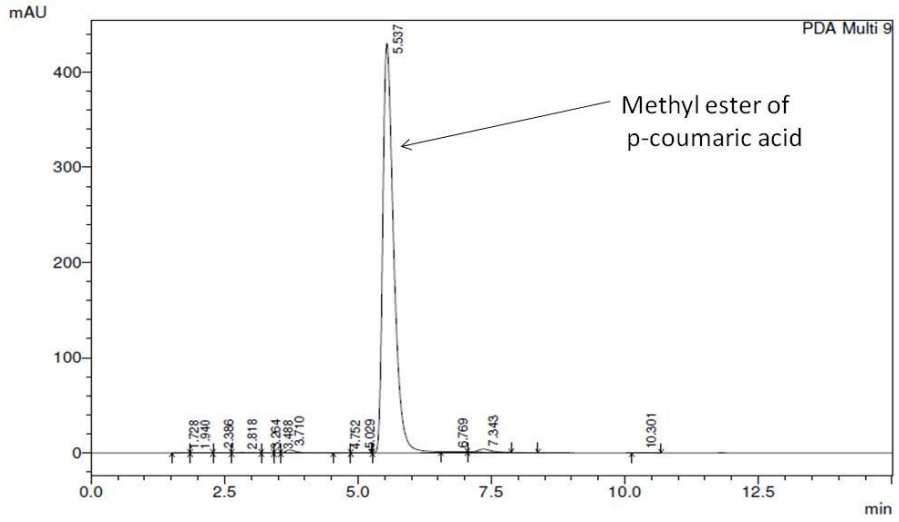
5.3.8 Feruloyl esterase typing

The separation of methyl esters of hydroxycinnamic acids from their respective acids through preparatory TLC can be visualized through Figure 5.8.The enzyme was found to be active on methyl ferulate and methyl sinapate . The enzyme preferentially acted on esters of phenolic acids with bulky methyl groups on the ortho position. This type of feruloyl esterase can be therefore classified as type A (Crepin, Faulds et al. 2004). The representative chromatograms can be seen in Figure 5.9 and 5.10.

Figure 5.8 Preparatory TLC of methyl esters of hydroxycinnamic acids

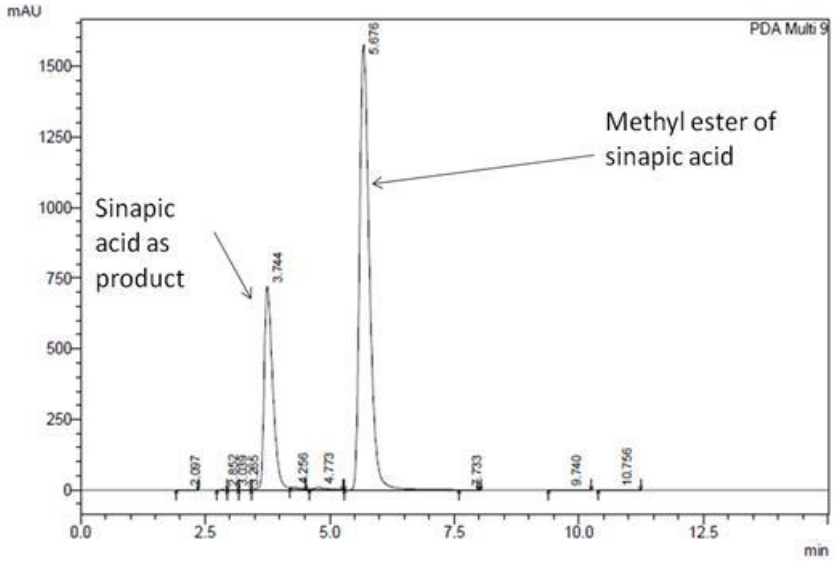
Methyl ester encircled in grey and free un-reacted hydroxycinnamic acid encircled in black after etherification reaction with methanol. **Lane 1** p-coumaric acid methyl ester **Lane 2** caffeic acid methyl este **Lane 3**- sinapic acid methyl ester

Figure 5.9 HPLC Chromatogram of reaction mixture with methyl ester of p-coumaric acid as the substrate



Methyl ester of p-coumaric acid retention time 5.537min

Figure 5.10 HPLC chromatogram positive reaction on methyl ester of sinapic acid.



Sinapic acid retention time 3.744 min and methyl ester of sinapic acid retention time 5.676 min

5.4 Summary

To summarize, the enzyme displayed an optimum pH of 6.0 and was fairly thermostable as it retained ~45% of activity after incubation at 90^{o}C or 20 mins. Metal ions copper and zinc reduced the activity of the enzyme, while nickel and cobalt increased the activity of the enzyme. Detergents reduced the activity of the enzyme with inhibition in the order SDS>Tween 80> Guanidine HCl. Solvents adversely effected the activity of the enzyme with inhibition in the order butanol>octanol>propanol>ethanol>methanol, but PEG enhanced the activity of the enzyme. The enzyme was found to be a type A feruloyl esterase, hence AnFaeA.

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Chapter VI

Extraction of ferulic acid from destarched wheat bran using feruloyl esterase

6.1 Introduction

The abundance of ferulic acid in plant materials urges the desire to find means to extract this useful compound for its exploitation. Wheat bran is an agricultural byproduct, which possessed little economic value till recent times. The presence of compounds with anti-oxidant and anti tumor activities has forced us to re-evaluate the value that this byproduct adds to our diets (Ruby, Kuttan et al. 1995, Qu, Madl et al. 2005, Srinivasan, Sudheer et al. 2007, Bagheri, Asl et al. 2017, Kilani-Jaziri, Mokdad-Bzeouich et al. 2017). What makes a biological process lucrative is the fact that most of them are benign, with little or no effective disturbance of the eco system. Extraction of phenolic acids like ferulic acid is very much possible using caustic bases(Kim, Tsao et al. 2006, Ou, Luo et al. 2007, Tilay, Bule et al. 2008, Torre, Aliakbarian et al. 2008, Buranov and Mazza 2009, Aarabi, Mizani et al. 2016), but handling bases is dangerous at large scales and is not an environmentally safe process as neutralizing bases with acids would only lead to production of salts, which when introduced into water streams would increase the salinity of the surrounding soils leading to soil infertility issues and other ecological changes. Other methods for extraction of phenolic acids from plant material have also been devised which include ultrasonic assisted extraction, microwave assisted extraction methods as well as pressurized solvent extraction methods(Liu, Wang et al. 2006, Sun and Wang 2008, Buranov and Mazza 2009, Quan, Sun et al. 2009),which are energy intensive methods. Therefore, it becomes essential to involve biocatalysts viz. enzymes and in this particular case feruloyl esterase from *Aspergillus niger* (AnFaeA).Although wheat is not the only source of ferulic acid, other sources like sugar beet and corn bran with higher amounts of bound ferulic acid are also available. However, since the enzyme was produced using destarched wheat bran, using the same enzyme for the de-construction and hence extraction of ferulic acid would be favorable as the organism expresses the said enzyme for the very purpose. Since the enzyme is classified as a type A

enzyme, there are lesser chances of it releasing diferulates and oligoferulates which are not of interest. To make the process benign and effective, strategies to effectively separate the released ferulic acid must be applied. Ferulic acid has been purified by using adsorbents of different kinds in the past and the current work uses the same strategy of using adsorbents albeit with the difference of the use of a semi permeable membrane to separate the enzyme from the adsorbent so as to avoid loss of enzyme activity.

6.2 Materials and Methods

All major chemicals used in the chapter have heir details listed in Table 2.1

6.2.1 Determination of total Alkali extractable ferulic acid present in destarched wheat bran

Alkali extractable ferulic acid was determined by treating 1 g dry destarched wheat with 1 N NaOH at 25^{o}C for 12h. The resulting slurry was then acidified using 5N HCl till the pH dropped to 3.5. The acidified slurry was then extracted with three volumes of ethyl acetate. The extracted ethyl acetate volume was reduced by rotary evaporation (R210, Buchi, Switzerland), and the yellowish liquid residue was then dissolved in 10 mL of dry HPLC grade methanol (Sigma Aldrich, USA) and analyzed through HPLC.

6.2.2 Determination of significant parameters affecting the release of ferulic acid from destarched wheat bran

A Plackett-Burman design was adopted to test significant (at 5% significance) parameters that affected ferulic acid release from the substrate. The design was made using Design-Expert software, Stat-Ease, USA. The design made such that one could understand the effects of change in temperature using two data points viz. 35and 45^{o}C. Reaction pH effects were tested at 3.5 and 6.5 units were studied. It was also critical to find the best enzyme to substrate ratio (U/gm) or the enzyme loading; hence enzyme loading ratio from 50 to 150 U/g was tested. The time required for the reaction was also crucial as it is necessary to

strike a balance between reaction completion and enzyme deactivation. Therefore, reactions with two end point times were studied with 3h and 6h. The design was made so as to include 12 runs with the given set of parameters. All reactions were carried out in 100 mL capacity screw cap flasks to avoid water loss by evaporation. Citrate buffer 10 mM was used to maintain the two different pH points. The solid loading of destarched wheat bran with respect to the buffer was 5 % with a reaction volume of 30 mL. All reactions were carried out with forced mixing at 200 rpm in a hot water bath with linear shaking (SW 22, Julabo, Germany). Equation 6.1 given below is the representative of the final form of the linear equation that one obtains, where Y is the response variable (% ferulic acid released) and X_i represents the i^{th} variable that is being studied. Equation 6.2 on the other hand is used to calculate the effect of each variable X_i, where $\sum M_{i+}$ is the sum of all responses (percent ferulic acid release) when variable X_i, is set to higher level and $\sum M_{i-}$ is the sum of all responses (percent ferulic acid release) when variable X_i, is set to lower level.

Equation 6.1 First order polynomial used in Plackett-Burman design.

$$Y = \beta_0 + \sum \beta_i X_i$$

Equation 6.2 Effect of individual variables(Plackett-Burman).

$$\boldsymbol{E(X_i) = \frac{\sum M_{i+} - \sum M_{i-}}{N}}$$

6.2.3 Steepest Ascent and Central Composite Design to optimize the levels of the significant parameters for the release of ferulic acid from destarched wheat bran

Steepest ascent method was used, by using the linear equation in the Plackett-Burman design and a step size of 0.75 with 8 runs in total. The runs were designed using a statistical software Minitab 17 trial (Minitab Inc. USA), by

keeping the temperature constant at 45^{o}C and the ph constant at 3.5. The parameters from the runs which were close to maximal release of ferulic acid from destarched wheat bran were then used to determine the range of the parameters to be studied using Central composite design. The following table (Table 6.1)gives the details of the chosen parameters.

Table 6.1 Range of parameters selected for central composite design

Parameter Name	Units	Low	Mid	High
FAE enzyme loading	U/g	325	437.5	550
pH	units	3	3.5	4
Reaction temperature	C	40	45	50
Reaction time	hours	6.5	9	11.5

The central composite design was made so that the four parameters were tested at three different levels, viz. +1, 0, -1. Since there were four parameters at three levels, the design had a total of 30 runs. The statistical analysis of the experiment was carried out by means of analysis of variance to the obtained results using Design Expert 7.0 trial (Stat-Ease USA). The significance of the parameters was tested at 5 % level of significance. A quadratic model with the parameters as variables and the percentage release of ferulic acid as the output is generated from the given data using the same software. Equation 6.3 given below was used to correlate the response (percent release of ferulic acid) with the significant variables.

Equation 6.3 second order quadratic equation used to determine the yield Y with respect to variables used in study(FAE enzyme loading, pH, reaction temperature, reaction time)

$$\boldsymbol{Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \sum_{i<j=1}^{n} \beta_{ij} X_i X_j}$$

6.2.4 Packed column process for extraction of ferulic acid from destarched wheat bran

An apparatus for the process was set up so that a reservoir of buffer with the enzyme was constantly maintained at 42^{o}C, using a static hot water batch (Jiotek, Lab Companion BW-20G, South Korea)which is continuously passed through a packed column containing destarched wheat bran, using a peristaltic pump (F430B,Gilson USA). The buffer containing the enzyme and released ferulic acid is passed through a column housing the adsorbent resin in a semi permeable 14kDa cutoff cellulose acetate membrane (Sigma Aldrich, USA). After the set time for hydrolysis was over, the membrane containing the adsorbent resin was washed with excess of methanol to extract the adsorbed ferulic acid. The schematic for the process is given below (Figure 6.1) with the parameters included in Table 6.2.

Figure: 6.1 Schematic diagram of apparatus for packed column process.

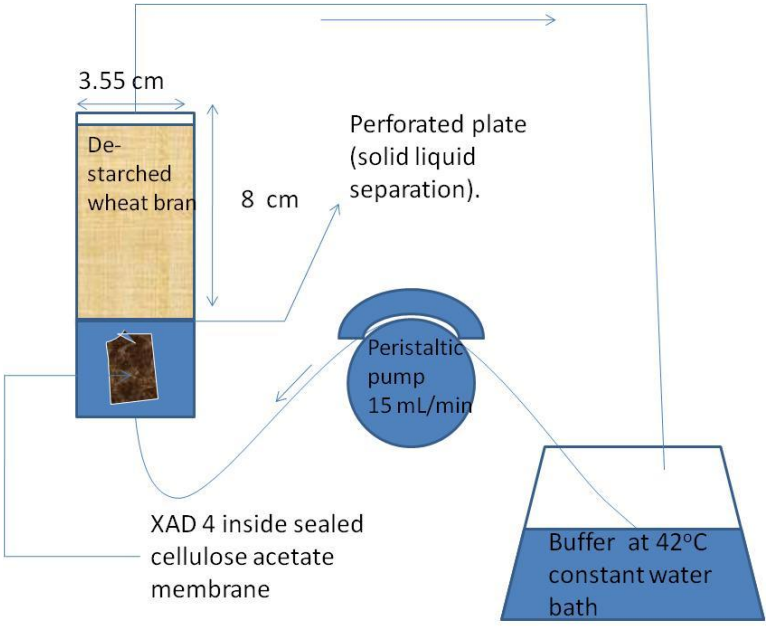


Table 6.2 Parameters for packed column ferulic acid extraction from destarched wheat bran.

Parameter	Value
FAE enzyme loading	550 U/g
pH	3.5
Reaction temperature	42^{o}C
Reaction time	11.5 hrs
Flow rate	15 mL/min
Destarched wheat bran (mass)	15 g

6.2.5 Determination of adsorption capacity of different adsorbents

Adsorbents of two categories were studied, adsorbents that would be used for extracting the ionized form of ferulic acid and the ones that would be used specifically for the non ionized form. Since the pKa of ferulic acid is 4.58, adsorbents used to study the extraction in the non ionized form were suspended and equilibrated with 50 mM citrate buffer at pH 4.5 and citrate buffer 50 mM at pH 5.0 was used to study the extraction of the ionized form viz. ferulate ion. Functionalized cross linked resins AMBERLITE XAD2, AMBERLITE XAD4, AMBERLITE XAD16 (Sigma Aldrich, USA) were used as non ionic ferulic acid adsorbents, while chitosan, powdered activated charcoal (PAC) and anionic resin SRA 400 (Sisco Research Laboratories, India) were used as adsorbents for ferulate ion adsorbtion . The experimental conditions for the non ionic ferulic acid adsorbents and ionic ferulate adsorbents are described in Table 6.3 and Table 6.4. The duration of contact of the adsorbents was 6h and samples were withdrawn every 2h after the first hour sample. The withdrawn samples were then assayed through HPLC for the concentration of ferulic acid remaining in the supernatant. All experiments were carried out at 40^{o}C

Table 6.3 Experimental parameters for non ionic ferulic acid adsorption

Parameter	**Value**
Ferulic acid concentration	2 mg/mL
Buffer	Citrate(50mM) pH 4.5
Mixing	Yes (Rotamix)
Volume of ferulic acid solution	7mL
Mass of adsorbent	100 mg

Table 6.4 Experimental parameters for ionic ferulate ion adsorption.

Parameter	**Value**
Ferulic acid concentration	2 mg/mL
Buffer	Citrate(50mM) pH 5.0
Mixing	Yes (Rotamix)
Volume of ferulic acid solution	7mL
Mass of adsorbent	1000 mg

6.2.6 Determination of enzyme loss due to adsorption and ease of desorption of selected adsorbents

To determine the loss of enzyme activity in the supernatant due to adsorption of the enzyme on the adsorbents used for extraction of ferulic acid, all adsorbents were suspended in their respective buffers as mentioned above with an additional component, viz. enzyme content of 50 U/mL feruloyl esterase. The parameters for the experiment are given below in Table 6.5 and Table 6.6. All experiments were carried out at 40^{o}C. The duration of the whole experiment was 8h. Start and endpoint samples were taken to determine total enzyme loss after the time required for complete adsorption of ferulic acid (6h).

Table 6.5 Experimental parameters for enzyme adsorption by non ionic ferulic acid adsorbents

Parameters	**Value**
Enzyme concentration	50 U/mL
Buffer	Citrate(50mM) pH 4.5
Mixing	Yes (Rotamix)
Volume of ferulic acid solution	6mL
Mass of adsorbent	100 mg

Table 6.6 Experimental parameters for enzyme adsorption by ionic ferulate ion adsorbents

Parameters	**Value**
Enzyme concentration	50 U/mL
Buffer	Citrate(50mM) pH 5.0
Mixing	Yes (Rotamix)
Volume of ferulic acid solution	6mL
Mass of adsorbent	1000 mg

Ease of desorption was studied for AMBERLITE XAD 4, chitosan and SRA 400 previously suspended in respective buffer solutions containing ferulic acid as described in Table 6.1 and 6.2 for 6h. The adsorbents were separated by centrifugation at 5000 rpm for 5 minutes, and were then separated from the supernatant so as to be used for the evaluation of desorption. Methanol was used as desorption solvent for AMBERLITE XAD 4 , 0.05 N HCl in methanol for chitosan(owing to solubility of chitosan at higher acid concentrations), while 1 N HCL in distilled water was used as desorption solvent for SRA 400. The duration

for the process was set as 8h. The suspensions were continuously mixed by rotamix and endpoint samples were drawn and analyzed by HPLC to determine the amount of ferulic acid that was released by the respective eluent solvents.

6.3 Results and Discussion

6.3.1 Determination of total Alkali extractable ferulic acid present in destarched wheat bran

It was observed that the particular batch of wheat bran that was used in the study had a total alkali extractable ferulic acid of 6.21 ±0.28 mg/g

6.3.2 Determination of significant parameters affecting the release of ferulic acid from the substrate

The data for the experimental runs from the Placket-Burman design can be seen in Table 6.7. Run six gave the highest percentage of ferulic acid released from destarched wheat bran. ANOVA table for the design was generated by the statistical software and parameters with p-values less than 0.05 were determined to be significant (linearly) for the release of ferulic acid from destarched wheat bran (Table 6.8). All the studied factors were significant for the release of ferulic acid from destarched wheat bran as suggested by the pareto chart, where the t – value levels of the factors were observed to be above the t-value limit marked in red in Figure 6.2.

Table 6.7 Plackett-Burman design for understanding significant parameters for ferulic acid extraction from destarched wheat bran.

Run number	Blocks	Temperature (^{o}C)	pH	Enzyme loading (FAE U/g destarched wheat bran)	time of hydrolysis (Hr)	Percent ferulic acid release (%)
1	Block 1	45	6.5	50	6	1.85
2	Block 1	45	3.5	50	3	4.17
3	Block 1	35	3.5	50	3	4.41
4	Block 1	35	6.5	150	6	5.49
5	Block 1	35	3.5	50	6	7
6	Block 1	45	3.5	150	6	**13.82**
7	Block 1	45	3.5	150	6	13.38
8	Block 1	35	6.5	50	6	1.76
9	Block 1	45	6.5	50	3	1.55
10	Block 1	35	6.5	150	3	3.46
11	Block 1	35	3.5	150	3	8.92
12	Block 1	45	6.5	150	3	5.28

Table 6.8 ANOVA table for variables tested using the Plackett-Burman design.

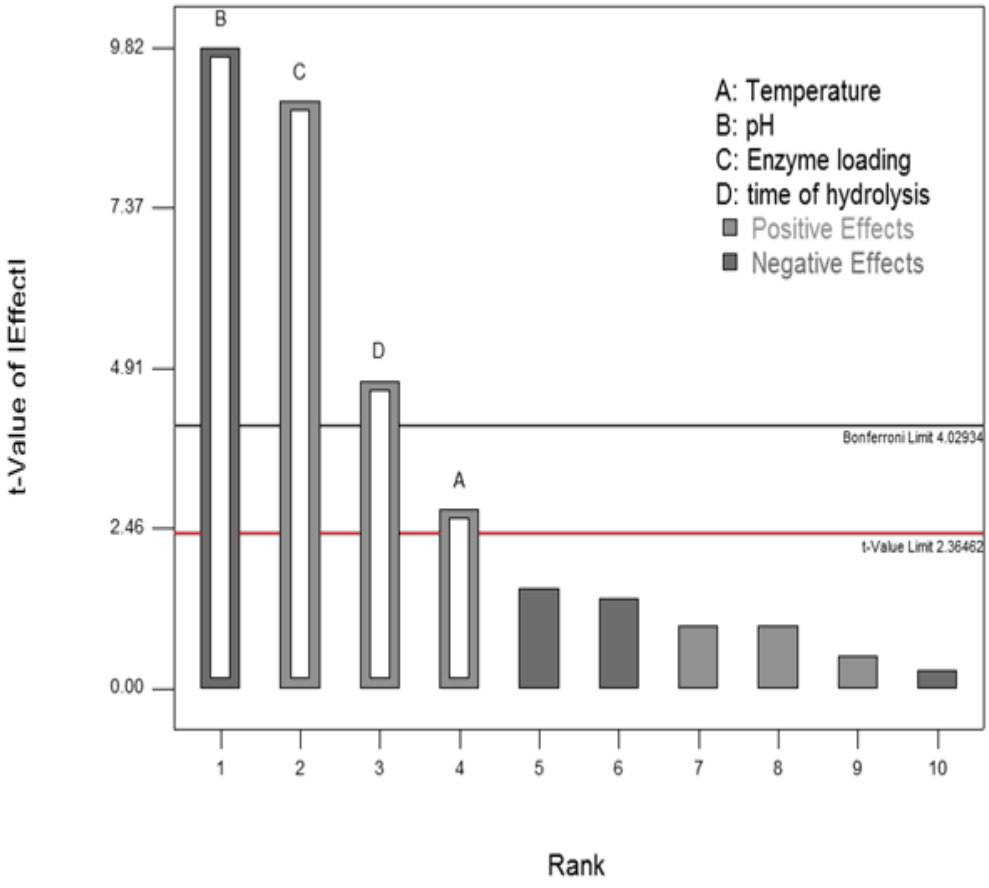
Terms	Sum of Squares	Degrees of freedom	Mean	F value	p-value probablity >F	
Model	186.87	4	46.72	51.786	< 0.0001	significant
A-Temperature	6.765	1	6.765	7.499	0.0290	
B-pH	86.995	1	86.99	96.433	< 0.0001	
C-Enzyme loading	73.063	1	73.06	80.99	< 0.0001	
D-time of hydrolysis	20.047	1	20.05	22.222	0.0022	
Residual	6.3149	7	0.902			
Lack of Fit	6.2181	6	1.036	10.706	0.2298	not significant
Pure Error	0.0968	1	0.097			
Cor Total	193.18	11				

The generated equation is given as per Equation 6.4. The equation has R^2 = 0.9673, AdjR^2 = 0.9486 and Pred R^2 = 0.9039. The agreement of all three R^2 values indicate that the fit of the equation is not due to noise.

Equation 6.4: linear regression equation generated by using Plackett-Burman design.

*Percent ferulic acid release = 0.15017 * Temperature -1.79500 * pH +0.049350 * Enzyme loading +0.86167 * time of hydrolysis + 0.080000*

Figure 6.2 Pareto chart of selected variables with percent ferulic acid release as a response.



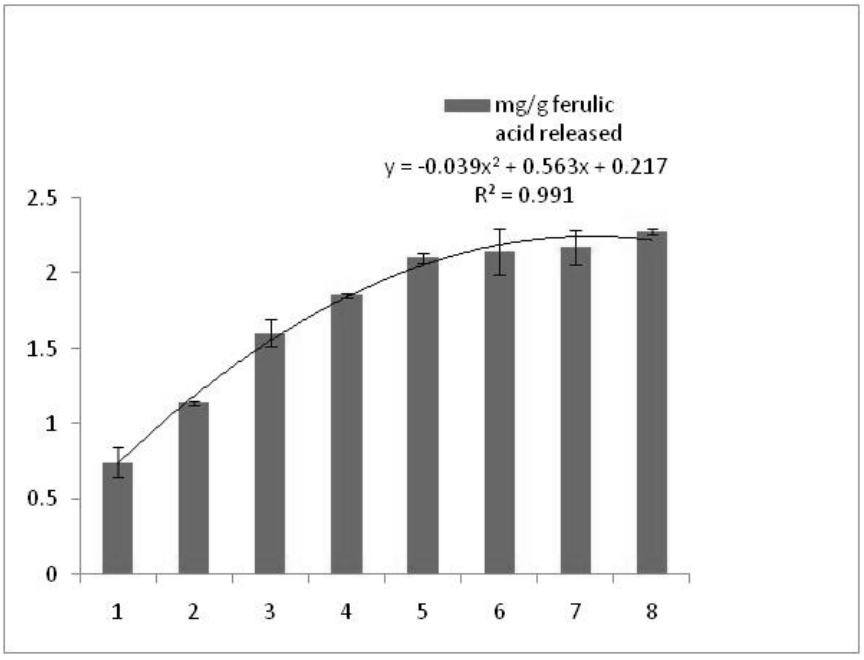
6.3.3 Steepest Ascent and Central Composite Design to optimize the levels of the significant parameters for the release of ferulic acid from destarched wheat bran

Steepest ascent method for finding the limits of enzyme loading and time of reaction was carried out according to the data given in Table 6.9. Since quadratic models work best for variable values that give a dome shaped or parabolic curve of the response enzyme variable values from run three and run six were chosen as base values to be used in the central composite design, as observed from Figure 6.3.

Table 6.9 Steepest ascent runs and results.

Temperature (^oC)	pH	enzyme loading (U/g)	reaction time (h)	ferulic acid released (mg/g)	percent ferulic acid release
45	3.5	100	4.5	0.746	10.64
45	3.5	175	5.68	1.141	16.28
45	3.5	250	6.86	1.606	22.91
45	3.5	325	8.04	1.857	26.49
45	3.5	400	9.21	2.106	30.05
45	3.5	475	10.39	2.149	30.66
45	3.5	550	11.57	2.174	31.01
45	3.5	625	12.75	2.281	32.54

Figure 6.3 Plot of ferulic acid released with respect to run number of steepest ascent method.



The experimental results of ferulic acid released from destarched wheat bran using the central composite design experimental design can be observed through Table 6.10.

Table 6.10 Central composite design for deriving second order polynomial model for the release of ferulic acid from destarched wheat bran.

Run number	**Enzyme loading**	**pH**	**Temper-ature(^{o}C)**	**Hydro-lysis time (Hr)**	**Ferulic acid released mg/g**	**percent ferulic acid released**
1	325	4	40	6.5	1.21	19.43
2	437.5	3.5	45	9	1.88	30.16
3	550	3	40	11.5	**2.06**	**33.08**
4	437.5	3.5	45	9	1.82	29.28
5	550	3	50	11.5	2.02	32.41
6	437.5	3.5	45	9	1.79	28.85
7	437.5	3.5	55	9	1.01	16.27
8	325	3	40	11.5	1.54	24.81
9	325	3	40	6.5	1.29	20.75
10	325	3	50	6.5	1.44	23.12
11	325	4	50	6.5	0.94	15.14
12	437.5	4.5	45	9	1.34	21.5
13	437.5	3.5	45	9	1.78	28.63
14	550	4	50	6.5	1.34	21.59
15	550	3	40	6.5	1.74	27.94
16	437.5	3.5	35	9	1.45	23.26
17	437.5	3.5	45	4	1.39	22.28
18	325	4	50	11.5	1.18	19.01
19	550	4	40	11.5	1.97	31.66
20	437.5	3.5	45	14	2.13	34.26
21	325	4	40	11.5	1.54	24.79
22	437.5	3.5	45	9	1.84	29.53
23	212.5	3.5	45	9	1.16	18.73
24	662.5	3.5	45	9	**2.16**	**34.67**
25	550	4	40	6.5	1.62	25.98
26	437.5	2.5	45	9	1.83	29.41
27	325	3	50	11.5	1.56	25.07

28	437.5	3.5	45	9	1.85	29.67
29	550	3	50	6.5	1.83	29.48
30	550	4	50	11.5	1.53	24.61

Run 24 gave the highest release of ferulic acid of 34.67 %, however one of the points of run 25 is an axial point (enzyme loading), which is beyond the selected range of parameters. Run three was the run that gave the highest release of ferulic acid, without any axial points. The ANOVA table that is given as a standard output for central composite design by the statistical package used has been expressed through Table 6.11. I was known previously that all four factors are significant individually as can be observed from the p-values of linear terms, which are all bellow 0.05. The only significant interaction terms were the terms for pH*Temperature and Temperature*Time of hydrolysis. The model itself had a p-value less than 0.005 (0.0001), and the lack of fit term was found to have a p-value of 0.1064 which indicates again that the model is sound.

Table 6.11 ANOVA table for central composite design model equation

Terms	**Sum of Squares**	**degree of freedom**	**Mean Square**	**F-Value**	**p-value Prob> F**	
Model	811.3	14	57.95	75.87	< 0.0001	significant
A-FAE	311.8	1	311.8	408.3	< 0.0001	
B-pH	105.4	1	105.4	137.9	< 0.0001	
C-Temperature	42.65	1	42.65	55.84	< 0.0001	
D-Time of hydrolysis	130.6	1	130.6	171	< 0.0001	
AB	0.846	1	0.846	1.108	0.3091	
AC	0.614	1	0.614	0.804	0.3842	
AD	0.147	1	0.147	0.192	0.6672	
BC	39.21	1	39.21	51.33	< 0.0001	
BD	0.921	1	0.921	1.206	0.2895	
CD	4.49	1	4.49	5.878	0.0284	
A^2	12.82	1	12.82	16.79	0.0010	
B^2	27.12	1	27.12	35.51	< 0.0001	
C^2	160.1	1	160.1	209.7	< 0.0001	

D^2	2.305	1	2.305	3.017	0.1029	
Residual	11.46	15	0.764			
Lack of Fit	9.903	10	0.99	3.186	0.1064	not significant
Pure Error	1.554	5	0.311			

The second order polynomial Equation 6.5 that describes the relationship between the process parameters and the final percent yield of ferulic acid from destarched wheat bran is given bellow.

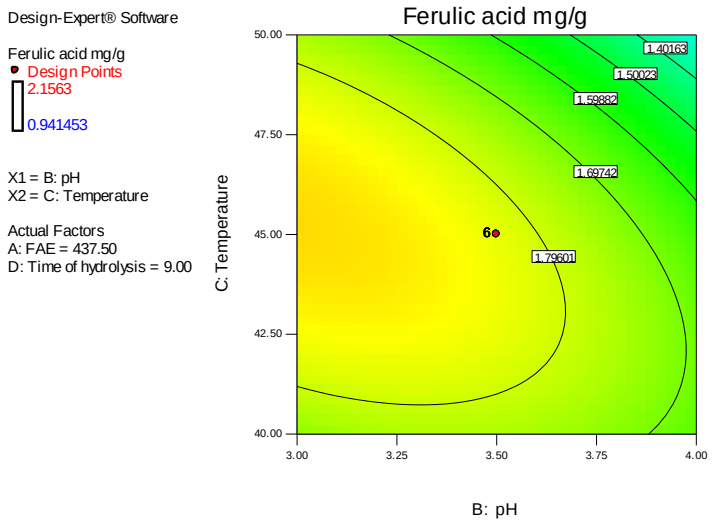
Equation 6.5: second order polynomial regression equation generated by using central composite design

*Percent ferulic acid released = 0.10622 * FAE + 51.89275 * pH + 11.15742 * Temperature + 2.85426 * Time of hydrolysis - 4.08899E-003 * FAE * pH - 3.48207E-004 * FAE * Temperature + 3.40700E-004 * FAE * Time of hydrolysis - 0.62616 * pH * Temperature + 0.19192 * pH * Time of hydrolysis - 0.042378 * Temperature * Time of hydrolysis - 5.40216E-005 * $(FAE)^2$ -3.97776 *$(pH)^2$ - 0.096653 * $(Temperature)^2$ - 0.046378 * $(Time\ of\ hydrolysis)^2$ - 346.45783*

It was observed that the model equation has R^2=0.9861, Adjusted R^2=0.9731, Predicted R^2=0.9280, which are all in fair agreement with each other hence it can very well be ascertained that the high regreesion that is observed is not due to noise. The model can thus predict the out come of the process (given that they are within the studied range) with 98.61% accuracy.

It was evident from the ANOVA table (Table 6.11) that the interaction between temperature and pH is significant. The interaction contour plot for the two parameters can be seen in Figure 6.4

Figure 6.4 Contuor plot displaying interaction between reaction temperature and reaction pH , with percent release of ferulic acid being represented by colored contours.



To make the process more feasible it was necessary that lowest possible temperature and highest possible pH maybe applied to reduce the consumption of energy and to ensure that the enzyme remains active for a longer amount of time. It was therefore decided to find a solution for highest release of ferulic acid from destarched wheat bran by solving the polynomial equation so that the temperature is set to 42^{o}C and the pH to 3.5. The most desirable solutions obtained through the exercise are included in Table 6.12.

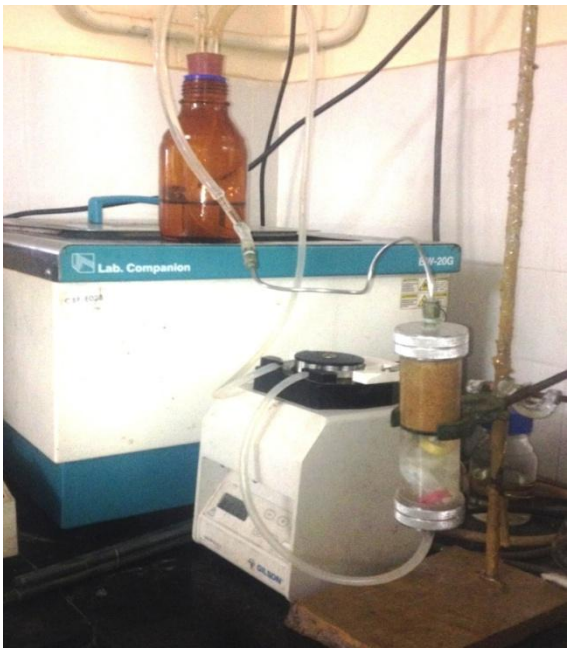
Table 6.12 Possible solutions to the polynomial equation.

Number	Enzyme loading	pH	Temper-ature(^{o}C)	Hydro-lysis time (Hr)	Ferulic acid released mg/g	percent ferulic acid released
1	547.8	3.5	42	11.45	2.16	34.69
2	548.9	3.5	42	11.43	2.16	34.69
3	549.8	3.5	42	11.38	2.16	34.67
4	546.8	3.5	42	11.5	2.16	34.71
5	545.5	3.5	42	11.49	2.16	34.67
6	550	3.5	42	11.44	2.16	**34.73**
7	548.3	3.5	42	11.49	2.16	34.73
8	549.6	3.5	42	11.4	2.16	34.68

6.3.4 Packed column process for extraction of ferulic acid from destarched wheat bran

Parameters similar to run six of Table 6.12 were used to carry out release of ferulic acid from destarched wheat bran in packed in a column. The final yield of the whole experiment was 2.02 mg ferulic acid per gram of destarched wheat bran. This accounts for 32.54% of total releasable ferulic acid from destarched wheat bran. The loss of yield compared to the predicted value maybe due to differences in the experimental setups. The Figure 6.5 below shows the actual setup used for release of ferulic acid from destarched wheat bran in a packed column.

Figure 6.5 Experimental setup for extraction of ferulic acid from destarched wheat bran.

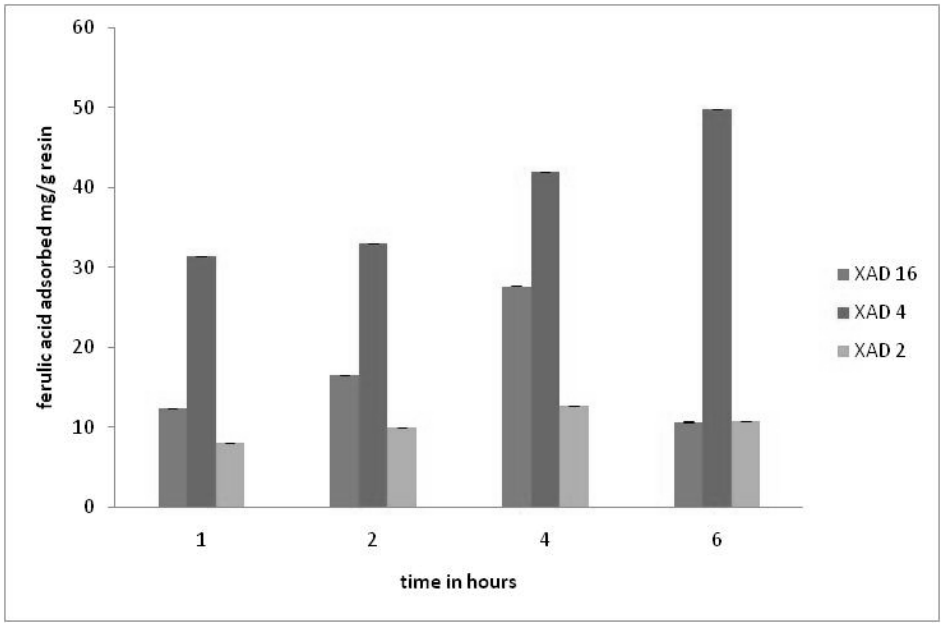


6.3.5 Deteremination of adsorption capacity of different adsorbents

The groups of adsorbents were tested. Among the adsorbents for the non ionic ferulic acid adsorbents, AMBERLITE XAD 4 had shown the maximal

adsorption capacity of 49.98 mg ferulic acid/g (s.d. < 1%) during the 6th hour, while AMBERLITE XAD 2 had a maximal adsorption of 12.7 mg ferulic acid/g during the 4th hour and AMBERLITE XAD 16 which was the adsorbent of choice for previous studies (Couteau and Mathaly 1997, Tilay, Bule et al. 2008, Barberousse, Kamoun et al. 2009) registered a capacity of 27.6 mg ferulic acid /g in the given set of conditions. AMBERLITE XAD 4 was therefore established as a superior adsorbent compared to AMBERLITE XAD 2 and AMBERLITE XAD 16 as can be observed from Figure 6.6.

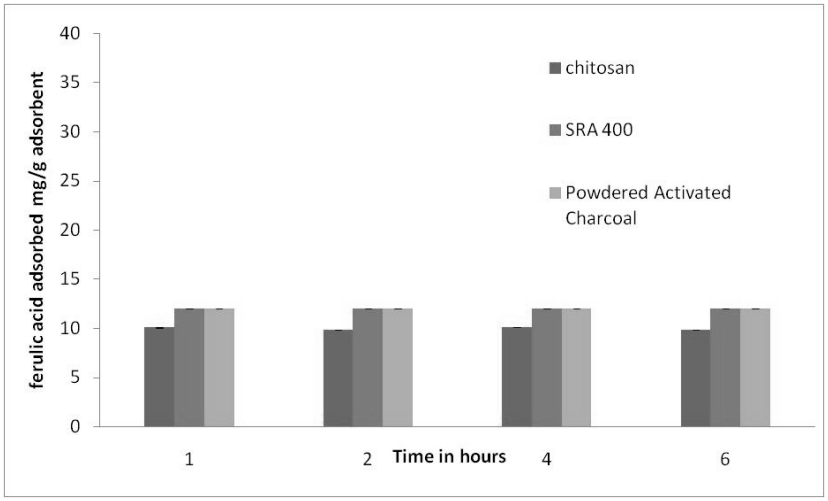
Figure 6.6 Ferulic acid adsorption capacities for AMBERLITE XAD 16, XAD 4, XAD 2.



Among the adsorbents for ionic ferulate form, SRA 400 and powdered activated charcoal (PAC) adsorbed all of the ferulic acid present in the buffer within the first hour, and chitosan had a capacity of 10 mg ferulic acid /g. Even though powdered activated charcoal had higher capacity for adsorption compared to chitosan, solid liquid separation using centrifugation at 10000 rpm for 10 minutes was not sufficient for clarifying the solution, using additional methods only increased the losses of total yield for ferulic acid in the final form, hence it

was decided that powdered activated charcoal was not to be used further as an adsorbent. The summary of the study can be visualized in Figure 6.7.

Figure 6.7 Ferulic acid adsorption capacity for chitosan, SRA 400 and PAC.



6.3.6 Determination of enzyme loss due to adsorption and ease of desorption of selected adsorbents

All adsorbents, whether for the ionic ferulate form or for the non ionic ferulic aid form were not specific adsorbents because of the fact that all of the adsorbents caused a significant loss of free enzyme activity in the buffer when directly in contact with each other. As can be observed from Figure 6.8 all AMBERLITE XAD resins caused a drop in enzyme activity to levels below 9% of the original activity after 8h of direct contact. From Figure 6.9 it is evident that powdered activated charcoal was further disadvantageous as it reduced the free enzyme activity to 10.5%, after eight hours of direct contact. Strong anion exchanger SRA 400 reduced the activity of free enzyme to 8.65 % of the initial activity after direct contact for 8h. The final goal of this study was to understand whether there is a possibility of re using the residual free enzyme after a cycle of extraction of ferulic acid from destarched wheat bran and also to find the most specific adsorbent for ferulic acid so that there would be minimal protein contamination in the final product. It was therefore evident from the said study

Figure 6.8 Loss of enzyme activity due to contact with non ionic ferulic acid adsorbents.

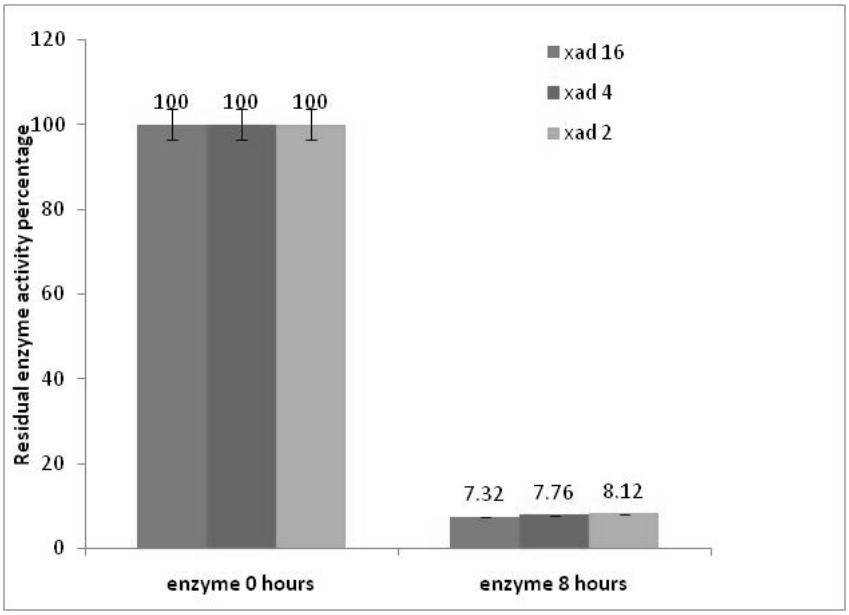
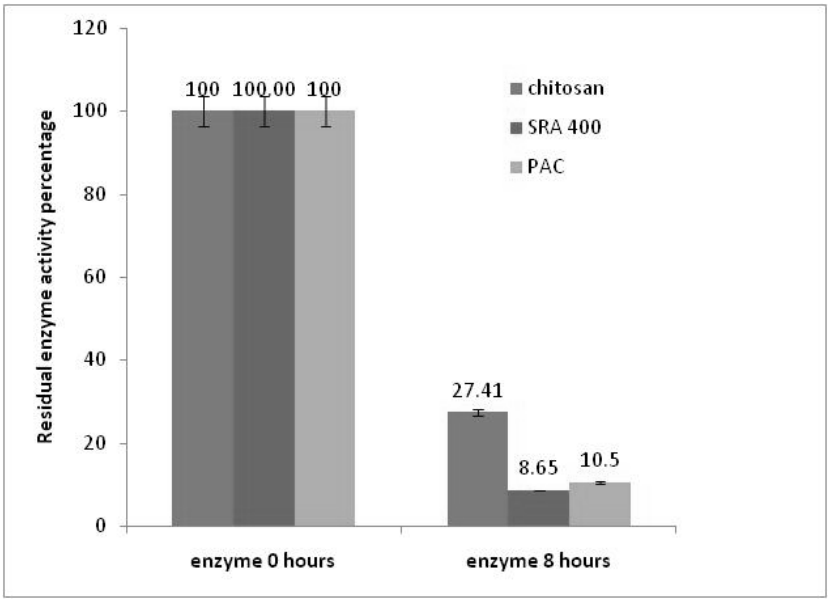


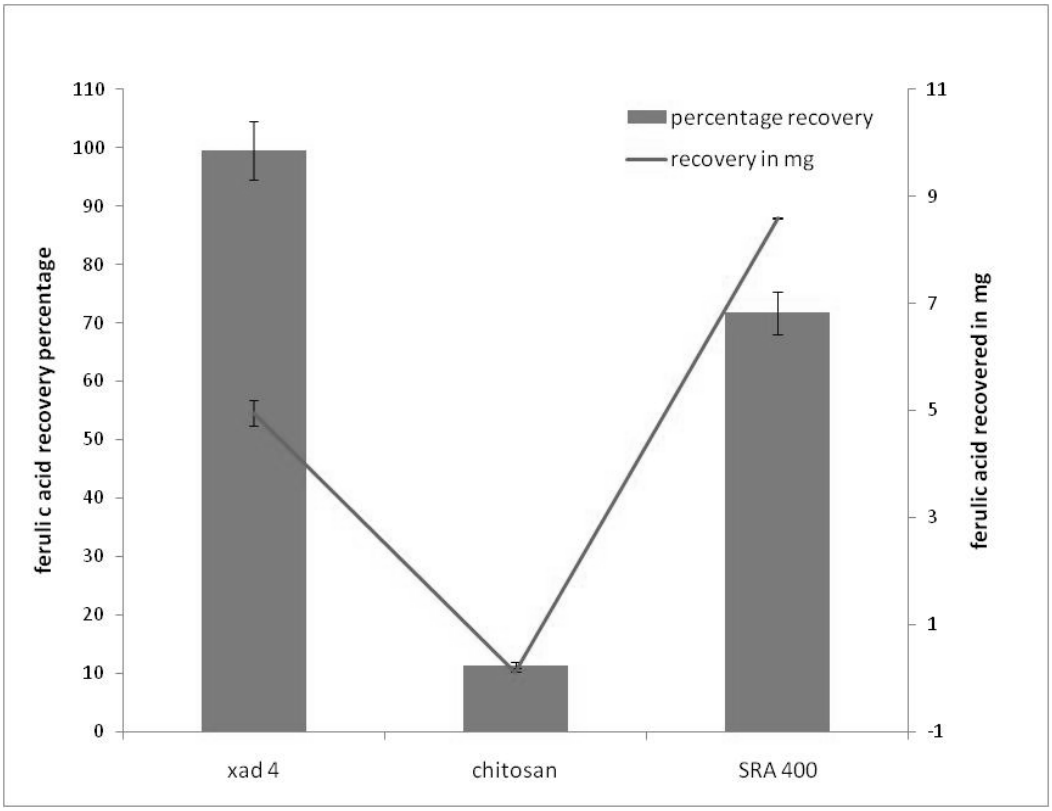
Figure 6.9 Loss of enzyme activity due to contact with ionic adsorbents.



Desorption of ferulic acid from the adsorbents gave different profiles. AMBERLITE XAD 4 had the lesser capacity to adsorb ferulic acid compared to SRA 400 anionic exchanger, the XAD 4 displayed a favorable trait which involved complete desorption of ferulic acid bound on to the resin within the time allotted for the process. Chitosan, the adsorbent which displayed lesser affinity for the enzyme compared to other adsorbents performed poorly, as observed in Figure 6.10 with the adsorbent releasing only 11.3 % of the adsorbed ferulic acid from the adsorption cycle. AMBERLITE XAD 4 displayed almost complete desorption of the adsorbed ferulic acid with a percent desorption of 99.5 %. SRA 400 the anion exchanger was able to release 71.7 % of the total ferulic acid that it adsorbed within the given time frame. The choice for the appropriate adsorbent was therefore to be between SRA 400 and AMBERLITE XAD 4.

Since AMBERLITE XAD 4 displayed almost no loss of adsorbed ferulic acid and also used methanol, a solvent of lower vapor pressure compared to water (hence easier to purify ferulic acid, with solvent recycling), without any additives like HCl in case of SRA 400; it was decided that AMBERLITE XAD 4 was a better choice of adsorbent for purification of ferulic acid. The lower capacity of AMBERLITE XAD 4 compared to SRA 400 can be easily compensated for by increasing the amount of resin used for the process.

Figure 6.10 Recovery of ferulic acid by desorption from different adsorbents.



6.4 Summary

In summary, a robust model for the recovery of ferulic acid from destarched wheat bran using feruloyl esterase from Aspergillus niger ATCC 13497 was devised. Enzyme loading of 550 U/g, reaction time of 11.5 h, and reaction temperature at 42^{o}C, using citrate buffer at pH 3.5 (10 mM) gave the highest release of ferulic acid with 2.02 mg/g ferulic acid released from destarched wheat bran, which accounts to 32.54% of alkali releasable ferulic acid. AMBERLITE XAD 4 was found to be the best adsorbent for ferulic acid in terms of capacity (45 mg/g) as well as ease of desorption and percentage desorption (99.5%). Both methods were combined to set up a packed column reactor.

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Chapter VII

Studies on selected applications of feruloyl esterase and ferulic acid.

7.1 Introduction

The enzyme Feruloyl esterase and ferulic acid both are products of the process that is described in this work. The dual value addition of wheat by product gives leverage for associated 2nd generation fuel processes to improve the economics of the process, which is a step towards sustainable energy. The applications of feruloyl esterase have been discussed in detail in Section 1.2 and in section 3.1 of this thesis. These applications include pulp bleaching, nutraceutical preparations with the enzyme, enzyme cocktail preparation for efficient hydrolysis of lignocellulosic materials, and preparation of feruloyl esters of different substrates including alcohols and sugars(Giuliani, Piana et al. 2001, Topakas, Stamatis et al. 2003, Sørensen, Pedersen et al. 2005, Tabka, Herpoël-Gimbert et al. 2006, Vafiadi, Topakas et al. 2009, Panagiotou, Topakas et al. 2010). Alkyl esters of ferulic acid are easily soluble in lipids and hence can be used as effective preparations of UV blocks with a cream base, while ferulic acid ester with arabinose has better solubility in aqueous solvents with properties that render it anti-mycobacterial (Vafiadi, Topakas et al. 2007). Feruloyl esterases are similar to lipases, which have a special property of interfacial activation when suspended in mixtures of organic solvents and water (van Tilbeurgh, Egloff et al. 1993). Feruloyl esterases are comparatively more hydrophilic but since they are so similar to lipases, they can still be used for trans esterification reactions (Hermoso, Sanz-Aparicio et al. 2004).

Ferulic acid has been discussed thoroughly within the thesis in sections 1.2 and 6.1. The biological activity of the molecule makes it a lucrative target for the cosmecutical preparations for its functions as a UV block, anti-ageing agent (Svobodová, Psotová et al. 2003). Ferulic acid is esterified to plant cell walls and hence is an indispensable part of the human diet as discussed in section 1.2. The presence of ferulic acid in daily food material therefore makes it a safe compound for food packaging and food preservation applications. Ferulic acid has been established as preservative for food product like fish(Maqsood, Benjakul et al. 2013).

There are applications with ferulic acid as a crosslinker and hardener for various biopolymer blend membranes and films (Ou, Wang et al. 2005, Mathew, Abraham et al. 2007).

The current chapter deals with the problem of production of ferulic acid ester with arabinose using AnFaeA as per the method of (Vafiadi, Topakas et al. 2005) and immobilization of the enzyme in mesoporous silica particles followed by assessment of the immobilized enzyme for the preparation of the arabinose ester. The chapter also deals with incorporation of ferulic acid into blended films of a dextran like exo-polysaccharide and chitosan.

7.2 Materials and Methods

All Major chemicals used in the chapter are listed in Table 2.1

7.2.1 Esterification of ferulic acid with arabinose using free enzyme

Purified feruloyl esterase was obtained after hydrophobic interaction chromatography as explained in section 4.2.2 and then the dialyzed enzyme solution was cooled to -20^{o}C overnight and subjected to the process of lyophilization at -80^{o}C for 24 h. The lyophilized powder was then stored at 4^{o}C till further use. Butanol containing 6mM ferulic acid was prepared, and MOPS buffer 10 mM with 2mM arabinose was added to this mixture and finally hexane was added so that these three solutions were in different ratios as per Table 7.1. The reaction was maintained at 37^{o}C for 8 h after the addition of 2 mg of lyophilized enzyme per mixture. The initial ratios of the mixture were used as per the literature information from Vafiadi, Topakas et al. (2005). The reaction with ratios which displayed an extra band compared to all the corresponding blanks were taken as positives and the positives were further confirmed by repeating the reaction using the same reaction conditions as before but with a reaction time of 24 h. The reaction components were separated on Merck F254 silica plates using chloroform: methanol: acetic acid: water (5:2:2:1) according to (Couto, Karboune

et al. 2010) using HPTLC system (CAMAG Switzerland). The bands were visualized under UV (366 nm). Arabinose was not visible under UV light hence initially to confirm separation and reaction success, the bands were visualized by spraying 3% H_2SO_4 in methanol on the developed plate followed by heating to char the components at 100^oC. The plates visualized by methanol sulphhuric acid reagent were scanned by the CAMAG TLC scanner at 400 nm (charred bands absorption).

7.2.2 Preparation of mesoporous silica and immobilization of enzyme on aminated mesoporous silica for esterification of ferulic acid with arabinose

Mesoporous silica particles were produced using a method modified from Björk (2016). Solution I is prepared by mixing 10 g tetraethyl orthosilicate (Merck India) with 25 g of dry ethanol (Merck India) and 0.1N HCl and stirred to homogeneity for 1 h. Solution II is prepared by mixing pluronic 123(Sigma Aldrich USA) with 15 g of dry ethanol and is stirred to homogeneity. Solution II is then added drop wise to solution 1 with stirring. This solution is held at 60^oC for 24 h to get a 12.5g residue, which is mixed with 10g glycerol. The mixture is stirred and the remaining ethanol is removed by vacuum drying. 5g of this mixture is then mixed with 5 ml of enzyme solution (HIC purified enzyme as per section 4.2.2). This mixture is then dried under vacuum to give dried flakes of Pluronic123 with silica composites embedded. The solids thus obtained are washed with 70 % ethanol to remove excess of pluronic 123. The washed solid particles are then calcined at 550^oC for 8h using a muffle furnace (Pathak electrical works India). The solid mesoporous silica particles that are obtained are aminated by suspending 200mg of the mesoporous silica in 6mL of toluene along with 200µL of APTES (3 amino propyl triethoxy silane , Sigma Aldrich, USA) in a sealed round bottom flask with constant stirring with the temperature set at 70^oC. The aminated mesoporous silica thus obtained is suspended in a solution of purified enzyme and mixed by gentle stirring for 1h in phosphate buffer pH 6.5. The solids are separated by centrifugation at 10000 rpm for 10 minutes. The solids are then suspended in 0.2% glutaraldehyde solution (3 mL) (Merck, India)

and gently mixed for 1h by continuous stirring. The mesoporous silica enzyme composite particles are then checked for feruloyl esterase assay as per the method presented in sections 2.2.3 and 2.2.4. 10mg of the composite material is then substituted for the lyophilized enzyme mentioned in method 7.2.1 to check for the efficiency of the immobilized enzyme for esterification of arabinose with ferulic acid.

7.2.3 Preparation of dextran chitosan blend films as a cross linker

2% w/v chitosan (Himedia, India) solution in 1% v/v acetic was prepared by continuous stirring of the solution. The homogenous solution was then degassed by incubating the solution in an ultrasonicating bath at 30oC for 1h. Dextran like exopolysaccharide purified from a food grade lactic acid bacterium at MPTD , CSIR-NIIST and the method for preparation of the said exopolysaccharide is listed by Vasanthakumari, Harikumar et al. (2015). 2%w/v solution of the exopolysaccharide was prepared by dissolving it in water and degassing it like the chitosan solution. The film was prepared by casting a 1:1 solution of the above solutions with the addition of 8,16,24,32 and 40 mg of ferulic acid dissolved in methanol (per 8 mL of mixture) and 1% H2O2 v/v, on to 5cm x 6cm boats of poly ethylene terephthalate films. The casting set up was then placed invacuum at 40^{o}C to evaporate the solvent. The film thus obtained was used for further testing.

7.2.4 Tensile strength and thickness of blend films

The thickness of the film was measured using a Screw gauge. The value for the thickness measurement used for tensile strength calculations was obtained by measuring the thickness of the film samples at three positions along the length of 20 x 110 mm films casted specifically for calculating the tensile strength of the film and using a mean value. The precision of the thickness measurements was ±5%. The tensile strength of the material was given by tested using Universal Testing Instrument Model H5KS (Tinius Olsen, Horsham, USA) fitted with a 100N static load cell, according to standard testing method (ASTM 1995)

crosshead speed of 50mm/min and extension of 100mm and the initial grip separation was 50mm.. The films were cut into strips 10mm wide and 110mm long and mounted between cardboard grips (40mm x 30mm) using adhesive.

7.2.5 Thermogravimetric Analysis of blend films

The thermal behaviors of the films were studied by thermo gravimetric analysis using TG-DTA 6200 (SII Nano-technology Inc., Japan). The substance (9mg) was subjected to a temperature range of 30–300 °C under normal atmosphere at a rate of 15°C/min and the corresponding weight loss was determined.

7.2.6 Scanning electron microscopy of blend films

The surface characteristics of the edge of the broken films were studied by JSM-5600 LV scanning electron microscope of JEOL, Tokyo, Japan. The dried samples were mounted on a metal stub and sputtered with gold in order to make the sample conductive, and the images were taken at an accelerating voltage of 10KV.

7.2.7 Solid state UV-visible spectroscopy of blend films

10mmx10mm film samples were subjected to solid state UV-Visible spectroscopy using a Shimadzu 2100 UV-Visible spectrophotometer. The transparency of the film plays a crucial role in the acceptability of the film for food packaging uses, hence making this test important.

7.2.8 Water transfer rate of blend films

Water transfer rate of the chitosan dextran blend films (control and with addition of ferulic acid) were tested according to ASTM E96 method, specifically the wet cup method. The test involved addition of 10mL de –ionized water to a 150mL screw cap bottle and which is sealed using a hollow cap and paraffin wax. The set up is then transferred to a desiccator chamber with dry silica gel. The

weights of each of the screw cap bottles were recorded every two hours and the loss in weight of the water from the vessels is ultimately used to calculate the water transfer rate for each film.

7.3 Results and Discussion

7.3.1 Esterification of ferulic acid with arabinose using free enzyme

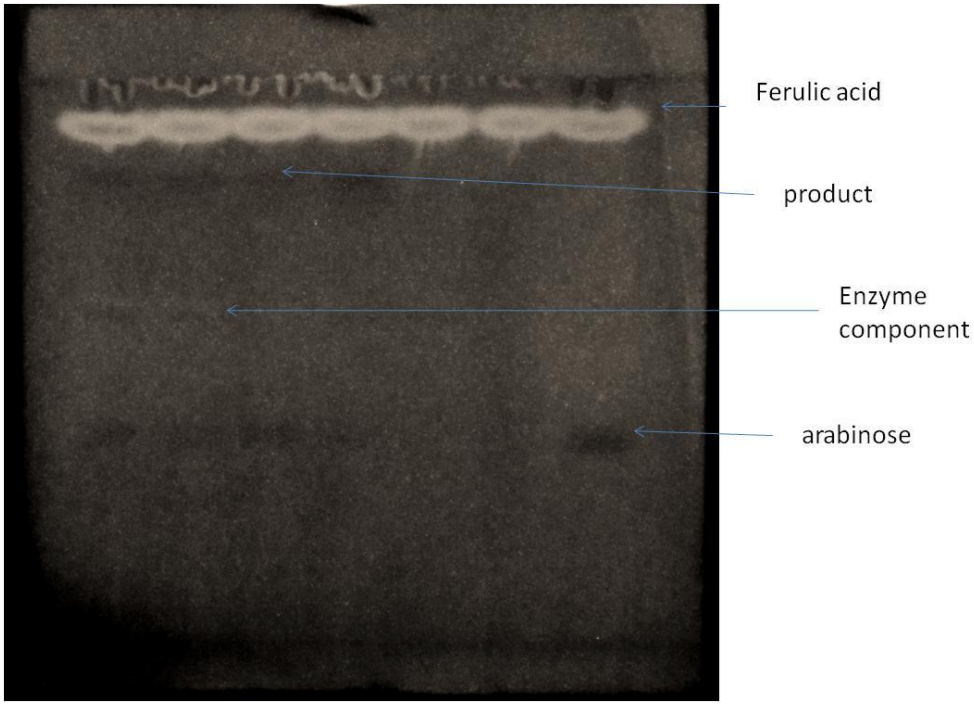
The results of testing of different ratios of hexane: butanol: buffer on the outcome of the esterification reaction can be observed in Table 7.1. Ratios hexane: butanol: buffer 51:46:3, 52:46:3, 51:47:3 were positive. The ratio was not the same as that reported (Topakas, Stamatis et al. 2003, Vafiadi, Topakas et al. 2005).This can be because of the differences in the enzyme used for the esterification reaction. A typical TLC developed for testing the outcome of the reaction can be seen in figure 7.1

Table 7.1 Effect of solvent composition on the outcome of the esterification reaction

Hexane	Butanol	Buffer	Reaction.
46	46	3	negative
47	46	3	negative
48	46	3	negative
49	46	3	negative
50	46	3	negative
51	46	3	positive
52	**46**	**3**	**positive**

53	46	3	negative
54	46	3	negative
55	46	3	negative
56	46	3	negative
51	41	3	negative
51	42	3	negative
51	43	3	negative
51	44	3	negative
51	45	3	negative
51	**46**	**3**	**positive**
51	**47**	**3**	**positive**
51	48	3	negative
51	49	3	negative
51	50	3	negative
51	51	3	negative
51	46	1	negative
51	46	2	negative
51	46	3	positive

Figure 7.1 Typical TLC of positive samples with appropriate controls



Legend :**Lane 1** positive 1 **Lane 2** positive 2 Lane 3: positive 3 **Lane 4**positive 4 **Lane 5** control w/o arabinose **Lane 6** control w/o arabinose. **Lane 7** control w/o enzyme.

Table 7.2 Peaks assigned in TLC scanned at 400 nm.

Rf	**Area**	**Area %**	**Assigned substance**
0.32	936	7.54	arabinose
0.54	2341	18.87	enzyme
0.78	1409.3	11.36	feruloylated arabinose
0.85	7720.1	62.22	ferulic acid

Table 7.2 shows the Rf values of the different bands present in the positive sample, while the chromatogram for different representative lanes can be observed in Figure 7.2. It is evident that only in presence of all the reaction components and enzyme, a band with Rf 0.78 is observed which was assigned as feruloylated arabinose.

Figure 7.2 Chromatograms of TLC lanes scanned at 400 nm with positive and controls

Figure 7.2a Lane 1 of Fig 7.1- positive reaction

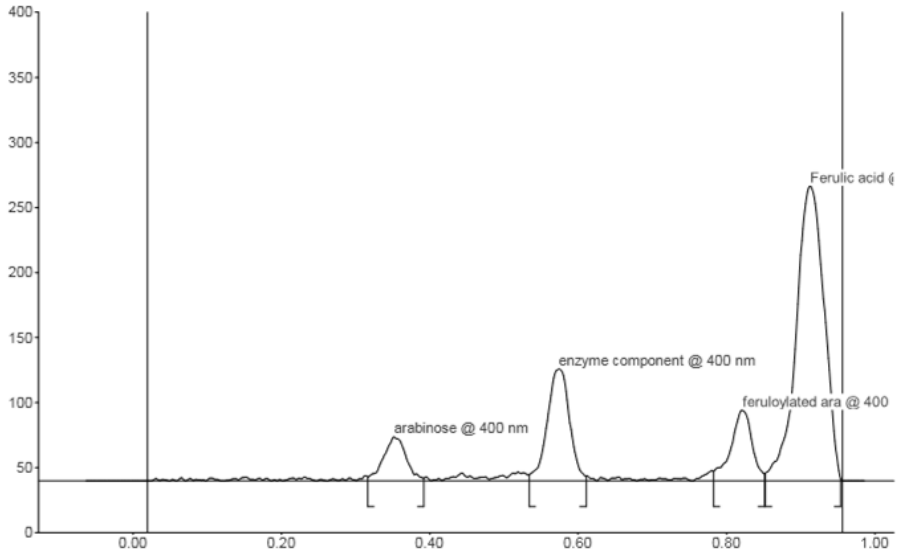


Figure 7.2b Lane 7 of Figure 7.1 Control reaction without enzyme

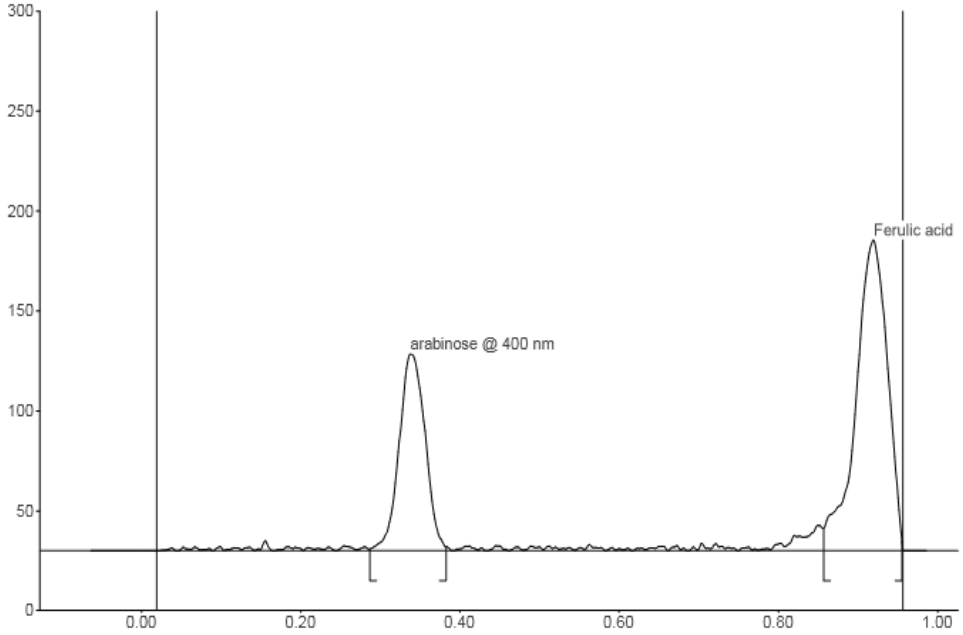
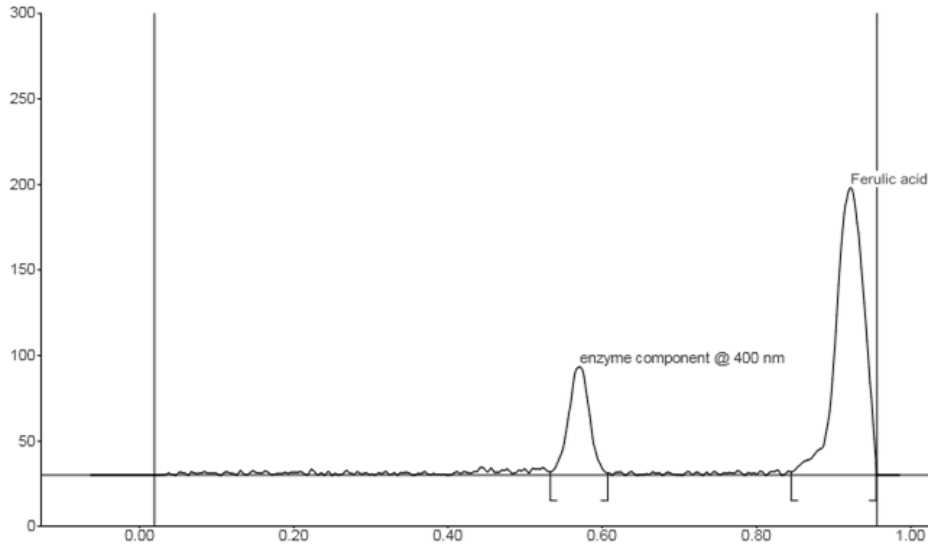
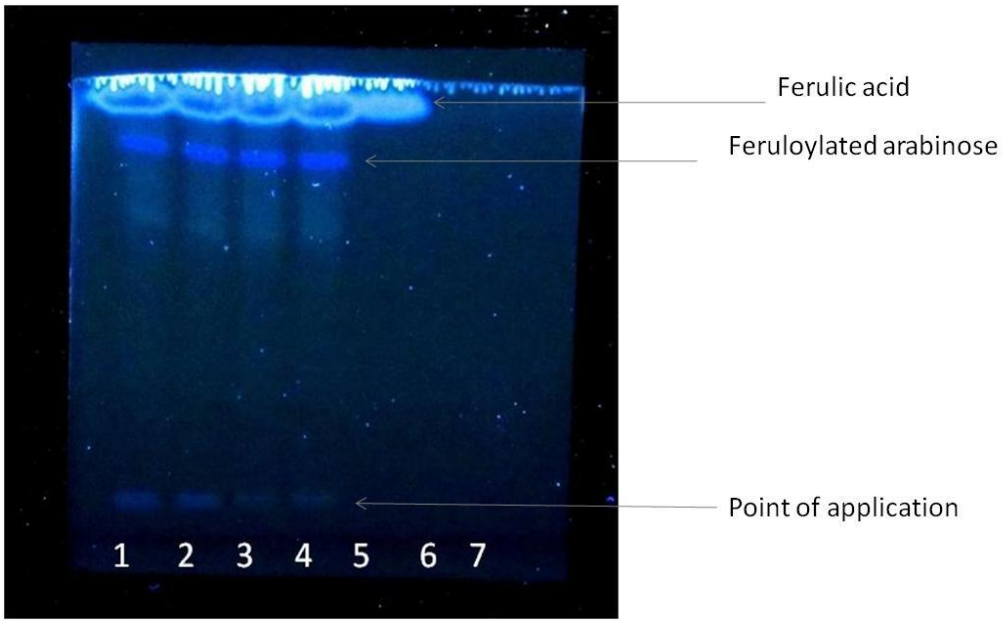


Figure 7.2c Lane 5 of Figure 7.1 Control reaction without arabinose



Confirmation of positive esterification reaction using ternary mixture ratio of hexane:t-butanol:buffer (51:47:3) can be seen in Figure 7.3. The intensity of the charred spots reduce with time and it becomes essential that the TLC plate be scanned immediately after charring with the methanol sulphuric acid reagent. Since the spot intensity varies with time, the method cannot be used to quantify the amount of arabinose consumed or the ferulic acid ester produced.

Figure 7.3 TLC of positive samples with controls under UV



Legend **Lane 1-4** Positive reactions, **Lane 5** Ferulic acid in butanol

Lane 6 Arabinose in buffer pH 4.5 **Lane 7** Enzyme in buffer pH 4.5

7.3.2 Preparation of mesoporous silica and immobilization of enzyme on aminated mesoporous silica for esterification of ferulic acid with arabinose

Aminated mesoporous silica particles have porous that are big enough for interactions with the enzyme molecules and amino groups displayed on the surface for cross linking reaction of enzyme with glutaraldehyde. Immobilization was successfully carried out with aminated mesoporous silica particles and the particles had feruloyl esterase hydrolytic activity of 2.00 U/mg as observed in Table 7.3. This was considerably less compare to the specific activity of the purified protein (528.67 U/mg) as reported in section 4.3.2. The esterification reaction was attempted using the immobilized enzyme however the band with Rf 0.78 (Rf of ferulic acid esterified arabinose) was absent indicating that the reaction had failed. The rationale for using mesoporous silica was to create a hydrophilic milieu near the enzyme so as to increase the activity of the enzyme(Thörn, Gustafsson et al. 2011). The reaction was undetectable probably

because of the minuscule specific activity of the immobilized enzyme compared to free enzyme.

Figure 7.4 Mesoporous silica particles and enzyme composite

Table 7.3 Enzyme immobilization progress

Enzyme	**Enzyme Activity**
Pre immobilization concentrated enzyme	1015.3U/mL ±24.5 U/mL
Post immobilization supernatant enzyme	893.5U/mL ±14.2 U/mL
Immobilized enzyme mesoporous silica composites	30.0U/mL ±2.3 U/mL 15 mg material used for reaction **2.0U/mg** of composite material

7.3.3 Tensile strength of blend films

As can be seen from table 7.4 Dextran chitosan blend film incorporated with 40 mg ferulic acid displayed a slight increase in tensile strength, compared to the control. All other treatments did not improve the tensile strength of the film.

Table 7.4 Tensile strength of the blend films

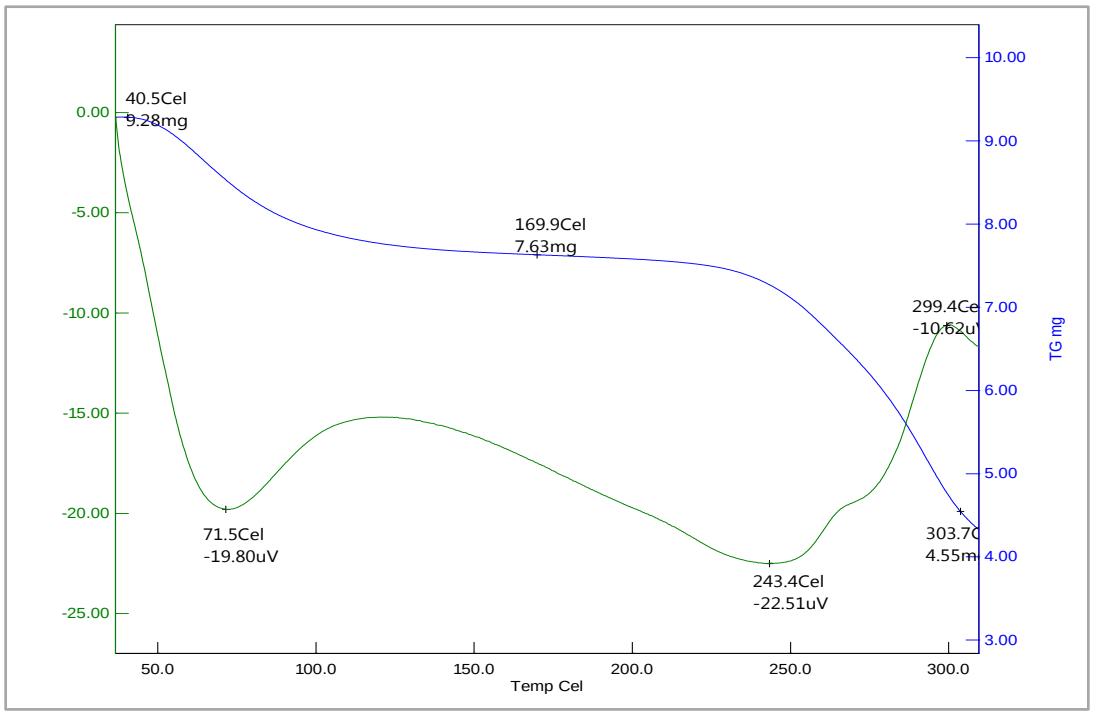
Ferulic acid per film	Tensile Mpa	Max force N	Elongation %	Stress at break Mpa	Thickness	width
Blend film with ferulic acid (40mg)	121.25	4.9	16.02	21.25	0.004	10
Conrol	83.75	3.4	18.91	25.00	0.004	10

7.3.4 Thermo gravimetric Analysis of blend films

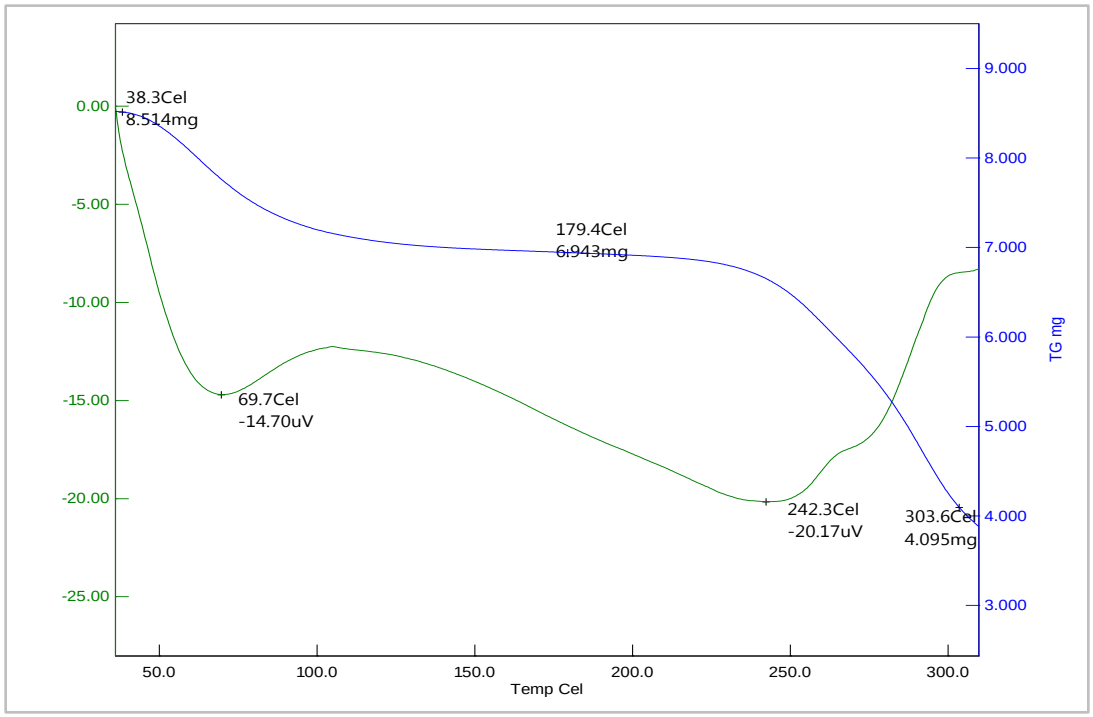
There was no change in the thermogravimetric profiles of the blend film without and with ferulic acid. There was no heat absorbed in the range 168-171^{o}C as can be seen from the DTA curve in Figure 7.5. This would indicate that there is no free ferulic acid in the film and that it has undergone covalent attachment with the components of the film. The film starts decomposing close to 250^{o}C for both the control and film containing ferulic acid. Ferulic acid was used as an additive earlier in guar gum so as to reduce its thermal degradation as discussed in section 1.7 earlier; however no such effect was seen for dextran chitosan blend films.

7.5 Thermo gravimetric analysis of representative films.

7.5a TG and DTA curves of dextran chitosan blend control film



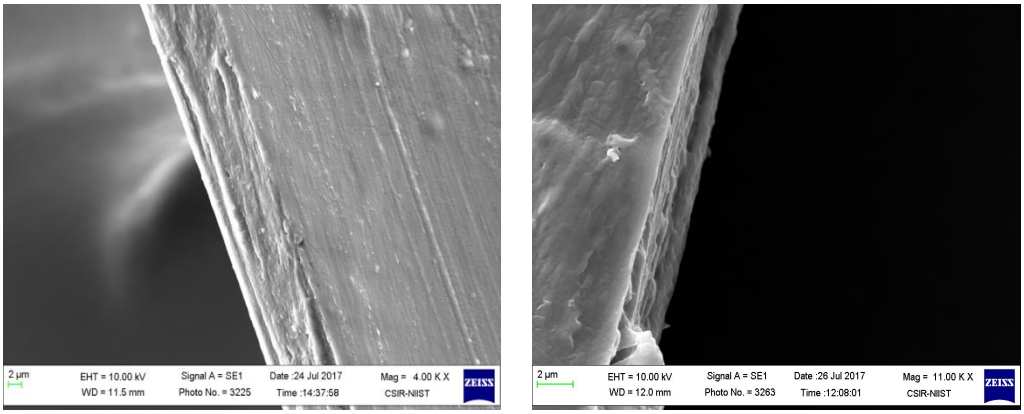
7.5b TG and DTA curves of dextran chitosan blend films with 40 mg ferulic acid.



7.3.5 Scanning electron microscopy of blend films

The scanning electron micrographs of the break in the film displayed minute differences in structure, as can be observed from Figure 7.6 with the film containing ferulic acid comparatively more compact than the control film (blend film without ferulic acid) with the film being composed of multiple layers while the control film appeared like a single cohesive unit. The compaction of the film and the layering maybe due to the cross linking property of ferulic acid.

Figure7.6 Comparative scanning electron micrographs for control and test films



Control Film Film containing 40mg ferulic acid

7.3.6 Solid state UV-visible spectroscopy of blend films

As seen in Figures7.7 and 7.8, it can be concluded that ferulic acid incorporation occurred due to the absorbance of the film at 340 nm while the control film did not show any signs of absorbance increase at 340 nm. Ferulic acid has absorbance maximum at 322 nm there is a red shift with blend film incorporated ferulic acid due to the formation phenoxy moieties due to the oxidation of ferulic acid for crosslinking by the action of hydrogen peroxide(Pan,

Spencer et al. 1999). This indicates that ferulic acid is thoroughly blended with the dextran chitosan film.

Figure 7.7 Optical absorbance profile of control film (dextran chitosan blend film without ferulic acid)

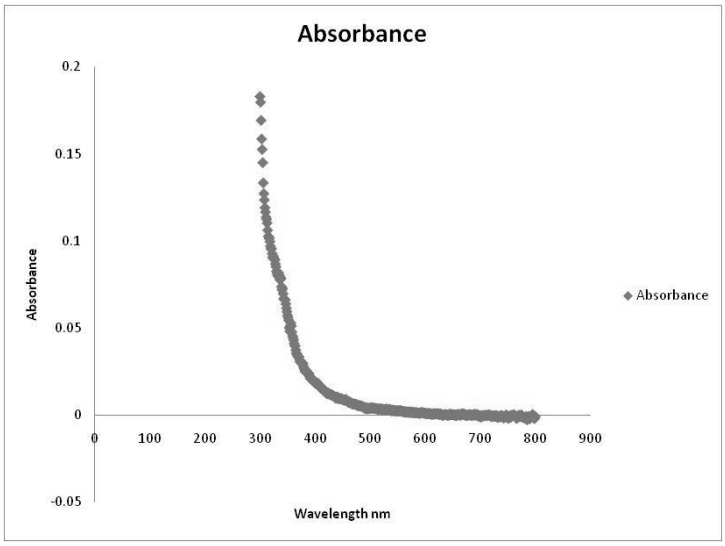
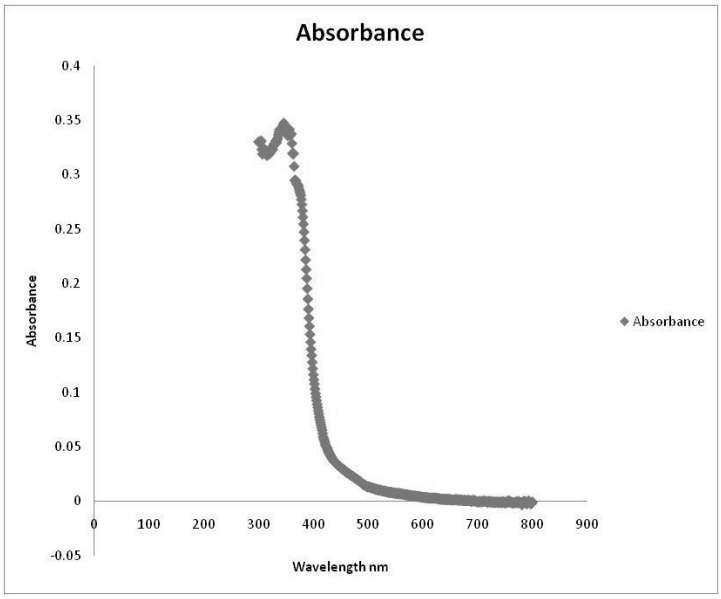


Figure 7.8 Optical absorption of dextran chitosan blend film containing ferulic acid



Photographs of control film and film with ferulic acid can be seen in Figure 7.9. Both films appear transparent under visible light as seen in Figure 7.9 a, while fluorescence intensity for both films are higher under 366nm compared to 254nm while film containing ferulic acid always fluoresces with higher intensity (Figure 7.9b,c). We may therefore be able to use the films for packaging pharmaceuticals which require UV protection for stability.

Figure 7.9 Photographs of films under UV and visible light.

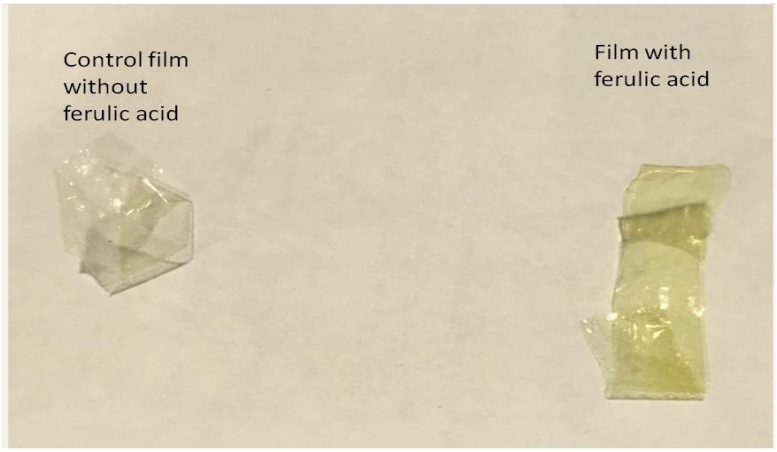


Figure 7.9a Films under visible light

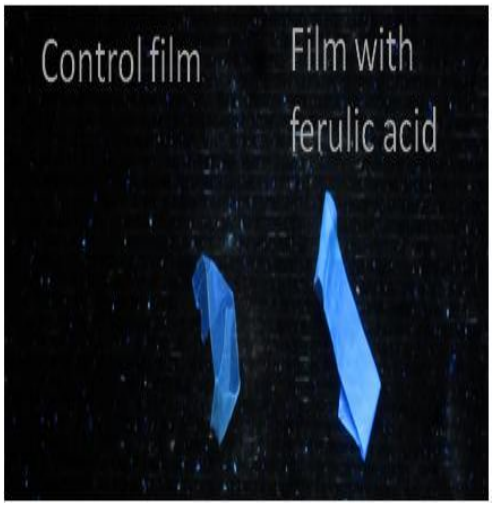


Figure 7.9b Films under UV 366nm

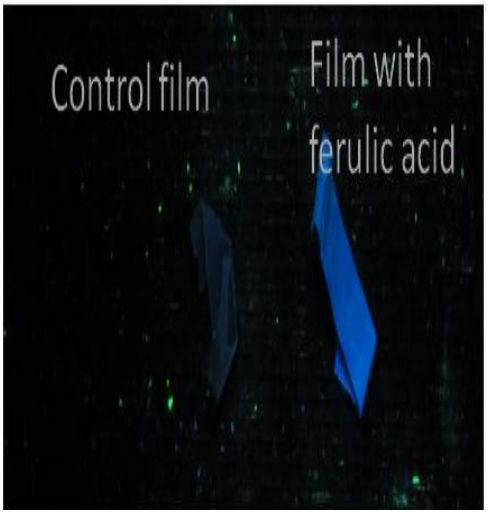


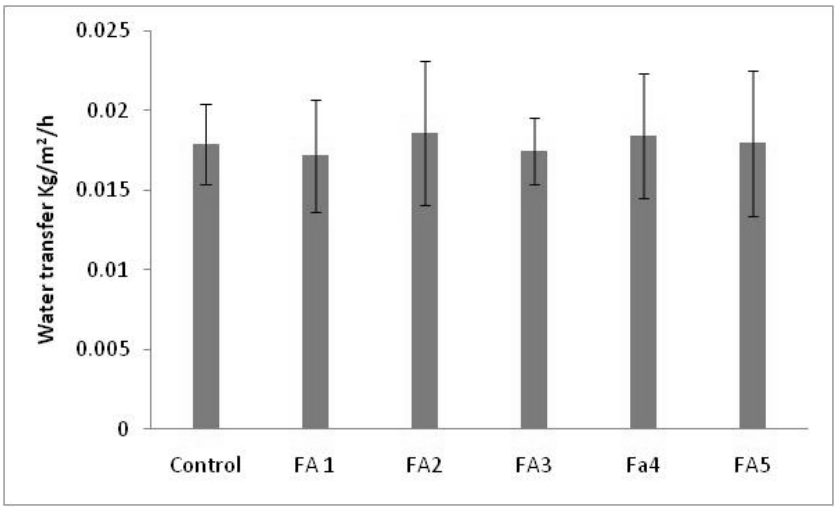
Figure 7.9c Films under UV 254nm

7.3.7 Water transfer rate of blend films

The water transfer rate for control film and the corresponding treatment films with all levels of ferulic acid were at levels very similar to each other. One of the main goals of introducing ferulic acid into the film was to decrease the water transfer rate of the film. Among the different levels studied (2, 8,16,32,40 mg ferulic acid per film) there was no significant difference between the films.

All the fims had transfer rates similar to the control with a value of 0.01791 $Kg/m^2/h$ for thickness in the range of 3-5 µm. (low density polythene 1mm thickness has a transfer rate of0.0009 $Kg/m^2/h$)

Figure 7.10 Water vapor transmission rate of films



Legend FA1 film with 2mg ferulic acid, **FA2** film with 8mg ferulic acid **FA3** film with 16mg ferulic acid **FA4** film with 32mg ferulic acid **FA5** film with 40mg ferulic acid

7.4 Summary

Free enzyme was successfully used to prepare ferulic acid ester with arabinose using a ratio of 51:47:3 of hexane: t-butanol: buffer. Immobilization of enzyme on aminated mesoporous silica particles though successful was not found to be efficient for synthesis of arabinose ferulic acid ester. Film with 40 mg

ferulic acid per film displayed altered microscopic properties and increased tensile strength compared to control films without ferulic acid. Films with ferulic displayed increased optical absorbance at 340nm while remaining transparent in the visible, indicating good acceptability with an added property of being UV absorbent. Thermogravimetric tests and water vapor transmission did not show any significance difference between dextran chitosan blend films with and without ferulic acid.

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Chapter VIII

Summary and Conclusion

During the current study, production of feruloyl esterase employing solid state fermentation of agro residual substrate wheat bran was scrutinized with focus on parameters affecting the enzyme production and its purification, followed by study of characteristics of the enzyme. The current study also dealt with the problem of efficiently biorefining destarched wheat bran with the use of the said enzyme for maximizing the recovery of ferulic acid from destarched wheat bran. The use of this enzyme for a green synthesis of arabinose ferulic acid ester has also been studied. They study has also shed light on the effect of ferulic acid in dextran chitosan blend films.

Among the different agro residual substrates that were probed for their content of alkali releasable ferulic acid, sorghum showed the highest amount, while chili stalk had the lowest. Even though sorghum had the highest content of alkali releasable ferulic acid, it was not the best biomass for the production of feruloyl esterase through solid state fermentation. It was determined that destarched wheat bran was the best substrate for the production of the enzyme. Productivity as high as 166.4 U/gds/h (11984.1 U/gds in 72h) was recorded with optimum conditions of pH 5 of the solid medium (destarched what bran) and the initial moisture set as 67%, using *Aspergillus niger* ATCC 13497, in flask levels with 3g of destarched wheat bran. A Comparable yield was obtained at tray level fermentation (50g) yielding 10957 U/gds of feruloyl esterase. Wheat is an indispensible part of the Indian agrarian economy and the Indian diet which indicates that wheat is produced and consumed in our country in large quantities, which makes it imperative to use wheat bran, a byproduct to its fullest so as to aid the economy, and using destarched wheat bran for producing feruloyl esterase and ferulic acid is one of the methods to do so.

The crude extract that was obtained from fermented destarched wheat bran was subjected to fractionation using ammonium sulphate and the 60-90% saturation fraction gave the highest activity. This fraction was used for hydrophobic interaction chromatography based fractionation and resulted in a purified fraction of feruloyl esterase with a specific activity of 528 U/mg. This purified enzyme was found to have a molecular weight of 35 kDa.

The purified enzyme was subjected to various biochemical analysis for understanding its characteristics and it was found that the enzyme displayed an optimum pH of 6.0 and was fairly thermostable as it retained ~45% of activity after incubation at 90^{o}C or 20 mins and up to 50%

activity at 70^{o}C for 20 min. Some metal ions like Cu^{+2}, Zn^{+2} and Ba^{+2} were inhibitory to the enzyme , while Ni^{+2} and Co^{+2} stimulated its activity .Detergents were detrimental to the activity of the enzyme with inhibition in the order SDS>Tween 80. Solvents adversely effected the activity of the enzyme with inhibition in the order butanol>octanol>propanol>ethanol>methanol, but PEG enhanced the activity of the enzyme. The enzyme was typed as a type A feruloyl esterase, hence it was found to be AnFaeA. These biochemical parameters essentially define the working constraints of the enzyme and will pave the way towards convenient setup of parameters for processes which employ the enzyme. This information may be particularly useful for future studies involving blends of feruloyl esterase with other lignocellulose deconstruction enzymes for higher synergy.

A quadratic model for the predicting the release of ferulic acid from destarched wheat bran was derived. Enzyme loading of 550 U/g, reaction time of 11.5 h, and reaction temperature at 42^{o}C, using citrate buffer at pH 3.5 (10 mM) gave the highest release of ferulic acid with 2.02 mg/g ferulic acid which is as high as 32.54% of the total alkali releasable ferulic acid. AMBERLITE XAD 4 was found to be the best adsorbent for ferulic acid in terms of capacity (45 mg/g) as well as ease of desorption and percentage desorption (99.5%). To demonstrate feasibility of the method for larger scales a packed column reactor was used to validate the same parameters for the same process. Understanding that parameters optimized using a actively mixed batch reactor mini setup may be scaled up to a packed column process without a big loss in accuracy of the model urges similar studies to scale up enzymatic processes involving insoluble substrates. One may even argue that sequential use of enzymes for fractionation of lignocelluloses can be employed in the packed column setup just like a refinery process.

Purified enzyme was able to esterify ferulic acid with arabinose. While most of tests did not show a difference between dextran chitosan blends with and without ferulic acid, the optical properties of the films differed with respect to an additional absorption maximum of 340nm for the film with ferulic acid. There was improved tensile strength recorded only for films with 40mg ferulic acid per film. The electron micrographs of the films had minute differences where the blend films with ferulic acid displaying a layered structure. Finding newer packaging materials is a challenge and even though the current studies do not encourage the use of this particular blend to make food packaging, it still opens up avenues. Even in small quantities

ferulic acid did make the film almost UV opaque which begs the question , can we incorporate ferulic acid or its derivatives to make plastics that are resistant to UV light, hence only opening up broader and bigger avenues for research.

New reports of feruloyl esterase producing microorganisms are being published and research towards the same continues. However, studies related to performance of feruloyl esterase along with enzymes other than xylanase for lignocellulosic hydrolysis remain yet to be explored. The correct combination of pretreatment strategies, along with optimized enzyme mixtures and optimal process flow designs would serve the goal of sustainable energy. With efficient and exhaustive research focused towards sustainable energy, work on feruloyl esterase plays a small but significant part in the equation, which is yet to be unraveled. With developments in molecular cloning and genetic manipulations, performance of the enzyme can be tuned to fit the role that it is required to play. There is a gap in research regarding the improvement in enzyme activity using tools like site directed mutagenesis and directed evolution which may be addressed by upcoming researchers. Phenolic acids like ferulic acid could be one of the paradigm shifting value added platform chemicals which can be generated in association with lignocellulosic biofuel industry. Even though FAEs has been a subject of research for as long as three decades, excavating further, understanding the biochemistry and exploring the diversity of this group of enzymes has only opened up new avenues for applications of this enzyme and its product.

Annexure I Microbiological Media

(a) Mineral media composition used for wetting solid substrate

Vogel's minimal medium was used as a wetting medium, without the addition of biotin. The following was the composition of the minimal medium.

Table 2.1: Major salts of Vogel's minimal medium

Salt/Component	Final concentration g/L
$C_6H_5Na_3O_7 . 2 H_2O$	2.4
KH_2PO_4	5
NH_4NO_3	2
$MgSO_4. 7 H_2O$	0.2
$CaCl_2. 2 H_2O$	0.1

The salts were dissolved in de ionized water to obtain 50 x concentration major salt stock solution

Table 2.2:Minorsalts of Vogel's minimal medium

Salt/Component	Final concentration g/L
$HOC(COOH)(CH_2COOH)_2 \cdot H_2O$	0.005
$ZnSO_4. 7 H_2O$	0.005
$(NH_4)_2SO_4$	0.001
$CuSO_4. 5 H_2O$	0.00025
$MnSO_4.H_2O$	0.00005
H_3BO_3	0.00005
$Na_2MoO_4. 2 H_2O$	0.00005

The salts were dissolved in de ionized water to obtain 1000 x concentration for the minor salts stock solution.

(b) Potato dextrose agar

Salt/Component	Final concentration g/L
Potatoes, infusion from	200
Dextrose	20
Agar	20

The salt solutions were appropriately diluted and mixed before they were sterilized using an autoclave. All media including solid media were autoclaved using a Labline autoclave (Labline,India), by holding the material at 121^{o}C for 15 minutes at 15 psi.

Annexure II List of instruments

Instruments	Make and Model
Autoclave	LABLINE, India
Centrifuge	REMI (India) , KUBOTA 7780 (Japan)
Cold Room	Rinac Pvt. Ltd ,India
Heating water bath	B20G, Lab companion, South Korea
HPLC	Shimadzu modular UFLC, with Shimadzu SPDM20A PDA detector
HPTLC system	CamagLinomat V applicator, Camag HTPLC Scanner, Switzerland
Laminar Air flow	Clean Air (India), Model CAV sentinel gold(BSL 2) Esco Micro (Singapore)
Magnetic stirrer	Spinot, Tarsons India
Microscope	Leica DM 2000, Germany
Moisture analyser	A&D MX 50, Japan
Nanodrop Nanospectrophotometer	ND1000, Thermo Fisher Scientific, India
pH meter	EUTECH, Thermo Fisher Scientific, India
Poly acrylamide gel electrophoresis system	Protean Mini, Bio-Rad USA
Protein Purification system	BioLogic LP low-pressure chromatography system,Bio-Rad USA
Rotavapour	Buchi, Switzerland
Shaking incubator	INFORS HT Multitron stanadard, Switzerland
Shaking water bath	SW22, Julabo, Germany
Static incubator	LABLINE Fungal incubator, India
Thermal Cycler	MyCycler, Bio-Rad USA.

Annexure III AcSIR Course work

Level 100			
Course number	**Title**	**Credits**	**Status**
BIO-NIIST-1-001	Biostatistics	1	Completed
BIO-NIIST-1-002	Bioinformatics	1	Completed
BIO-NIIST-1-003	Basic Chemistry	1	Completed
BIO-NIIST-1-004	Research Methodology, Communication, Ethics & Safety	1	Completed

Level 200			
Course number	**Title**	**Credits**	**Status**
BIO-NIIST-2-001	Biotechniques and Instrumentation	1	Completed
BIO-NIIST-2-256	Basic and Applied Microbiology	2	Completed
BIO-NIIST-2-257	Basic Molecular Biology	2	Completed

Level 300			
Course number	**Title**	**Credits**	**Status**
BIO-NIIST-3-001	Seminar Course	1	Completed
BIO-NIIST-3-381	Bioprocess Technology	2	Completed
BIO-NIIST-3-382	Enzymology and Enzyme Technology	2	Completed

Level 400			
Course number	**Title**	**Credits**	**Status**
BIO-NIIST-4-001	Review Writing	1	Completed
BIO-NIIST-4-002	Project Proposal	1	Completed

CSIR-800		**Completed**

Annexure IV List of Publications

Gopalan Nishant, Kesavan Madhavan Nampoothiri, George Szakacs, Binod Parameswaran, and Ashok Pandey. "Solid-state fermentation for the production of biomass valorizing feruloyl esterase." *Biocatalysis and Agricultural Biotechnology* 7 (2016): 7-13.

Gopalan, Nishant, L. V. Rodríguez-Duran, G. Saucedo-Castaneda, and K. MadhavanNampoothiri. "Review on technological and scientific aspects of feruloylesterases: a versatile enzyme for biorefining of biomass." *Bioresource technology* 193 (2015): 534-544.

Gopalan, N., and K. M. Nampoothiri. "Biotechnological Production of Enzymes Using Agro-Industrial Wastes: Economic Considerations, Commercialization Potential, and Future Prospects." *Agro-Industrial Wastes as Feedstock for Enzyme Production: Apply and Exploit the Emerging and Valuable Use Options of Waste Biomass* (2016): 313. (Eds: Gurpreet S. Dhillon and SurinderKaur),Academic Press/Elsevier ,Netherlands,pp313-330

Varsha, KonthamKulangara, **Gopalan Nishant**, Srambikal Mohandas Sneha, GanesanShilpa, LeenaDevendra, SulochanaPriya, and KesavanMadhavan Nampoothiri. "Antifungal, anticancer and aminopeptidase inhibitory potential of a phenazine compound produced by Lactococcus BSN307." *Indian journal of microbiology* 56, no. 4 (2016): 411-416.

V.Gopinath, M**.** Anusree, **N.Gopalan** and K.S Dharand Nampoothiri,K.M.*(2013), Amino-based Product from Biomass and Microbial Amino Acid Production.In: Bioenergy Reserch: Advances &Applications (Eds: V. K. Gupta, M. Tuohy, C.P. Kubicek, J. Saddler and FengXu , Elsevier ,Netherlands,pp337-352.

Annexure IV Conference Proceedings

Nishant Gopalan, and K Madhavan Nampoothiri (2015) Statistical optimization for the production of feruloyl esterase using Aspergillusniger ATCC 13497 through solid state fermentation. Presented at New Horizons in Biotechnology (NHBT 2015), IB-80, Proceedingsof New Horizons in Biotechnology and XIIconvention of the Biotech Research Society of India, Nov 22-25, Thiruvananthapuram, Kerala.

Nishant Gopalan, and K Madhavan Nampoothiri (2014),Production of feruloyl esterase using Aspergillusniger by solid state fermentation. Presented at International Conference on Emerging Trends in Biotechnology2014.IBF-30, P I10,Proceedings of the international conference on Emerging trends in Biotechnology (ICETB 2014), XI convention of the Biotech Research Society of India & Indo-Italian Workshop on Industrial Pharmaceutical Biotechnology , Nov 6-9, School of Environmental sciences, Jawaharlal Nehru University, New Delhi.

Arjun Prasad, Akanksha Jadhav**, Nishant Gopalan**, Ashok Pandey and Binod Parameswaran (2012), A biorefinery approach for the production of bioethanol and amino acids from sorghum biomass. Presented at International Conference on Industrial Biotechnology (ICIB-2012) and Indo-Italian Workshop on Food Biotechnology: Industrial Processing, Safety & Health, Nov 21-23, Punjabi University, Patiala, Punjab.

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Solid-state fermentation for the production of biomass valorizing feruloyl esterase

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ABSTRACT

Aspergillus niger ATCC 13497 has been used to produce feruloyl esterase type A enzyme. The process parameters were optimized to attain a maximum of 11984.1 U/gds of enzyme activity within a period of 72 h using destarched wheat bran as a substrate. The purified enzyme studied in the present work displays a pH optimum of 5, and a temperature optimum of 37 °C. The enzyme has a size of 35 kDa and exhibited moderate thermostability, with 41% of activity retained at 90 °C. Ni $^{+2}$ and Co^{+2} ions were found to have stimulatory activity on the enzyme with 66% and 78% increase in activity respectively. Cu+2 inhibited the activity by 88%. Statistical optimization enhanced the enzyme activity by 2.47 fold.

1. Introduction

Agro-industrial biomass are generated as byproducts of agricultural practices. These materials are of lower economic value or are regarded as wasteful pollutants in certain cases. To alleviate the effects of carbon emissions caused by non-renewable petroleum based, greener sources of bio based fuels are being researched. The United Nations set in motion a program, Sustainable Energy for All or SE4ALL in 2012. One of the objectives of the program is to increase the share of renewable energy, among the global energy generation (Wilson, 2012). Therefore, using agro-industrial waste/byproducts for generation of energy is being considered by converting the lignocellulosic agricultural by-products to fermentable sugars, followed by conversion of the fermentable sugars to fuel alcohols like ethanol and butanol or gases like hydrogen and methane (Sun and Cheng, 2002). Chemical processes to convert lignocelluloses to fuel through gasification or cracking are also methods which are being considered (Santos et al., 2010). Bioethanol produced through second and third generation technologies is one such promising fuel, however, in order to economically produce bioethanol, the main process must be valorized by subsidary processes. Therefore, it becomes essential to produce high value low volume by-products so as to offset the cost of the fuel.

Feruloyl esterases (EC#3.1.1.73) is a group of versatile enzymes that can be used as xylanase accessory enzymes to increase the efficiency of hydrolysis of lignocellulosics or can also be used as a synthetic catalyst to produce various esters of ferulic acid and the ferulic acid released from different sources can be converted by controlled bio-oxidation to flavor compounds vanillin and vinyl guaiacol (Bonnin et al., 2002; De Vries et al., 2002; Faulds and Williamson, 1995; Laszlo et al., 2006; Mastihubová et al., 2006; Mathew et al., 2007). Cinnamoyl ester hydrolase, ferulic acid esterase and hydroxycinnamoyl esterase are alternative names to the said enzyme. Feulic acid, a hydroxycinnamic acid first detected in the oleo resin of an arid plant *Ferula asafetida* is a good antioxidant molecule, with UV absorbance in the UV-B region, making it a valuable molecule for the cosmeceutical market (Graf, 1992; Hlasiwetz and Barth, 1866). Feruloyl esterases aid in the release if ferulic acid from various lignocellulosic feedstocks without the use of alkali, hence the process can be categorized as a green process. Traditionally, feruloyl esterases are assayed by detecting the ferulic acid released from a methyl/ethyl ester by HPLC or by the

Abbreviations: AnFaeA, *Aspergillus niger* feruloyl esterase Type A; AtFaeA, *Aspergillus tubingensis* feruloyl esterase Type; AwFaeA, *Aspergillus awamori* feruloyl esterase Type A; CCD, Central Composite Design; DMSO, Dimethyl Sulphoxide; min, minutes; MOPS, 3-(N-morpholino) propanesulfonic acid; rpm, Rotations per minute; SMF, Submerged fermentation; SSF, Solid state fermentation; U/gds, Enzyme units per gram dry substrate; U/mL, Enzyme units per milliliter

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Review

Review on technological and scientific aspects of feruloyl esterases: A versatile enzyme for biorefining of biomass

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HIGHLIGHTS

- Feruloyl esterases release hydroxycinnamates from lignocellulosic biomass.
- Enzymatic extraction of hydroxycinnamates from less explored agroresidual biomass.
- Hydroxycinnamates as value added chemicals for a cost effective biorefinary.
- Molecular cloning of feruloyl esterase for enzymatic process.

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ABSTRACT

With increasing focus on sustainable energy, bio-refining from lignocellulosic biomass has become a thrust area of research. With most of the works being focused on biofuels, significant efforts are also being directed towards other value added products. Feruloyl esterases (EC. 3.1.1.73) can be used as a tool for bio-refining of lignocellulosic material for the recovery and purification of ferulic acid and related hydroxycinnamic acids ubiquitously found in the plant cell wall. More and more genes coding for feruloyl esterases have been mined out from various sources to allow efficient enzymatic release of ferulic acid and allied hydroxycinnamic acids (HCAs) from plant-based biomass. A sum up on enzymatic extraction of HCAs and its recovery from less explored agro residual by-products is still a missing link and this review brushes up the achieved landmarks so far in this direction and also covers a detailed patent search on this biomass refining enzyme.

1. Introduction

Hydroxycinnamic acids (HCAs) are components of plant cell walls. These compounds exhibit various biological activities which are of interest to the pharmaceutical industry, making them high-value chemicals. HCAs like ferulic acid, caffeic acid, *p*-coumaric acid, sinapic acid, etc. (Fig. 1) have excellent antioxidative properties, due to the presence of the para hydroxy group on the phenolic acid moiety. These compounds can form stable phenoxy moieties thus serving as good antioxidants, the unsaturation of the aliphatic side chain and its bulk also assists in the antioxidant activity of these compounds (Razzaghi-Asl et al., 2013). HCAs may occur as free phenolic acids, as soluble conjugates or covalently linked to cell wall polymers (Figs. 2 and 3). Ferulic acid is the most plentiful HCA in nature, and it is found mainly linked to hemicellulosic carbohydrate moieties and to lignin components (Manach et al., 2004; Shahidi and Chandrasekara, 2010).

HCAs have absorption maxima in the UV-B range, from 310 to 360 nm, which also makes these compounds useful in the preparation of skinceuticals. HCAs have been indicated as beneficial for clinical conditions like Alzheimer's and is known to be a protective agent against colon cancers. Ferulic acid is also used as precursors for biotransformation into vanillin. Caffeic acid and its phenethyl esters have been identified as the active pharmaceutical component of propolis (Russo et al., 2002).

Release of HCAs from plant material is typically facilitated by alkaline hydrolysis of the plant-derived biomass. Alkaline hydrolysis is not preferred, and green methods akin to enzymatic

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ORIGINAL ARTICLE

Antifungal, Anticancer and Aminopeptidase Inhibitory Potential of a Phenazine Compound Produced by *Lactococcus* BSN307

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Abstract A bioactive compound was purified from the culture medium of a new strain of *Lactococcus* BSN307 by solvent extraction followed by chromatographic techniques. This bioactive compound was identified to belong to phenazine class of compounds by MS, NMR and FTIR. The phenazine compound showed antifungal activity against *Aspergillus niger*, *Penicillium chrysogenum* as well as *Fusarium oxysporum* by disc diffusion assay in addition to antioxidant potential as demonstrated by DPPH scavenging assay. The compound demonstrated selective cytotoxicity against cancer cell lines HeLa and MCF-7 where IC_{50} was achieved with 20 and 24 μg/mL respectively. At the same time no cytotoxicity was occurred in normal H9c2 cells. The bioactive found to be inhibitory to both leucine and proline aminopeptidases and thus revealed its potential as metalloenzyme inhibitor. This study, for the first time reports the production of phenazine class of compounds by lactic acid bacteria.

Keywords Anticancer · Antifungal · Bioactive · Phenazine · *Lactococcus*

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Introduction

Lactic acid bacteria (LAB) are thoroughly studied to genome level for their production of therapeutic compounds including antimicrobials. Phenazines which are nitrogen containing heterocyclic compounds that differ in their properties based on the type and position of functional groups are extensively studied because of their wide range antibiotic properties [1]. Members of the phylum Proteobacteria and Actinobacteria, most importantly *Pseudomonas* and *Streptomyces* are the major producers of phenazine compounds and are not yet reported to be produced by any member of phylum Firmicutes.

Here, we report the purification, identification and biological activity evaluation of a phenazine compound produced by a new strain of *Lactococcus* BSN307 (DSMZ 100577, MCC 2824). This novel LAB strain was closely related to *Lactococcus garvieae* as identified by 16S rRNA gene (KM261818) sequencing but showed phenotypic, chemotaxonomic and molecular level differences and stands separate from the type strain of *L. garvieae*^{T} (data not provided). This compound showed antifungal, antioxidant and anticancer properties along with aminopeptidase enzyme inhibition potential. We have previously reported the identification of organic acids and a bioactive phenolic compound in the cell free supernatant of the same LAB strain [2, 3]. The advantage of LAB metabolites having both antifungal and antioxidant properties is that they can be developed as food preservatives with dietary antioxidant properties once incorporated into food. As far as we know, this is the preliminary report about the production of phenazine class of compound by LAB or any member of phylum Firmicutes as well as the aminopeptidase inhibitory potential of any LAB metabolite.

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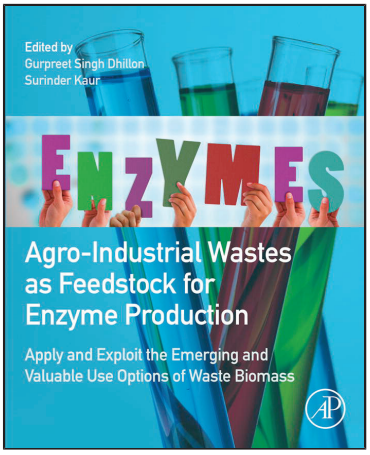

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CHAPTER 14

Biotechnological Production of Enzymes Using Agro-Industrial Wastes: Economic Considerations, Commercialization Potential, and Future Prospects

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INTRODUCTION

Industrial processes are purely driven by the economics of the given process. The viability of a product is governed mainly by the gap in demand and supply and the percentage profit that comes out of the whole process. Just like any other bioprocess, enzyme production also consists of upstream, fermentation/bioproduction and downstream processes. The whole economics of the process is governed by the cost involved in executing all three stages, as well as the demand and acceptability of the final product. Enzymes are considered as green alternatives to industrial catalysts. However, in the modern biotechnology era, enzymes are used in special niche areas, where enzymes are the most essential components to the core process, viz., stereo selective synthesis (aldolases, hydrolases, nitrilase), detergent formulations (lipase and proteases), juice liquefaction (pectinase, xylanase), therapeutics (asparaginase for cancer treatment), diagnostics (glucose oxidase for glucometers) (Kasturi et al., 1998; Wang, 2008; Clapés et al., 2010; Pal and Khanum, 2011). The final use of the enzyme dictates the purity of the enzyme required and hence the price of the enzyme preparation. Technical enzymes are employed in four major areas, viz., food and beverages, detergents, animal feed, biomass hydrolyzing, or biofuel along with other subsidiaries, such as leather industries, textile, paper-pulp and biopharmaceuticals (Kirk et al., 2002). While the largest industry segment that acts as consumers for enzymes still remains the food and beverage industry, there has been a shift of highest-selling enzymes from proteases until 2012 to carbohydrases in 2013–14. The increase in the sales of carbohydrase enzymes were accounted for by increasing adoption of enzymes in the food and beverage industry as well as active research and technological thrust toward the production of second-generation biofuels and allied products. The United Nations and World Bank led Sustainable Energy for All (SE4All) initiative and it

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CHAPTER

19

Amino-Based Products from Biomass and Microbial Amino Acid Production

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OUTLINE

AMINO ACIDS

The amino acid industry has shown an exponential growth since its infancy in the 1950s. It has grown from extracting flavor enhancers from seaweeds, to fermenting high-purity, optically active forms in hundreds and thousands tons. The isolation of a bacterial strain producing glutamic acid and an efficient screening method to identify the highest producer by the Japanese researchers of the Kyowa Hakko Kogyo Co. was the key event in the amino acid fermentation industry. Until then, there was no suitable commercial process for the mass production of amino acids. Later on, it received further boost when the workers of the same organization reported a homoserine auxotrophic lysine producer. This discovery led to the development of a commercially viable fermentation process for lysine fermentation with a conversion efficiency of 26% from glucose. Bioprocess engineering and strain improvement methods have contributed to the massive growth of the industry.

The essential amino acids hold a major place in the global amino acid market, as these cannot be

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