STUDIES ON FERULIC ACID, A BIOACTIVE COMPOUND AND ON FERULOYL ESTERASE INVOLVED IN ITS PRODUCTION

THESIS SUBMITTED TO THE
UNIVERSITY OF KERALA
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN BIOTECHNOLOGY UNDER THE FACULTY OF APPLIED SCIENCE

By
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UNDER THE SUPERVISION OF
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Dedicated to Jehovah, Jireh without whom this work would have been impossible and who made it a wonderful experience of seeking and learning

DECLARATION

I, **Sindhu Mathew**, do hereby declare that the matter embodied in this thesis entitled "**Studies on ferulic acid, a bioactive compound and on feruloyl esterase involved in its production**" is a bonafide record of the investigation carried out by me in the Chemical Sciences and Technology Division of the Regional Research Laboratory, Trivandrum under the guidance of Dr. T. Emilia Abraham and no part of this thesis has been submitted elsewhere for the award of any other degree or diploma.

Dated: 29.4.2006 (**Sindhu Mathew**)

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CERTIFICATE

Certified that the work embodied in this thesis entitled "**Studies on ferulic acid, a bioactive compound and on feruloyl esterase involved in its production**" is a record of bonafide work carried out by **Ms. Sindhu Mathew** under my supervision in fulfillment of the requirements for the degree of **Doctor of Philosophy in Biotechnology** of the University of Kerala and the same has not been submitted elsewhere for any other degree or diploma.

Dated: 29.4.2006

Dr. T. Emilia Abraham
(Supervising Guide)

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Sindhu Mathew

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Abbreviations

ABTS	2, 2'-Azinobis (3-ethyl benzthiazoline sulfonic acid)
DMSO	Dimethyl sulfoxide
o-	ortho
p-	para
SEM	Scanning electron microscopy
AFM	Atomic force microscopy
FAE	Ferulic acid esterase
U/ml	Units/milliliter
U/mg	Units/milligram
CoA	Coenzyme A
YEPG	Yeast extract peptone glucose
NMR	Nuclear magnetic resonance
TGA	Thermogravimetric analysis
DTG	Differential thermogravimetric analysis
DSC	Differential scanning calorimetry
FTIR	Fourier transform infra red
XRD	X-ray diffraction
TS	Tensile strength
S.D	Standard deviation
MPa	Mega Pascal

Abbreviations

Pr	Protocatechuic acid
Gu	Guaiacol
Ca	Caffeic acid
Fe	Ferulic acid
Co	p-Coumaric acid
Ga	Gallic acid
V.Ac	Vanillic acid
Si	Sinapinic acid
PG	Propyl gallate
Ve.Ald	Veratraldehyde
BHA	Butylated hydroxyl anisole
Ve.Alc	Veratryl alcohol
Va.Alc	Vanillyl alcohol
Py	Pyrogallol
Be	Benzoic acid
BHT	Butylated hydroxyl toluene
CLE	*Cinnamomum verum* leaf extract
CBE	*Cinnamomum verum* bark extract
DS	Degree of substitution
DPPH	Diphenyl picryl hydrazine

PREFACE

Ferulic acid (4-hydroxy-3-methoxy cinnamic acid) is present at a relatively high concentration in the cell walls of several plants, including Monocots and Dicots and is found covalently linked to the polysaccharides by ester bonds and to components of lignin through ether or ester linkages. Maize bran with 3.1% (w/w) ferulic acid is one of the most promising sources of this antioxidant compound. Feruloyl esterases are a subclass of the carboxylic acid esterases that hydrolyse the ester bond between hydroxycinnamic acids and sugars present in plant cell walls and they act synergistically with xylanases and pectinases and facilitate the access of hydrolases to the backbone of wall polymers. In the manufacture of animal feed, the ferulic acid esterases can act as accessory enzyme to increase the total yield of sugars. It also enhances the paper pulping process by making the solubilisation of lignin-carbohydrate complexes easier.

The present study was aimed at the production of feruloyl esterase enzyme by microorganisms and on the potential applications of ferulic acid, including its biotransformation to a highly value added product, namely 4-vinyl guaiacol and its antioxidant activity.

The thesis comprises of six chapters. The first chapter presents a brief over view about ferulic acid, its dimerization and occurrence in the plant kingdom and its application in food, health and medicine. It also gives an outline of the various feruloyl esterase enzymes reported from microbial and plant sources.

The second chapter deals with the pretreatment of different growth substrates such as wheat, rice and maize bran and sugarcane bagasse and screening of microorganisms for feruloyl esterase enzyme production by submerged fermentation on different growth substrates.

The third chapter deals with the biotransformation of ferulic acid by the isolated strain JCS-3 identified as the yeast *Debaryomyces hansenii* and assigned the number 539 by MTCC, Chandigarh, to yield higher value added products possessing GRAS (Generally regarded as safe) status. The transformed products have been separated and identified by TLC, HPLC, ^{1}H and ^{13}C NMR spectroscopy.

Comparison of the free radical scavenging properties of some phenolic acids including ferulic acid is made in chapter four. This chapter also deals with the free radical scavenging and antioxidant properties of *Cinnamomum verum* bark and leaf extracts rich in phenolic acids.

Free ferulic acid does not enter the enterohepatic circulation and therefore through oral or intravenous administration, ferulic acid cannot easily reach the colon. However enzyme resistant starch is a type of dietary fiber that is almost completely fermented in the colon and has been reported to be a satisfactory carrier of ferulic acid. The synthesis, physico-chemical, thermal and biological characterization of starch ferulate is explained in chapter five.

Chapter six deals with the preparation, characterization and microstructural imaging of starch-chitosan blend films containing ferulic acid. The thermal and mechanical properties of the various blend films were studied. The films were characterized by FT-IR and XRD. The swelling properties of the films were studied as a function of pH. The water vapor transmission rate, oxygen transmission rate and lipid peroxide inhibition activity of ferulic acid incorporated blend films were also studied. The Summary and Conclusion are given at the end.

CHAPTER I

INTRODUCTION

1. 1 Introduction

Ferulic acid (4-hydroxy-3-methoxy cinnamic acid) is present at a relatively high concentration in the cell walls of several plants, including Graminaceae among Monocots (Hartley and Ford, 1989) and Solanaceae (Fry, 1982) and Chenopodiaceae (Rombouts and Thibault, 1986) among Dicots (Fry, 1979; Hartley and Harris, 1981). They are derived from the general phenylpropanoid pathway that is ubiquitous in plants. In cereal grains, ferulic acid is essentially found in the bran (Wetzel et al., 1988; Seitz, 1989) and hence it is a suitable raw material for the production of ferulic acid (Ralet et al., 1990). It is also found esterified to the glucuronoarabinoxylans in pineapple cell walls (Smith and Harris, 2001). In dicots, ferulic acid is present in high amounts in sugar beet pulp, another potential raw material for the production of ferulic acid (Rombouts and Thibault, 1986) and in the cell walls of Spinach (*Spinacia oleracea*) (Fry, 1982), Glass wort (*Salicornia ramosissima*) (Renard et al., 1993), Chinese water chestnut (*Eleocharis dulcis*) (Parr et al., 1996), Pine hypocotyls (*Pinus pinaster*) (Sanchez et al., 1996) and Carrot (*Daucus carota*) (Massiot et al., 1988).

Ferulic acid constitutes about 0.14% (w/w, dry weight) in barley grains (Nordkvisk et al., 1984), 0.66% in wheat bran (Smith and Hartley, 1983), 0.8% in sugar beet pulp (Micard et al., 1994), 0.9% in rice endosperm cell wall (Shibuya, 1984) and 3.1% in maize bran (Saulnier et al., 1995). Among the agricultural by-products, that are potential sources of ferulic acid, maize bran is one of the most promising sources. In barley and wheat, ferulic acid is concentrated mainly in the aleurone layer of the bran (Pussayanawin and Wetzel, 1987). Wheat straw contains approximately 30% more of ester linked cinnamic acids than wheat bran and contains approximately equal amounts of ferulic

acid and p-coumaric acid. In contrast, wheat bran contains a very small amount of p-coumaric acid (Lequart et al., 1999). Ferulic acid dominates the monomeric phenolic fraction (7.2 mg/g) of cold alkali treated cell walls of Chinese water chestnut (Parr et al., 1996).

1. 2 Location of ferulic acid

1.2.1 Occurrence of feruloylated oligosaccharides in Monocots

The arabinoxylans consist of β-1, 4 linked xylopyranosyl backbone, substituted with α-L-arabinofuranosyl residues, linked to C-3 or C-2 of the xylosyl units (Figure 1.1) (Smith and Hartley, 1983). Isolated feruloylated oligosaccharides from Gramineae cell walls, showed that ferulic acid was attached at the C-5 hydroxyl group of some arabinofuranose residues of the arabinoxylans (Hartley et al., 1990; Borneman et al., 1990). The major oligomers include : 3-O-(5-O trans feruloyl-α-L- arabinofuranosyl) D-xylose (Kato et al., 1983; Smith and Hartley, 1983; Kato and Nevins, 1985), O-[5-O-(transferuloyl)-α-L-arabinofuranosyl]–(1-3)-O-β-D xylopyranosyl (1-4) D xylopyranose (Mueller-Harvey et al., 1986) and O-β-D-xylopyranosyl-(1-4)-O-[5-O-(trans-feruloyl-α-L-arabinofuranosyl-(1-3)]–O-β-D-xylopyranosyl-(1-4)-D-xylopyranose (Kato et al., 1987).

Maize bran and wheat bran are rich in heteroxylans in which β-(1-4) linked xylose residues form the backbone (xylans), and arabinose residues or glucuronic acid are attached at O-2 or O-3. Ferulic acid is found linked to the O-5 of these arabinose residues (Wende and Fry, 1997). The maize bran cell walls are very resistant to enzymatic degradation, probably due to the highly branched character of the heteroxylan, high level of crosslinking and high levels of esterified ferulic acid, which in fact restricts enzyme accessibility (Faulds et al., 1995).

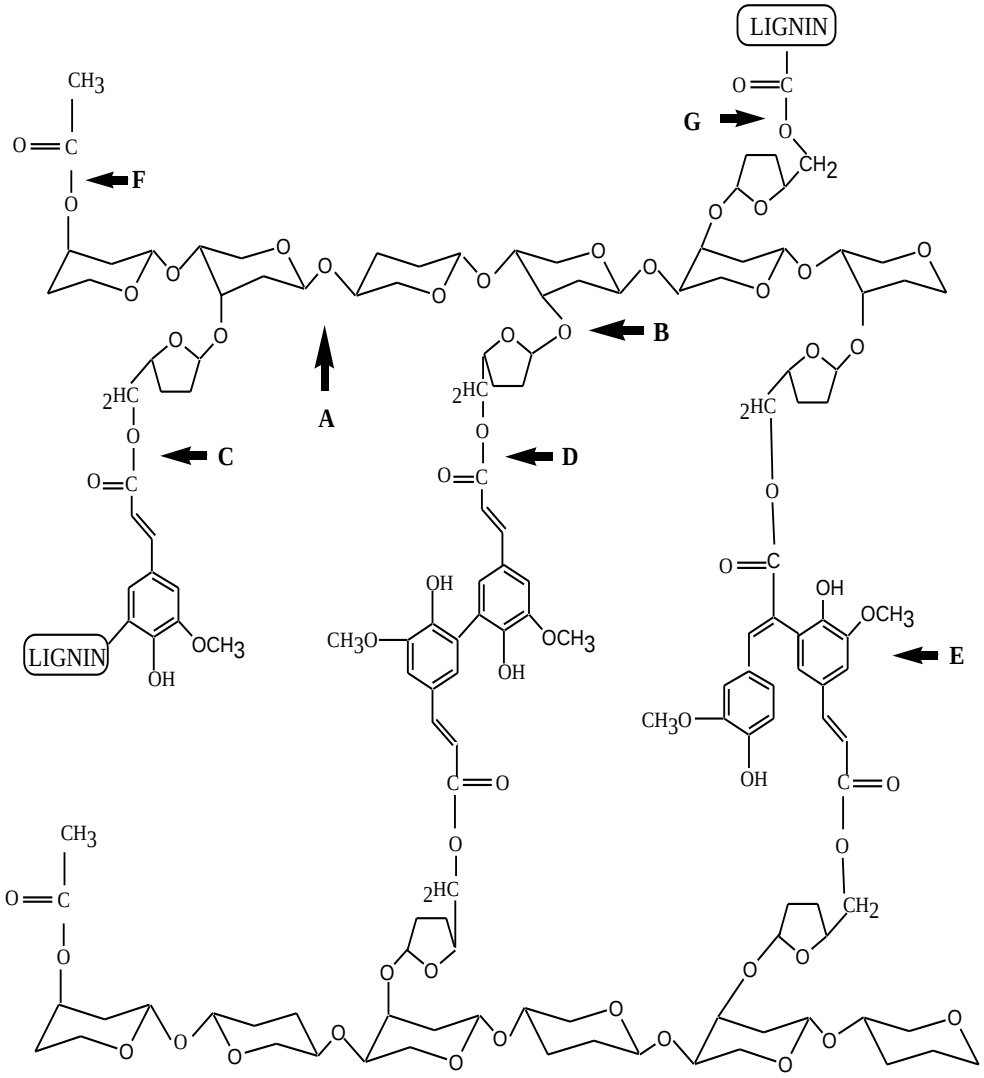


Figure 1.1: Generalised structure of arabinoxylan, the main component of the hemicellulosic part of monocotyledonous plants. A: β-1-4 linked xylan backbone, B: Xylose-arabinose linkage, C: 5-O-feruloyl lignin, D: 5-O-diferuloyl group (5-5'linked dimer), E: 5-O-diferuloyl group (8-5'dimer), F: 3-O-acetyl group, G: arabinose-lignin.

1.2.2 Occurrence of feruloylated oligosaccharides in Dicots

In Dicots, ferulic acid is associated with the pectic polysaccharides. In sugar beet pulp, ferulic acid is found ester linked to

either the C-2 hydroxyl group of arabinofuranose or C-6 hydroxyl group of galactopyranose residues of the pectic side chains (Figure 1.2) (Colquhoun et al., 1994). The feruloylated oligosaccharides: 3-O-(3-O-feruloyl-α-L-arabinopyranosyl)-L-arabinose and 4-O-(6-O-feruloyl-β-D galactopyranosyl) D-galactose were isolated from the pectic fraction of the cell wall (Fry, 1982).

In Sugar beet pulp, the main polysaccharide is pectin which comprises of 'smooth' regions and 'hairy regions'. The smooth regions (or homogalacturonans) are composed of α-1, 4 linked galacturonic acid residues while the 'hairy' regions are composed of α-1, 2 linked rhamnogalacturonans, bearing arabinan or arabinogalactan side chains linked to rhamnose residues (Renard et al., 1995; Saulnier and Thibault, 1999). Structural and compositional analysis of the feruloylated arabinose oligomers, derived from arabinan side chains of pectins, indicates that ferulic acid is esterified to the O-2 of arabinofuranose residues, which are part of the α-(1-5) linked arabinan chains (Guillon and Thibault, 1989). This main chain is also substituted on O-3 by arabinofuranose residues, indicating that the feruloyl groups are linked to the main core of the arabinan and not to the arabinose residues in the side chains. For the feruloylated galactose oligomers derived from galactan side chains of pectins, ferulic acid is linked to the O-6 of galactopyranose residue, linked to another galactose by a β-(1-4) linkage. This suggests that the feruloyl groups are linked to the main core of the β-(1-4) linked type I galactan (Guillon et al., 1989). In sugar beet pulp, approximately 50-55% of the ferulic acid is linked to the O-2 of arabinose residues and 45-50% to the O-6 of galactose residues (Ralet at al., 1994). The pectins in sugar beet pulp are easily degraded by enzymes, possibly due to their much lower content of ferulic and diferulic acids (Micard et al., 1994). The different dehydrodimers represented > 0.1% of the cell wall and the main dehydrodimers are 5-

5', 8-O-4', 8-5', and 8-8', the most abundant derivative being 8-5' (Micard et al., 1997).

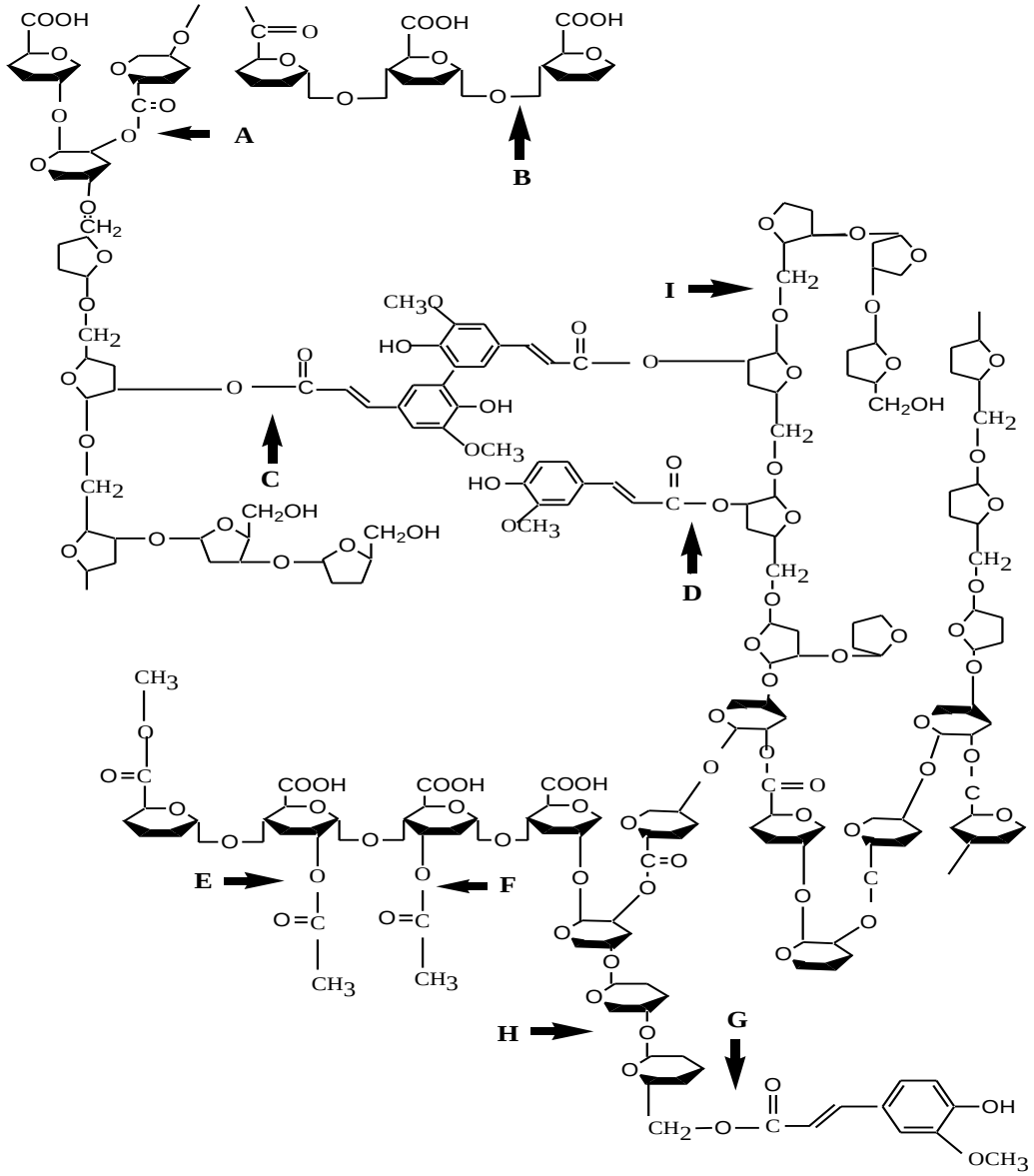


Figure 1.2: Generalised structure of rhamnified pectin from sugar beet. Backbone formed of alternating residues of Galacturonic acid and Rhamnose (Hairy region)- A, B: (Smooth region)- α, 1-4 linked galacturonic acid residues, C: Diferuloyl group attached to C-2 of arabinose, D: Feruloyl group attached to C-2 of arabinose, E: 2-O-acetyl group, F: 3-O-acetyl group, G: Feruloyl group attached to

C-6 of galactose, H: β, 1-4 linked galactose, I: α -(1, 5) linked arabinan chains.

1.3 Ferulic acid dimers and its role in cross linking

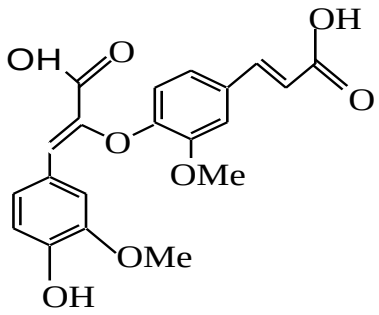
Ferulic acid is found covalently linked to the polysaccharides by ester bonds (Mueller Harvey et al., 1986) and to the components of lignin by ether or ester bonds (Scalbert et al., 1985). Upon saponification of the cell walls, five ferulate dimers (dehydrodiferulic acid, diFA) were released (in the order of abundance): 8-O-4'diFA[(Z)-β-{4-[(E)-2-carboxyvinyl]-2-methoxyphenoxy}-4-hydroxy-3-methoxycinnamicacid], 5-8'BenDi[trans-5-[(E)-2-carboxyvinyl]-2-(4-hydroxy-3-methoxyphenyl)-7-methoxy-2-3-dihydrobenzofuran-3-carboxylic acid], 5-5'diFA[(E,E)-4-4'-dihydroxy-5,5'-dimethoxy-3,3'-bicinnamic acid], 5-8'diFA [(E,E)-4-4'-dihydroxy-3,5'-dimethoxy-β-3'-bicinnamic acid] and 8-8'ArylD,[trans-7-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-6-methoxy-1,2-dihydronaphthalene-2,3-dicarboxylicacid] (Fig.3) (Bartolome et al., 1997). Peroxidase catalyses the oxidation of ferulic acid *in vivo*, resulting in the formation of ferulic acid dehydrodimers. It also catalyses the polymerization of the lignin precursors in to a wall bound polymer, that closely resembles lignin, believed to enhance the rigidity and strength of the cell wall (Ward et al., 2001). It is thought to participate in the regulation of cellular expansion, since it can be dimerised *in vitro* under oxidative conditions and thereby lead to crosslinks between cell wall polymers (Biggs and Fry, 1987). Such crosslinking, mediated by oxidation in the presence of peroxidases and hydrogen peroxide, may contribute to the control of cell growth (Ishii and Hiroi, 1990) and reduce cell wall digestibility (Borneman et al., 1992) by pathogens (Kondo et al., 1990) and ruminant secreted enzymes (Graham and Aman, 1984). In these reactions, the phenoxy radicals may react to form various dehydrodimer isomers like 5-5', 8-O-4', 8-5' and 8-8' (Ralph et al., 1994). In model

systems, using cell walls (Grabber et al., 1995) or feruloylated arabinoxylans (Figueroa-Espinosa and Rouau, 1998) the 8-5' derivative is preferentially formed, although other dimers are also observed. In maize cell walls, about 45% of the dehydrodimers are coupled by 8-5 linkages while 8-8', 5-5' and 8-O-4' coupled dehydrodimers each comprise 10-25% of the total (Grabber et al., 1995). Suspension cultured corn, cocksfoot and switch grass contain five dimers including 5-5'diFA, 8-O-4'diFA and 5-8'diFA (Ralph et al., 1994). A diferuloyl arabinoxylan hexasaccharide, containing 5-5' linked diferulic acid has been obtained by the enzymatic hydrolysis of bamboo shoot cell walls (Ishii, 1991). It has been claimed that phenolic dimers are the "molecular equivalent of spot welding a steel mesh frame" (Liyama et al., 1994).

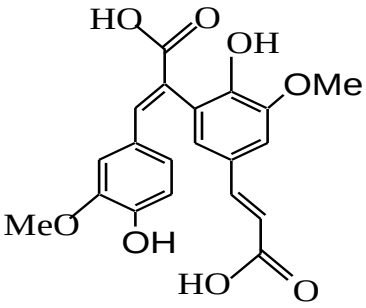
In maize, the diferulate content is 25 g/kg and in sugar beet pulp it is 1.7g/kg (Saulnier and Thibault, 1999) and in Chinese water chestnut, the diferulate level is 4.5g/kg (Parr et al., 1996). Crosslinking of maize cell wall polymers by esterified hydroxycinnamic acids, in particular by diferulate cross linkages has recently been proven to be a limiting factor, during enzymic dissolution of plant cell walls (Grabber et al., 1998). Indeed both the rate and extent of cell wall hydrolysis appear to decrease with an increased concentration of diferulate cross links.

Ferulic acid undergo transitions *in vitro* under the action of daylight, whereby they isomerise from the E-form(trans) to the Z-form (cis) and to a lesser extent in the reverse direction, to form an equilibrium mixture in which the (E)-form predominates (Hartley and Jones, 1975; Towers and Abeysekera, 1984). (E)-ferulic acid is also reported to dimerise under certain circumstances (Ford and Hartley, 1989). It is not absolutely clear whether these dimers are formed in the plant, by the action of light or are synthesized enzymatically. There is

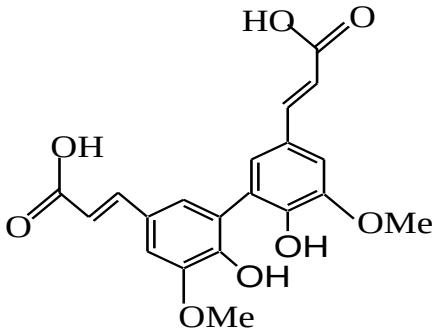
some evidence, that light may be directly responsible for the presence of (Z)-dimers in plant cell walls (Yamamoto and Towers, 1985) however, there is no conclusive evidence for this.



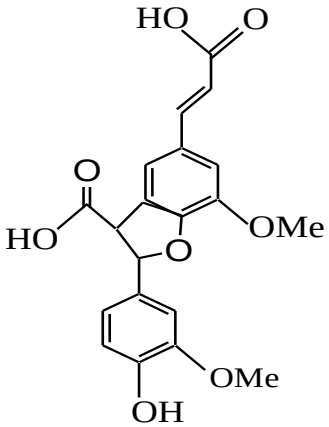
8-O-4' di FA



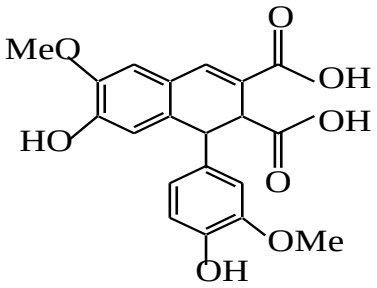
8-5' di FA



5-5'diFA



5-8' BenDi



8-8' Aryl D

Figure 1.3: Structure of the different dehydrodimers of ferulic acid, isolated from plant cell walls.

1.4 Additional roles of ferulic acid in plants and its degradation in the rumen

Ferulic acid plays an important role in the plant cell walls, including protection against pathogen invasion (Hartley and Jones, 1977). E-feruloyltyramine, a derivative of ferulic acid, is synthesized in response to wounding, and these restrict enzymic dissolution of the cell wall polymers (Pearce et al., 1998). Besides its inhibiting action on plant growth (Einhellig et al., 1985), ferulic acid can also inactivate plant viruses (Sridhar et al., 1979) and its direct toxicity can be used against insects and for many antibiotic purposes (Beschia et al., 1982; Cabrera et al., 1995).

1.5 Release of ferulic acid from natural sources by chemical hydrolysis

Plant cell walls may be exposed to extremes of pH to break certain linkages within the cell wall. Alkali treatment breaks the ester bond, linking phenolic acids to the cell wall. The total alkali extractable content of ferulic acid in barley spent grain is 0.32% (w/w dry weight), and in destarched wheat bran it is 1% (w/w dry weight) (Bartolome and Gomez–Cordoves, 1999). Acid treatment breaks the glycosidic bonds and solubilises the sugars but leave the ester bonds generally intact. These chemical treatments however require high concentrations of acid or base and such treatments lead to modifications of other components and often bring about unwanted chemical changes in the plant cell wall. A gentle and more specific method is to go for enzymatic hydrolysis.

Phenolic acids are common constituents of forage fed to ruminants. Particularly ferulic acid and p-coumaric acid, mainly in the trans configuration are abundant in graminaceous plants which represent a major component of the ruminant diets. These phenolic acids are released during ruminal degradation of plant cell walls (Akin, 1988). Since these compounds are inhibitory to ruminal microorganism's *in*

vitro (Akin et al., 1993; Borneman et al., 1986) and putatively inhibit the digestion of plant cell walls in the rumen by these microorganisms, they are considered an antinutritional factor in the forage. Therefore the modifications of ferulic and p-coumaric acids, by rumen microorganisms (Healey et al., 1980) can have an important antitoxicity role.

1.6 Feruloyl esterases and their substrate specificity

Phenolic acid esterases were first detected by Deobald and Crawford in 1987, when ferulic acid esterase was identified in the extracellular enzyme preparations from *Streptomyces viridosporus* (Deobald and Crawford, 1987). Cinnamoyl or feruloyl esterases or cinnamic acid hydrolases are a subclass of the carboxylic ester hydrolases (E.C 3.1.1.1) that hydrolyse the ester bond between hydroxy cinnamic acids like ferulic acid and the hemicellulose present in the plant cell walls, resulting presumably in an increased accessibility for the enzymatic attack on hemicellulose (Mackenzie et al., 1987).

Feruloyl esterases can be isolated from a wide range of microorganisms (Kroon and Williamson, 1996; Donaghy and Mc Kay, 1997) when they are grown, on complex substrates such as xylan, pectin, wheat bran or sugar beet pulp and recently these enzymes have been purified and partially characterized from *Streptomyces olivochromogenes* (Faulds and Williamson, 1991; 1993a) *Pseudomonas fluorescens* subsp.cellulosa (Faulds et al., 1995), *Neocallimastix* MC-2 (Borneman et al., 1992), *Penicillium pinophilum* (Castanares et al., 1992), *Schizophyllum commune* (Mackenzie and Bilous, 1988) and from several species of *Aspergillus* (Faulds and Williamson, 1994; Tenkanen et al., 1991).

Over recent years, the number of microbial cinnamoyl esterases identified has reached over 30, with 10 genes sequenced. Some esterases are part of a modular complex, (Laurie et al., 1997; Blum et

al., 2000a) contain cellulose binding modules (Kroon et al., 2000) or exist only as a catalytic domain (De Vries et al., 1997). A crystal structure of the cinnamoyl esterase domain of Xyn Z, from the anaerobic bacteria *Clostridium thermocellum* has been reported recently (Blum et al., 2000b). The primary structural analysis of FAE's has shown that they display α/β hydrolase fold, with an Asp/His/Ser catalytic triad at their active site (Kroon et al., 2000). The *Pseudomonas* esterase has been sequenced and shown to contain a cellulose binding domain, located at the N–terminus (Ferreira et al., 1993). Hermoso have described the first crystal structure of the non-modular type-A feruloyl esterase from *Aspergillus niger* (AnFaeA). Crystallographic and site directed mutagenesis studies identified the catalytic triad, (Ser133-His247-Asp194) that forms the catalytic machinery of this enzyme. The active-site cavity is confined by a lid (residues 68–80), on the analogy of lipases, and by a loop (residues 226–244) that confers plasticity to the substrate-binding site. The lid presents a high ratio of polar residues, which in addition to a unique N-glycosylation site stabilizes the lid in an open conformation, conferring the esterase character to this enzyme. Comparison with functionally related proteins points to a functional convergence after evolutionary divergence within the feruloyl esterase family. Its protein scaffold is based on an α/β hydrolase fold, and consists of a major nine-stranded mixed β-sheet, two minor two-stranded β-sheet arrangements and seven helices. In the central β-sheet system of nine strands, eight of them are ordered with respect to the sequence. Only the N-terminal strand (residues 2–4) which is next to the C-terminal strand (residues 224–227), breaks this pattern. The central five strands (β4–β8) are all parallel, while the two terminal pairs are antiparallel to the others. All the α–β connections are right-handed, which consequently generates at one face of the central β sheet, a series of linking helices and segments defining a complex structure. Three

disulphide bridges are located along the structure, generating a more compact fold. One of them, Cys 91–Cys 94, near the flap region (residues 68–80), generates an extremely tight turn as the two cysteine residues are only two residues apart. The bridge Cys 29–Cys 258 maintains the C-terminal end of the protein close to the structural core, creating a hydrophobic pocket in which the C-terminal Trp residue is buried. The last bridge, Cys 22–Cys 234, links the β9 to the extended C terminus. From the structure, it is evident that Cys 235 is not involved in disulphide bridge formation (Hermoso et al., 2004).

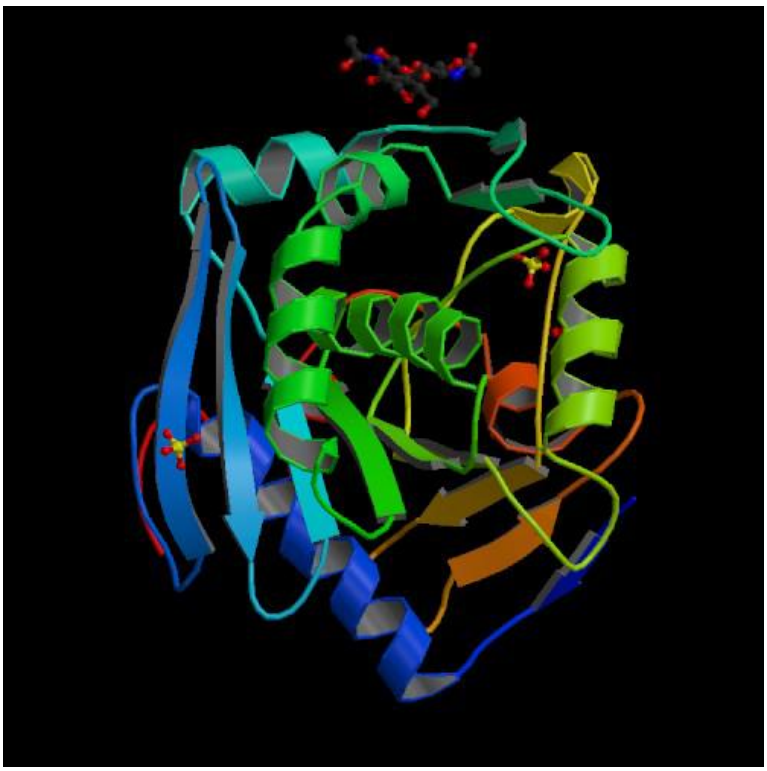


Figure 1.4: Crystallographic structure of feruloyl esterase from *Aspergillus niger* (Protein data bank code: 1USW)

1.6.1 Enzymes from *Aspergillus niger*

Various ferulic acid esterases (FAE) are produced in the presence of different substrates (Brezillon et al., 1996). FAE activity was detected only in the supernatants from cultures grown in the presence of lignocellulose derived carbon sources. FAE A from *A. niger* is able to release 5-5' diferulic acid from presolubilised barley, wheat bran and sugar beet pulp. It also releases 8-O-4 diferulate. Two *A. niger* esterases (FAE-I and II) were isolated from a commercial pectinase preparation (Table 1.1) (Faulds and Williamson, 1993b). Both of these were active on methyl ferulate, but only FAE-I was able to cleave the ester linkages present in sugar-beet as well as those found in wheat bran. FAE-II released more ferulic acid from destarched wheat bran than FAE-I, the inverse of the activity on methyl ferulate. This could be due

to the steric hindrance caused by the size of the enzyme. Each enzyme recognizes specific moieties on the phenolic ring and access to the feruloylated groups on the plant cell walls may depend on steric hindrance. FAE-II was specific for the feruloylated substrate FAXX (O-[5-O-(trans-feruloyl)-α-L-arabinofuranosyl]-(1-3)-O-β-D-xylopyranosyl–(1-4)-D-xylopyranose while the relative rate of hydrolysis of FAXX and PAXX (O-[5-O-trans-p-coumaroyl)-α-L-arabinofuranosyl]-(1-3)-O-β-D-xylopyranosyl–(1-4)-D-xylopyranose) by FAE-I were 3:1. Studies on the thermal and chemical stabilities of FAE-III from *A. niger* have shown that it is sensitive to guanidine hydrochloride unfolding and is most thermostable between pH 5 and 6 (Williamson and Vallejo, 1997). Methyl sinapate is a better substrate for FAE III than methyl ferulate. Growth of *A. niger* on oat spelt xylan, induces production of FAE-III, which is able to release ferulic acid from wheat bran derived feruloylated oligosaccharides and from methyl ferulate, but is inactive on sugar beet pulp derived feruloylated oligosaccharides and methyl caffeate (Faulds and Williamson, 1994). Although arabinoxylans in barley (Vietor et al., 1992), wheat bran (Stevens and Selvendran, 1988) and maize (Chanliaud et al., 1995) consist of β-(1-4) xylans in which xylose residues are substituted with arabinose at C-2, C-3 or both, the composition and structure are different. Less substituted xylan substrates (Destarched wheat bran<Barley spent grain<maize bran) are better substrates for FAE-III, either in the absence or presence of xylanase. Therefore substituents may restrict the accessibility of the esterase to the feruloyl groups (Sancho et al., 2001).

From these studies, it is clear that different enzymes are needed depending on the origin of the substrate (cereal or sugar beet pulp) since the nature of linkages between ferulic acid and sugar moiety depends on the source (Micard et al., 1994). However it has been

recently suggested that feruloyl esterases are able to release ferulic acid, from feruloylated arabinoxylan oligosaccharides and sugar beet pulp although the ester linkages of ferulic acid to the sugar moiety in arabinoxylan and pectin are different (Bonnin et al., 2002). Thus feruloyl esterase specificity, seem not to be related to the type of ester linkage in the carbon source and it is likely that the genes coding for polysaccharide degrading enzymes are co-regulated, and a low level of constitutive transcription produces enzymes that release cell wall polysaccharide fragments, which further promote the transcription of different genes coding for polysaccharide degrading enzymes, including feruloyl esterases.

The feruloyl esterase A (Fae A) encoding gene (fae A) from *Aspergillus niger* has been cloned and induction of this gene has been studied at the protein level (De Vries et al., 1997). Growth on crude substrates like sugar beet pulp resulted in higher extracellular Fae A levels than growth on xylan or pectin, possibly due to the presence of easily metabolisable compounds in the crude substrates, resulting in enhanced growth. However, when ferulic acid was added to oat spelt xylan, Fae A activity was even higher than on the crude substrates. fae A expression in *A. niger* is affected by at least three regulatory systems: a xylan/xylose specific system for induction (Xln R); a ferulic acid specific system for induction and carbon catabolite repression mediated by CreA (De Vries and Visser, 1999). A heterologous expression system using the cDNA encoding *A. niger* cinnamoyl esterase (faeA) has been established in the yeast *Pichia pastoris*. This organism possesses a number of attributes that render it an attractive host, for the expression and production of cinnamoyl esterase. It can be grown conveniently to high density levels in a simple and inexpensive medium, it is able to carry out certain post translational modification events such as proteolytic maturation, glycosylation and disulfide bond formation and

most importantly it can secrete proteins to very high levels, under the control of the efficient and highly regulated promoter of the alcohol oxidase gene, AOX (Hollenberg and Gellissen, 1997; Cereghino and Cregg, 2000). Moreover *Pichia* does not secrete any endogenous esterase in the medium. Heterologous expression of fae A will enable the production of sufficient quantities of a single enzyme for structural and biotechnological studies (Juge et al., 2001). The fae A gene has been expressed in both *A. niger* and *Aspergillus tubingensis*, however very low levels of secretion were obtained using these systems mainly due to proteolytic degradation of the recombinant enzyme in the medium (De Vries et al., 1997).

1.6.2 Enzymes from other sources

A further example of substrate selectivity is a feruloyl esterase from *Penicillium pinophilum*, which was found to release ferulic acid from wheat bran substrate but not from rye grass (Castanares et al., 1992). The esterase from *Pseudomonas fluorescens* was shown to have a molecular weight of 59,000 and liberated ferulic acid from destarched wheat bran in the presence of endoxylanases (Ferreira et al., 1993). *Talaromyces stipitatus* grown on wheat bran or sugar beet pulp was able to produce Type-A (Ts FaeA) and Type-B (TsFaeB) feruloyl esterases with activity profile, similar to those obtained for the characterized esterases of *Aspergillus niger* in addition to another esterase Ts FaeC distinct from Type-A and Type-B classes of feruloyl esterase by a broader substrate specificity (Garcia-Conesa et al., 2004).

The direct action of pure ferulic acid esterase on the raw material is much less than on the soluble substrates, implying that an extensive degradation of the cell wall polysaccharide is first required. The specificities of the enzymes for the methyl esters of cinnamic acids, acting as model substrates show that the *Pseudomonas* esterase and two isoforms from *Aspergillus niger* (Faulds and Williamson, 1993a) are

specific for compounds with different substitutions on the phenolic ring of the cinnamic acid. Furthermore, these enzymes could not hydrolyse the methyl esters of benzoic acids, (Vanillate and syringate) showing the importance of the C=C bond in the recognition of the substrates. The distance between the phenolic ring and the ester bond and the number and position of methoxy and hydroxyl substitutions on the benzene ring are critical in determining the catalytic efficiency of ferulic acid esterase (Kroon et al., 1997). The apparent affinity of feruloyl esterases for cinnamate substrates is greatly enhanced, when the cinnamic acid is esterified to sugars compared to alkyl esters (Williamson et al., 1998).

Table 1.1

Properties of ferulic acid esterases

Source	Enzyme	M_r. (KDa)	pI	Action	Reference
A. niger	FAE- I	63[a]	3.0	Acts on both O-2 feruloylated arabinose and O-6 ferulolytaed galactose & also on 1-5 linked arabinose; Active on methyl ferulate, methyl coumarate and methyl caffeate	Faulds and Williamson, 1993b; Ralet et al., 1994
A. niger	FAE-II	29[a]	3.6	Acts on O-5 feruloylated arabinose ;Active on methyl ferulate, methyl sinapate and methyl coumarate.	Faulds and Williamson, 1993b
A. niger CBS 120.49	FAE-III	36[a]	3.3	Specific for 1-5linked ferulic acid ,Methyl sinapate preferred over methyl ferulate ; Inactive on methyl caffeate	Faulds and Williamson, 1994;1995; Micard,Renard and Thibault, 1994.
S.olivochromogenes	FE	29[a]	7.9; 8.5	Acts on methyl ferulate ,methyl sinapate and methyl coumarate	Faulds and Williamson, 1991.
A. niger CS -180	CinnAE	145[b]	4.8	Acts on O-2 feruloylated arabinose	Kroon, Faulds and Williamson, 1994
A. oryzae VTT-D-85248	FE	30[a]	3.6	Acts on methyl ferulate and methyl sinapate	Tenkanen et al., 1991
A. awamori	FE	35[a]	3.8	Acts on α- napthyl esters	Koseki et al., 1998
Neocallimastix strain MC-2	FAE-I	69[a]	4.2	Active on FAXX and PAXX	Borneman et al., 1992
	FAE-II	24[a]	5.7	Specific for FAXX	Borneman et al., 1992;1993
Streptomyce avermitilis UAH-30	FAE	-	-	Acts on destarched wheat bran, oat spelt xylan and sugarcane bagasse	Garcia et al., 1998
Penicillium pinophilum CMI 87160ii	FE	57[a]	4.6	Acts on methyl ferulate, methyl coumarate and wheat straw xylan.	Castanares, Mc Crae and Wood, 1992

[a] Determined by SDS -PAGE
[b] Determined by Native PAGE

1.6.3 Synergistic interactions between enzymes

Feruloyl esterases act synergistically with other hemicellulases such as xylanases and pectinases to maximize the microbial degradation of plant cell walls (Brezillon et al., 1996). A battery of enzymes such as endoxylanases, β-xylosidases, α-arabinofuranosidases and esterases are involved in the degradation of arabinoxylans (Coughlan et al., 1993). The presence of main chain polysaccharide hydrolases, such as arabinofuranosidases and xylanases [β-(1-4) D-xylan xylanohydrolases] (EC 3.2.1.8) enhance the action of side chain acting hydrolases such as α-glucuronidases, acetyl xylan esterases and feruloyl esterases. Thus a reciprocal synergistic interaction occurs during xylanase catalysed breakdown of polysaccharides, in to low molecular weight fragments suitable as esterase substrate and also the esterase releases phenolic groups, facilitating the accessibility of xylanase to the xylan backbone (Bartolome et al., 1995). This type of bi-product heterosynergy occurring between a main chain and an accessory enzyme has been reported previously between ferulic acid esterases and xylanases from *Schizophyllum commune* (Mac Kenzie and Bilous, 1988), *Streptomyces olivochromogenes* (Faulds and Williamson, 1991), *Aspergillus awamori* (Mc Crae et al., 1994), *Pseudomonas fluorescens* (Faulds et al., 1994) and in particular between *A. niger* FAE–III and *Trichoderma viride* xylanase (Faulds and Williamson, 1994). On a laboratory scale, 95% of the total ferulic acid was released from wheat bran with FAE III and endoxylanase from *Trichoderma viride* (Faulds and Williamson, 1995). All the xylanases showed less activity on destarched wheat bran, than on oat spelt xylan, owing to the more branched and complex structure of the former. However, the ratio of the activity on the two substrates were relatively small for the *T. viride* xylanase, which may explain why this particular xylanase produced the highest level of destarched wheat bran hydrolysis compared to the other

xylanases (Bartolome et al., 1995). The yield of ferulic acid was highly dependent on the source of xylanase. Ferulic acid release increased as a function of endoxylanase concentration. The incubation of wheat and barley cell walls with ferulic acid esterase from *Aspergillus niger* (FAE-III) or *Pseudomonas fluorescens* (XylD) together with either xylanase I from *A. niger* or *Trichoderma viride* xylanase lead to the release of the ferulate dimer 5-5' diFA (Bartolome et al., 1997). The relative rates of hydrolysis of soluble low molecular weight feruloylated compounds by the XylD esterase, when studied in the absence of endoxylanase showed that the (1-5) linked substrate (Ara,F (1-5 linked) was hydrolysed 6.5 fold more rapidly than the corresponding (1-2 linked) substrate (Faulds et al., 1995).

Free ferulic acid induces *A. niger* and *Neocallimastix* cinnamoyl esterases (Faulds, et al., 1997). In both *A. niger* and *Streptomyces avermitilis* (Garcia et al., 1998) feruloyl esterase and xylanase induction appear to be under different regulatory mechanisms. In the case of A. *niger*, free ferulic acid inhibited the production of xylanase while inducing the feruloyl esterases. Ferulic acid esterase activity in *Streptomyces avermitilis* could be detected only in cultures supplemented with commercial xylanase preparation (Garcia et al., 1998).

1.6.4 Plant sources of feruloyl esterases

Plant enzymes may be an alternative to the use of microbial enzymes, in the release of ferulic acid from cell wall materials, in situations where the use of microbial enzyme may be undesirable. Cinnamoyl esterase activity has recently been detected in malt (Bartolome et al., 1996) and barley grains (Sancho et al., 1999). The crude barley extract exhibited activity against the methyl esters of ferulic, sinapic, p-coumaric and caffeic acid and the feruloylated disaccharide Ara_2F (O-[2-O-(transferuloyl)--L-arabinofuranosyl]-(1-5)-

L-arabinofuranose). Xylanase activity seems not to be present in the stored grain, but it is induced during germination (Slade et al., 1989).

1.7 Applications of ferulic acid

1.7.1 In food, health and medicine

Functional foods enriched in biologically active compounds are becoming increasingly available in many countries and the potential markets are enormous. Some Chinese herbal medicines, such as *Angelica sinensis* contain very high levels of ferulic acid (Wang and Peng, 1994) although the health benefits of hydroxycinnamates are as yet unproven in western medicine. Ferulic acid and isoferulic acid are active components of the rhizome of *Cimicifuga* species, used as anti-inflammatory drugs in Japanese oriental medicines (Hirabayashi et al., 1995). Ferulic acid is one of the bioactive components in *Carpobrotus edulis* (Sour fig) with antibacterial activity against *Bacillus subtilis* and *Streptococcus pneumoniae* (Van der Watt and Pretorius, 2001).

Ferulic acid is also reported as antihepatotoxic (Kiso et al., 1983) and offers various benefits for the cardiovascular system (Rukmini and Reghuram, 1991). Apart from its anti-inflammatory action (Chawla et al., 1987) ferulic acid as a constituent of synthetic lignins, may contribute to the defense against viral infections including AIDS (Lai et al., 1992; Nakashima et al., 1992). By antibody sandwich enzyme linked immunosorbent assay, it was found that ferulic acid and isoferulic acid can suppress the production of interleukin-8 (IL-8) which is the main cause of the local accumulation of neutrophils and modulates various inflammatory reactions. This may open a new approach to the treatment of chronic inflammatory diseases (Chawla et al., 1987).

The antioxidant activity of ferulic acid is well recognized (Castelluccio et al., 1995; Rice Evans et al., 1996; Graf, 1992; Scott et al., 1993). Topically applied antioxidant drugs, represent a successful strategy for protecting the skin against UV mediated oxidative damage.

However they can afford to the skin, a satisfactory photoprotection only if they are able to permeate through the stratum corneum, and thus reach deeper cutaneous layers. Ferulic acid is a good candidate for successful employment as topical protective agent against UV radiation induced skin damage. Ferulic acid dissolved in saturated aqueous solutions at pH 3 or 7.2 was found to permeate through the stratum corneum (Saija et al., 2000) and the capability of ferulic acid to permeate through the skin is justified by its higher lipophilicity (Shahrzad and Bitsch, 1996). It efficiently protects human phosphatidyl choline liposomal membranes from UV mediated oxidative damage (Saija et al., 1998). Furthermore it exhibits scavenging activity against nitric oxide secreted by the human skin keratinocytes in response to UV A and UV B radiation. The nitrite scavenging property of wheat bran was found to be due to the ferulic acid content (Moller et al., 1988). Ferulic acid reduces carcinogen-DNA adduct formation in cultured cells (Wargovich et al., 1985).

Ferulic acid acts as an effective electrophilic trapping agent (Newmark, 1987) and inhibits the occurrence of pulmonary cancers in mice (Lesea, 1983). It also exhibit inhibitory effect on 4- nitro quinoline oxide (4-QO) induced rat tongue carcinogenesis (Tanaka et al., 1993). Studies on the chemopreventive potential of ferulic acid on azoxymethane (AOM) induced colon carcinogenesis showed that ferulic acid increase the activities of detoxifying enzymes such as glutathione S transferase (GST) and quinone reductase (QR) (Kawabata et al., 2000). The redox potential of ferulic acid is lower than that of oxyradicals, such as the hydroxyl and superoxide radical which means that they are excellent scavengers of these oxyradicals (Wang, 1993). There are several mechanisms by which phenolic compounds exert biological activity. The most commonly cited mechanism is the binding of phenolics to the receptors (Zhu et al., 1997).

Thermal degradation of polysaccharide gums and starches can be an important factor in the texture of food products. This degradation can occur at neutral pH values and is thought to be due to oxidative-reductive depolymerisation (ORD). Ferulic acid protects guar gum and cassava starch from thermal degradation due to depolymerization by its ability to terminate free radical chain reactions (Hill and Gray, 1999).

Phenolic acids derived from plant cell walls have long been used as food preservatives to inhibit microbial growth (Davidson and Branen, 1981). The ability of ferulic acid to inhibit the peroxidation of fatty acids permits it to be utilized as a natural food preservative (Graf, 1992).

Ferulic acid rich pectic polysaccharides have been shown to undergo gelation in the presence of peroxidase and H_2O_2 (Thibault et al., 1991). Similarly an arabinoxylan ferulate polysaccharide can be converted with peroxidase and hydrogen peroxide, under controlled conditions to thermostable (sterilisable) cold-setting clear brittle gels (Greenshields and Rees, 1992). The properties of these natural biogels are such that they are of interest to the pharmaceutical and food industries in the development of new and diverse products. It also is a part of the gel matrix of wound dressings, in a chemical form similar to the diferuloylated cross-links between arabinoxylan polymers in the cell walls (Kennedy et al., 1999).

1.8 Potential uses of feruloyl esterases

After the traditional processing of cereal grains and sugar-beet roots, ferulic acid is recovered in the brans and in the pulp. The feruloylated polysaccharides, account for a large part of these cheap and abundant by-products, which are mainly used as cattle feed. In processes such as the manufacture of animal feed, where maximum degradation of cell wall is needed, the ferulic acid esterases can act as accessory enzyme to increase the total yield of sugars.

Feruloyl esterases enhance paper pulping process by removing substitutions and linkages, between the polymers during pulping, thus making the solubilisation of lignin-carbohydrate complexes easier (de Graaff et al., 1992). Esterases have potential as analytical aids in modern carbohydrate chemistry, and will provide tools in understanding the fine structure and linkage patterns which exist in the plant cell wall (Williamson et al., 1998). Ferulic acid is postulated to form cross links with the proteins in wheat (Hoseney and Faubion, 1981) which is important in the rheology of doughs and so the catalytic activity of cinnamoyl esterases may play a role in the fermentation and baking process.

Ferulic acid esterase from *Humicola insolens* has been found to successively catalyse the transesterifications of secondary alcohols with high enantioselectivity and may eventually lead to the use of this enzyme as a potent catalyst in stereoselective organic synthesis (Hatzakis et al., 2002).

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CHAPTER II

STUDIES ON THE PRODUCTION OF FERULOYL ESTERASE FROM CEREAL BRANS AND SUGAR CANE BAGASSE BY MICROBIAL FERMENTATION

2.1 Introduction

Plant cell walls constitute the largest source of renewable energy on earth. Ferulic acid is the most abundant hydroxycinnamic acid, present in the cell walls of several plants and found covalently linked to polysaccharides by ester bonds and to components of lignin through ether linkages (Jeffries, 1990). It plays an important role in maintaining the structural integrity of the plant cell wall matrix and represent factors which partially limit the biodegradability of non-lignified plant cell wall polysaccharides (Akin et al., 1990; Hartley et al., 1992). The high level of ferulic acid hydrolysis from plant cell wall materials, rich in this acid such as cereal brans (Faulds and Williamson, 1995; Faulds et al., 1995) or sugar beet pulp (Micard et al., 1994) would provide a sufficient natural source of ferulic acid. Feruloyl esterases, (FAE; E.C. 3.1.1.73) (Ralph et al., 1995) a subclass of the carboxylic acid esterases (E.C. 3.1.1.1) are involved in breaking the bond between arabinose and ferulic acid, releasing the covalently bound lignin from hemicelluloses (Williamson et al., 1998). They act as accessory enzymes for the complete saccharification of plant cell wall hemicelluloses. Feruloyl esterases, which cleave the ester cross-linkages and result in the subsequent release of free ferulic acid, were first described by Mac Kenzie et al. in 1987. Various ferulic acid esterases are produced in the presence of different growth substrates (Brezillon et al., 1996; Bartolome et al., 1995).

The characterisation of these enzymes may find use in many areas including animal nutrition, the pulp and paper industry and may facilitate the extraction of plant material and the textural modification of food products (Hoseney and Faubion, 1981). Feruloyl esterases can be used to process waste agricultural materials in to valuable products. A number of industrial and food applications have been reported for ferulic acid based on its microbial degradation to

vanillin (Gross et al., 1991) and its antioxidant properties (Graf, 1992). Ferulic acid derived from plant cell walls has long been used as food preservatives to inhibit microbial growth (Kroon and Williamson, 1999; Borneman et al., 1986). Production of vanillin through biocatalytic route is an attractive option, being the world's principal flavoring compound used extensively in the food industry.

2.2 Materials and methods

2.2.1 Isolation and maintenance of the microorganisms

Microorganisms from wheat straw and decaying plant barks were serially diluted and plated on to mineral media (Donaghy et al., 1998) of neutral and acidic pH containing wheat bran as the main carbon source. The microorganisms capable of utilizing bran as the sole carbon source were selected for further studies. The isolated pure cultures were maintained on potato dextrose agar and chloramphenicol rose bengal agar slants of pH 4 as majority of them were fungal cultures.

2.2.2 Strains and culture conditions employed for fermentation studies

Out of the ten isolated strains, the strains designated JCS-1, JCS-2, JCS-3, JCS-4, JCS-5 and JCS-6 isolated from mineral media plates containing wheat bran as the main carbon source were used for screening studies. The known strains employed were *Aspergillus flavipes*, *Phanerochaete* and *Trametes* sp. known to be producers of cellulolytic, ligninolytic and oxidative enzymes. They were grown on Czapek-Dox modified media ($NaNO_3$ - 6g/l, $MgSO_4$ - 0.5g/l, KCl - 0.5 g/l, KH_2PO_4 - 0.5g/l, $FeSO_4$ - 0.1 g/l) of p^H 6 with 1% (w/v) pretreated (destarched and defatted) plant cell wall preparations such as wheat bran/ maize bran/ rice bran/ sugar cane bagasse at an agitation speed of 120 rpm and a temperature of 28 ± 2ºC. A 48-hour old culture was used as the pre-inoculum in all the cases. The strains were maintained on Chloramphenicol Rose bengal agar slants.

2.2.3 Chemicals

α-Amylase (Termamyl 120 L) and glucoamylase (Amyloglucosidase) was procured from NOVO, Bagsvaerd, Denmark). Bovine serum albumin was purchased from Sigma, St.Louis, USA. Ethyl Ferulate was obtained from Apin Chemicals, Oxon, U.K.

2.2.4 Methods

2.2.4.1 Pretreatment of the growth substrates

The plant cell wall preparations were procured locally and subjected to destarching and defatting.

Destarching was achieved by treating the growth substrates (100g) with 1 ml of α-amylase (E.C 3.2.1.1) in 1 litre of distilled water containing 50 ppm of calcium, at p^H 6.5 for 2 hours and temperature of 90-95ºC followed by 1 ml of glucoamylase (E.C 3.2.1.3) at p^H 4.5 and 60ºC for 1 hour. The solution was then centrifuged at 4000 rpm for 15 minutes. The residue was repeatedly washed with warm water, centrifuged and oven dried at 40°C.

Defatting was achieved by treating the residue with hexane in a soxhlet extractor at 70°C for 6 hours. They were then ground and sieved to pass through a 425 μm mesh standard sieve.

2.2.4.2 Preparation of ferulic acid methyl ester

The methyl ester of ferulic acid, was synthesised by the method of Borneman (Borneman et al., 1990) slightly modified as follows. Ferulic acid (1.0 g) (Sigma, U.S.A) was dissolved in 10 ml dry methanol and six drops of conc. H_2SO_4 were added to it. The mixture was refluxed along with stirring in a soxhlet extractor containing activated molecular sieves (4 Aº) for six hours. About 2-3 ml of 6% sodium bicarbonate was added to the cooled reaction mixture until it became turbid and then the aqueous solution was extracted in a separating funnel, with freshly distilled diethyl ether (4 x 25 ml). The combined ether layers were neutralized by water wash and dried over activated sodium sulfate and

the solvent was evaporated off. The residue was recrystallised from ethyl acetate-petroleum ether mixture to give methyl ester at yields greater than 90%.

2.2.4.3 Preparation of dry methanol

Laboratory reagent grade methanol was used for the preparation of dry methanol by refluxing the methanol with freshly ignited and cooled calcium oxide followed by distillation. To the distillate was added magnesium turnings and iodine for activating the magnesium. The resulting yellowish brown solution was warmed for some time until the iodine was completely removed. Heating was continued until the magnesium was converted to methanolate, followed by refluxing and distilling to get dry methanol which was then stored on activated zeolites of 4 A° pore size (Brian et al., 1996).

$Mg + 2\ CH_3OH \rightarrow H_2 + Mg(O\ CH_3)_2$

$Mg(O\ CH_3)_2 + 2\ H_2O \rightarrow Mg(OH)_2 + 2\ CH_3OH$

2.2.4.4 Purification and analysis of methyl ferulate

The conversion of ferulic acid to methyl ferulate was confirmed by thin layer chromatography. The methyl ester was purified by silica gel column chromatography using a glass column of inner diameter 2.2 cm and length 30 cm. The ester was eluted out in a system of hexane and ethyl acetate and analysed by ^1H NMR Spectroscopy. NMR Spectra was recorded on a 300 MHz high field Spectrometer (Bruker Advance DPX 300) and tetramethyl silane was used as the internal standard. A homogenous suspension of the ester was prepared in deuterated chloroform and a 10 ppm scan width was used.

2.2.4.5 Determination of λ_{max} of ferulic acid and methyl ferulate

The absorption spectra of ferulic acid and methyl ferulate in 100 mM Morpholinopropane sulphonic acid buffer (MOPS buffer) and 0.1M glycine-NaOH buffer, pH 10 at a concentration of 33μM was recorded in the range 260-400 nm and their absorption maxima were determined.

2.2.4.6 Isomerisation and solubility studies and of ferulic acid

Trans-cis isomerism of hydroxycinnamic acids occur under UV irradiation and TLC was used for the separation of cis and trans-cinnamic acid derivatives. 0.25mm thick silicagel 60 F_{254} plates were used for the identification of the isomers by employing different solvent systems. The solubility of ferulic acid in different solvents in the eluotropic series was also studied.

2.2.4.7 Enzyme assay

Feruloyl esterase activity against methyl ferulate (MFA) was determined spectrophotometrically. After appropriate inoculation time, the cultures were centrifuged at 8000 rpm at 4°C for 15 minutes and the supernatant was used for the assays. The enzyme activities (U/ml) were assayed in triplicate and are expressed as μmol of ferulic acid released per minute at 37ºC and pH 6. The assay mixture used to measure free ferulic acid released from methyl ferulate consisted of a 33μM solution of methyl ferulate in dry methanol as the substrate, 20 μL of culture filtrate as the enzyme source and citrate buffer (0.05 M) of pH 6. The reaction mixture was incubated for 10 minutes at 37ºC and change in absorbance was monitored at 286 nm against a free acid standard in a UV-Visible Spectrophotometer (UV 2001 Shimadzu, Japan). Total protein was determined by Lowry's method using standard Bovine serum albumin for the calibration curve (Lowry et al., 1951).

2.2.4.8 Selection of microbial strains for ferulolyl esterase production based on agar plate assay method and by submerged fermentation.

The isolated strains designated as JCS-1, JCS-2, JCS-3, JCS-4, JCS-5 and JCS-6 as well as the known strains *Aspergillus flavipes, Phanerochaete* and *Trametes* sp. were screened for maximum enzyme production by submerged fermentation in Czapek Dox modified media containing 1% pretreated wheat bran as carbon source

over a 10-day period. The effect of the other pretreated growth substrates like maize bran, rice bran and sugar cane bagasse were also studied. Agar plate assay was performed using mineral media of the following composition: $(NH_4)_2SO_4$ - 1.3g/l, $KH_2PO_4^-$ 0.39g/l, $MgSO_4^-$ 0.25g/l, $CaCl_2.2H_2O$- 0.07g/l, $FeCl_3$ -0.03g/l, Yeast extract-1g/l, Agar-20g/l and pH 6.0. Ethyl ferulate (0.1 % w/v) from a stock solution of 10% (w/v) in dimethyl formamide was used as the sole carbon source and the diameter of the halo zones in the agar plate were measured.

2.3 Results and discussion

2.3.1 Synthesis of methyl ferulate and identification by ^{1}H NMR

A 7:3 (v/v) system of hexane and ethyl acetate gave the best separation of the compounds in silica gel plates. The ester was eluted out in a 6.8:3.2(v/v) system of hexane and ethyl acetate. The ester having less polarity than the acid gets separated well from the acid on using the appropriate combination of solvents.

The chemical shift values in ^{1}H NMR are reported in parts per million relative to tetra methyl silane as internal standard (Fig. 2.1). The methyl ester was compared to the published spectra of the free acid (Pouchert, 1976). The methoxy peak characteristic of methyl ester was obtained as a singlet at δ 3.93 and the other ring methoxy group also as a singlet at δ 3.92 ppm. The presence of a pair of down field doublets at δ 7.5 and 7.6 and δ 6.2 and 6.3 confirmed the presence of vinylic protons. The phenolic hydroxyl group was observed as a singlet at δ 5.8 and aromatic protons in the range 6.5 to 7.5.

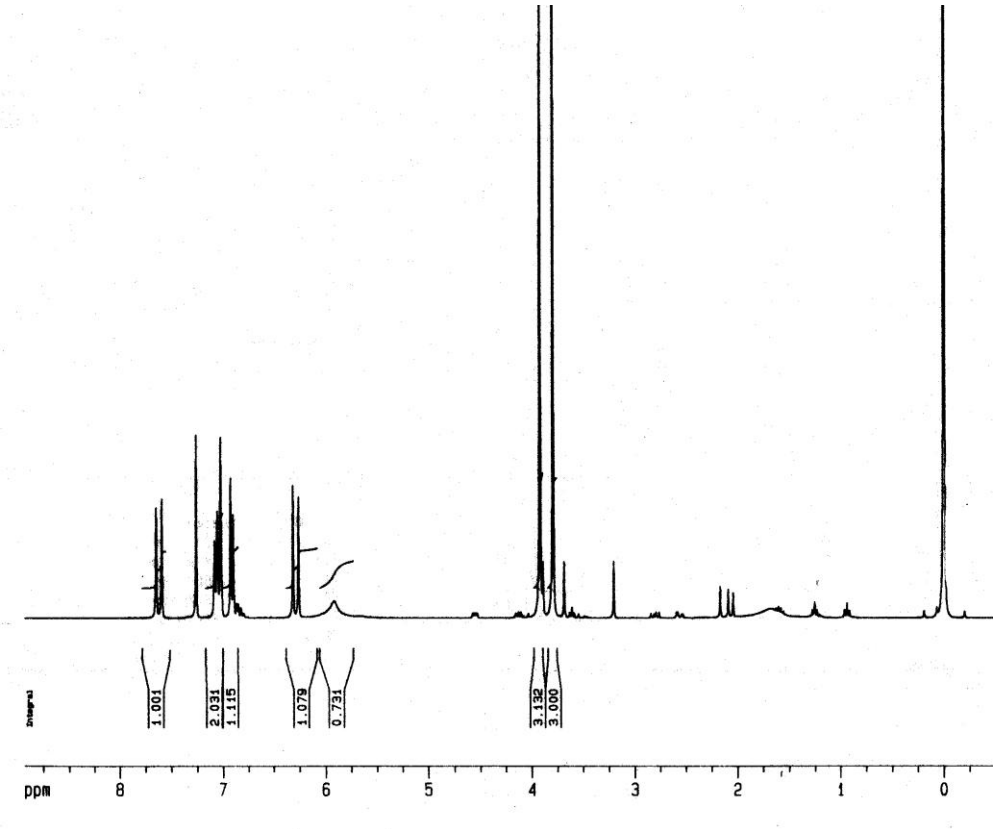

Figure 2.1: ^{1}H NMR Spectra of methyl ferulate in deuterated chloroform at 300 MHz.

2.3.2 Absorption maxima of ferulic acid and methyl ferulate

Ferulic acid has two pKa values of 4.6 and 9.4 (Henriken et al., 1999). Ferulic acid showed two absorption maxima, one at 286 nm and another at 310 nm at pH 6. At pH 10, the absorption maxima of free ferulic acid showed a hyperchromic shift towards 345 nm corresponding to the formation of sodium ferulate. Methyl ferulate gave absorption maxima at 322 nm and a shoulder at 300 nm at pH 6 while at pH 10, the absorption maxima of methyl ferulate shifted towards 375 nm (Fig. 2.2 and fig. 2.3) in accordance with published data (Faulds and Williamson, 1994). Absorption spectra of ferulic acid at neutral and alkaline pH showed different positions of absorption maxima in relation to the pH values and the ionic state of ferulic acid. Esters of ferulic acid have been

shown to absorb at longer wavelengths (Fry, 1982; Hanson and Zucker, 1963) compared to the free ferulic acid.

Phenolics absorb UV light in the wavelength range 250-380 nm. The absorption maxima and shape of the spectrum depends on the chemical nature of the phenolic compound and pH of the solvent. Simple phenolics have absorption maxima at about 250-280 nm at pH 7 and at 277-300 nm in alkali due to the ionization of the phenolic OH group. Phenolics with conjugated side chains such as cinnamic acid derivatives absorb at longer wavelengths shifting to 330-380 nm on addition of alkali (Kennedy et al., 1999). The extinction coefficient of ferulic acid and methyl ferulate in 100mM MOPS buffer, pH 6 were 8121.2 $M^{-1}cm^{-1}$ and 20,300 $M^{-1}cm^{-1}$ while that in 0.1 M glycine-NaOH buffer, pH 10 was 30,240 and 41, 390 respectively.

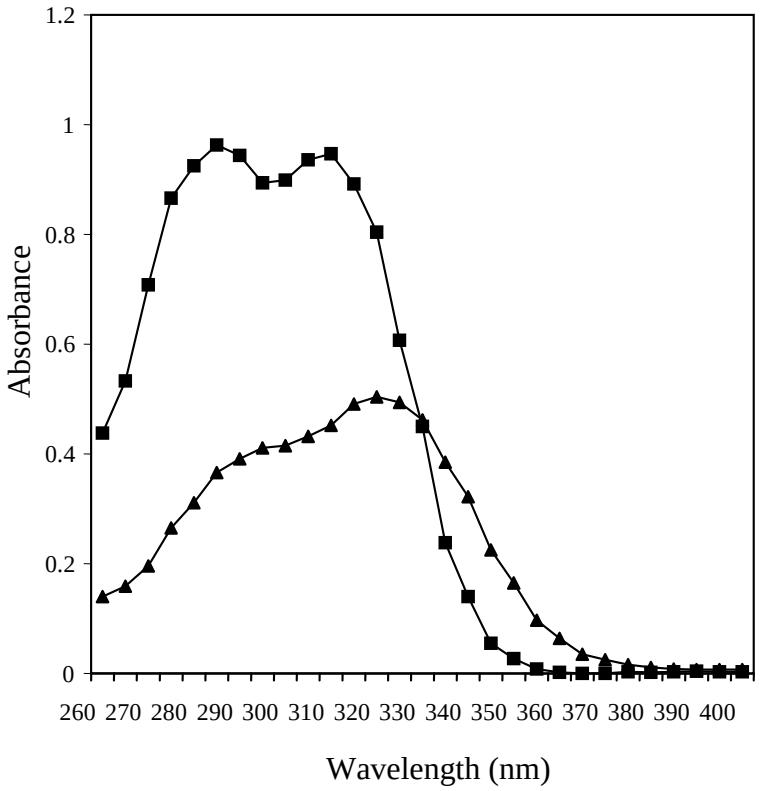

Figure 2.2: Absorption spectra of ferulic acid (■) and Methyl ferulate (▲) in MOPS buffer, pH 6 in the wavelength range 260-400 nm.

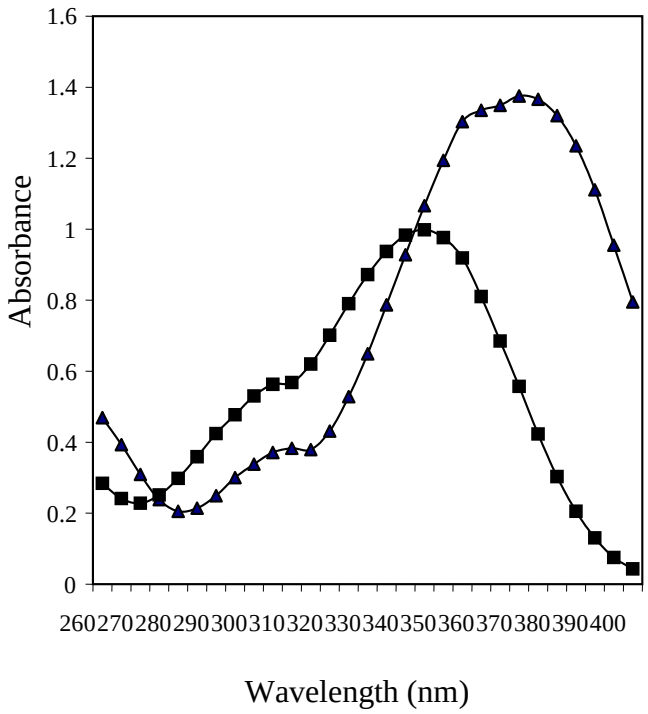

Figure 2.3: Absorption spectra of ferulic acid (■) and methyl ferulate (▲) in glycine-NaOH buffer, pH 10 in the wavelength range 260-400 nm.

2.3.3 Isomerisation and solubility profile

TLC helped to separate the two isomers. The cis form being a considerably less polar molecule than the trans-form had lower R_f value (Hartley and Jones, 1975). The cis-trans isomerism is favored towards the trans side. The Rf values for trans and cis ferulic acid were 0.40 and 0.33 respectively.

Ferulic acid being a highly polar hydroxycinnamic acid failed to solubilise in non polar solvents namely hexane (0.0), n-pentane (0.0) and iso-octane (0.1). It dissolved completely in polar solvents like methanol (6.6), ethanol (5.2), acetonitrile (5.9) and acetone (5.1) while the solubilisation was partial in solvents of medium polarity like

dichloromethane (3.4), chloroform (4.4), 1,4 dioxan (4.8) and dimethyl sulphoxide (4.8). The poor solubility of ferulic acid in the solvents lower down in the eluotropic series limits it use in enzyme mediated esterification reactions in non polar solvents.

2.3.4 Screening studies

The formation of a clearing zone around the point of inoculation indicated feruloyl esterase production in the plate assay (Donaghy et al., 1998). The isolated strain, JCS-3 gave the maximum halo zone which measured to 20 mm.

The screening studies (Fig. 2.4) showed that when grown on pretreated wheat bran, the isolated strain JCS-3 showed maximum specific activity for feruloyl esterase on the eighth day (10.80 U/mg protein) and maximum enzyme activity (6.54 U/ml) on the second day. Among the known strains *Aspergillus flavipes* exhibited the highest specific activity value on the sixth day (10.57 U/mg protein) with a corresponding enzyme activity maximum of 6.82 U/ml, closely followed by *Trametus* sp. with a specific activity maximum of 9.17 U/mg protein and corresponding enzyme activity maximum of 5.29 U/ml on the first day itself. Among the isolated strains JCS-3 was closely followed by JCS-2 with a specific activity maximum of 9.53 U/mg protein and a corresponding enzyme activity maximum of 7.07 U/ml on the second day following inoculation. JCS-4 showed maximum specific activity for the enzyme on the fifth day (9.46 U/mg protein) and an enzyme activity maximum of 6.98 U/ml on the sixth day followed by JCS-6 with maximum enzyme activity on the ninth day (5.32 U/ml) and specific activity maximum also on the same day (8.34 U/mg protein).

Hence further screening studies were confined to *Aspergillus flavipes* and JCS-3 on other pretreated growth substrates like maize bran, rice bran and sugar cane bagasse. Screening studies of *Aspergillus flavipes* and JCS-3 on pretreated maize bran, rice bran and

sugar cane bagasse showed JCS-3 to be a slightly better strain for the production of feruloyl esterase with regard to specific activity. On maize bran *Aspergillus flavipes* showed maximum specific activity (18.38 U/mg protein) and maximum enzyme activity on the third day (4.47 U/ml) compared to wheat bran having maximum specific activity on the second day (18.3 U/mg protein) with an enzyme activity maximum of 8.76 U/ml. When grown on sugar cane bagasse the maximum specific activity recorded was 24.84 U/mg protein on the fourth day and a corresponding enzyme activity of 6.15 U/ml (Fig. 2.5).

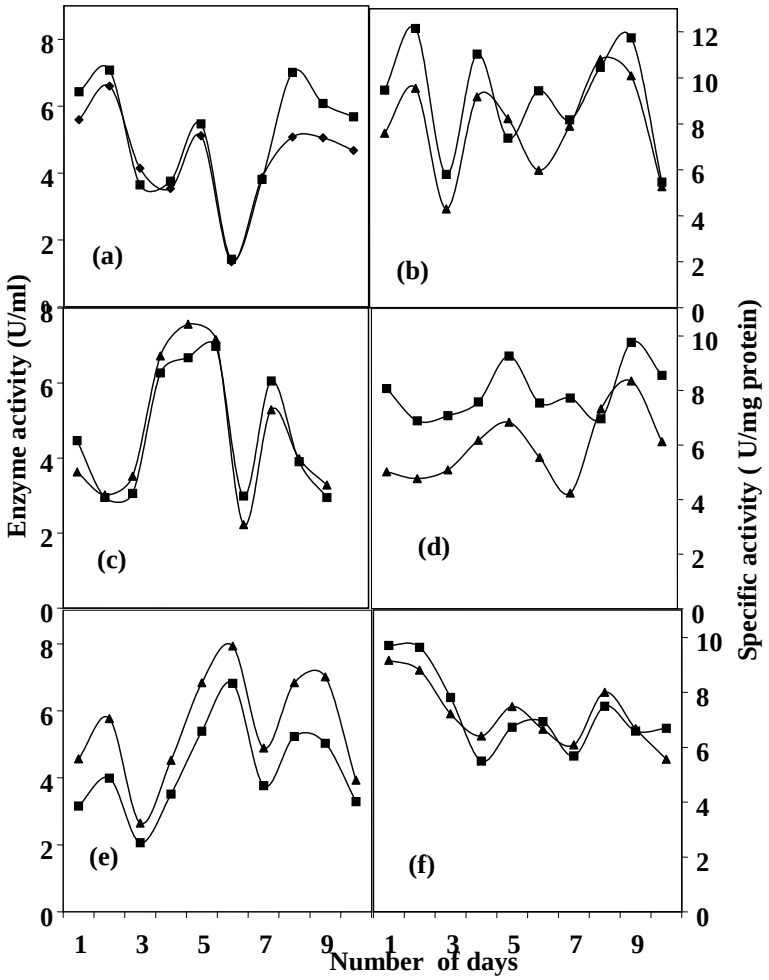

Figure 2.4: Feruloyl esterase profile of the known and isolated strains which gave high specific activity for feruloyl esterase enzyme on wheat bran ; Enzyme activity expressed in U/ml (■) and Specific activity in U/mg protein (▲);(a)- JCS-2 ; (b)-JCS-3 ; (c)-JCS-4 ; (d)-JCS-6 ; (e)- *Aspergillus flavipes* ; (f)- *Trametus* sp.

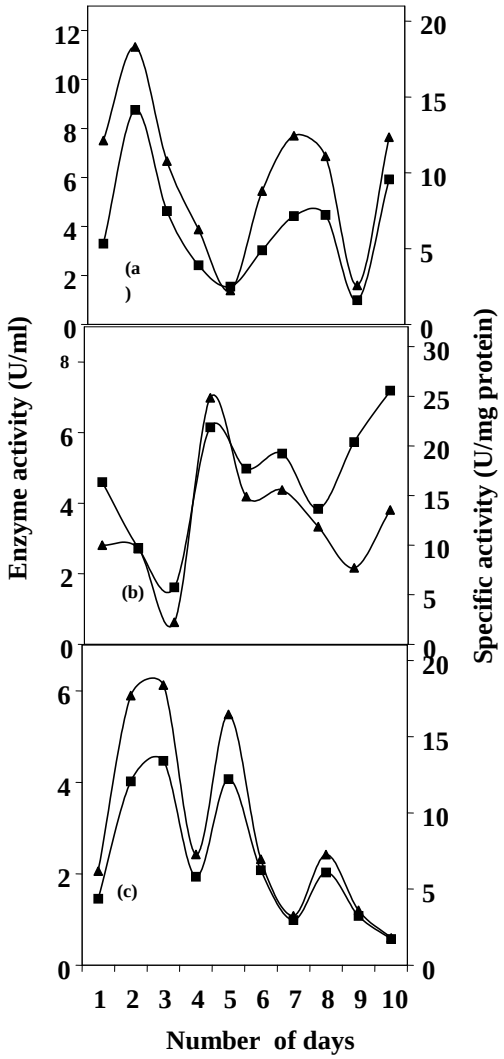

Figure 2.5: Feruloyl esterase profile of *Aspergillus flavipes* on pretreated maize bran, sugar cane bagasse and rice bran. (a)- rice bran; (b)-sugarcane bagasse; (c)-maize bran. Enzyme activity expressed in U/ml (■) and specific activity in U/mg protein (♦).

Among the different growth substrates employed, JCS-3 grown on pretreated maize bran supported maximum enzyme activity (7.17 U/ml) as well as specific activity (36.71 U/mg protein) on the eighth day, closely followed by rice bran, wheat bran and sugarcane bagasse. But JCS-3 grown on rice bran as growth substrate showed maximum enzyme activity (7.05 U/ml) and a corresponding specific activity value of 34.88 U/mg protein in four days (Fig. 2.6).

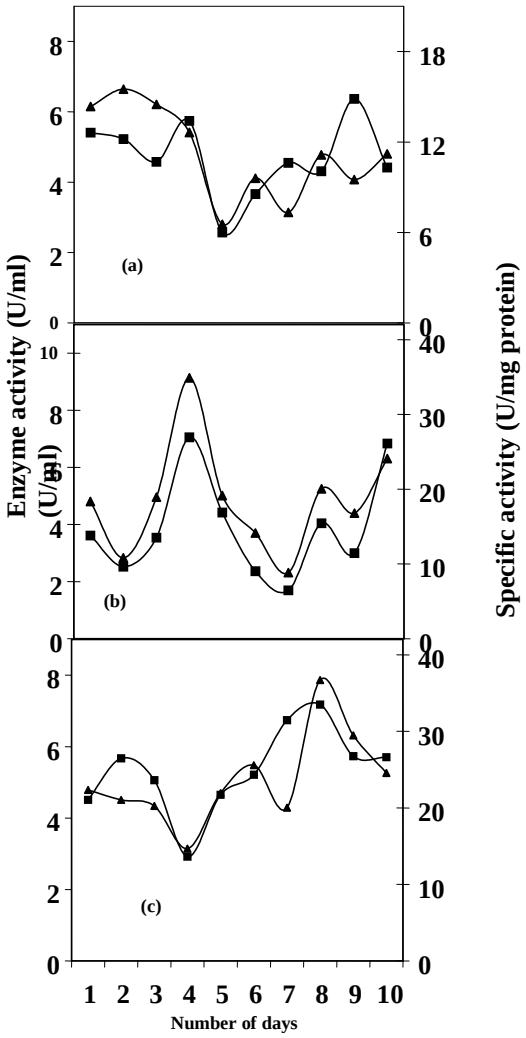

Figure 2.6: Effect of different cereal brans and sugar cane bagasse as carbon source in inducing feruloyl esterase production of the isolated strain, JCS-3. (a)-sugarcane bagasse; (b)-rice bran; (c)-

maize bran; Enzyme activity expressed in U/ml (■) and Specific activity in U/mg protein (♦).

Enzyme source	Carbon source	Incubation time	Enzyme activity U/ml	Specific activity U/mg protein	Reference

Hemicellulosic materials are probably important in the induction of ferulic acid esterases. The carbon source used in the culture should be enriched in ferulic acid in order to induce high levels of feruloyl esterase. Earlier studies have confirmed that feruloyl esterases are induced by components of the cell wall. Ferulic acid constitutes about 0.5% w/w of wheat bran (Ralet et al., 1990), 0.9% w/w of rice endosperm cell wall (Shibuya, 1984) and 3.1% w/w of maize bran (Saulnier et al., 1995). The results correlated well with the reported higher content of ferulic acid in maize bran. Comparison of the feruloyl esterase activity of *Aspergillus flavipes* and the isolated strain JCS-3 along with the already reported strains are given in Table 2.1. A relative comparison is not possible since the enzyme substrates used and the assay procedures are different; even then the isolated strain, JCS-3 and *Aspergillus flavipes* were found to be good producers of feruloyl esterase enzyme. Multiple forms of feruloyl esterase may be secreted during growth on cereals (Johnson et al., 1989) and hence not all of the esterase activity may be attributed to one enzyme.

In summary, the isolated strain JCS-3 was found to be a potent organism when grown on the pretreated cereal brans to produce feruloyl esterase activity when compared to the other organisms studied.

Table 2.1: Extracellular feruloyl esterase activities of various microbial sources.

JCS-3	1% W.B [a]	8 d	6.54	10.80 (crude)	Sindhu and Abraham, 2005
JCS-3	1% M.B [a]	8d	7.17	36.71 (crude)	Sindhu and Abraham, 2005
Aspergillus flavipes	1%W.B [a]	6d	6.82	10.57 (crude)	Sindhu and Abraham, 2005
Aspergillus flavipes	1% M.B [a]	3d	4.47	18.38 (crude)	Sindhu and Abraham, 2005
Aspergillus awamori VTT-D-71025	3% W.B	7d	0.09	-	Johnson et al., 1989
Aspergillus niger NRCC 401127	1% W.B [b]	4d	10.58	132.25	Johnson et al., 1989
Aspergillus niger VTT-D-77050	3% W.B	7d	0.13	-	Tenkanen et al., 1991

[a] Destarched and defatted wheat bran

[b] Starch free wheat bran

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CHAPTER III
BIOTRANSFORMATION OF FERULIC ACID

3.1 Introduction

Biotransformation is the conversion of a compound, from one form to another, by the action of microorganisms, enzymes or plant sources. As in chemical processing, the biocatalyst initiates or accelerates a reaction between two or more substances, while the catalyst remains unchanged and is recycled. Biocatalytic processes are now used for the preparation of fine chemicals, for the pharmaceutical, agricultural, food and chemical industries.

3.2 Biotransformation of ferulic acid – a brief review of literature

The sugar refining industry and the cereal processing industries produce several million tons of sugar beet pulp and cereal brans, every year. 20 million tons of sugar beet pulp and 150 million tons of cereal brans have been produced globally in 2004. These by-products, are extensively used as animal feed, but extensive research is needed to produce higher value added products from these.

3.2.1 Conversion of ferulic acid to vanillin: need for biotechnological routes

One of the intensely studied biotransformations of ferulic acid is its conversion to vanillin (Rosazza et al., 1995). Vanillin (3-methoxy-4-hydroxy benzaldehyde) is among the most important and widely used flavor in the food industry and is responsible for the characteristic aroma of vanilla bean extracts. The value of vanillin extracted from the vanilla pods varies between $ 1200- $ 1400 per kilo (Mulheim and Lerch, 1999; Lomascolo et al., 1999) which is about 100-fold more than the cost of vanillin, obtained by chemical synthesis, which comes to around $ 15 per kilo. The high price of “natural” vanillin is mainly due to the limited availability of vanilla pods, depending on climate-associated fluctuations of harvest yields, economical reasons, last but not least to the labor intensive cultivation, pollination, harvesting and curing of vanilla pods. The high cost of natural vanillin favored the production of

vanillin, through synthetic means (Clarke, 1990). There has been a worldwide increasing demand for natural vanilla flavor, which can no longer be accomplished by the available supply of vanilla pods, since the growing areas are limited and the production is stagnant. Therefore production processes, based on biotransformation has become more attractive.

Strong market demand for natural and environment friendly products has spawned efforts to produce vanillin, by microbial transformation from natural substrates such as ferulic acid (Mulheim and Lerch, 1999). Such biotransformations could be based on cultured vanilla plant cells or plant callus tissue, prokaryotic or eukaryotic microorganisms or isolated enzymes as biocatalysts. The high level of ferulic acid production by hydrolysis from plant cell wall materials such as cereal brans (Faulds and Williamson, 1995) and sugar beet pulp (Micard et al., 1994) rich in this acid would provide a sufficient natural source of ferulic acid. The EEC legislation incorporates under the term ‘natural products’ those produced from natural sources by living cells or their components, including enzymes, having GRAS (Generally regarded as safe) status. The ferulic acid, gained by chemical processes cannot be regarded as natural. Therefore attempts have been made to release ferulic acid enzymatically from lignin, employing feruloyl esterases and cinnamoyl esterases (Blum et al., 2000; Brezillon et al., 1996; Faulds and Williamson, 1995). A cinnamoyl esterase from *Aspergillus niger* (FAEA), inducible upon growth on cereal derived material, released almost 100% of the ferulic acid from wheat bran when used in conjunction with the xylanase from *Trichoderma viride* (Faulds and Williamson, 1995). Another cinnamoyl esterase from *Aspergillus niger* (CinnAE), in conjuction with arabinanase and arabinofuranosidase released high levels of ferulic acid from sugar beet pulp (Kroon and Williamson, 1996).

3.2.2 Other major transformations of ferulic acid

Ferulic acid is also a precursor of 4-vinyl guaiacol (3-methoxy 4-hydroxystyrene), the transformation being catalysed by the enzyme ferulic acid decarboxylase (Donaghy et al., 1999). 4-vinyl guaiacol and other vinyl phenols, may be produced from free phenolic acids and contribute to either the desirable or objectionable aroma of important food products (Maga, 1978).

Ferulic acid is also converted to caffeic acid, a more potent antioxidant compound (Vieira et al., 1998) by *Penicillium rubrum* (Tillet and Walker, 1990) and *Pseudomonas* sp. under anaerobic conditions (Taylor, 1983). Ferulic acid is also transformed in to 3-methoxy-4-hydroxy phenyl propionic acid by anaerobic bacteria like *Pseudomonas cepacia* (Grbic-Galic, 1985) and *Wolinella succinogenes* (Ohmiya et al., 1986), coniferyl alcohol, employed in the successful preparation of lignans, synthetic lignin model compounds, synthetic lignins or dehydrogenation polymers (DHPs) (Kim and Ralph, 2005) by *Trametes* (Nishida and Fukuzumi, 1978) and also in to vanillic acid by *Pseudomonas fluorescens* (Civolani et al., 2000).

3.2.3 General pathways of ferulic acid transformation

Six major pathways of ferulic acid transformation can be distinguished with respect to the initial reaction, namely non oxidative decarboxylation, side chain reduction, Coenzyme A independent deacetylation, Coenzyme A dependent deacetylation, demethylation and oxidative coupling. In the first mechanism, the initial step of ferulic acid catabolism is catalysed by a decarboxylase and 4-vinyl guaiacol is formed. This decarboxylation has been discovered in many fungi and yeast (Arfmann and Abraham, 1989; Huang et al., 1993a; Manitto et al., 1975; Turner and Rice, 1975) and also in some bacteria (Karmakar et al., 2000; Labuda et al., 1993).

One common mechanism of enzymatic carboxylic acid reduction, involves activation of carboxyl groups with ATP to yield highly reactive carbonyl-AMP intermediates which are readily reduced by NADPH to aldehydes. Both the adenylation and carbonyl-AMP reduction steps are catalysed by a single enzyme (Gross, 1972). A separate alcohol oxidoreductase, reduces the resulting aldehydes to their corresponding alcohol products (Li and Rosazza, 1997). The reductions of carboxylic acids to aldehydes are difficult to achieve by chemical means. Also the natural carboxylic acid starting materials are abundant and inexpensive and are soluble in aqueous media.

Elimination of an acetate moiety, from the unsaturated cinnamate side chain is one of the most common metabolic pathways of ferulic acid in bacteria, yeast and fungi. The proposed pathway for the cleavage of acetate from ferulic acid involved, the addition of water to the double bond to give rise to a hydroxy derivative which subsequently undergoes oxidation to the ketone form and ultimately in the removal of acetate group to give rise to vanillin (Rosazza et al., 1995). CoA independent deacetylation has been reported in *Pseudomonas acidovorans* (Toms and Wood, 1970), *Pseudomonas mira* (Jurkova and Wurst, 1993), *Streptomyces setonii* (Sutherland et al., 1983) and *Fusarium solani* (Nazareth and Mavinkurve, 1986) yielding vanillin by the elimination of acetate moiety from ferulic acid side chain.

The ferulic acid degradation in *Delftia acidovorans* proceeds via a coenzyme A-dependent non-β-oxidative pathway (Plaggenborg et al., 2001). A similar coenzyme A-dependent, non β-oxidative pathway for ferulic acid cleavage has been identified in *Pseudomonas fluorescens* AN 103 (Narbad and Gasson, 1998), *Pseudomonas* sp. strain HR 199 (Overhage et al., 1999b) and *Amycolatopsis* sp. strain HR 167 (Achterholt et al., 2000). A Coenzyme A (CoA) dependent mechanism has been suggested in the degradation of ferulic acid by *Rhodotorula*

rubra (Huang et al., 1993b) which involves the activation of ferulic acid to the CoA thioester (Overhage et al., 1999b) which is then hydrated and nonoxidatively, cleaved to vanillin and acetyl CoA.

Clostridium methoxybenzovorans, an anaerobic bacteria (Mechichi et al., 1999) and *Enterobacter cloacae*, (Grbic-Galic and La Pat-Polasko, 1985) a facultative aerobic bacteria, have been shown to grow on ferulic acid and transform it in to caffeic acid (Micard et al., 2002). These strains were likely to use different enzymatic pathways for the O-demethylation of ferulic acid in to caffeic acid. Caffeic acid thus obtained, by the microbial demethylation of ferulic acid, recovered from plant cell walls, could be of interest to produce very good natural antioxidant (Pratt, 1992).

It is well known (Mayer, 1987) that laccase, being one electron oxidases, leads to phenoxy radicals as the primary oxidation products. These radicals can undergo further reactions (presumably oligomerization) which complicate the identification of products generated from the enzymatic reactions. Generation of phenolic radicals can theoretically lead to a plethora of dimeric, oligomeric or polymeric products. The aromatic ring and the conjugated double bonds of the side chain present additional sites, where electrons generated enzymatically or chemically can be delocalized. Ferulic acid in the presence of laccase enzyme initially gave rise to two dimers of ferulic acid namely 5-8' Ben Di; trans-5-[(E)-2-carboxyvinyl]-2-(4-hydroxy-3-methoxy-phenyl)-7-methoxy-2,3-dihydrobenzofuran-3-carboxylic acid and 8-O-4'di FA; (Z)-β-{4-[(E)-2-carboxyvinyl]-2-methoxyphenoxy}-4-hydroxy-3-methoxy cinnamic acid (Federica et al., 2001).

Peroxidase/H_2O_2 mediated oxidation of trans ferulic acid esterified to the main polymers in plant cell walls can form several ferulic acid dehydrodimers : 8-5-, 8-O-4-, 5-5- and 8-8- (Ralph et al., 1994) which have now been identified and quantified in several plant cell walls

(Micard et al., 1997; Parr et al., 1996; Waldron et al., 1997). Ferulic acid was oxidized by wheat germ peroxidase, to form phenoxy radicals resulting in the formation of ferulate dimers (Rebecca et al., 2002). The oxidation of ferulic acid by lignin peroxidase has also been reported (Gary et al., 2001).

3.2.4 Microbial routes of ferulic acid degradation

3.2.4.1 Fungal mediated biotransformation

Microorganisms on account of their rapid growth rates and amenability to molecular genetics are much better targets and can be selected for their ability to grow on phenolic compounds as sole source of carbon and energy. Ferulic acid metabolism, by the white rot fungi has been largely reported to occur via a propenoic chain degradation to vanillic acid, which in turn was subsequently reduced to vanillin and vanillyl alcohol (Gupta et al., 1981). The production of vanillin from ferulic acid was observed in the case of the white rot fungus *Pycnoporus cinnabarinus* (Falconnier et al., 1994).

Filamentous fungi like *Fusarium solani* (Nazareth and Mavinkurve, 1986) and *Paecilomyces variotti* (Rahouti et al., 1989) have been shown to convert ferulic acid to vanillin via 4-vinyl guaiacol by non oxidative decarboxylation. In *Paecilomyces variotti*, the vinylguaiacol formed is in turn converted to vanillin, which is either oxidized to vanillic acid or reduced to vanillyl alcohol.

Tillet and Walker (1990) have reported the demethylation of ferulic acid to caffeic acid and the further side chain shortening to protocatechuic acid by *Penicillium rubrum*. *Trametes* sp. reduced ferulic acid to coniferyl alcohol which was further degraded to vanillic acid, vanillyl alcohol and methoxyhydroquinone (Nishida and Fukuzumi, 1978). Gupta et al. (1981) have reported high levels of reduced products such as coniferyl alcohol and small amounts of vanillin, vanillic acid

and methoxyhydroquinone from ferulic acid by *Sporotrichum pulverulentum*.

Ferulic acid derived from sugar beet pulp, was used as precursor in a biotechnological two step process to produce natural vanillin by employing the micromycete, *Aspergillus niger* and the basidiomycete, *Pycnoporus cinnabarinus* (Lesage Meesen et al., 1999). In the first step *A. niger* transformed ferulic acid to vanillic acid via propenoic chain degradation, to give rise to 920 mg/l of vanillic acid (molar yield 88%) and was subsequently decarboxylated to methoxyhydroquinone. In the second step vanillic acid was reduced to 237mg/l of vanillin (molar yield 22%) by *Pycnoporus* (Lesage Meesen et al., 1996), a laccase deficient strain since laccase activity was found to be associated with the formation of ferulic acid polymers (Lomascolo et al., 1999). The low levels of vanillin were due to the predominance of vanillic acid oxidative system. The enzyme vanillate decarboxylase, responsible for this conversion, has already been described in white rot fungi (Buswell et al., 1981). In most cases, the yields of vanillin were low and the time required for biotransformation reactions were long (Hagedorn and Kaphammer, 1994).

Resting cells of *Rhodotorula rubra* converted trans-ferulic acid to vanillic acid and then to guaiacol and protocatechuic acid under aerobic conditions and to vanillic acid and 4-hydroxy-3-methoxy styrene under an argon atmosphere (Huang et al., 1993b). Both the conversions involve quinoid and vinylogous β-keto acid intermediates which are prone to decarboxylation. *Saccharomyces cerevisiae* converted ferulic acid into 4-hydroxy- 3-methoxy styrene with 96% yield and 4-hydroxy-3-methoxy phenylpropionic acid with 54% yield under an atmosphere of argon (Huang et al., 1993a). *Brettanomyces anomalus* has been shown to decarboxylate ferulic acid into 4-vinyl guaiacol and 4-ethyl guaiacol (Duncan et al., 1995).

3.2.4.2 Bacteria as source of biocatalysts

Cell suspensions of *Acinetobacter calcoaceticus* DSM 586, were able to grow on ferulic acid as sole carbon source and oxidize it in to vanillic acid and further to protocatechuic acid (Delneri et al., 1995). Vanillic acid is an abundant, readily available precursor for the biocatalytic synthesis of vanillin and is another major product obtained by the β-oxidation of ferulic acid by a species of *Bacillus* (Gurujayalekshmy and Mahadevan, 1987) and *Pseudomonas* (Jurkova and Wurst, 1993).

A biotechnological production process with *Bacillus pumilus*, based on a buffer: hexane two phase systems has been developed which yielded 9.6 g/l of 4 vinyl guaiacol from 25 g/l ferulic acid, corresponding to a molar yield of 49.7% (Lee et al., 1998). The proposed mechanism for the decarboxylation catalysed by ferulic acid decarboxylase involves the initial enzymatic isomerisation of ferulic acid to a quinoid intermediate which decarboxylates spontaneously (Huang et al., 1993b).

3.2.4.3. Bioconversions brought about by Actinomycetes

Actinomycetes are known to metabolize different phenylpropenoic acids. Mulheim has reported a relatively high yield, in the direct conversion of ferulic acid to vanillin (6.4g/l; molar yield 68%) by cultures of *Streptomyces setonii* (Mulheim and Lerch, 1999). With this organism, the yields of vanillin were directly proportional to ferulic acid concentration in the culture media. The metabolism of ferulic acid by *Streptomyces setonii* has been studied in detail (Sutherland et al, 1983) and this microorganism degrades ferulic acid into vanillic acid via vanillin. The production of vanillic acid by conversion of ferulic acid with *S. halstedii* also has been studied (Brunati et al, 2004). The mycelium showed the highest productivity when grown in a medium containing 1 g/l of ferulic acid, furnishing 80% molar conversion after 20 hours, followed by its degradation. Low amounts of vanillin (7–8%

molar conversion) could be detected during the first hours of the biotransformation. Higher levels of production of vanillin from ferulic acid have been reported with two strains of *Amycolatopsis*, which were found to accumulate as much as 11.5g/l vanillin (Rabenhorst and Hopp, 1997).

3.2.4.4 Biotransformation mediated by microalgae

Microalgae are being increasingly used in biotransformations, leading to flavors and fragrances. Biotransformation of ferulic acid was studied in free and immobilized cell cultures of *Haematococcus pluvialis*, a fresh water green unicellular algae and resulted in the accumulation of vanilla flavor metabolites like vanillin, vanillic acid, vanillyl alcohol, protocatechuic acid, p-hydroxybenzoic acid, p-hydroxybenzaldehyde and p-coumaric acid. Immobilized cells fed with ferulic acid showed comparatively better yields than free cells of *H. pluvialis* cultures. Maximum accumulation of vanillin, vanillic acid, vanillyl alcohol and protocatechuic acid occurred on the fourth day while immobilized cultures showed maximum accumulation of all metabolites on the third day itself with 10.6mg/l vanillin, 5.4mg/l vanillic acid, 3.3mg/l vanillyl alcohol, 1.6mg/l protocatechuic acid, 1.3mg/l p-hydroxybenzoic acid and 1.1mg/l p-coumaric acid (Tripathi et al., 2002).

3.3 Objectives of the present study

The high cost of vanilla flavor has led to the large scale production of vanillin, through synthetic means (Clarke, 1990) and in the recent years there has been an upsurge in the demand, for natural vanillin. Vanillin obtained by a biotechnological process can be considered as natural, since the EEC and US Legislation incorporates under the term ‘natural products’ those produced from biological sources by living cells or their components and has a much lower production cost. There has been reports in the literature on the

biotranformation of ferulic acid by yeast strains like *Rhodotorula rubra* (Turner and Rice, 1975) and *Saccharomyces cerevisiae* (Huang et al., 1993a) but to the best of our knowledge no study has been conducted, so far on the biotransformation of ferulic acid by the yeast *Debaryomyces hansenii*. Hence attempts were made to study the products, obtained by the bioconversion of ferulic acid by the yeast *Debaryomyces hansenii*, which was isolated in the laboratory.

RAPID CONVERSION OF FERULIC ACID TO 4-VINYL GUAIACOL BY *DEBARYOMYCES HANSENII*

3.4 Biotransformation of ferulic acid to 4-vinyl guaiacol

Ferulic acid is a precursor of 4-vinyl guaiacol (3-methoxy 4-hydroxystyrene), the transformation being catalysed by the enzyme ferulic acid decarboxylase (Donaghy et al., 1999). Vinyl guaiacol may be biocatalytically transformed to acetovanillone and ethyl guaiacol used in perfumery. It has been chemically transformed in to more biodegradable, oxygenated polystyrenes which demonstrate superior mechanical strength compared to other styrenes (Iwabuchi et al.,1977; Hakakeyama et al., 1977) and also in to vanillin (Koseki et al., 1996).

Sutherland et al. in 1995 demonstrated that a number of yeast isolates from frozen concentrated orange juice could decarboxylate naturally occurring ferulic acid. Previous studies have reported the existence of yeasts with the ability to transform ferulic acid in to 4-vinyl guaiacol: *Saccharomyces cerevisiae* (Huang et al., 1993a) and *Rhodotorula* sp. (Turner and Rice, 1975).

Several reports have described the decarboxylation of ferulic acid to 4-hydroxy-3-methoxy styrene (Arfman and Abraham, 1989; Samejima et al., 1987) and further oxidization to vanillin and vanillic acid (Nazareth and Mavinkurve, 1986). Similar results have been

reported in *Paecilomyces variotti* and *Pestalotia palmarum* (Rahouti et al., 1989). Ferulic acid is sequentially degraded to vanillin, vanillic acid and protocatechuic acid by bacteria (Sutherland et al., 1983; Toms and Wood, 1970) and fungi (Henderson, 1961; Ishikawa et al., 1963). The high cost of vanilla flavor has led to the large scale production of vanillin through synthetic means (Clarke, 1990) and in the recent years there has been an upsurge in the demand for natural vanillin.

3.5 Materials and Methods

3.5.1 Chemicals

Ferulic acid (Sigma Chemical Co, St. Louis, Mo,USA), Vanillin (Fluka, Sigma Aldrich, USA), Vanillic acid (SD Fine Chemicals, Mumbai, India) Vanillyl alcohol and 4-vinyl guaiacol (Lancaster Synthesis Ltd, Lancashire, U.K) were of analytical grade and of the highest purity. Peptone and yeast extract, were obtained from Himedia Laboratories, Mumbai, India. Inorganic salts were of analytical grade. Solvents used were of HPLC grade.

3.5.2 Yeast strain

An yeast culture isolated in the laboratory and identified as *Debaryomyces hansenii* by MTCC, Chandigarh, India and assigned the number 539 was used for the study. The culture was maintained on Chloramphenicol Rose Bengal agar and stored at 4°C.

3.5.3.1 Medium and culture conditions

A two stage fermentation protocol (Betts et al., 1974) was followed for the biotransformation studies. Stage I cultures were grown in 30 ml of sterile YEPG medium held in 150 ml Fernbach flasks and shaken at 120 rpm and 30°C. YEPG medium contains glucose-20 g/l, peptone-5g/l, yeast extract-5g/l, KH_2PO_4-5g/l, NaCl-5g/l and distilled water-1000 ml, pH 7.0 and was autoclaved at 121°C for 15 minutes. A 5% inoculum derived from 48 hr old stage I culture was used to start stage II

cultures of 100 ml of sterile YEPG medium in 500 ml flasks and were incubated for 24 hours. Ferulic acid dissolved in N, N-dimethyl formamide was filter sterilized and added to the medium to a final concentration of 2g/l and incubated on a rotary shaker at 120 rpm for 72 hours.

3.5.3.2 Growth measurements

Growth was measured in terms of the optical density of the culture samples at 600nm in a UV-Visible Spectrophotometer (Shimadzu UV 2100).

3.5.3.3 Substrate utilization and transformation studies

Culture samples were periodically withdrawn at 30 minutes, 1, 2, 3, 4, 5, 6, 7, 8, 10, 15, 24, 48 and 72 hours respectively after the addition of the substrate for the determination of pH, turbidity and concentration of the transformed product. The disappearance of ferulate was monitored in the range 200-400 nm since ferulate absorbs maximally at 286 and 310 nm.

3.5.3.4 Enzyme assays

The culture supernatant collected after centrifugation was checked for the presence of laccase and peroxidase enzymes since they can catalyse the polymerization of aromatic precursors (Buswell et al., 1982) and thereby reduce the efficiency of breakdown of ferulic acid. The measurement of laccase activity was done using ABTS (2, 2'-azino-bis (3-ethylbenz-thiazoline-6 sulfonic acid) as substrate (5mM final concentration) (Eggert et al., 1996) in 100mM sodium acetate buffer, pH 5.0. The total reaction volume of 3ml comprised of 2 ml buffer, 0.750ml of 20mM ABTS and 0.250ml of culture supernatant and the optical density of the oxidation product was read at 420nm (ε_{max} =36000 $M^{-1}cm^{-1}$).

The peroxidase activity was also measured using ABTS as substrate (Childs and Bardsley, 1975). The assay mixture consisted of 0.6mM

ABTS and 1.2mM H_2O_2 in 67mM phosphate buffer, pH 6.0 and culture supernatant. Activity was calculated from the change in absorbance/min at 420nm.

3.5.3.5 Preparation of yeast resting cells and cell free extract

The yeast cells after 10 hours of incubation with the substrate were harvested by centrifugation at 10,000 x g at 4°C and the pellet was washed (X3) with 50mM Bis-Tris Propane buffer, pH 6.0. The resultant pellet were resuspended in 5 ml BTP buffer and referred to as yeast resting cells. A similar pellet of yeast cells were freeze dried (12 h), mixed with an equal volume of sand and ground using a mortar and pestle. The ground cells were resuspended in 50mM Bis-Tris Propane buffer, pH 6.0 (5.0 ml) and was centrifuged at 12,000x g for 20 minutes to remove the cell debris and the supernatant was referred to as cell free extract. The total protein concentration was determined by the Lowry's method with bovine serum albumin as standard.

The ferulic acid decarboxylase activities of the yeast cell free extract as well as the yeast resting cells were analysed. The assay reaction mixture contained 50mM BTP buffer, pH 6.0 containing ferulic acid at a concentration of 2mg/ml and 0.2ml cell free extract. Reaction was started by the addition of cell suspension/cell free extract (20%v/v) and incubating the mixture at 30°C for 30 minutes. 1 Unit (U) of activity was defined as the activity required for degrading 1µmol of ferulic acid per minute.

3.5.3.6 Analytical methods and instrumentation

4 ml of the culture medium was removed at different time intervals, centrifuged to remove the yeast cells and extracted thrice with equal volumes of ethyl acetate:propanol mixture (9:1v/v) and the combined extracts were dried over sodium sulfate and concentrated in vacuo in a rotavapor at 50±1°C (Buchi Model R-205, Germany).

For preparative scale experiments, stage II incubations were carried out in 100 ml of media held in 1 litre flasks. The samples were then centrifuged at 10,000 x g for10 minutes, 10 hours after the addition of the substrate. The culture supernatants were extracted in to organic solvents and dried over sodium sulfate. The combined organic layers were concentrated in vacuo in a rotavapor at 50±1°C.

3.5.3.6.1 TLC analyses

TLC analyses were carried out on 0.25 mm thick silica gel GF_{254} plates (Merck). The plates were air dried and activated at 120°C for 1 hour before use. The developing solvents employed were toluene-acetic acid (11.5:0.4 v/v) mixture and benzene: dioxane: acetic acid (90:25:4 v/v/v). The Developed chromatograms were directly visualized under 254 and 366 nm UV light to observe the quenching of fluorescence by the compounds. Aromatic aldehydes were detected on TLC plates by spraying with 2, 4 dinitrophenyl hydrazine in 30% perchloric acid. The components in the organic extract were separated by silica gel column chromatography (60-120 mesh) using a glass column of inner diameter 2.2 cm and length 30 cm and hexane: ethyl acetate system as the eluting system.

3.5.3.6.2 Spectral analyses

The unidentified compound eluted out from silicagel column using 1% ethyl acetate was analysed by nuclear magnetic resonance spectroscopy (NMR). NMR spectra were recorded on a high field spectrometer (Bruker Advance DPX 300) operating at 300MHz for ^{1}H and at 75 MHz for ^{13}C NMR. A homogenous suspension of the isolated 4-vinyl guaiacol was prepared in deuterated chloroform and a 10 ppm scan width was used for ^{1}H NMR. Tetramethyl silane was used as the internal standard. Chemical shift values are reported in parts per million and coupling constants (joule values) are given in hertz. Abbreviations for NMR are as follows: s, singlet; d, doublet; t, triplet; m, multiplet.

3.5.3.6.3 HPLC Analyses of the phenolic metabolites

Ferulic acid and the transformed products were analysed by reverse phase HPLC on a Lichrocart 250-4mm ODS C_{18}column (Merck, Darmstadt, Germany) using a HPLC model LC 10AD, Shimadzu at room temperature equipped with dual pumps and a SPD-10A UV/VIS detector. The mobile phase comprised of water, methanol, acetic acid (70:30:1 v/v/v) and the flow rate was 1ml/min. The UV detector was maintained at 280 nm. The samples were filtered through 0.22μm PTFE membrane before analysis and the injected volume was 20 μl. The peaks were identified by comparison with the known standards. The standard deviations of the analyses were less than 5%. The chromatograph was connected to the CSL Data Processing software and the quantification was performed using external standards.

3.6 Results and Discussion

3.6.1 Growth and preliminary analyses

Ferulic acid added to yeast growing cells showed distinct changes in the UV absorption pattern (Figure 3.1). The decarboxylation of ferulic acid to 4-vinyl guaiacol leads to a hypsochromic shift in the peak maximum. The λ_{max} of vinyl guaiacol in methanol corresponded to 265 and 226 nm which explains that the shift in the absorption maxima (λmax 210, 260 nm) was due to the degradation of ferulic acid to vinyl guaiacol. The pH changed from the initial value of 7 to 5.6 with the advancement of growth and addition of ferulate. More than 90% of the substrate degradation was observed within 10 hours of growth, with the accumulation of 4-vinyl guaiacol as the major degradation product and low amounts of vanillin, vanillic acid and vanillyl alcohol.

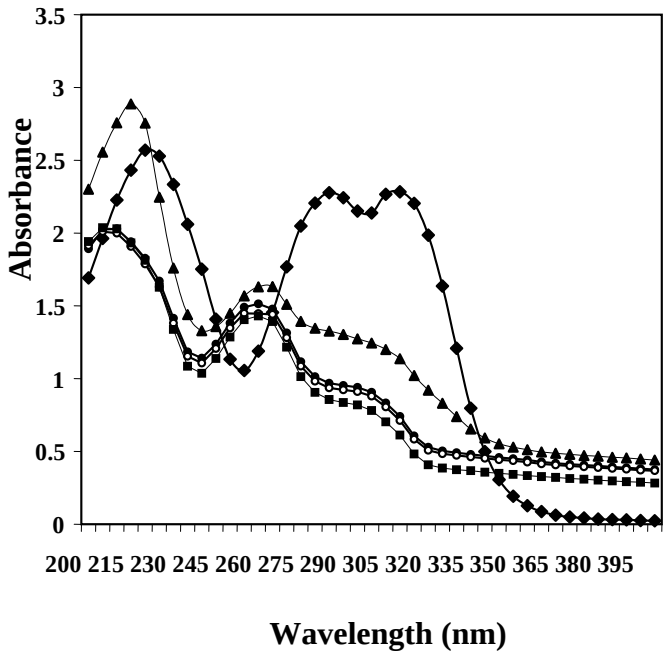


Figure 3.1: UV spectra of the YEPG medium inoculated with *Debaryomyces hansenii* and supplemented with ferulic acid at different hours of growth. (♦-0 hour; ○- 0.5 hour; ● - 6hours; ▲- 10 hours; ■ – 24 hours).

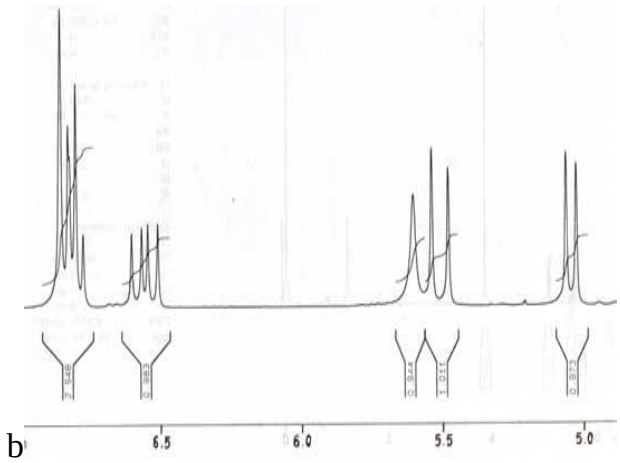
3.6.2 TLC Analyses

TLC analyses of the concentrated culture extracts identified vanillin (R_f 0.27), ferulic acid (R_f 0.18), vanillic acid (R_f 0.19) and an unidentified compound at R_f 0.60 in the toluene acetic acid system which was later identified as 4-vinyl guaiacol. Aromatic aldehyes gave an orange coloration with phenyl hydrazine. The major component which eluted out in 1% ethyl acetate from silica gel column was later identified as 4-vinyl guaiacol.

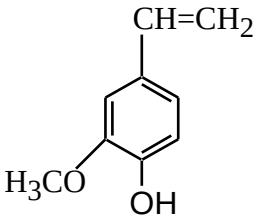
3.6.3 Ferulic acid metabolism

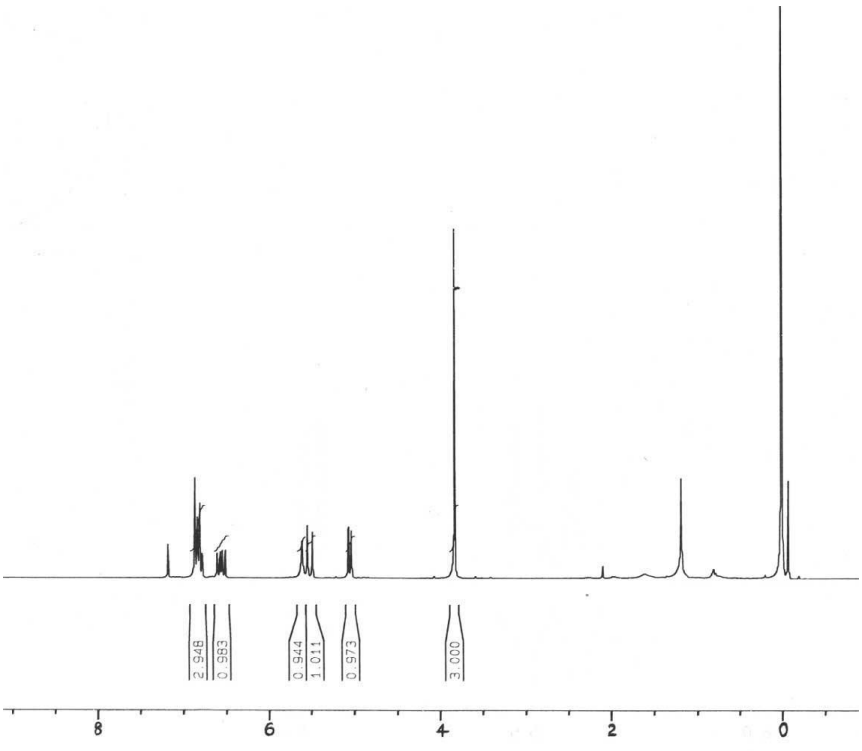
The ^1H NMR spectrum (Fig. 3.2) indicated one methoxyl group (δ 3.82, s, 3H), one hydroxyl group (δ 5.60, s, 1H), three aromatic protons

(δ 6.8, m, 3H) and the olefinic protons at (δ 5.06, d, 1H, J_{B-X} =10.8 Hz), (δ 5.53, d, 1H, J_{A-X}=17.5 Hz) and (δ 6.65, m, 1H, J_{A-X}=17.5 Hz). The ^{13}C NMR spectra comprised of peaks at 77.5 ($CDCl_3$),55.9 (O-CH_3),108.9 (C-2'),110.0 (C-2),115.0 (C-5'),120.0 (C-6'),130.2 (C-1'),136.8 (C-1),145 (C-4'),147.5 (C-3') identifying the compound as 4-vinyl guaiacol. These values were nearly identical to those reported elsewhere (Nazareth and Mavinkurve, 1986; Rahouti et al., 1989; Karmakar et al., 2000).



b



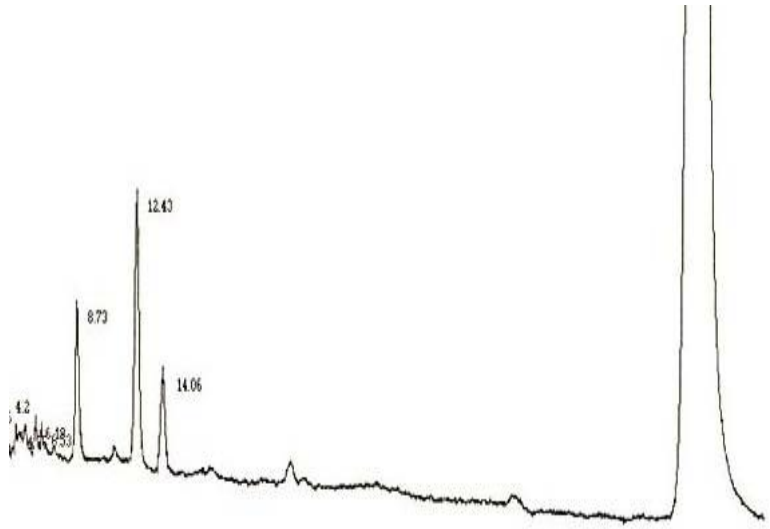


a

Figure 3.2: a: ^{1}H NMR spectrum of 4-vinyl guaiacol; b: Expanded region from 5-7 ppm

3.6.4 HPLC Analyses

In the HPLC analyses the compounds eluted in the following order. The R_t values were Vanillyl alcohol (4.2), Vanillic acid (6.1), Vanillin (8.7), Trans ferulic acid (12.4), Cis ferulic acid (14), 4-vinyl guaiacol (36) and two unidentified compounds (Fig.3.3). The formation of 4-vinyl guaiacol increased steadily over the first few hours after ferulic acid supplementation, reaching a maximum of 1470.8 mg/l and a corresponding molar yield of 95.07% at the tenth hour (Fig. 3.4). The maximum decrease in ferulic acid concentration (50% reduction) was observed in the first few hours after supplementation. The rate of degradation of ferulic acid in most of the earlier reported cases were very slow with low yield of metabolites (Nazareth and Mavinkurve, 1986; Rahouti et al., 1989; Huang et al., 1993a).



Retention time (minutes)

Figure 3.3: HPLC chromatogram of the ethyl acetate extract. The retention times were vanillyl alcohol (4.2), vanillic acid (6.5) vanillin (8.7), trans-ferulic acid (12.4), cis-ferulic acid (14) and 4-vinyl guaiacol (36)

The measurement of phenolic compounds showed that in addition to vinyl guaiacol, vanillin, vanillic acid and vanillyl alcohol were also detected but in lower quantities. Vanillin was detected in the culture supernatant starting from 30 minutes and reached a maximum at the fifth hour (169.09 mg/l) while vanillic acid started to accumulate from the second hour and reached its maximum at the fifth hour (93.2 mg/l). The latter observation can be explained by the oxidation of the reactive aldehyde to the corresponding acid. This is in line with the previous findings that vanillin is an intermediate of ferulic acid degradation to vanillic acid (Toms and Wood, 1970). After the depletion of ferulic acid, both the vanillin formed and the residual vanillic acid was degraded. The presence of vanillyl alcohol was detected only from the fourth hour and reached a maximum of 14.74 mg/l at the tenth hour. The

biotransformation of ferulic acid by *Debaryomyces hansenii* showed that the ferulic acid decarboxylation system was more predominant. *Debaryomyces hansenii* degraded vanillic acid via a reductive pathway leading to vanillin and vanillyl alcohol.

The increase in vinyl guaiacol coincided with a decrease in ferulic acid, detected in the culture supernatant. These intermediates in the culture media may be occurring either as a result of C_2 cleavage of ferulic acid side chain or due to the cleavage of the vinyl bond of 4-vinyl guaiacol produced in the culture media as result of C1 cleavage of the ferulic acid side chain. The metabolism of ferulic acid, with 4-vinyl guaiacol being one of the products, occurs in many bacteria and fungi (Rosazza et al., 1995). Decarboxylation function represents a step of secondary metabolism that could have evolved in response to the toxicity of the compounds (Borneman et al., 1986).

It has been previously reported that *Pycnoporus* converts vanillic acid to vanillin and vanillyl alcohol through a similar reductive pathway (Lesage Meesen et al., 1996). Vanillic acid is also important for biotechnological applications since it is used as a starting material in the chemical synthesis of vanillin (Rosazza et al., 1995). Vanillin dehydrogenase activity has been found to be responsible for the conversion of vanillin to vanillic acid in *Pseudomonas* sp. Strain HR199 (Overhage et al., 1999a).

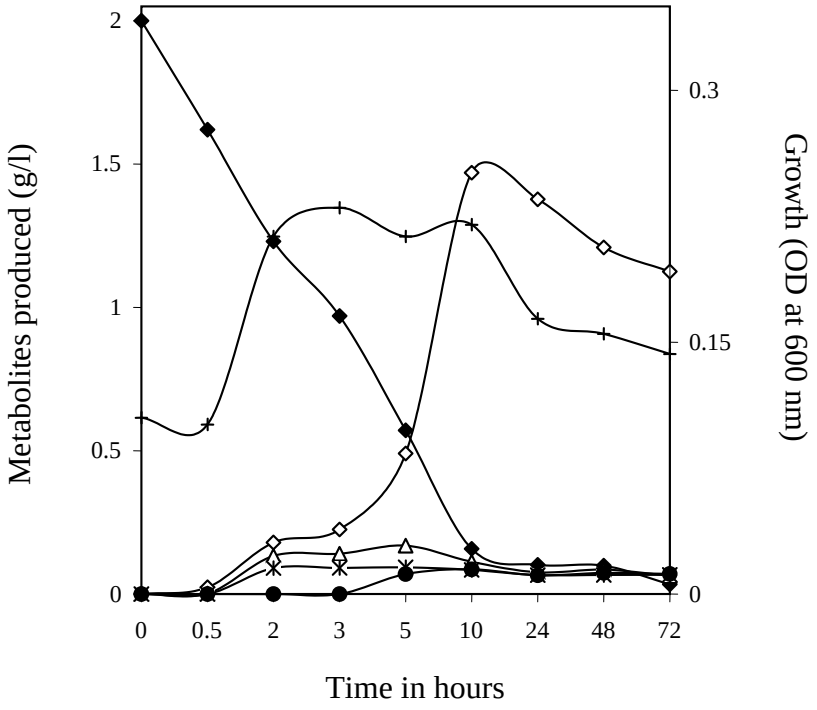


Figure 3.4: Growth (+) and ferulic acid breakdown by *Debaryomyces hansenii* in YEPG medium supplemented with 2mg/ml of ferulic acid. Concentrations of 4-vinyl guaiacol (□), vanillin (Δ), vanillic acid (*) and vanillyl alcohol as measured by HPLC

A tentative scheme for the catabolism of ferulic acid by *Debaryomyces hansenii* 539 has been given in fig. 3.5, the major one being the decarboxylation step.

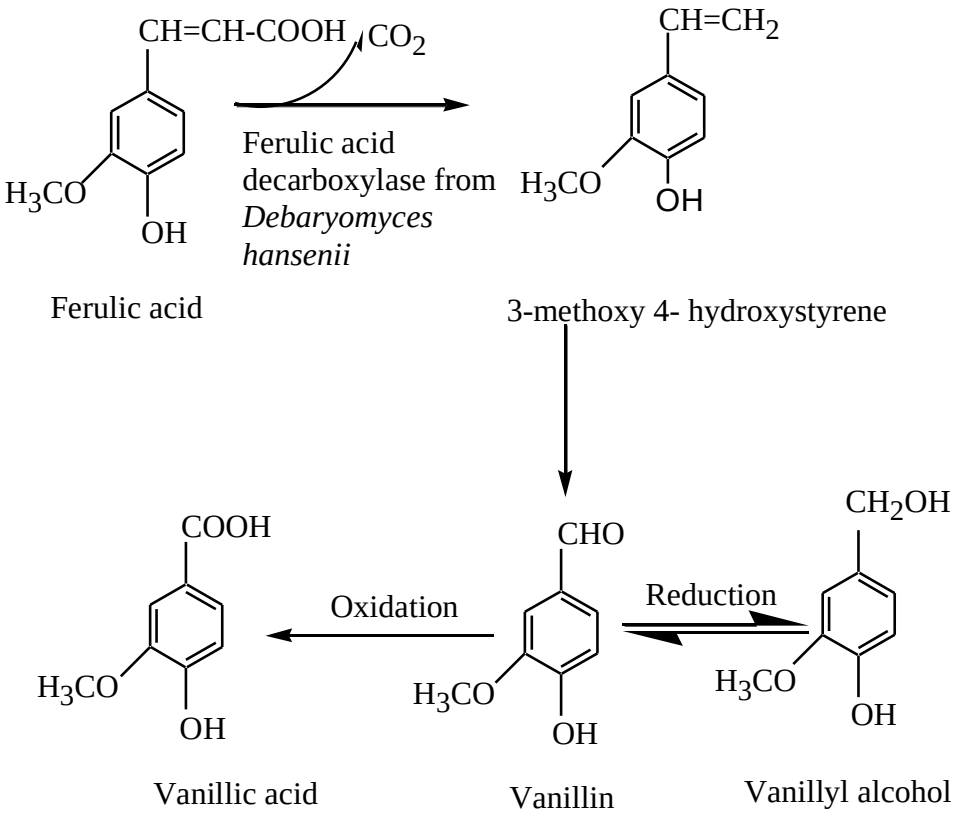


Figure 3.5: Proposed pathway for the catabolism of ferulic acid by *Debaryomyces hansenii* 539

3.6.5 Enzyme activity

Laccase and peroxidase activity was found to be absent in the culture supernatant. Ferulic acid decarboxylase activity was not detected in the culture supernatant; however the cell free extract showed ferulate decarboxylase activity with the ability to transform ferulic acid in to 4-vinyl guaiacol via a nonoxidative decarboxylation. The specific activity of ferulate decarboxylase enzyme was found to be 1.02 U/mg protein.

The production of this enzyme seems to be induced by the presence of ferulic acid in the culture broth (Degrassi et al., 1995)

Huang in 1993 (Huang et al., 1993a) found that the ferulic acid decarboxylase of *Saccharomyces cerevisiae* was associated with the resting cells of yeast which support the absence of decarboxylase activity in the culture supernatant. It would appear that the enzyme is membrane associated or might be intracellular and therefore released by the grinding of yeast cells.

The biotransformation of ferulic acid through decarboxylation represents a step of secondary metabolism that could have evolved in response to the toxicity of the compound (Borneman et al., 1986). Experiments indicated that the conversion of ferulic acid to 4-vinyl guaiacol was a relatively high yielding biotransformation. Microbial and enzymatic bioconversion processes are considered natural; thus if natural ferulic acid obtained from cereal brans/sugar beet pulp is used as a substrate in the bioconversion process catalysed by *Debaryomyces hansenii*, the products vanillin and vinyl guaiacol could also be regarded as natural. This biotransformation not only could be important in improving the ruminal digestive function but could also be of interest as a means of generating value added chemicals such as guaiacol derivatives and flavor compounds from ferulic acid widely distributed in the plants.

The key advantage of biocatalysis is that enzymes and microorganisms catalyse reactions specifically under mild conditions thereby saving energy (Faber, 1992). The role and utility of this reaction needs further investigation. A better control over the fungal metabolic pathway involved would lead to the feasibility of the process industrially.

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CHAPTER IV

FREE RADICAL SCAVENGING PROPERTIES AND ANTIOXIDANT ACTIVITIES OF PHENOLIC ACIDS AND PLANT EXTRACTS

4.A. FREE RADICAL SCAVENGING PROPERTIES OF PHENOLIC ACIDS

4.1 Introduction

The protective effects of diets, rich in vegetables and fruits against various forms of cancer and cardiovascular disease (Block, 1992; 1994) have been attributed to the antioxidant nutrients and plant phenolics such as flavonoids and phenylpropanoids. Very little attention has been paid towards the antioxidant activity of simple phenolic acids, the derivatives of benzoic and hydroxycinnamic acids. These compounds are ubiquitous in plant food (Herrman, 1989) and therefore form a part of our daily diet. In addition to free radical scavenging capacity (Kandaswami and Middleton, 1994) plant phenolics have multiple biological activities (Ho et al., 1992) including vasodilatory (Kinsella et al., 1993) anticarcinogenic, anti-inflammatory, antibacterial, immune stimulating, anti-allergic, antiviral and estrogenic effects as well as being inhibitors of phospholipase A_2, cycloxygenase, lipoxygenase (Jovanovic et al., 1992), glutathione reductase (Elliott et al., 1992) and xanthine oxidase (Chang et al., 1993). Plant polyphenols can act as reducing agents, hydrogen donating antioxidants, and singlet oxygen quenchers. In some cases, metal chelation properties have also been proposed.

The current tendency is to replace synthetic phenols like BHA, BHT etc. with phenols extracted from natural sources with comparable antioxidant power and better safety attributes (Moure et al., 2001) Ferulic acid has been claimed to lessen the side effects of chemo or radiotherapy of carcinomas by increasing the natural immune defense. It also exhibits strong anti-inflammatory property and inhibits chemically induced carcinogenesis (Graf, 1992). Caffeic acid is a well known natural phenol found in seeds, fruits, tubers and herbaceous parts of many vegetable species and its antioxidant activity is well

substantiated scientifically (Yanishlieva and Marinova, 1995). It was found to have better stabilizing effect than BHA on the thermal oxidation of cod liver oil (Leonardis and Macciola, 2003). Caffeic acid and Gallic acid suppress chromosomal breakage and sister chromatid exchange induced by some carcinogens or UV light in mammalian cells (Haidle et al., 1985).

The antioxidant activity of phenolic acids is reasonably related to their structure namely the substitutions on the aromatic ring and the structure of the side chain (Shahidi and Wanasundara, 1992). In this study, the capacity of derivatives of benzoic acid, benzaldehyde, benzyl alcohol, phenol, hydroxycinnamates and synthetic antioxidants like BHA and propyl gallate in their free radical quenching capacity, reducing power, hydroxyl and superoxide radical scavenging ability has been compared. Most of these compounds are widely distributed in nature and have been shown to possess antioxidative properties. The compounds employed differ in the pattern of hydroxylation and methoxylation of their aromatic ring (Figure 4.1).

4.2 Materials and methods

4.2.1 Chemicals

1,1-diphenyl-2-picrylhydrazyl(DPPH), 2, 2'-azinobis-3-ethylbenzthiazoline-6-sulfonic acid(ABTS), Phenazine methosulphate (PMS), β-Nicotinamide adeninedinucleotide(NADH), 3,4-Dihydroxy cinnamic acid (Caffeic acid), 4-Hydroxy cinnamic acid (p-Coumaric acid), 3,5 Dimethoxy-4-hydroxy cinnamic acid (Sinapinic acid), 4-Hydroxy-3-methoxy cinnamic acid (Ferulic acid), 3,4-Dimethoxy benzyl alcohol (Veratryl alcohol) and 3,4-Dihydroxy benzoic acid (Protocatechuic acid) was obtained from Sigma (MO,USA). 6-hydroxy-2, 5, 7, 8-tetramethyl chroman-2-carboxylic acid (Trolox) and 4-Hydroxy 3-methoxy benzaldehyde (Vanillin) was purchased from Sigma Aldrich Chemical Co. (Milwaukee, WI). 4-Hydroxy 3-methoxy

benzyl alcohol (Vanillyl alcohol) from Lancaster, Morecambe, UK and 1, 2, 3-trihydroxybenzene (Pyrogallol) from BDH, Lab Supplies, Poole, UK. Butylated hydroxyanisole (BHA), n-Propyl 3,4,5 trihydroxy benzoate (Propyl Gallate) and Benzoic acid from SD Fine Chemicals (India), Thiobarbituric acid (TBA) and 3,4 Dimethoxy benzaldehyde (Veratraldehyde) from CDH (India), Deoxyribose, Nitroblue tetrazolium (NBT), Trichloroacetic acid (TCA), Pottasium persulphate, 2-Methoxy phenol (Guaiacol), 4-hydroxy 3-methoxy benzoic acid (Vanillic acid) and Gallic acid from Sisco Research Lab (India). All the other chemicals employed were of standard analytical grade.

4.2.2 Evaluation of antioxidant activity

4.2.2.1 DPPH free radical scavenging assay

The antioxidant activity of all the sixteen compounds were measured in terms of hydrogen donating or radical scavenging ability using the stable $DPPH^{\bullet}$ method (Brand-Williams et al., 1995) as modified by Sanchez-Moreno et al. (Sanchez-Moreno et al., 1998). A methanolic solution (0.1 ml) of the compounds at 15 μM concentration was added to 2.9 ml of $DPPH^{\bullet}$ (60μM) solution. When $DPPH^{\bullet}$ reacts with an antioxidant compound, which can donate hydrogen it gets reduced and the resulting decrease in absorbance at 517 nm was recorded up to 30 minutes using a UV-Visible Spectrophotometer (Shimadzu UV-Vis. 2100) and the mean values were obtained from triplicate experiments.

The remaining concentration of $DPPH^{\bullet}$ in the reaction medium was calculated from a calibration curve, determined by linear regression: $A_{515\ nm} = 0.0209\ [DPPH^{\bullet}]_T - 0.0078$ and $r = 0.9992$. The percentage of remaining $DPPH^{\bullet}$ ($(DPPH^{\bullet})_R$) was calculated as

$(DPPH^{\bullet})_R(\%) = (DPPH^{\bullet})_T/(DPPH^{\bullet})_{T=0} \times 100$ where $(DPPH^{\bullet})_T$ is the concentration of $DPPH^{\bullet}$ at 30 minutes time and $(DPPH^{\bullet})_{T=0}$ is the concentration at zero time (initial concentration).

4.2.2.2 ABTS radical cation decolorisation assay

The experiments were carried out using an improved ABTS decolorisation assay (Re et al., 1999) and it involves the generation of $ABTS^{+\bullet}$ chromophore by the oxidation of ABTS with potassium persulfate. It is applicable for both hydrophilic and lipophilic compounds.

The $ABTS^{+\bullet}$ solution was diluted to an absorbance of 0.7±0.05 at 734 nm (Shimadzu UV-Vis. Spectrophotometer, Model 2100). Absorbance was measured 1 hour after the initial mixing of methanolic solutions of standard compounds (15μM) with 1 ml of $ABTS^{+\bullet}$ solution. All determinations were carried out in triplicate.

4.2.2.3 Superoxide anion scavenging activity

Measurement of superoxide anion scavenging activity of the compounds was based on the method described by Liu, Ooi and Chang (Liu et al., 1997) with slight modification (Oktay et al., 2003). Superoxide radicals are generated nonenzymatically in PMS-NADH systems by the oxidation of NADH and assayed by the reduction of NBT. In this experiment, the superoxide radicals were generated in 1 ml of Tris-HCl buffer (16 mM, pH 8.0) containing NBT (50 μM) solution and NADH (78 μM) solution. The reaction was started by the addition of PMS solution (10 μM) to the mixture. The reaction mixture was incubated at 25˚C for 5 minutes, and the absorbance at 560 nm in a spectrophotometer was measured against blank samples. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. The percentage inhibition of superoxide anion generation was calculated using the following formula:

% Inhibition= $[(A_0-A_1)/A_0 \times 100]$

where A_0 was the absorbance of the control and A_1 was the absorbance of the compound.

4.2.2.4 Reductive potential

The reductive potential of the compounds were determined according to the method of Oyaizu (Oyaizu, 1986). The standard compounds (500μM) in 1 ml of distilled water was mixed with phosphate buffer (2.5 ml, 0.2M, pH 6.6) and potassium ferricyanide $[K_3Fe(CN)_6]$ (2.5 ml, 1%w/v). The mixture was incubated at 50˚C for 20 min. A portion (2.5 ml) of trichloroacetic acid (10%w/v) was added to the mixture, which was then centrifuged for 10 minutes at 1000g. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5ml) and $FeCl_3$ (0.5ml, 0.1%w/v), and the absorbance was measured at 700 nm in a spectrophotometer. Higher absorbance of the reaction mixture indicated greater reductive potential.

4.2.2.5 Hydroxyl ($OH^{\bullet}$) radical scavenging activity

The hydroxyl radical scavenging activity was measured by the deoxyribose method (Halliwell et al., 1987). The reaction mixture which contained the standard compounds (25μM), deoxyribose (3.75mM), H_2O_2 (1mM), Potassium phosphate buffer (20mM, pH 7.4), $FeCl_3$ (0.1mM), EDTA (0.1mM) and Ascorbic acid (0.1mM) were incubated in a water bath at 37±0.5˚C for 1 hour. The extent of deoxyribose degradation was measured by the TBA method (Ohkawa et al., 1979). 1 millilitre of TBA (1%w/v) and 1 millilitre of TCA (2.8%w/v) were added to the mixture and heated in a water bath at 100˚C for 20 minutes. The absorbance of the resulting solution was measured spectrophotometrically at 532 nm. All the analyses were done in triplicates and the average values were taken. Inhibition (I) of deoxyribose degradation in percent was calculated according to the equation

$I= (A_0-A_1/A_0) \times 100$

where A_0 is the absorbance of the control reaction and A_1 is the absorbance of the test compound.

4.2.2.6 Statistical analysis

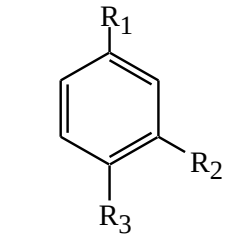
The experimental results are expressed as mean ± standard deviation (S.D) of triplicate measurements. The results were processed using Microsoft excel 2000.

4.3 Results and discussion

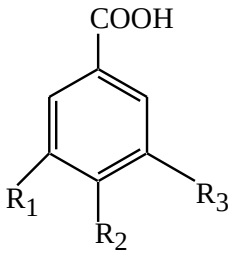
4.3.1 DPPH$^{\bullet}$ scavenging activity

The effect of antioxidants on the DPPH radical was thought to be due to their hydrogen donating ability (Blois, 1958). A freshly prepared DPPH$^{\bullet}$ solution exhibits a deep purple color with absorption maximum at 517 nm. Antioxidant molecules can quench DPPH free radicals by providing hydrogen atoms or by electron donation, via a free-radical attack on the DPPH molecule and convert them to a colorless/bleached product (i.e., 2, 2-diphenyl-1-hydrazine, or a substituted analogous hydrazine) a stable diamagnetic molecule (Matthaus, 2002) resulting in a decrease in the absorbance at 517 nm. In the DPPH test, most of the compounds employed were able to reduce the stable radical of DPPH to the yellow-colored diphenylpicrylhydrazine. The method is based on the reduction of alcoholic DPPH solution in the presence of a hydrogen-donating antioxidant due to the formation of the non-radical form DPPH-H by the reaction. Out of the sixteen compounds tested, the DPPH radical scavenging efficiency in the prescribed time period of 30 minutes was found to be in the order:

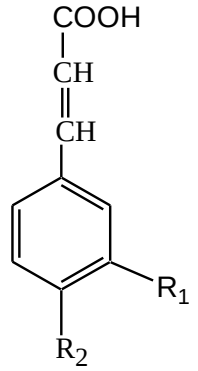
Ca>Pr>Ga>PG>Py>BHA>Si>Fe>Va.alc>Gu>V.ac>Co>Be followed by veratraldehyde, vanillin and veratryl alcohol with very negligible scavenging activity (Figure 4.2). This is in agreement with the results obtained by von Gadow et al. (1997). The DPPH$^{\bullet}$ scavenging efficiency of the compounds at different time intervals are shown in figure 4.3.



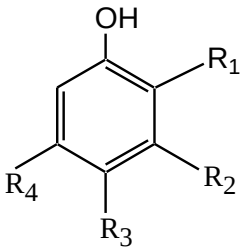
Vanillin - R_1-CHO, R_2-OCH_3, R_3-OH
Vanillyl alcohol - R_1-CH_2OH, R_2-OCH_3, R_3-OH
Veratryl alcohol - R_1-CH_2OH, R_2 & R_3 -OCH_3
Veratraldehyde - R_1-CHO, R_2 & R_3 -OCH_3



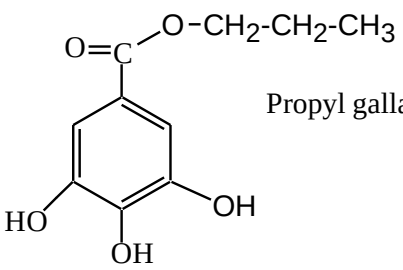
Benzoic acid - R_1, R_2 & R_3- H
Gallic acid - R_1, R_2 & R_3-OH
Protocatechuic acid - R_1-H, R_2 & R_3-OH
Vanillic acid - R_1-H, R_2-OH, R_3-OCH_3



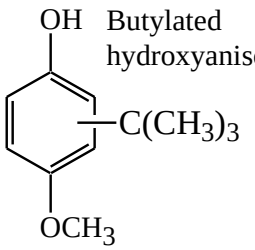
Caffeic acid - R_1 & R_2-OH
Ferulic acid - R_1-OCH_3 , R_2-OH
p-Coumaric acid - R_1-H, R_2-OH
Sinapinic acid - R_1-OCH_3, R_2 -OH, R_3-OCH_3



Guaiacol - R_1-OCH_3, R_2-H
Pyrogallol - R_1 & R_2-OH



Propyl gallate



Butylated hydroxyanisole

Figure 4.1: Structures of the different compounds used for the study

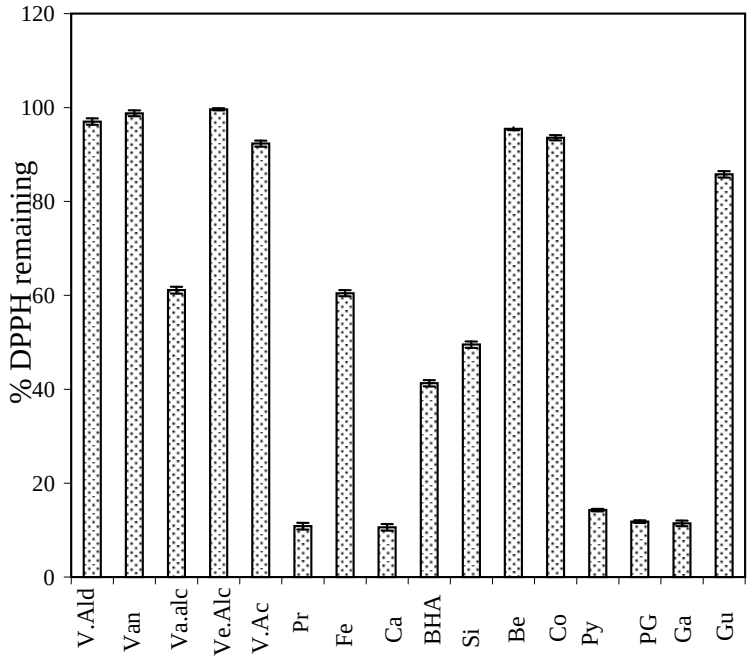


Figure 4.2: DPPH radical scavenging capacity of the different compounds. For legend abbreviations please see page no. 107

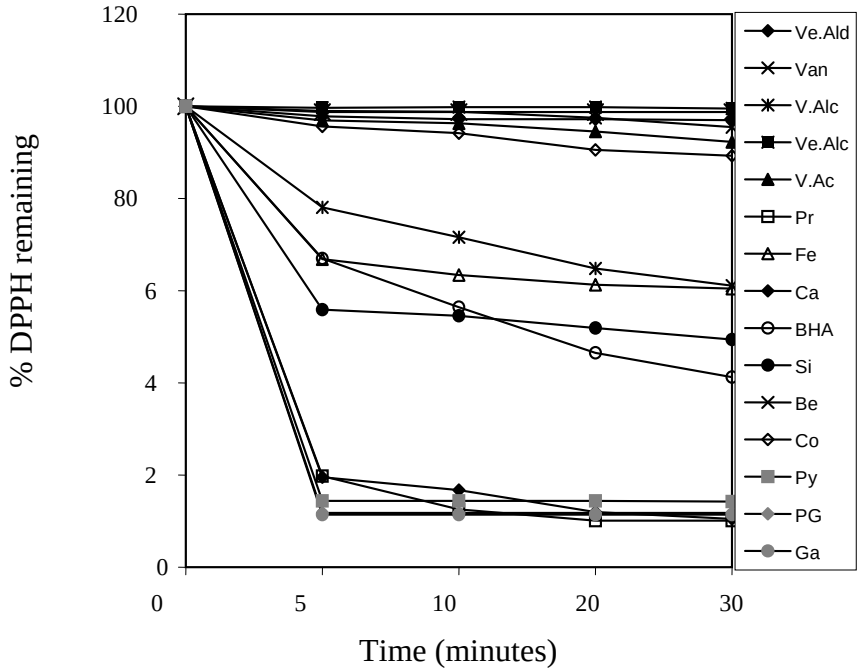


Figure 4.3: Reaction kinetics of the different compounds with DPPH radical

From this sequence it can be concluded that the hydroxylated cinnamates in general are more effective than their benzoic acid counterparts (ferulic acid > vanillic acid ; caffeic acid > protocatechuic acid). This can be explained in terms of the -CH=CH-COOH group, which participates in stabilizing the radicals of cinnamic acid derivatives by resonance (Cuvelier et al., 1992). Brand Williams et al. (1995) also found the cinnamic acid derivatives to be better scavengers of the DPPH radical than their benzoic acid analogues. However, the electron withdrawing properties of the carboxylate group in benzoic acid has a negative influence on its H-donating ability and thereby on its scavenging ability. The benzoic acid derivative, vanillic acid therefore has a lower antioxidant activity than ferulic acid influenced by the adjacency of the carboxylate groups to the phenyl ring. Brand Williams et al. (1995) and von Gadow et al. (1997) also found that p-coumaric acid and vanillic acid react poorly with DPPH radical and their hydrogen donating ability was lower than that of ferulic acid and BHA (Figure 4.2). The presence of –CH=CH-COOH groups in cinnamic acid ensures greater hydrogen donating ability and subsequent radical stabilization than the carboxylate group in benzoic acids.

The antioxidant activity of phenolic acids has been shown to depend on the number of hydroxyl groups in the molecule (Dziedzic and Hudson, 1983) which in fact accounts for the high scavenging activity of Gallic acid, Propyl gallate and Pyrogallol with three hydroxyl groups. This activity is strengthened by the presence of a second hydroxyl group, as in Protocatechuic and Caffeic acid, through the formation of an intramolecular hydrogen bond. The antioxidant capacity of phenolic compounds is generally ascribed to the reaction with oxidants to form resonance stabilized phenoxy radicals (Baum and Perun, 1962). When hydrogen abstraction occurs, compounds with a para or ortho position heteroatom can form a quinone or semiquinonoid

free radical and then form an o-benzoquinonoid structure through resonance (Friedman and Jurgens, 2000) which can also stabilize the radical to a certain extent. Another possible explanation of the activity difference is that electronic effects of ortho and para heteroatoms as well as the subtle equilibrium between intra and intermolecular hydrogen bonds might contribute to the high activity (Mac Faul et al., 1996). The semiquinonoid free radicals of the molecules produced after hydrogen abstraction can be stabilized by forming one or more intramolecular hydrogen bonds with OH at the ortho position (Cheng et al., 2003). The partition coefficients of the phenolics as well as their rates of reaction with the relevant radicals may define the antioxidant activities (Rice Evans et al., 1996). Factors such as lipophilicity, iron chelation, presence of bulky groups near OH group and solubility have been found to determine the antioxidant activity.

Substitution with a hydroxyl group was found to be more effective (Marinova and Yanishlieva, 1992) than with a methoxy group (protocatechuic acid > vanillic acid; caffeic acid > ferulic acid > p-coumaric acid). Caffeic acid had the highest hydrogen donating capacity toward the DPPH radical while veratryl alcohol had the poorest. Gallic acid and propyl gallate had no significant difference among them. Kikuzaki et al., in 2002 has reported similar order of scavenging ability towards DPPH radicals: caffeic acid > sinapic acid > ferulic acid > p-coumaric acid.

The evolution of the different reaction kinetics depends on the nature of the compound tested. Results from kinetic studies showed that PG, Py, Ca and Pr exhibit a faster reaction rate compared to V.alc, Fe, BHA and Si with intermediate reaction rate and Ve.alc, Van, Ve.ald, Be, V.ac and Co with slow reaction rate (Figure 4.3). In most of the cases, the compounds exhibited two phases in their interaction with the radical, the first phase exhibiting a rapid interaction

in the first five minutes followed by a slower phase in the next twenty five minutes.

4.3.2 ABTS radical scavenging capacity

The ABTS radical scavenging capacity of the different compounds at different time intervals are given in figure 4.4. Here also the compounds exhibited a biphasic behavior with a rapid interaction with the radical in the first one minute followed by a relatively slower phase. In the ABTS radical scavenging assay, the scavenging efficiency was found to be in the in the order Py > Ga > Fe > PG > Ca > BHA > Gu > Pr > V.alc > Si > Co > V.ac. Veratryl aldehyde, Vanillin, Benzoic acid and Veratryl alcohol exhibited very little or no ABTS scavenging activity (Figure 4.4). Pyrogallol and Gallic acid, the 3, 4, 5 trihydroxy benzoic acid had an antioxidant capacity corresponding to the three available hydroxyl groups and the antioxidant response is dependent on the relative positions of the hydroxyl groups in the ring. The alkyl group substitution in PG had a negative impact on the radical scavenging activity when compared to gallic acid. All of these studies rather consistently indicated that the antioxidant activity of phenolic acids is reasonably related to the structure, namely the substitutions on the aromatic ring and the structure of the side chain (Shahidi and Wanasundara, 1992). Still, the data are scanty or not comparable because of the different model systems used to determine the antioxidant activity. The double bond of propenoic derivatives like Fe, Ca and Si probably participates in stabilizing the radical by resonance and in ferulic acid, the efficiency is increased by one methoxy substitution in position ortho to the OH group. Ferulic acid is expected to be more effective than p-coumaric acid as the electron donating methoxy group allows increased stabilization of the resulting aryloxy radical through electron delocalization after hydrogen donation by the hydroxyl group (Rice Evans et al., 1996). Substitution of the 3-hydroxyl group of caffeic

acid by a methoxy group in ferulic acid has enhanced the total antioxidant activity. In the aqueous phase ferulic acid is 150% as efficient as caffeic acid. Substitution of the 3 and 5 hydroxy groups with methoxy groups in sinapic acid demonstrates a reduction in antioxidant activity (Figure 4.5). Ortho-substitution with electron donating alkyl or methoxy groups increases the stability of the aryloxy radical in the case of BHA and Guaiacol and hence its antioxidant potential (Rice Evans et al., 1996).

The antioxidant capacities of phenolic acids depend on the number of hydroxyl groups in the molecule that would be strengthened by steric hindrance (Dziedzic and Hudson, 1983). Electron donating functions and substituents, which provide appreciable hyper conjugation interaction are generally considered as the main inducing factors of radical stabilization. Some chemical reaction(s) (e.g., dimerization) following the oxidations of a polyphenol regenerates the oxidizable-OH moieties in the oxidation product. Thus, subsequent chemical reaction(s) most probably enhance the antioxidant activities of the polyphenols (Hotta et al., 2002).

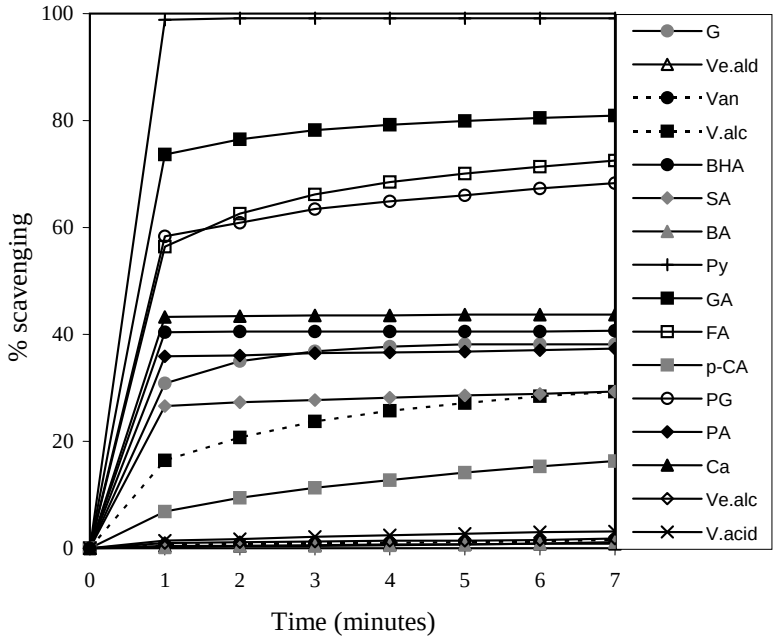


Figure 4.4: Reaction kinetics of different compounds with ABTS radical cation

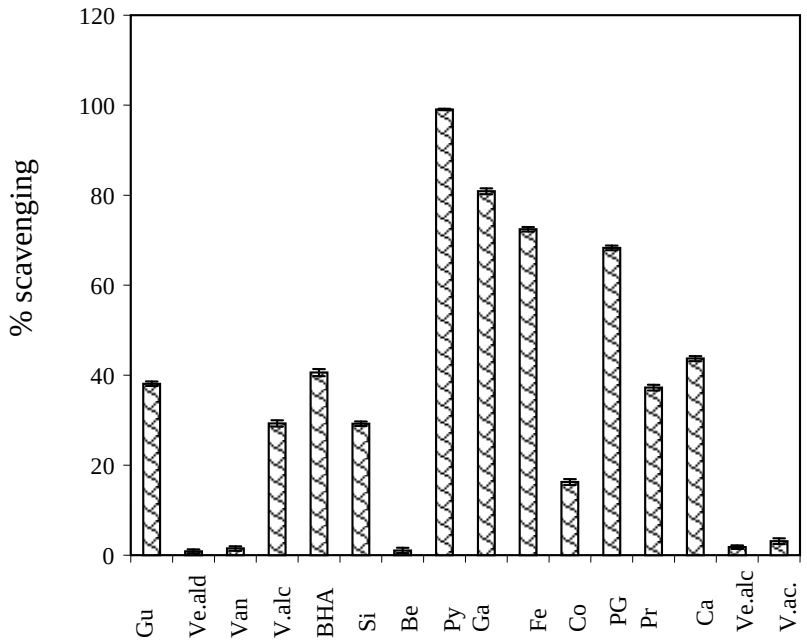


Figure 4.5: ABTS radical cation scavenging capacity of the different compounds

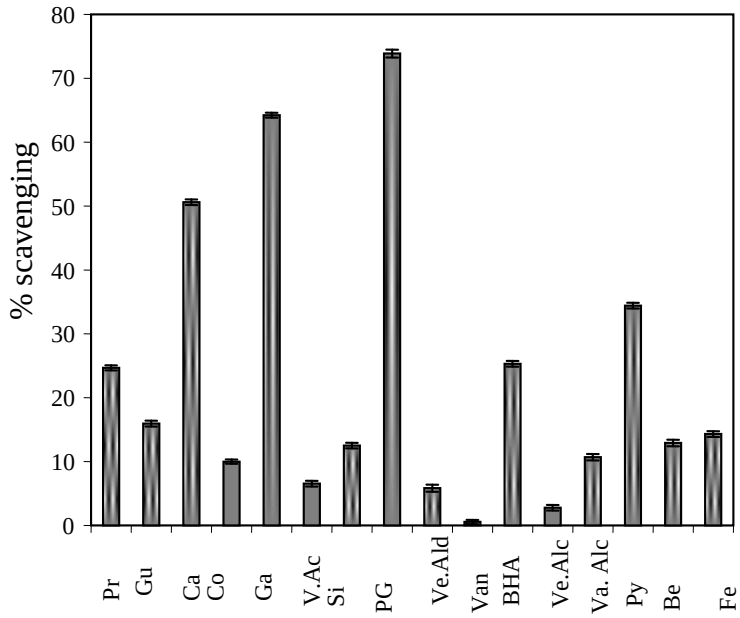


Figure 4.6 Superoxide radical scavenging capacity of the different compounds

4.3.3 Superoxide radical scavenging capacity

Superoxide anions are a precursor to active free radicals that have the potential of reacting with biological macromolecules and thereby induce tissue damage (Halliwell and Gutteridge, 1984). Superoxide anion plays an important role in the formation of other reactive oxygen species such as hydrogen peroxide, hydroxyl radical, and singlet oxygen which induce oxidative damage in lipids, proteins, and DNA (Pietta, 2000). Superoxide anion derived from dissolved oxygen by PMS–NADH coupling reaction reduces NBT in this system. In this method, superoxide anion reduces the yellow dye (NBT) to produce the blue formazan which is measured spectrophotometrically at 560 nm.

Phenolic antioxidants are reported to quench oxygen derived free radicals as well as the substrate derived free radicals by donating a hydrogen atom or an electron to the free radical (Wanasundara and Shahidi, 1996). The redox potentials of hydroxycinnamic acid derivatives are lower than that of oxy-radicals such as the hydroxyl

radical E_7=1.9V, superoxide radical anion E_7=0.94V (Wardman, 1989) which means that they are excellent scavengers of these oxy radicals. Compounds that possess more than one hydroxyl group in their aromatic ring like propyl gallate, gallic acid, caffeic acid, pyrogallol and protocatechuic acid exhibited stronger inhibitory potency than monohydroxyl substituents like p-coumaric acid and ferulic acid (Figure 4.6).

4.3.4 Reducing power

The redox potentials of hydroxycinnamic acid derivatives are dependent on the electron donating property of the substituents in the benzene ring. In this assay, the yellow color of the test solution changes to various shades of green and blue, depending on the reducing power of each compound. The presence of reductants causes the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form. Therefore, measuring the formation of Perl's Prussian blue at 700 nm can monitor the Fe^{2+}concentration. The reducing potential of the different compounds were found to be in the order Py > Ga > PG > Ca > Pr > Si > BHA > Va. Alc > Fe > Gu > V.ac > Co. Veratryl alcohol, Vanillin, Veratryl aldehyde and Benzoic acid exhibited very low reducing power (Figure 4.7). Pyrogallol and gallic acid are very strong reducing agents, owing to the presence of three hydroxyl groups. The antioxidant efficiency of monophenols was increased substantially by methoxy substitution at the o-position relative to the hydroxyl. Sinapic acid was more efficient than ferulic acid which in turn was more efficient than p-coumaric acid. Several authors have shown that ortho substitution with electron donating methoxy or alkyl groups increases the stability of the aryloxyl radical and thereby its antioxidative action. However in the case of acid phenols, methoxy substitution was far from equivalent to the addition of a hydroxyl group. The reduction potential of radicals derived from 3, 4 dihydroxy benzoate derivatives decrease with the electron donating

power at C-1. For example dihydroxybenzoate radicals have a higher reduction potential than dihydroxycinnamate radicals (Jovanovic et al., 1994).

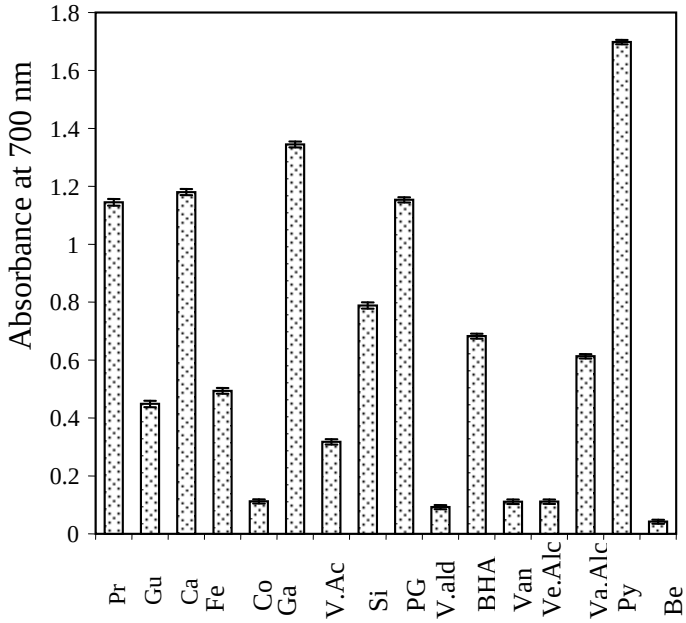


Figure 4.7: Reducing power of the different compounds

4.3.5 Hydroxyl radical scavenging capacity

Phenolic compounds tested in this study were able to form complexes with Fe^{3+} according to the EDTA assay of deoxyribose degradation. This general chelating ability of phenolics is probably related to the high nucleophilic character of the aromatic rings rather than to specific chelating groups within the molecule (Gutteridge et al., 1981). The hydroxyl radical scavenging efficiency was found to be in the order Si > PG > Co > Ve.ald =Ga> Ca > Ve.alc > Gu > Py > BHA > Pr > Va.alc > V.ac >Van> Be > Fe (Figure 4.8). Phenolics may also inhibit oxidation by chelating divalent metal ions and thus reduce the formation of free radicals induced by the Fenton reaction. The

monohydroxy benzoates (vanillic acid) are however effective hydroxyl radical scavengers (Grootveld and Halliwell, 1986) due to their propensity to hydroxylation and the high reactivity of the hydroxyl radical. The increase of methoxy groups substantially increased the antioxidant activity of the compounds by further stabilizing the phenoxyl radical which is responsible for the high hydroxyl radical scavenging capacity of Sinapic acid. The studies confirmed that the antioxidant efficiency of monophenols is strongly enhanced by the introduction of a second hydroxy group and is increased by one or two methoxy substitutions in positions ortho to the OH group as in the case of Sinapic acid.

The phenolic acids differed in their abilities to react with and quench $OH^{\bullet}$, $O_2^{\bullet+}$, $ABTS^{\bullet+}$ and $DPPH^{\bullet}$. The presence of an additional methoxyl group in the ortho position of the OH group showed a strong influence on the chelating property of phenolic acids and their radical scavenging capacity against $O_2^{\bullet+}$, $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ but not on their $OH^{\bullet}$ scavenging activity in the case of ferulic acid. The monohydroxybenzoates show no antioxidant activity in the ortho and para positions in terms of hydrogen donating capacity against radicals generated in the aqueous phase (For ex. Vanillic acid) but the meta hydroxyl acid has antioxidant activity consistent with the electron withdrawing potential of the single carboxyl functional group on the phenolic ring affecting the ortho and para positions. The dihydroxy benzoic acid derivatives show an antioxidant response dependent on the relative positions of the hydroxyl groups in the ring.

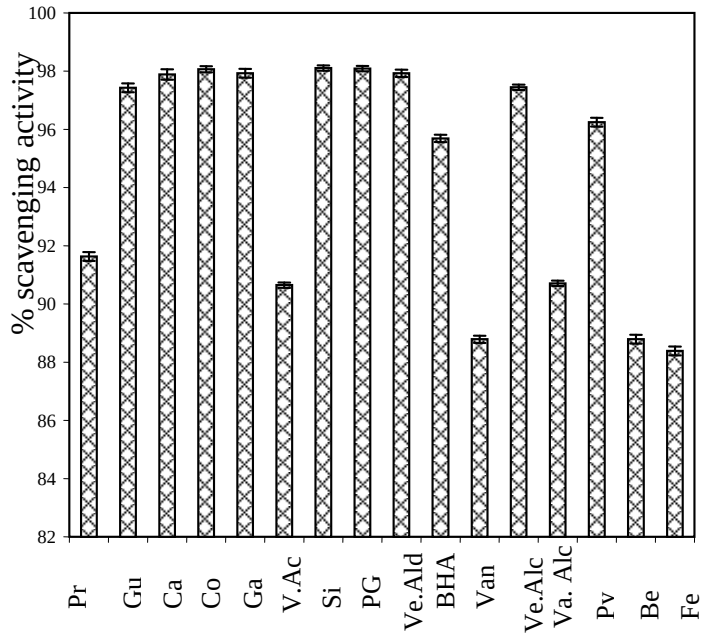


Figure 4.8: Hydroxyl radical scavenging capacity of the compounds

4.4 Conclusion

The antioxidant activity of the phenolic compounds has been evaluated by a series of in vitro tests: scavenging of ABTS•+ and DPPH•, reducing power and scavenging of hydroxyl radical and superoxide anion radical generated by non-enzymatic system. Radical scavenging activities are very important, due to the deleterious role of free radicals in foods and in biological systems. The cinnamic acid derivatives have been pointed out as preventive chain breaking antioxidants in the oxidation of LDL, probably through their radical scavenging ability which is related to phenoxyl radicals (Laranjinha et al., 1995). They usually react with free radicals formed during initiation or propagation steps of the oxidation process thus acting as chain breakers. However other mechanisms of action may be involved such as

chelation of transition metals such as copper or iron which are well known catalysts of oxidative stress.

Remarkably hydroxycinnamic acid derivatives are the most widely represented phenolic acids in food and vegetables strenghtening their potential role as nutritional antioxidants. In conclusion the results obtained in the present study have shown that out of the sixteen compounds studied except Veratraldehyde, Veratryl alcohol and benzoic acid most of them can effectively scavenge free radicals of one type or the other including superoxide anion, hydroxyl radicals and other free radicals under in vitro conditions. It is proposed now days that the use of more than one condition of oxidation is required to evaluate antioxidants. Electrochemical measurements need to be carried out to obtain information about the physicochemical parameters of polyphenols such as redox potential, number of electrons, electron-transfer rate constant etc. which will give a better explanation for the difference in antioxidant activities.

Abbreviations

Pr: Protocatechuic acid; Gu: Guaiacol; Ca: Caffeic acid; Fe: Ferulic acid Co: p-Coumaric acid; Ga:Gallic acid;V.Ac: Vanillic acid; Si:Sinapinic acid; PG:Propyl gallate; Ve.Ald:Veratraldehyde; BHA: Butylated hydroxy anisole; Ve.Alc:Veratrylalcohol; Va.Alc:Vanillyl Alcohol; Py:Pyrogallol; Be: Benzoic acid

4.B. In Vitro Antioxidant activity and Scavenging effects of *Cinnamomum verum* leaf extract assayed by different methodologies

4. 5 Introduction

The role of free radicals and active oxygen in the pathogenesis of human diseases including cancer, aging and atherosclerosis has been recognised (Halliwell et al., 1992). Electron acceptors, such as molecular oxygen, react rapidly with free radicals to become radicals themselves, and are also referred to as reactive oxygen species (ROS). The ROS include superoxide anions (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^{\bullet}OH$) (Grisham and Mc.Cord, 1986). Lipid peroxidation, which involves a series of free radical mediated chain reaction processes, is also associated with several types of biological damage. Therefore much attention has been focused on the use of antioxidants, especially natural antioxidants to inhibit lipid peroxidation and to protect them from damage due to free radicals.

The medicinal properties of plants have been investigated, in the light of recent scientific developments, through out the world, due to their potent pharmacological activities and economic viability. A great number of aromatic, spicy, medicinal and other plants contain chemical compounds, exhibiting antioxidant properties. Sources of natural antioxidants are primarily, plant phenolics that may occur in all parts of plants such as fruits, vegetables, nuts, seeds, leaves, roots and barks (Pratt and Hudson, 1990). Many of these antioxidant compounds possess anti-inflammatory, anti-atherosclerotic, antitumor, antimutagenic, anticarcinogenic, antibacterial or antiviral activities to a greater or lesser extent (Halliwell et al., 1994; Mitscher et al., 1996; Owen et al., 2000; Sala et al., 2002). Crude extracts of fruits, herbs, vegetables, cereals and other plant materials rich in phenolics are increasingly of interest in the food industry, because they retard the

oxidative degradation of lipids and thereby improve the quality and nutritive value of food (Kähkönen et al., 1999; Rice Evans et al., 1995). The extraction, characterization and utilization of natural antioxidants that may serve as potent candidates in combating carcinogenesis and aging process are in progress (Namiki, 1990).

Cinnamomum verum belongs to the family Lauraceae and Cinnamaldehyde, one of the components in it has been found to possess significant antiallergic, antiulcerogenic, antipyretic, anaesthetic (Chopra et al., 1980; Kurokawa et al., 1998) and antimutagenic activities (Sharma et al., 2001). *Cinnamomum cassia* has been used traditionally for treating dyspepsia, gastritis, blood circulation disturbances and inflammatory disease in both Eastern and Western countries (Ahn, 1998). The leaves and bark are used as spices and condiments (Joy et al., 1998). The chief constituents of leaf oils were found to be Eugenol, Benzyl benzoate (Guenther, 1953; Rao et al., 1988), Cinnamaldehyde, Cinnamyl acetate, Cinnamyl alcohol and Linalol (Variyar and Bandopadhayaya, 1989).

Several analytical methods have been proposed for determining the total antioxidant activity of biological extracts in order to evaluate the total antioxidant capacity of biological samples (Cano et al., 1998; Cao et al., 1993; Whitehead et al., 1992).

The present study deals with the free radical scavenging and antioxidant activities of the methanolic extract of *Cinnamomum verum* leaves.

4.6 Materials and methods

4.6.1 Chemicals

(3-(2-Pyridyl)-5, 6-diphenyl-1, 2, 4-triazine-4', 4"-disulfonic acid(Ferrozine) and Cinnamaldehyde was purchased from Sigma Chemical Co. (St. Louis, MO,USA). Ethylene diamine tetraacetic acid (EDTA) and Tween 20 from E-Merck (India) Ltd, Ascorbic acid, Gallic

acid, Eugenol, Folin Ciocalteau reagent and Linoleic acid from Sisco Research Lab (India). All the other chemicals used were of standard analytical grade and solvents were of HPLC grade.

4.6.2 Plant material

The plant, *Cinnamomum verum* is distributed through out tropical and subtropical India and leaves from a healthy plant was used for the preparation of the methanolic extract.

4.6.2.1 Preparation of extracts

The leaves from a *Cinnamomum verum* plant, planted in a courtyard were collected during the month of April, average temperature being 28-34°C. They were shade dried initially, freeze dried and then ground to a fine powder. Four grams each of the powdered leaf sample from a single plant were then extracted separately (4g x 3) in methanol at room temperature (27±1°C) under stirring for 5 hours and the extraction process was repeated till the solvent became colorless for calculating the average yield percentage. The total solvent volume employed was 400 ml in each case. The solvent fractions from a single extraction process were pooled and then filtered through Whatman No.1 filter paper and concentrated in vacuo at 50±1°C in a rotavapor (Buchi, Model R-205, Germany), followed by lyophilisation (Hetosic, Model CD 2.5) to obtain the dry extract which was stored at 0°C and the lyophilized extract dissolved in methanol were filtered through 0.45µ syringe nylon filter before analysis (*Cinnamomum* leaf extract-CLE).

4.6.3 Evaluation of antioxidant activity

4.6.3.1 Determination of ABTS radical cation decolorisation capacity

The experiments were carried out using an improved ABTS decolorisation assay (Re et al., 1999) and it involves the generation of $ABTS^{+\bullet}$ chromophore by the oxidation of ABTS with potassium

persulfate. It is applicable for both hydrophilic and lipophilic compounds.

The ABTS radical cation ($ABTS^{+\bullet}$) was produced by reacting 7 mM stock solution of ABTS with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark for at least 6 hours at room temperature before use. The $ABTS^{+\bullet}$ solution was diluted to an absorbance of 0.7 ± 0.05 at 734 nm (Shimadzu UV-Visible Spectrophotometer, Model 2100). Absorbance was measured 7 min after the initial mixing of different concentrations of the methanolic leaf extracts (final concentration 12.5-150μg/1.1ml) with 1 ml of $ABTS^{+\bullet}$ solution. The corresponding concentrations per milliliter of the solution were 11.3, 22.7, 45.4, 68.1, 90.9 and 136 μg respectively. The $ABTS^{+\bullet}$ scavenging capacity of the extract was compared with that of BHA and gallic acid. Trolox, the water soluble analogue of Vitamin E, was used as a reference standard. A standard curve was prepared by measuring the reduction in absorbance of the $ABTS^{+\bullet}$ solution at different concentrations of trolox over a period of 7 minutes. The Trolox equivalent antioxidant capacity (TEAC) of an extract represents the concentration of trolox solution that has the same antioxidant capacity as the extract. The TEAC values were determined as follows:

$$\Delta A_{trolox} = (A_{t=0\ Trolox} - A_{t=6min\ Trolox}) - \Delta A_{solvent\ (0\text{-}6\ min)} \quad [1]$$

$$\Delta A_{trolox} = m.\ [Trolox] \quad [2]$$

$$TEAC_{extract} = (\Delta Aextract / m).\ d \quad [3]$$

where ΔA=reduction of absorbance, A=absorbance at a given time, m=slope of the standard curve, [Trolox] =concentration of trolox, d=dilution factor

All determinations were carried out in triplicate.

4.6.3.2 Determination of DPPH radical scavenging capacity

The antioxidant activity of CLE was measured in terms of hydrogen donating or radical scavenging ability using the stable $DPPH^{\bullet}$ method (Brand-Williams et al., 1995) as modified by Sanchez-Moreno et al. (1998). A methanolic solution (0.1 ml) of the sample at various concentrations (final concentration 12.5-150μg/3ml) was added to 2.9 ml of DPPH (60 μM) solution. The corresponding concentrations per milliliter of the solution were 4.16, 8.3, 16.6, 25.0, 33.3, 41.6 and 50.0 μg respectively. When $DPPH^{\bullet}$ reacts with an antioxidant compound that can donate hydrogen, it gets reduced and the resulting decrease in absorbance at 517 nm was recorded at 10 minute intervals up to 30 minutes using a UV-Visible Spectrophotometer (Shimadzu UV-Vis. 2100) and the mean values were obtained from triplicate experiments.

The remaining concentration of $DPPH^{\bullet}$ in the reaction medium was calculated from a calibration curve, determined by linear regression: $A_{515\ nm} = 0.0209\ (DPPH^{\bullet})_T - 0.0078$ and $r = 0.9992$. The percentage of remaining $DPPH^{\bullet}$ $[(DPPH^{\bullet})_R]$ was calculated as

$(DPPH^{\bullet})_R\ (\%) = (DPPH^{\bullet})_T / (DPPH^{\bullet})_{T=0} \times 100$ where $(DPPH^{\bullet})_T$ is the concentration of $DPPH^{\bullet}$ at 30 minutes time and $(DPPH^{\bullet})_{T=0}$ is the concentration at zero time (initial concentration). The percentage of remaining $DPPH^{\bullet}$ was plotted against the sample or standard concentration to obtain the amount of antioxidant necessary to decrease the initial concentration of $DPPH^{\bullet}$ to 50% (EC_{50}). A lower EC_{50} value indicates greater antioxidant activity.

A kinetic study was also conducted to evaluate the free radical scavenging properties of CLE using stable $DPPH^{\bullet}$.

4.6.3.3 Determination of metal chelating activity

The chelation of ferrous ions by the extract was estimated by the method of Dinis et al. (1994) with slight modifications and compared with that of EDTA, BHA and Ascorbic acid. Different concentrations of

the extract (final concentration 200-1000µg/ml) were added to a solution of 1mM $FeCl_2$ (0.05 ml). The reaction was initiated by the addition of 1mM ferrozine (0.1 ml) and the mixture was finally quantified to 1ml with methanol, shaken vigorously and left standing at room temperature for 10 minutes. The final concentrations of $FeCl_2$ and ferrozine in the reaction mixture were 0.05 and 0.10mM respectively. After the mixture had reached equilibrium, the absorbance of the solution was measured spectrophotometrically at 562 nm. All tests and analyses were done in triplicate and average values were taken. The percentage of inhibition of ferrozine–Fe^{2+} complex formation was calculated using the formula given below

% Inhibition = $[(A_0-A_1)/A_0 \times 100]$

where A_0 is the absorbance of the control and A_1 is the absorbance in the presence of the sample of CL extract.

4.6.3.4 Determination of Hydroxyl ($OH^{\bullet}$) radical scavenging activity

The hydroxyl radical scavenging activity of CLE was measured by the deoxyribose method (Halliwell et al., 1987) and compared with that of gallic acid. The reaction mixture containing CLE (final concentration 12.5-125µg/ml), deoxyribose (3.75mM), H_2O_2 (1mM), Potassium phosphate buffer (20mM, pH 7.4), $FeCl_3$ (0.1mM), EDTA (0.1mM) and Ascorbic acid (0.1mM) were incubated in a water bath at 37±0.5°C for 1 hour. The extent of deoxyribose degradation was measured by the TBA method (Ohkawa et al., 1979). All the analyses were done in triplicates and average values were taken. Inhibition (I) of deoxyribose degradation in percent was calculated according to the equation

$I= (A_0-A_1/A_0) \times 100$

where A_0 is the absorbance of the control reaction and A_1 is the absorbance of the test compound.

4.6.3.5 Determination of reducing power

The reductive potential of the extract was determined according to the method of Oyaizu (1986) as described earlier. The different concentrations of the extract and the standard compounds Ascorbic acid, BHA and Gallic acid (125-1000 µg) in 1 ml of distilled water was mixed with phosphate buffer (2.5 ml, 0.2M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5ml, 1%w/v).The concentrations of the extract and standard compounds in the final mixture were 6.6, 13.3, 26.7, 40.1 and 53.4 µg/ml respectively. Higher absorbance of the reaction mixture indicated greater reducing power.

4.6.3.6 Determination of Antioxidant activity in linoleic acid emulsion system

The antioxidant activity of the CL extract was determined by the thiocyanate method (Duh et al., 1997). Different concentrations of the sample in methanol were mixed with linoleic acid emulsion in potassium phosphate buffer (0.02 M, pH 7.0). Fifty millilitre linoleic acid emulsion was prepared by mixing and homogenising 155µl linoleic acid, 175µg Tween 20 as emulsifier and 0.02M phosphate buffer. The reaction mixture was incubated at 37±1° C. Aliquots of 0.1 ml were taken at various intervals during incubation. The degree of oxidation was measured by sequentially adding ethanol (5ml, 75%v/v), ammonium thiocyanate (0.1 ml, 30 %w/v), and ferrous chloride (0.1 ml, 0.02 M in 3.5% HCl v/v) to sample solution (0.1 ml) and the absorbance was read at 500 nm. Solutions without the added extracts were used as blank samples. The degree of oxidation was measured every 24 hours and the data used are the average of triplicate analyses. The inhibition of lipid peroxidation in percent was calculated by the following equation:

LPI (%) = 100-[(A_1/A_0) x 100]

where A_1 is the absorbance at 500 nm in the presence of sample and A_0 was the absorbance of the control at 500 nm.

4.6.3.7 Determination of total phenolics

The extracts were diluted with the same solvent, used for extraction to a suitable concentration for analysis. Total phenolic content of the extract was assessed approximately by using the Folin-Ciocalteau phenol reagent method (Singleton and Rossi, 1965). To two hundred microlitres of the sample extracts were added 1.0 millilitre of Folin-Ciocalteau reagent and 0.8 millilitre of sodium carbonate (7.5 %w/v) and the contents were mixed and allowed to stand for 30 minutes. Absorption at 765 nm was measured in a UV-Visible Spectrophotometer. The total phenolic content was expressed as gallic acid equivalents (GAE) in milligrams per gram of sample, using a standard curve generated with gallic acid.

4.6.3.8 Statistical analysis

The experimental results are expressed as mean ± standard deviation (S.D) of triplicate measurements. The results were processed using Microsoft excel 2000 and Microcal Origin 6.0 and the data were subjected to one way analysis of variance (ANOVA) and the significance of differences between sample means were calculated by Duncan's multiple range test using SPSS for Windows, Standard Version 7.5.1, SPSS. Inc., Chicago, IL. P values ≤ 0.05 were regarded as significant and P values ≤ 0.01 as very significant.

4.7 Results and discussion

4.7.1 ABTS radical cation scavenging activity

The average yield of the lyophilized extract obtained from the CL powder was 1.05± 0.02g, corresponding to a recovery of 26.2%. Fig. 4.9 depicts the absorption spectra of ABTS radical cation at various time intervals. The relative antioxidant ability to scavenge the radical $ABTS^{+\bullet}$ has been compared to the standards Trolox, BHA, Ascorbic acid and Gallic acid and is an excellent tool for determining the

antioxidant activity of hydrogen donating antioxidants and of chain breaking antioxidants.

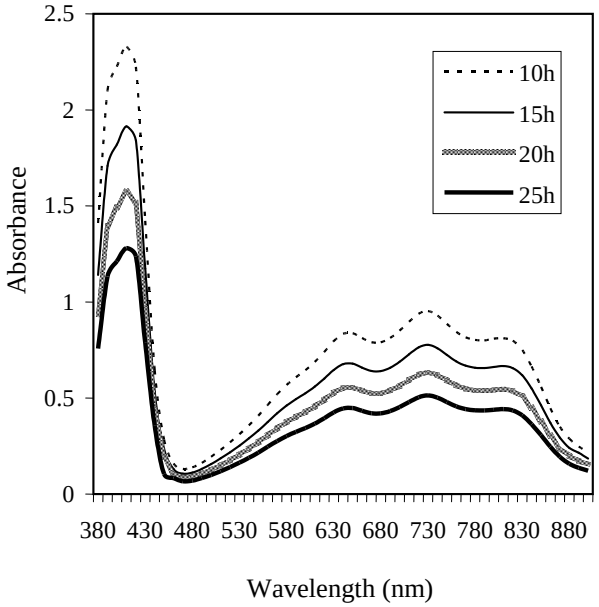


Figure 4.9: Absorption spectra of ABTS radical cation at various time intervals

Fig. 4.10 depicts a steady increase in the ABTS radical scavenging capacity of CLE up to a concentration of 75µg/1.1 ml followed by a relatively low increase, with further increase in concentration.

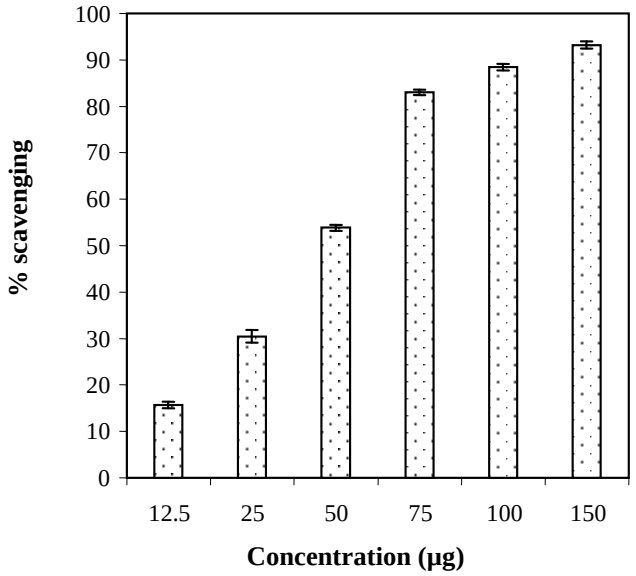


Figure 4.10: ABTS radical scavenging capacity of CLE. P value ≤ 0.01

The percentage scavenging of $ABTS^{+\bullet}$ by the CLE with time is shown in fig. 4.11. Both dose and time effects were observed. Higher concentrations of the extract were more effective in quenching free radicals in the system (Liangli et al., 2002). When CLE was added to $ABTS^{+\bullet}$, a biphasic reaction was observed. This reaction pattern implies that for the reaction of $ABTS^{+\bullet}$ with CLE, the reduction in absorbance and thus the TEAC depends on the time point at which absorbance is read. The TEAC at time less than 1 minute gives the fast reaction, however the TEAC was determined at 7 minutes as it includes the greater part of the slow reaction.

The TEAC value is concentration dependent and a higher concentration resulted in a lower TEAC. The TEAC value for CLE at the maximum concentration studied was found to be 18.1 ± 0.6. The reaction between $ABTS^{+\bullet}$ and the phenols give stoichiometric coefficients between one and two. The rate constant is almost unrelated

to the structure of the phenol, while the number of $ABTS^{+\bullet}$ scavenged by each phenol molecule has been shown to increase with para-substitution which can be explained in terms of a fast reversible electron transfer followed by self combination of the phenoxy radicals or by their reaction with another ABTS derived radical (Campos and Lissi., 1999).

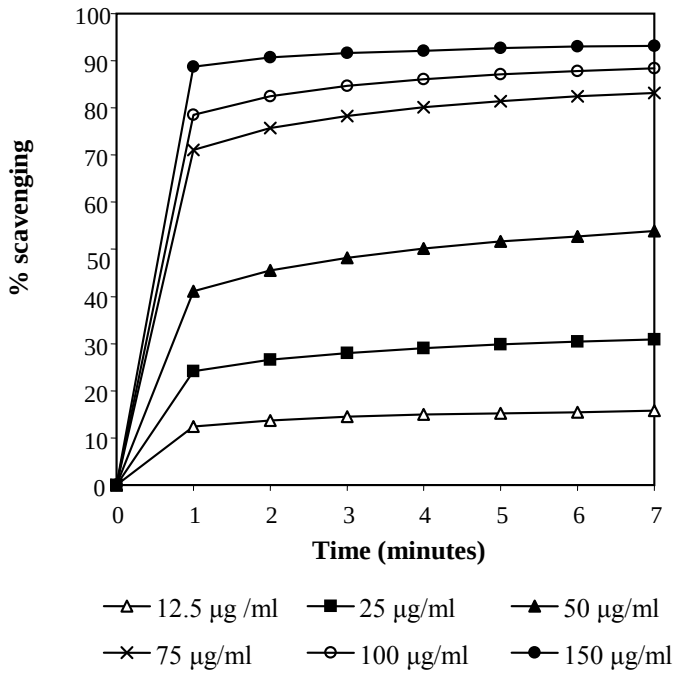


Figure 4.11: Reaction kinetics of different concentrations of CLE with ABTS radical cation

From the different concentrations of Gallic acid, Ascorbic acid, BHA and CLE tested for scavenging activity, the EC_{50} value corresponding to

the concentration of the compound which brings about 50% scavenging was found to be 0.71μg/ml, 2.33μg/ml, 2.35μg/ml and 42.4μg/ml respectively, clearly showing that the scavenging potential followed the order Gallic acid>Ascorbic acid>BHA>CLE. In gallic acid, the inductive effect of the three hydroxyl groups is an important factor that enhances the activity.

4.7.2 DPPH radical scavenging activity

The effect of antioxidants on $DPPH^{\bullet}$ is thought to be due to their hydrogen donating ability (Baumann et al., 1979). Fig. 4.12 shows the decrease in concentration of $DPPH^{\bullet}$ with time. Fig. 4.13 illustrates a significant decrease in the concentration of $DPPH^{\bullet}$ with increase in concentration of the extract. Significant DPPH radical scavenging activity was evident at all the tested concentrations of CLE. The scavenging activity increased with increasing concentration of CLE up to 125 μg/3ml and then almost levelled off with further increase in concentration.

The kinetic behaviour of CLE shows that the reaction approached almost steady state in 30 minutes time. The different relative scavenging capacity of CLE against different testing radicals may be due to the different mechanisms involved in the radical-antioxidant reactions. The stoichiometry of reactions between the antioxidant compounds in the extract and the $ABTS^{+\bullet}$ and $DPPH^{\bullet}$ is different and this may be one reason for the difference in their scavenging potential. Also the one electron reduction potential of the $DPPH^{\bullet}$ and $ABTS^{+\bullet}$ with respect to the compounds in the extract are different which is also responsible for their difference in response time. Other factors like stereoselectivity of the radicals or the solubility of the extract in different testing systems, may also affect the capacity of the CLE to react and quench different radicals (Yu et al., 2002). Significant difference in scavenging properties were observed in the case of

resinous exudates from *Heliotropium* on employing $ABTS^{+\bullet}$ and $DPPH^{\bullet}$ scavenging assay (Lissi et al., 1999). Wang et al. (1998) found that some compounds which have $ABTS^{+\bullet}$ scavenging activity, did not show $DPPH^{\bullet}$ scavenging activity. Therefore, a correlation between these two models may not be obvious, among biological samples containing a variety of antioxidants. The residual concentration of $DPPH^{\bullet}$ depends exclusively on the structure of the phenolic compound, since there are two theoretical termination reactions: one between DPPH radicals and the other between $DPPH^{\bullet}$ and phenol radical (Phe $O^{\bullet}$). However the former reaction is forbidden due to the steric hindrance and the latter reaction competes with the Phe $O^{\bullet}$ coupling termination reaction (Sanchez-Moreno et al., 1998). The accessibility of the radical center of $DPPH^{\bullet}$ to each polyphenol could also influence the order of the antioxidant power. The EC_{50} value of CLE was found to be 22.4μg/ml and that of Gallic acid, Ascorbic acid and BHA was 0.90, 1.26 and 2.46 μg/ml respectively and is inversely related to antioxidant capacity. Gallic acid was found to be approximately 25 times more efficient than CLE.

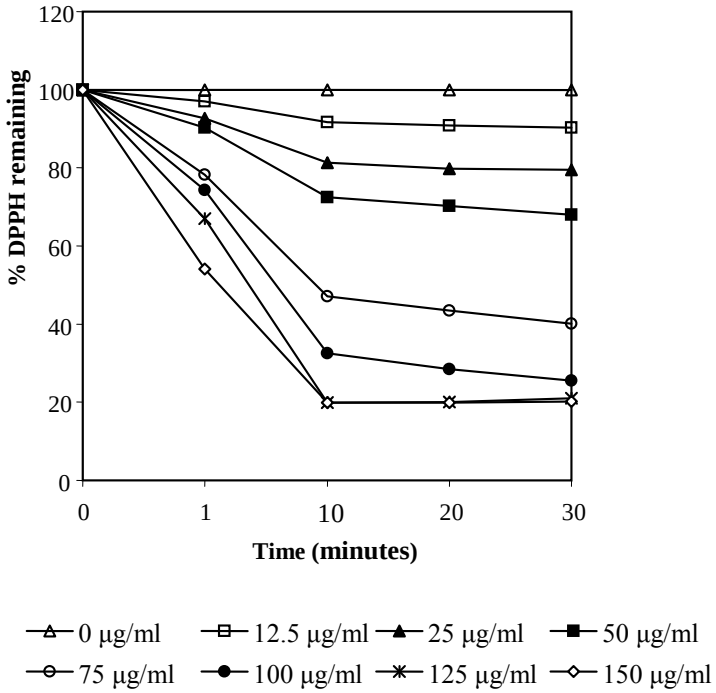


Figure 4.12: Reaction kinetics of different concentrations of CLE with DPPH radical.

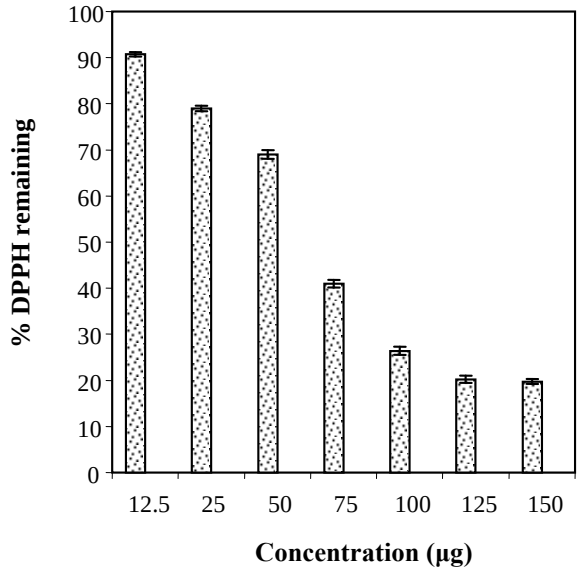


Figure 4.13: Free radical scavenging capacity of CLE as determined by the DPPH method. P value ≤ 0.05

4.7.3 Metal chelating activity

Transition metals have been proposed to catalyze the formation of the first few radicals to start the propagation of radical chain reaction in lipid peroxidation. Chelating agents may inhibit lipid oxidation by stabilising transition metals.

Ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of other chelating agents, the complex formation is disrupted with the result that the red color of the complex is decreased. As shown in fig. 4.14, the formation of the ferrozine-Fe^{2+} complex is not complete in the presence of CLE, indicating its ability to chelate the iron. The absorbance of ferrozine-Fe^{2+} complex decreased linearly in a dose dependent manner (200-1000 µg/ml). The metal chelating activity

of CLE was evaluated against Fe^{2+} and expressed as EDTA equivalents (16.5mg/g extract). The chelating ability was approximately sixty times lower than that of EDTA. The standard compounds BHA and ascorbic acid did not exhibit any metal chelating activity at the tested concentrations (200-1000μg/ml). Reaction of Ascorbic acid and Gallic acid with $FeCl_2$ might enhance the degradation of ascorbic acid and gallic acid and increase the ascorbyl and gallic acid radical concentrations (Satoh and Sakagami, 1997).

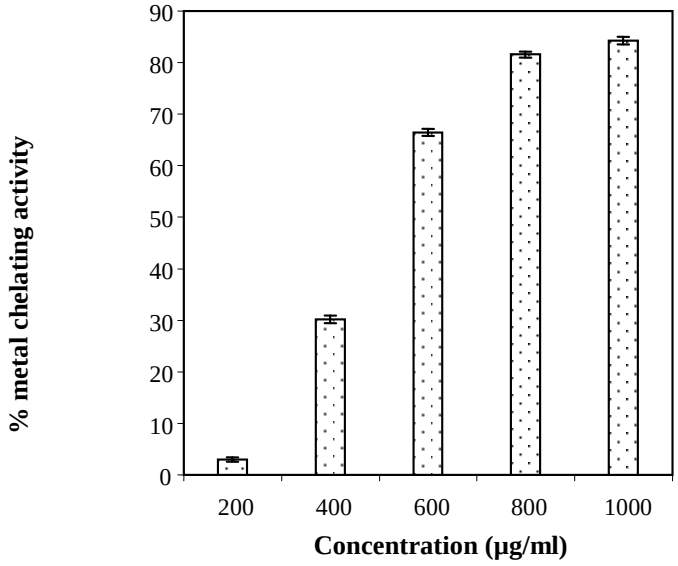


Figure 4.14: Metal chelating activity of different concentrations of CLE. P value ≤ 0.01

4.7.4 Hydroxy radical scavenging

The sample exhibited hydroxyl radical scavenging activity in a dose dependent manner in the range of 12.5-125μg/ml in

the reaction mixture with 95% scavenging at a concentration of 125µg/ml (Fig. 4.15). Generally molecules that inhibit deoxyribose degradadation are those that can chelate the ferrous ions and thereby prevent them from complexing with the deoxyribose and render them inactive in a Fenton's reaction (Smith et al., 1992). Overall, the scavenging activities of phenolic substances might be due to the active hydrogen donating ability of the hydroxyl substitutions. Since phenolic compounds present in the extract are good electron donors, they may accelerate the conversion of H_2O_2 in to H_2O. As shown in figure 4.14, CLE exhibited chelating effect on ferrous ions, suggesting that they minimize the concentration of metal in the fenton reaction, though to a smaller extent compared to EDTA. The antioxidant effect of several polyphenols that acts as inhibitors of hydroxyl radical formation and lipid peroxidation has been correlated with iron chelating properties (Ohnishi et al., 1994). Gallic acid at lower concentrations exhibited pro-oxidant effect, which may be due to the reduction of Fe^{3+} to Fe^{2+} which stimulated the $OH^{\bullet}$ formation and at higher concentrations may markedly scavenge $OH^{\bullet}$ and reduce the oxidative damage of deoxyribose (Yen et al., 2002). The prooxidant properties of phenolic acids like gallic acid are derived from their iron recycling reactivity (Cheng et al., 2003).

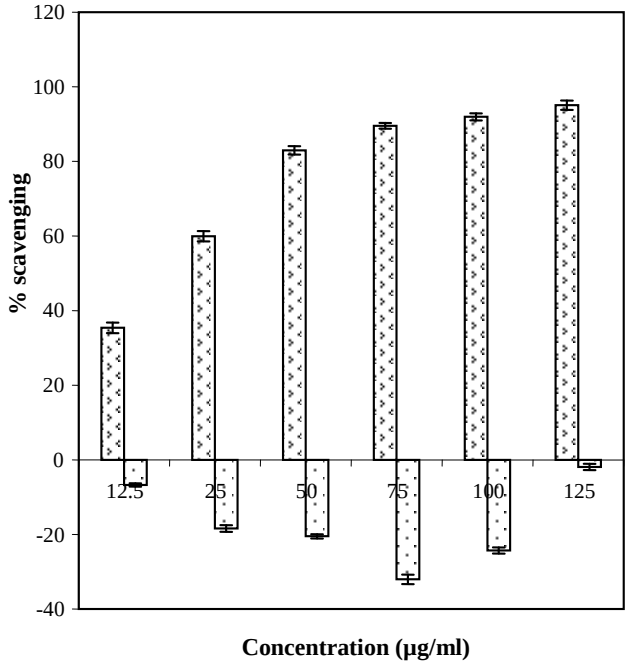


Figure 4.15: Hydroxy radical scavenging capacity of CLE. Prooxidant activity of Gallic acid at similar concentrations shown on the negative axis. P value ≤ 0.05

4.7.5 Reducing Power

The reducing power of the CLE and the reference compounds Ascorbic acid, Gallic acid and BHA increased steadily with the increasing concentration (Fig. 4.16). In general, the reducing power observed in the present study was in the following order Gallic acid >Ascorbic acid >BHA >CLE. The reducing powers (absorbance at 700 nm) of CLE, BHA and Ascorbic acid were 0.57, 2.89 and 3.53 at a dose of 1mg extract. The reducing power of CLE might be due to the di and monohydroxyl substitutions in the aromatic ring, which possess potent

hydrogen donating abilities as described by Shimada et al. (1992). The reducing properties are generally associated with the presence of reductones (Pin-Der Duh, 1998), which have been shown to exert antioxidant activity by breaking the free radical chain by donating a hydrogen atom.

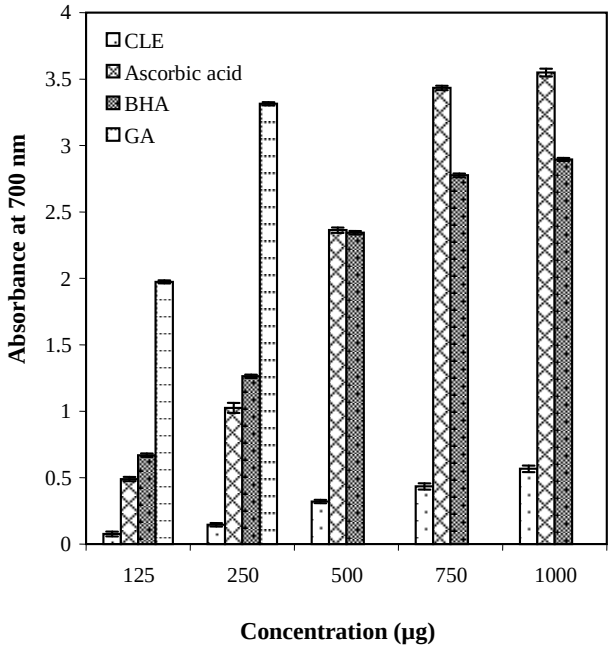


Figure 4.16: Reducing power of CLE, Gallic acid, BHA and Ascorbic acid. P value ≤ 0.01.

4.7.6 Total antioxidant determination in linoleic acid emulsion system

Lipid peroxidation leads to the rapid development of rancid and stale flavors and is considered as a primary mechanism of quality

deterioration in lipid foods and oils (Güntensperger et al., 1998). Synthetic antioxidants, such as butylated hydroxyanisole (BHA) are added in food, during processing to suppress lipid peroxidation and consequently to improve food quality and stability.

The antioxidant effects of the CLE on the peroxidation of linoleic acid were investigated and the results are represented in fig. 4.17. CLE exhibited antioxidant activity with 82.4% inhibition of linoleic acid peroxidation at a concentration of 125µg/ml and 24 hour time interval while that of the positive control, BHA was 89.7% at similar concentration and time. The peroxidation inhibition of CLE and BHA was found to decline with time and reached 52.7 and 68.2% at 144 hours. The extract exhibited effective antioxidant activity at the concentration tested, revealing its ability to deter lipid peroxidation. The phenolic compounds and other chemical components present in the extract may suppress lipid peroxidation through different chemical mechanisms, including free radical quenching, electron transfer, radical addition or radical recombination.

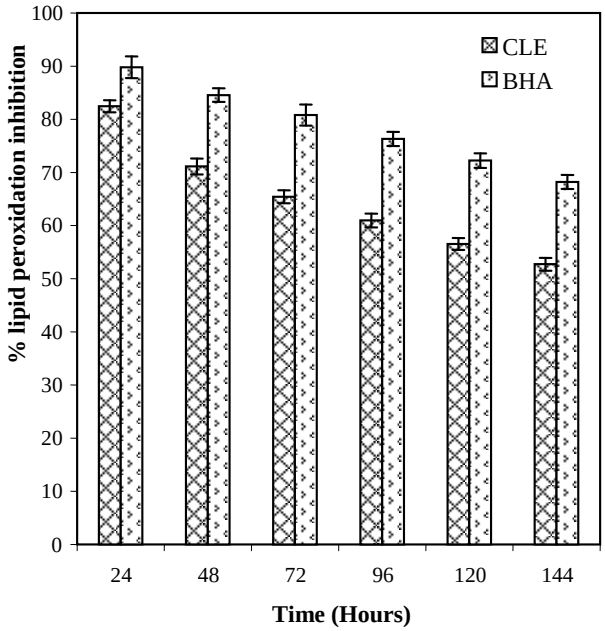


Figure 4.17: Antioxidant activity of CLE and BHA in the linoleic acid emulsion system, using the thiocyanate method. P value ≤ 0.05.

The total phenolic content of the CLE was estimated to be 116 ±1.9 mg Gallic acid equivalents/g of the plant extract from triplicate measurements.

4.7.7 Conclusion

In conclusion, the results obtained in the present study has shown that the methanolic extract of CL contain a number of antioxidant compounds which can effectively scavenge reactive oxygen species including hydroxyl radicals as well as other free radicals under *in vitro* conditions. Moreover the hydrogen donating ability of all the experimental compounds and the extract has been proven through the assessment of reducing power and $DPPH^{\bullet}$ scavenging activity. Even though, CLE are weak metal chelators and exhibit relatively low

reducing power, their free radical scavenging capacity and ability to inhibit lipid peroxidation is of considerable interest. CLE possess antioxidant properties, which are concentration dependent. The active principles of spices such as eugenol, cinnamaldehyde, cinnamic acid, cineol etc. were responsible for the antioxidant activity in Cinnamon (Nagababu and Lakshmaiah., 1992; Jayaprakasha et al., 2003). They have also been shown to inhibit human polymorphonuclear leucocytes 5-Lipoxygenase activity, the key enzyme involved in leukotriene synthesis, which can reduce the production of inflammatory mediators (Prasad et al., 2004). The results of this study show that the extract of CL can be used as an easily accessible source of natural antioxidants or in pharmaceutical industry. It can also be used in stabilizing food preparations against oxidative deterioration.

Antioxidant properties of botanical extracts should be evaluated in a variety of model systems using several different indices because the effectiveness of such antioxidant material is largely dependent upon the chemical and physical properties of the system to which they are added. A single analytical protocol adopted to monitor lipid oxidation may not be sufficient to make a valid judgement. The ability of the extract to retard lipid oxidation is attributable to the ability of its phenolic constituents to quench reactive oxygen species. There are however, reports of phytophenolics exhibiting antioxidant/prooxidant activities, which depend on factors like metal reducing potential, chelating behaviour, pH, solubility characteristics etc (Decker et al., 1997).

4.C Studies on the antioxidant activities of Cinnamon (*Cinnamomum verum*) bark extracts, through various in vitro models

4.8 Introduction

Free radicals and other reactive oxygen species, collectively known as ROS are generated continuously via normal physiological processes, more so in pathological conditions. Reactive oxygen intermediates (ROIs) are partially reduced forms of atmospheric oxygen (O_2). They typically result from the excitation of O_2 to form singlet oxygen (O_2^1) or from the transfer of one, two or three electrons to form superoxide radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) or a hydroxy radical ($HO^{\bullet}$) respectively. They are simultaneously degraded to non-reactive forms by enzymatic and non-enzymatic antioxidant defence mechanisms.

Autoxidation of polyunsaturated fatty acids not only lowers the nutritional value of food (Farag et al., 1989) but is also associated with membrane damage, aging, heart disease and cancer in living organisms (Cosgrove et al., 1987). Oxidative damage in the human body plays an important causative role in disease initiation and progression (Jacob and Burri, 1996). Damage from free radicals and reactive oxygen species has been linked to some neuro-degenerative disorders (Floyd, 1999) and cancers (Goodwin and Brodwick, 1995) and oxidation of low-density lipoprotein is a major factor in the promotion of coronary heart disease (CHD) and atherosclerosis (Frankel et al., 1993).

The addition of antioxidants to food products has therefore become popular as a means of increasing its shelf life and to reduce the wastage and nutritional losses by inhibiting and delaying oxidation (Tsuda et al., 1994). Synthetic antioxidants such as 2, 3 tert-butyl-4-methoxy phenol (BHA) and 2, 6-di-tert-butyl–4-methyl phenol (BHT)

are widely used in the food industry. However there are serious concerns about the carcinogenic potential of these substances (Branen, 1975) and there has been a general desire to replace the synthetic food additives with natural alternatives (Howell, 1986). Therefore intensive research is being carried out on the extraction, characterization and utilization of natural antioxidants that may serve as potent candidates in combating carcinogenesis and aging process.

Sources of natural antioxidants are primarily plant phenolics that may occur in all parts of the plants such as fruits, vegetables, nuts, seeds, leaves, roots and barks (Pratt and Hudson, 1990). Plant phenolics are multifunctional and can act as reducing agents (free radical terminators), metal chelators and singlet oxygen quenchers. Studies have shown that consumption of foods and beverages, rich in phenolic content is correlated with reduced incidents of heart disease (Verhagen, 1989). The most common plant phenolic antioxidants include flavonoid compounds, cinnamic acid derivatives, coumarins, tocopherols and polyfunctional organic acids (Hertog et al., 1993).

Several methods are used to measure the antioxidant activity of a biological material. The most commonly used for their ease, speed and sensitivity are those involving chromogen compounds of a radical nature to stimulate RONS (Reactive oxygen and nitrogen species). The presence of the antioxidant leads to the disappearance of these radical chromogens, the two most widely used being the $ABTS^{+\bullet}$ and the DPPH radicals. DPPH is a free radical that is acquired directly without preparation while $ABTS^{+\bullet}$ must be generated by enzymatic or chemical reactions (Miller and Rice Evans, 1997; Brand Williams et al., 1995).

Cinnamomum verum belongs to the family Lauraceae and possess significant antiallergic, antiulcerogenic, antipyretic and anaesthetic activities (Kurokawa et al., 1998). The bark yields an

essential oil containing cinnamaldehyde and eugenol. Several biological activities such as peripheral vasodilatory, antitumor, antifungal, cytotoxic and antimutagenic activities has been attributed to Cinnamaldehyde (Koh et al., 1998; Bullerman et al., 1977; Kwon et al., 1998; Shaughnessy et al., 2001).

4.9 Materials and methods

4.9.1 Chemicals

Trolox (6-hydroxy- 2, 5, 7, 8-tetramethyl chroman-2- carboxylic acid) and Cinnamaldehyde was purchased from Sigma Aldrich Chemical Co.(Milwaukee, WI). Butylated hydroxyanisole (BHA) from SD Fine Chemicals (India), Thiobarbituric acid (TBA) from CDH (India), Ethylene diamine tetraacetic acid (EDTA) and Tween-20 from E-Merck (India) Ltd, Deoxyribose, Nitroblue tetrazolium (NBT), Trichloroacetic acid (TCA), Pottasium persulphate, Ascorbic acid, Eugenol, Gallic acid and Linoleic acid from Sisco Research Lab (India). All the other chemicals employed were of standard analytical grade.

4.9.2 Plant material

The plant, *Cinnamomum verum* is widely distributed through out tropical and subtropical India and barks from healthy plants were collected for the preparation of methanolic extract.

4.9.2.1 Preparation of extracts

The barks of *Cinnamomum verum* were shade dried initially, freeze dried and then ground to a fine powder. Four grams of the powdered sample was then extracted for 5 hours with methanol under continuous stirring at room temperature (28˚C) and the extraction process was repeated till the solvent became colorless. The extracts were then concentrated in vacuo at 50±1˚C in a rotavapor (Buchi, Model R-205, Germany), followed by lyophilisation (Hetosicc, Model CD2.5) and the solid mass obtained was resuspended in methanol and stored at 0-4˚C.

4.9.3 Evaluation of antioxidant activity

4.9.3.1 Rapid screening of antioxidant by dot-blot and DPPH staining

An aliquot (3 µl) of each dilution of the methanolic plant extract and standard compounds like Cinnamaldehyde and Eugenol were carefully loaded on a 10 x 20 TLC layer (Silica gel 60 F_{254}; Merck) and allowed to dry. Drops of each sample were loaded in the order of increasing concentration along the row. The staining of the silica plate was based on the procedure of Soler-Rivas et al. (Solver-Rivas et al., 2000).

4.9.3.2 DPPH free radical scavenging assay

The antioxidant activity of CBE and the standard compound BHA was measured in terms of hydrogen donating or radical scavenging ability using the stable $DPPH^{\bullet}$ method (Brand-Williams et al., 1995) as modified by Sanchez-Moreno et al. (1998).

4.9.3.3 ABTS radical cation decolorisation assay

Generation of ABTS radical cation (Wolfenden and Willson, 1982) forms the basis of one of the spectrophotometric methods that have been applied to the measurement of the total antioxidant activity of solutions of pure substances, aqueous mixtures and beverages widely used for the assessment of antioxidant activity of various substances. The experiments were carried out using an improved ABTS decolorisation assay (Re et al., 1999) and it involves the generation of $ABTS^{+\bullet}$ chromophore by the oxidation of ABTS with potassium persulfate. It is applicable for both hydrophilic and lipophilic compounds.

4.9.3.4 Superoxide anion scavenging activity

Measurement of superoxide anion scavenging activity of extracts was based on the method described by Liu, Ooi, and Chang (1997) with slight modification (Oktay et al., 2003) as described earlier.

4.9.3.5 Reductive potential

The reductive potential of the extract was determined according to the method of Oyaizu (Oyaizu, 1986) as described earlier. Higher absorbance of the reaction mixture indicated greater reductive potential.

4.9.3.6 Metal chelating activity

The chelation of ferrous ions by the extracts and standard was estimated by the method of Dinis et al. (1994).

4.9.3.7 Antioxidant activity in linoleic acid emulsion system

The antioxidant activity of the CBE and BHA was determined by the thiocyanate method (Duh et al., 1997) as described earlier.

4.9.3.8 Hydroxyl ($OH^{\bullet}$) radical scavenging activity

The sugar deoxyribose on exposure to hydroxyl radicals, generated by the Fenton reaction model system degrades in to fragments and generates a pink chromogen on heating with TBA at low pH (Halliwell et al., 1987).

The hydroxyl radical scavenging activity was measured by the deoxyribose method (Halliwell et al., 1987) as described earlier.

4.9.3.9 Total phenolics

The extracts were diluted with the same solvent used for extraction, to a suitable concentration for analysis. Total phenolic content of extracts was assessed approximately by using the Folin-Ciocalteau phenol reagent method (Singleton & Rossi, 1965).

4.9.3.10 Statistical analysis

The experimental results are expressed as mean ± S.D of three parallel measurements. The results were processed using Microsoft excel 2000 and the data were subjected to one way analysis of variance (ANOVA) and the significance of differences between sample means were calculated by Duncan's multiple range test using SPSS for Windows, Standard Version 7.5.1, SPSS. Inc., Chicago, IL. P values $\leq$ 0.05 were regarded as significant and P values $\leq$ 0.01 as very significant.

4.10 Results and discussion

4.10.1 DPPH radical scavenging activity

Cinnamon bark extract showed good free radical scavenging capacity at all the concentrations studied. $DPPH^{\bullet}$ is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule (Soares et al., 1997). Stained silica layer revealed a purple background with yellow spots at the location of the drops, which showed radical scavenging activity in all the three cases. The intensity of the yellow color depends on the amount and nature of radical scavenger present in the sample and standard compounds. Eugenol exhibited a faster reaction rate and stronger intensities of white-yellow spots compared to Cinnamaldehyde and CBE (Fig. 4.18).

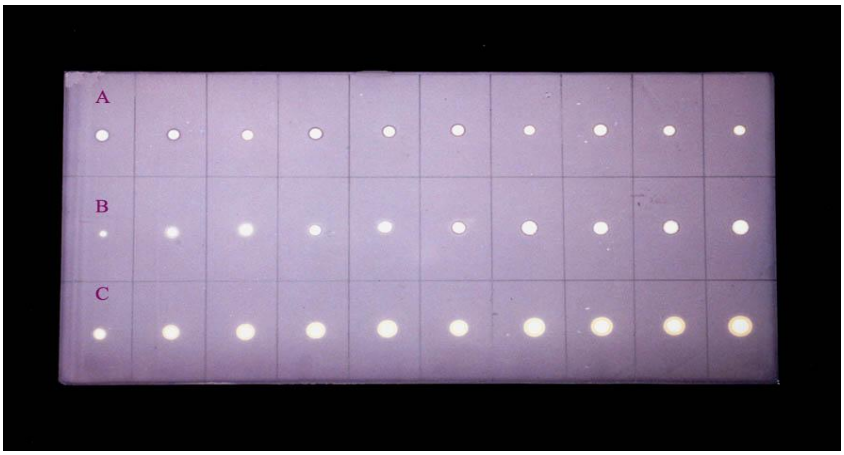


Figure 4.18: Dot blot assay on a silica sheet stained with DPPH solution in methanol; First row (A) CBE in the order of increasing concentration (200-2mg/ml); Second row (B) Cinnamaldehyde (10-100µg/ml); Third row (C) Eugenol (10-100µg/ml).

There was a significant decrease in the concentration of DPPH$^{\bullet}$ due to the scavenging ability of the methanolic extracts of CB (Fig 4.19). Significant radical scavenging activity was evident at all the tested concentrations of CB. The scavenging activity increased with increasing concentration of CBE and BHA up to 12.5 µg/ml and then levelled off with further increase in concentration. The EC_{50} value of CBE was found to be 4.21µg/ml and that of BHA 5.79µg/ml and is inversely related to antioxidant capacity.

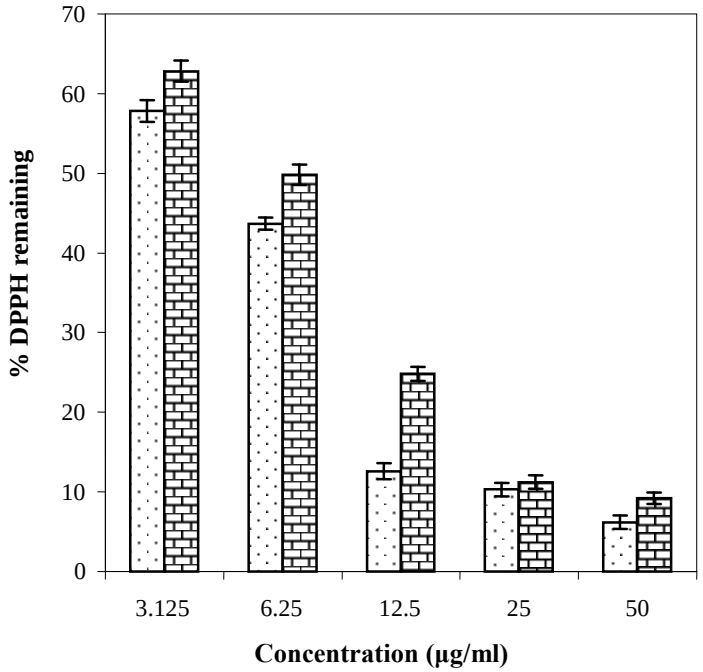


Figure 4.19: Free radical scavenging capacity of CBE (white bar) and BHA (checker bar) as determined by the DPPH method. P value ≤ 0.05.

4.10.2 ABTS radical cation scavenging activity

This method measures the relative antioxidant ability to scavenge the radical $ABTS^{+\bullet}$ as compared with a standard amount of Trolox, and is an excellent tool for determining the antioxidant activity of hydrogen donating antioxidants and of chain breaking antioxidants. Fig 4.20 depicts a steady increase in the ABTS radical scavenging capacity of CBE up to a concentration of 25 μg/ml followed by a levelling off with further increase in concentration. The TEAC value for CBE at the maximum concentration studied was found to be 18.45 ± 0.6.

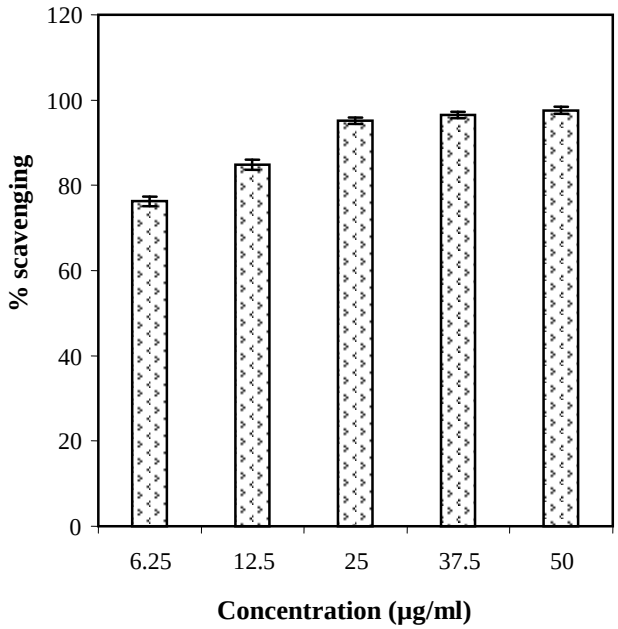


Figure 4.20: ABTS radical scavenging capacity of CBE. P value ≤ 0.05

4.10.3 Superoxide anion scavenging activity

Superoxide is biologically important since it can be decomposed to form stronger oxidative species such as singlet oxygen and hydroxyl radicals (Korycka-Dahl and Richardson, 1978).

From the investigations on the superoxide radical scavenging capacities, it was found that the CBE inhibit superoxide radical in a dose dependent manner. In the PMS/NADH-NBT system, superoxide anion derived from dissolved oxygen by PMS/NADH coupling reaction reduces NBT. The decrease of absorbance at 560 nm with antioxidants thus indicates the consumption of superoxide anion in the reaction mixture. The methanol extract of CB exhibited superoxide scavenging activity (Fig 4.21) at all the concentrations (12.5-100µg/ml). However, the reference compound, Ascorbic acid at similar concentrations exhibited pro-oxidant effect. Ascorbic acid is a potent reducing agent and acts as a free radical scavenger. However, it may act as a pro-oxidant in the presence of metals (Bendich et al., 1986).

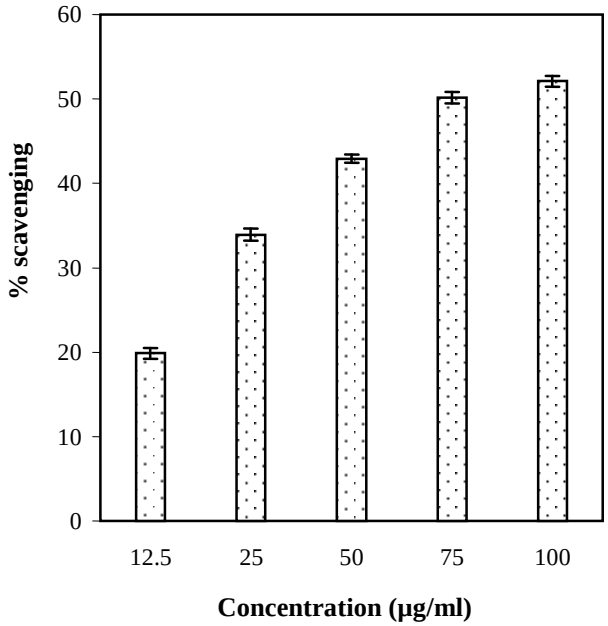


Figure 4.21: Superoxide radical scavenging capacity of methanol extracts of CB determined by the PMS/NADH-NBT method. P value ≤ 0.05.

4.10.4 Reducing Power

The reducing power of the CBE and the reference compound, Ascorbic acid increased steadily with increasing concentration (Fig 4.22). The reducing powers (absorbance at 700 nm) of CBE and Ascorbic acid were 2.727 and 3.610 at a dose of 1mg showing that the CBE can act as electron donors and can react with free radicals to convert them to more stable products and thereby terminate radical chain reaction. The reducing power of CBE might be due to the di and monohydroxyl substitutions in the aromatic ring which possess potent hydrogen donating abilities as described by Shimada et al., (Shimada et al., 1992).

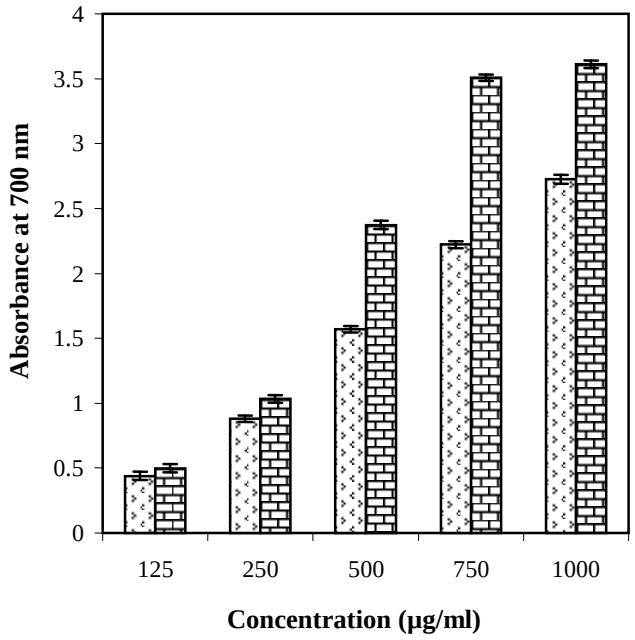


Figure 4.22: Reducing power of CBE (white bar) and ascorbic acid (checker bar). P value ≤ 0.01

4.10.5 Metal chelating activity

The chelating properties of CBE were examined against Fe^{2+}. Ferrozine can quantitatively form complexes with Fe^{2+}. In the presence

of other chelating agents, the complex formation is disrupted with the result that the red color of the complex is decreased. As shown in Fig. 4.23, the formation of the ferrozine-Fe^{2+} complex is not complete in the presence of CBE, indicating that it can chelate the iron. The absorbance of ferrozine-Fe^{2+} complex decreased linearly in a dose dependent manner (750-1750µg/ml). However the chelating ability was much lower than that of EDTA.

Metal chelating activity was significant since it reduced the concentration of the catalyzing transition metal in lipid peroxidation (Duh et al., 1999). It has been reported that chelating agents which form σ bonds with a metal are effective as secondary antioxidants because they reduce the redox potential, thereby stabilizing the oxidized form of the metal ion (Gordon, 1990).

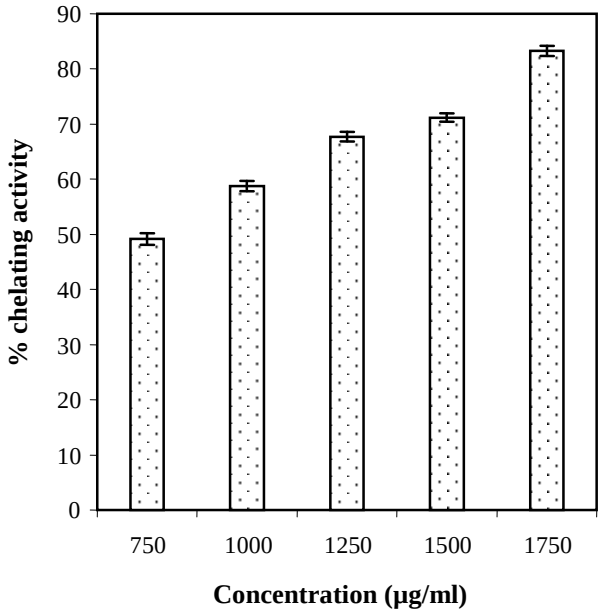


Figure 4.23: Metal chelating activity of different concentrations of CBE. P value ≤ 0.01

4.10.6 Total antioxidant determination in linoleic acid emulsion system

The CBE exhibited effective and powerful antioxidant activity at all the concentrations tested. The effect of various concentrations of the CBE (25-200µg/ml) on peroxidation of linoleic acid emulsion is represented in Fig. 4.24. The antioxidant activity of the extract increased with the increasing concentration. The percentage inhibition of peroxidation in linoleic acid system by 25, 50, 75, 100 and 200µg/ml was found to be 81.75%, 82.35%, 84.5%, 86.48% and 93.34% respectively at 48 hours. The percentage inhibition of 200µg/ml concentration of BHA was found to be 89.78%.

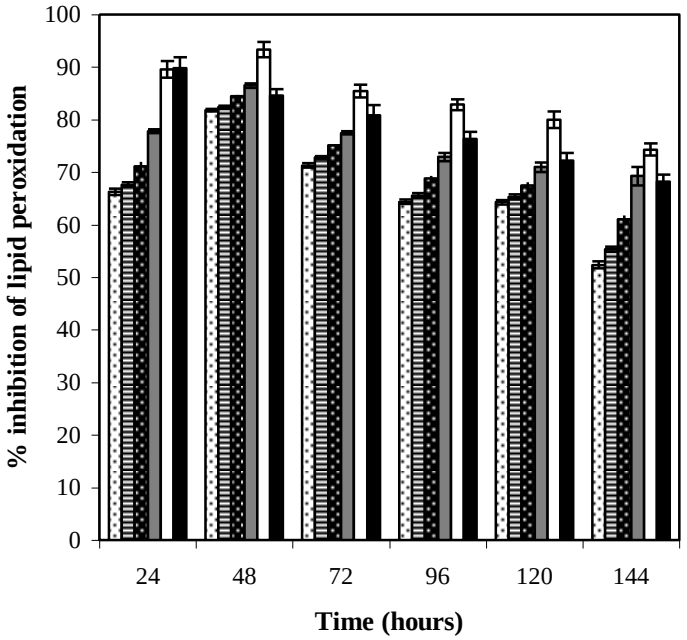


Figure 4.24: Antioxidant activity of CBE at different concentrations -25µg/ml (white spotted bar); 50µg/ml (bar with horizontal lines); 75µg/ml (black spotted bar);100µg/ml (grey bar); 200µg/ml (white bar) and BHA-200µg/ml (black bar) in the linoleic acid emulsion system using the thiocyanate method.

4.10.7 Hydroxy radical scavenging

The highly reactive ·OH can cause oxidative damage to DNA, lipids and proteins (Spencer et al., 1994). As is the case for many other free radicals, ·OH can be neutralised if it is provided with a hydrogen atom. The sample exhibited hydroxyl radical scavenging activity in a dose dependent manner in the range of 15-250µg/ml in the reaction mixture (Fig 4.25) Overall, the scavenging activities of phenolic substances might be due to the active hydrogen donating ability of hydroxyl substitutions.

The total phenolic content of the CBE was estimated to be 288.96±2.2 mg Gallic acid equivalents/g of plant extract from triplicate measurements.

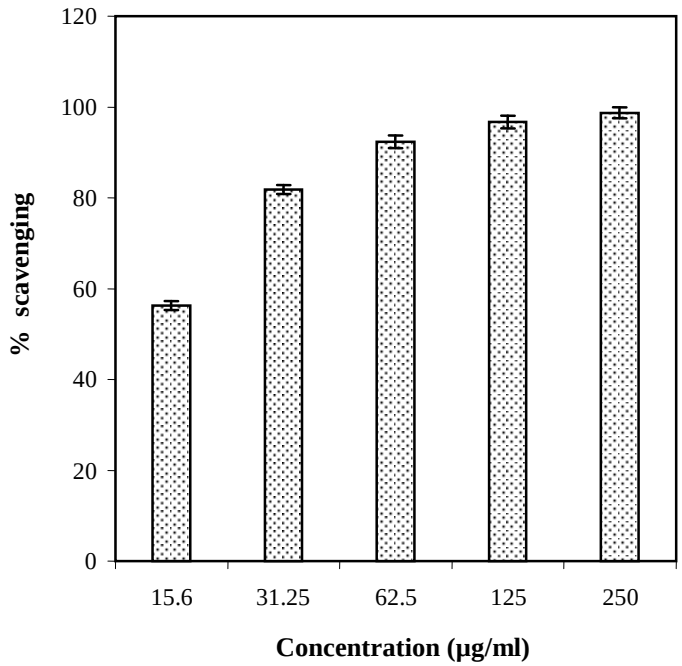


Figure 4.25: Hydroxy radical scavenging capacity of CBE. P value ≤ 0.05.

4.10.8 Conclusion

In conclusion, the results obtained in the present study has shown the methanolic extract of CB which contain a number of antioxidant compounds can effectively scavenge reactive oxygen species including superoxide anion and hydroxyl radicals as well as other free radicals under in vitro conditions. Moreover the hydrogen donating ability of all the experimental compounds and the extract has been proven through the assessment of reducing power and $DPPH^{\bullet}$ scavenging activity. Eventhough CBE are weak chelators of metal ions their free radical scavenging capacity is comparable to that of synthetic antioxidants such as BHA. CBE possess antioxidant properties, which are concentration dependent.

Antioxidant properties of botanical extracts should be evaluated in a variety of model systems using several different indices because the effectiveness of such antioxidant material is largely dependent upon the chemical and physical properties of the system to which they were added and a single analytical protocol adopted to monitor lipid oxidation may not be sufficient to make a valid judgement. The ability of the extract to retard lipid oxidation is attributable to the ability of its phenolic constituents to quench reactive oxygen species.

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CHAPTER V

SYNTHESIS AND CHARACTERIZATION OF THE PHYSICO-CHEMICAL PROPERTIES OF STARCH FERULATE

5.1 Introduction

Starch is an attractive raw material due to its abundant supply, low cost, biodegradability, biocompatibility and ease of chemical modification. Indeed, modified starches have approval for food use, in which they act as thickeners, gelling agents, as sizing agents in textiles and as adhesives for paper and paper products. In recent years, a number of authors (Aburto et al., 1999; Fang et al., 2002) have reported the preparation of modified starches to higher degrees of substitution, relying on the use of organic solvents. Reactions on starches to prepare highly substituted derivatives are not easy, mainly because of the almost impossible proposition of dissolving granular starch in a suitable medium without significant degradation (Sagar and Merill, 1995). The introduction of an ester group into polysaccharides constitutes an important synthetic task, because it permits to modify their original hydrophilic nature and to obtain enhanced or new thermal and mechanical properties (Aburto et al., 2000). Maize and potato starch esters have been prepared under heterogeneous reaction conditions, using acid anhydride, and pyridine via a nucleophilic acyl substitution reaction, in dimethylformamide (Aburto et al., 2000).

Functional foods, enriched in biologically active compounds are becoming increasingly available in many countries and the potential markets are enormous. Ferulic acid is one of the most abundant hydroxy cinnamic acid, present at relatively high concentrations in the cell walls of several plants (Hartley and Ford, 1989; Hartley and Harris, 1981) and the antioxidant activity of ferulic acid is well recognized (Rice Evans et al., 1996) and it is also reported as antihepatotoxic (Kiso et al., 1983) and offers various benefits for the cardiovascular system (Rukmini and Reghuram, 1991). Apart from its anti-inflammatory action (Chawla et al., 1987) ferulic acid as a constituent of synthetic lignins, may contribute to the defense against viral infections including AIDS (Lai

Ohhara et al., 1992). Ferulic acid has been reported to suppress experimental carcinogenesis in the lungs (Lesca, 1983), skin (Kaul and Khanduja, 1998) and colon (Mori et al., 1999). Ferulic acid at a concentration of 100 mM was found to significantly suppress inducible nitric oxide synthase expression by 38% and obstruction of these biochemical phenomena is anticipated to be a reasonable strategy for chemoprevention (Murakami et al., 2002).

However, free ferulic acid does not enter the enterohepatic circulation (Chang et al., 1993) and therefore through oral or intravenous administration, ferulic acid does not easily reach the colon. Moreover, the dietary fibre bound ferulic acid is only partly released by the microorganisms in the colon. At such low levels, it is unable to act as a chemopreventive agent (Ou et al., 1999). Enzyme resistant starch is a type of dietary fiber that is almost completely fermented in the colon (Baghurst et al., 1996) and may be a satisfactory carrier of ferulic acid. The release of ferulic acid from starch ferulate by colon microorganisms has been found to be much higher than with dietary fibers from wheat bran (Ou et al., 2001). Resistant starch like potato starch escape digestion in the small intestine and pass in to the large bowel (Sievert and Pomeranz, 1989) and improve cholesterol metabolism and reduce the risk of colon cancer (Yue and Waring, 1998).

The effect of feruloyl oligosaccharides on the growth of *Bifidobacterium bifidum* F-35 had been investigated *in vitro* and the biomass yield of the *B. bifidum* was found to increase with the increasing concentration of feruloyl oligosaccharides. This indicated that the growth of *B. bifidum* was promoted by the feruloyl oligosaccharides from wheat bran insoluble dietary fiber, and not suppressed by the ferulic acid moiety (Yuan et al., 2005).

The present study deals with the preparation, physicochemical and thermal characterization and microstructural imaging of starch ferulates.

5.2 Materials and methods

5.2.1 Materials

Potato starch was isolated and purified from the fresh tubers of potato (*Solanum tuberosum*) using the modified procedure of Willinger (Willinger, 1964). The amylose and amylopectin contents of starch were determined simultaneously (Landers et al., 1991). The ash, protein and fat content of the starch were determined according to the standard AOAC methods (AOAC, 1995). Ferulic acid was procured from Sigma (St. Louis, USA). Thionyl chloride, DMSO and Pyridine were obtained from Merck, India, Sisco Research Laboratories and CDH, India respectively. All the reagents used were of analytical grade.

5.2.2 Esterification

The esterification of starch was carried out in two steps. The first step involved the synthesis of ferulic acid chloride at 80°C for 3 hours by refluxing ferulic acid with thionyl chloride. Upon completion of the reaction, the thionyl chloride was removed by repeated distillation using chloroform and the acid chloride formed was dried under nitrogen. Stoichiometric quantities of the acid chloride were added to fixed moles of potato starch dissolved in DMSO, followed by pyridine, added in a 2:1 ratio with respect to the acid chloride and refluxed at 110°C for 1 hour under nitrogen and continuous stirring. The product was precipitated using 95% alcohol (300ml x 3). The precipitate was then centrifuged, washed with 70% alcohol followed by vacuum drying at 55°C to determine the degree of substitution (Table 5.1).

5.2.3 Determination of the degree of substitution (DS)

Starch ferulate (0.25g) was extracted in 25ml solution of 1:1 mixture of 1% (w/v) of $NaBH_4$ and 8% (w/v) solution of NaOH so that the final

concentrations are 0.5% and 4% respectively. The extracts were then acidified with HCl to pH 2.5 and extracted with 2 volumes of ethyl acetate (X3). The content of free ferulic acid was then determined spectrophotometrically at 320 nm using a UV-Visible spectrophotometer (Shimadzu, UV 2100) (Rybka et al., 1993) and ethyl acetate as blank.

DS value was determined titrimetrically according to the method of Wurzburg (Wurzburg, 1964) with slight modifications. Powdered starch ferulate (0.125g) was weighed accurately and to it was added 6.25 ml of 75% ethanol, warmed at 50°C and held at that temperature for 30 minutes followed by addition of 5 ml of 0.5 N NaOH. The flask was stoppered and allowed to stand 72 hours with occasional shaking. The excess NaOH was back titrated against 0.5 N HCl using phenolphthalein as indicator. A blank was simultaneously titrated with native starch as sample. DS was calculated as:

$$\%\text{feruloyl} = \frac{[\text{ml.(blank)} - \text{ml.(sample)}] \times \text{normality of acid} \times 0.177 \times 100}{\text{Sample weight in grams (dry basis)}}$$

$$\text{Degree of Substitution (DS)} = \frac{162 \times \%\ \text{feruloyl}}{177 \times 1000 - (176 \times \%\ \text{feruloyl})}$$

where 162 = molecular weight of glucose unit; 177 = molecular weight of feruloyl group; 176 = molecular weight of feruloyl group – 1.

Reaction efficiencies were calculated as the ratio of measured DS to the theoretical DS (moles of starch ferulate/moles of anhydroglucose residue).

5.2.4 Determination of viscosity

The viscosities of the native and modified starch esters in dimethylsulfoxide (DMSO) were determined using a Synchro-lectric Brookfield viscometer (Model RVT, MA, USA) at three different rpm using spindle number 21 and expressed in Pa.s. Both starch and starch

ferulate (1%w/v) were dissolved in DMSO at 80°C under stirring and the viscosity was measured in triplicates at 30±1°C.

5.2.5 Thermal analyses

5.2.5.1 Thermogravimetric analyses

Thermogravimetric analyses were performed in a Simultaneous DTA-TG Apparatus (DTG-60; Shimadzu, Japan). Samples (2-8 mg) were heated at a rate of 20°C/min from ambient temperature to 800°C. Nitrogen was used as the purge gas at a flow rate of 20 ml/min. In order to determine the thermal stability of native starch and the starch ester, the following values were determined: $T_{x\%}$- temperature corresponding to x% mass loss.

5.2.5.2 Differential scanning calorimetry (DSC) analyses

Thermal properties of the native potato starch and the starch ester were characterized using a Perkin-Elmer Pyris DSC 6 (Perkin Elmer, Boston, MA). Nitrogen at the rate of 30 ml/min was used as the purge gas. 5-9 mg of powdered material were crimped close in aluminum pans and heated from 20°C up to 200°C in the case of starch ester and up to 250°C in the case of native starch at the rate of 10°C per minute, followed by a cooling cycle back to 20°C at the same rate.

5.2.6 Fourier transform infrared (FTIR) spectroscopy

The FTIR spectra of the native starch and starch ester were recorded in an IR spectrometer (Nicolet Magna 4R 560, Minnesota, USA) using potassium bromide (KBr) discs prepared from powdered samples mixed with dry KBr in the ratio 1:200.

5.2.7 X-ray diffraction studies

X-ray diffraction patterns of native potato starch, ferulic acid and starch ferulate were analyzed using an X-ray diffractometer (XPERT, Philips, Eindhoven, The Netherlands) with Nickel filtered Cu Kα radiation (λ=0.154 nm) at a voltage of 40 kV and current of 30 mA. The diffractometer was equipped with an automatic divergence slit and the

scattered radiation was detected in the angular range of 5–40 (2θ) with a scanning speed of 2°(2θ)/min and a step size of 0.06 (2θ).

5.2.8 Microstructure studies by SEM

The morphological structures of the native potato starch and starch ferulates of different DS 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 15KV and magnification of 350X and 2500X.

5.2.9 Determination of solubility

The solubility of the starch ferulates were measured at 5% (w/v) concentration in a range of organic solvents with stirring at room temperature and under heating.

5.2.10 Spectrophotometric analyses

The UV-Visible spectra of starch, starch ferulates of different DS in DMSO solution (at a concentration of 100mg/ml) and ferulic acid in ethanol (10μg/ml) were obtained in an UV 2100 spectrophotometer (Shimadzu, Kyoto, Japan).

5.2.11 Evaluation of antioxidant activity

5.2.11.1 Rapid screening of radical scavenging capacity of starch ferulates by dot-blot and DPPH staining

An aliquot (5 μl) of each dilution of the starch ferulate and the standard compound, ferulic acid were carefully loaded on a 10 x 20 TLC plate (Silica gel 60 F_{254}; Merck) and allowed to dry. Drops of each sample were loaded in the order of increasing concentration along the row. The staining of the silica plate was based on the procedure of Soler-Rivas et al. (Solver-Rivas et al., 2000).

5.2.11.2 Determination of ABTS radical cation decolorisation capacity

The experiments were carried out using an improved ABTS decolorisation assay (Re et al., 1999) and it involves the generation of $ABTS^{+\bullet}$ chromophore by the oxidation of ABTS with potassium persulfate as described earlier.

Absorbance was measured 7 min after the initial mixing of different concentrations of starch ferulate with 1 ml of $ABTS^{+\bullet}$ solution. All determinations were carried out in triplicate.

5.3 Results and discussion

5.3.1 Starch ferulate synthesis

The solubilisation of the swollen starch in DMSO activates the exposed hydroxyl groups to attack by electrophilic reagents. Pyridine acts not only as a base but also as a nucleophilic acylation catalyst. The hydrochloric acid formed as a by-product reacts with the excess pyridine to form the pyridinium salt (Fang et al., 2002). The percentage yield of starch ferulates obtained is given in Table 5.1. The formation of the starch ferulate was confirmed by IR.

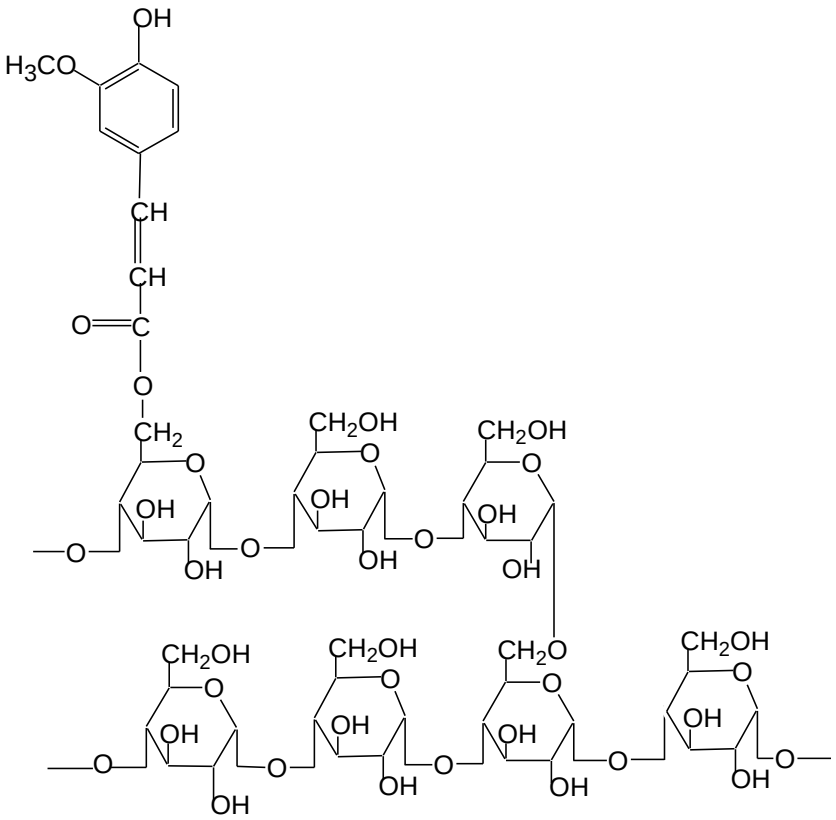


Figure 5.1: Structure of starch ferulate with ferulic acid attached to the 6th carbon atom of the glucose unit of starch

Type	No. of moles of ferulic acid chloride	No. of moles of starch	Theoretical DS of starch ferulate	DS obtained by		Reaction efficiency (%)	Yield (%)
				Alkaline saponifi-cation	By Spectrophotometry		
1	0.0072	0.014	0.514	0.150	0.125	29.18	96.2
2	0.0102	0.014	0.728	0.360	0.344	49.56	94
3	0.014	0.014	1.00	0.50	0.519	50	97
4	0.0224	0.014	1.60	0.776	0.757	48.53	97

Table 5.1: Esterification yields and degree of substitution of the starch esters

5.3.2 DS of starch ferulate

The DS of a starch derivative is defined as the number of hydroxyl (OH) groups substituted per D-glucopyranosyl structural unit of the starch polymer. Since each glucose unit possesses three reactive hydroxyl groups, the maximum possible DS value is 3. The primary OH group on C6 is more reactive and is esterified more readily than the secondary ones on C2 and C3 due to steric hindrance. DS varies with the source of starch, amylose and amylopectin fraction, chemical amounts and reaction time. The esterification of starch with organic acids results in thermoplastic and hydrophobic materials when the DS is high enough (Rudnik et al., 2005)

The DS of the starch ferulates increased with higher ratios of acid chloride to starch (Table 5.1). Higher ferulic acid chloride concentration result in a higher rate of molecular collision and a greater availability of ferulic acid chloride molecules in the vicinity of starch (Xu et al., 2004). The reaction efficiency however decreased slightly at higher molar concentrations of acid chloride as the esterification reactivity of the hydroxyl goups in anhydroglucose unit is different. Hydrophobicity of starch increases with increasing DS which increases the miscibility of starch with other hydrophobic polymers (Thiebaud et al., 1997).

5.3.3 Viscosity

The viscosity profile of both native potato starch and the synthesized starch ferulates, when spindle number 21 was used are shown in Table 5.2. On esterification, the starch exhibited a reduction in viscosity, supporting an enhanced hydrophobicity for the starch ester compared to the native starch. Chemical modification cause rupturing of some or all of the starch molecules, thereby weakening them and decreasing their capacity to swell. Also a certain degree of depolymerization which must have occurred during the esterification reaction might also have contributed to the reduction in viscosity. The

viscosity was found to decrease with increasing rpm revealing the shear thinning behavior and pseudoplastic nature of both native starch and the starch ester.

Sample	Brookfield rpm	Brookfield viscosity in Pa.s
Native potato starch	10 20 50	0.055 0.047 0.034
Starch ferulate 1	10 20 50	0.025 0.020 0.010
Starch ferulate 2	10 20 50	0.025 0.020 0.009
Starch ferulate 3	10 20 50	0.020 0.015 0.007
Starch ferulate 4	10 20 50	0.017 0.012 0.005

Table 5.2: Viscosity profile of potato starch and starch ferulates

5.3.4.1 TGA

The TG spectra were used to determine the weight loss of the material on heating. The TGA and differential thermogravimetric (DTG) curves for the native starch and starch ferulate are shown in figure 5.2 and figure 5.3. The starch and starch esters of low DS showed a three stage weight loss, the first minor one, corresponding to the loss of water around 60-100°C and the other two corresponding to the decomposition which was more sharp and pronounced for the native starch. The native starch underwent 50% weight loss at 302°C while in the case of starch

ferulate it ranged between 332-370°C for esters with different DS (Table 5.3). The DTG curve for native starch showed three peaks, the first one corresponding to the loss of water at around 60-100°C followed by a two stage decomposition. The second major peak had a maximum at 298°C for native starch corresponding to its decomposition. With increase in DS, there was a shifting of peak maxima towards higher temperatures. The starch ferulates were found to be more thermally stable than native starch. Since the main decomposition mechanism of starch is the dehydration reaction between starch hydroxyls, the lower the amount of hydroxyl groups remaining the better is the thermal stability of the starch esters (Rudnik et al., 2005).

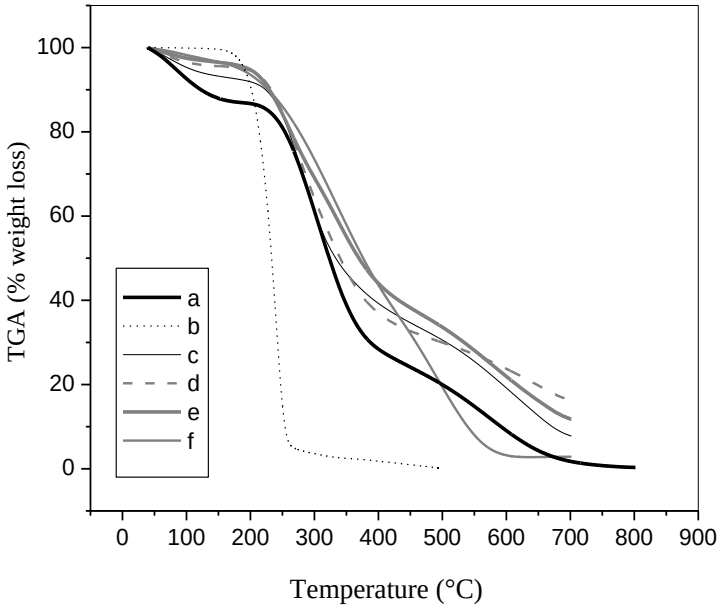


Figure 5.2: TGA thermogram of native starch (a), ferulic acid (b) and starch ferulates of different DS- St. fer. 1(c); St. fer. 2 (d); St. fer. 3 (e); St. fer. 4 (f).

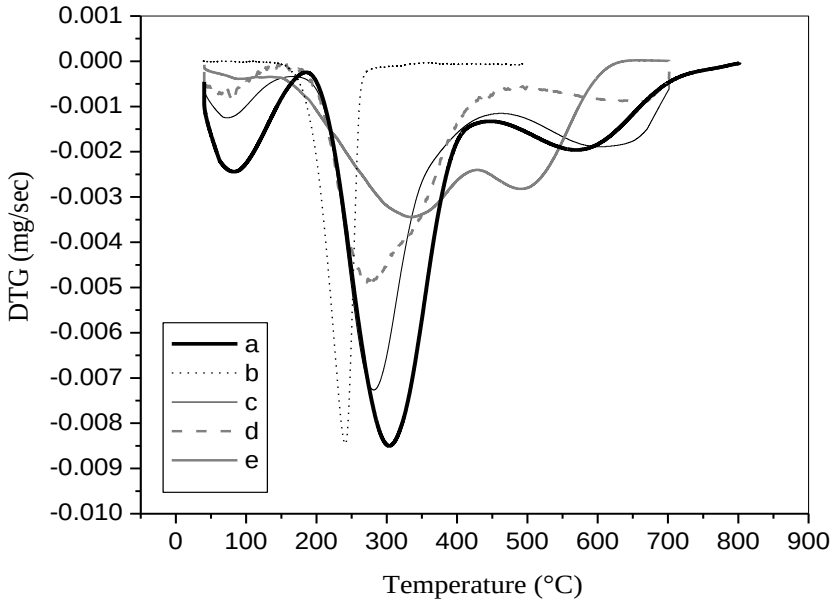


Figure 5.3: Differential thermogravimetric curves of native starch (a), ferulic acid (b) and starch ferulates of different DS- St. fer. 1 (c); St. fer. 2 (d) St. fer. 4(e).

Sample	$T_{5\%}$	$T_{25\%}$	$T_{50\%}$
Starch	81.5	267.4	321.0
Ferulic acid	190.4	216.4	234.3
Starch ferulate 1	105.3	271.9	332.5
Starch ferulate 2	191.1	271.9	340.1
Starch ferulate 3	196.7	279.5	370.3
Starch ferulate 4	198.5	292.2	375.4

Table 5.3: Thermal characteristics of starch and starch ferulates

5.3.4.2 DSC

DSC was used to measure the presence of exothermal or endothermal changes with increase in temperature. (Fig. 5.4) However the DSC profile of native starch and starch ferulate did not reveal any significant data except for the broad endothermal peak around 60-100°C corresponding to the evaporation of water as supported by the TGA and DTG data.

On gelatinization, the hydrogen bonding between the adjacent glucose units in the swollen granule of starch is disrupted and the crystallinity is destroyed (St. Pierre et al., 1997).

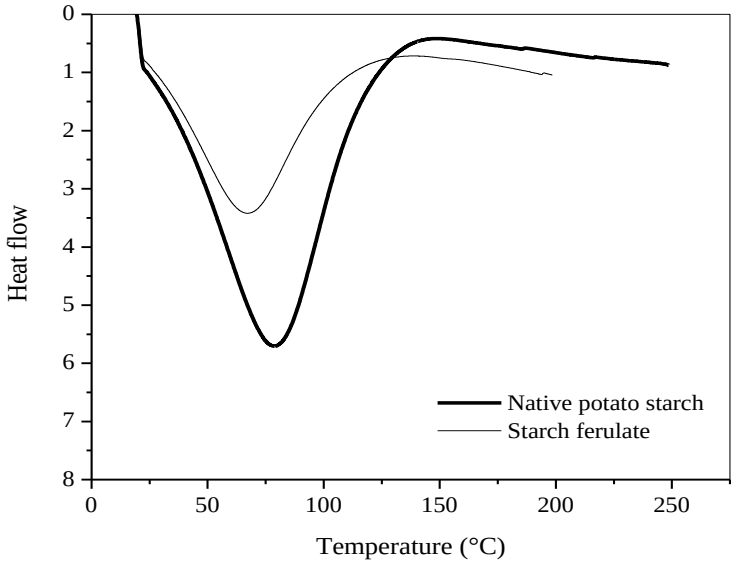


Figure 5.4: DSC thermogram of native starch and starch ferulate.

5.3.5 FTIR

In Potato starch, the finger print region of the spectrum consists of three characteristic peaks between 923 and 1162 cm^{-1} attributed to the C-O bond stretching (Figure 5.5) (Goheen and Wool, 1991). The bands at 1659 cm^{-1} and 1467 cm^{-1} are assigned to the δ (O-H) bendings of water and CH_2 respectively (Mano et al., 2003). The sharp band at 2926 cm^{-1} is characteristic of C-H stretches associated with the ring methine hydrogen atoms. An extremely broad band occurs at 3400 cm^{-1} due to the hydrogen bonded hydroxyl groups that contribute to the complex vibrational stretches associated with free inter and intra-molecular bound hydroxyl group which make up the gross structure of starch (Fang et al., 2002).

The spectrum of ferulic acid consisted of a sharp peak at 3436 cm^{-1} corresponding to the hydroxyl group. The sharp bands at 1619, 1593, 1513, 1434 cm^{-1} are due to C-C skeletal vibrations and

characteristic of the aromatic ring. The sharp bands at 804 and 850 cm^{-1} are due to the two adjacent hydrogen atoms. The formation of the acid chloride was confirmed by the presence of carbonyl absorption in the FT-IR spectrum at 1732 cm^{-1} and absence of the broad hydroxyl band of the acid in the region of 3500-3000 cm^{-1}. Esterification resulted in the formation of a carbonyl signal in the FT-IR spectrum around 1726 cm^{-1} (Figure 5.6) distinct from the carbonyl signals of both unreacted acyl chloride around 1732 cm^{-1} or the hydrolysis products, carboxylic acids around 1700 cm^{-1} and their salts around 1640 cm^{-1}. The occurence of two peaks of strong intensities at 2926 and 2860 cm^{-1} in the spectra is attributed to the methyl and methylene C-H stretching associated with the feruloyl substituents. In the starch ferulate, the broad peak around 3400 cm^{-1} reduced in intensity owing to the reduction in the number of hydroxyl groups. The intensity of the carbonyl group peak at 1726 cm^{-1} also increased with increase in DS (Figure 5.6).

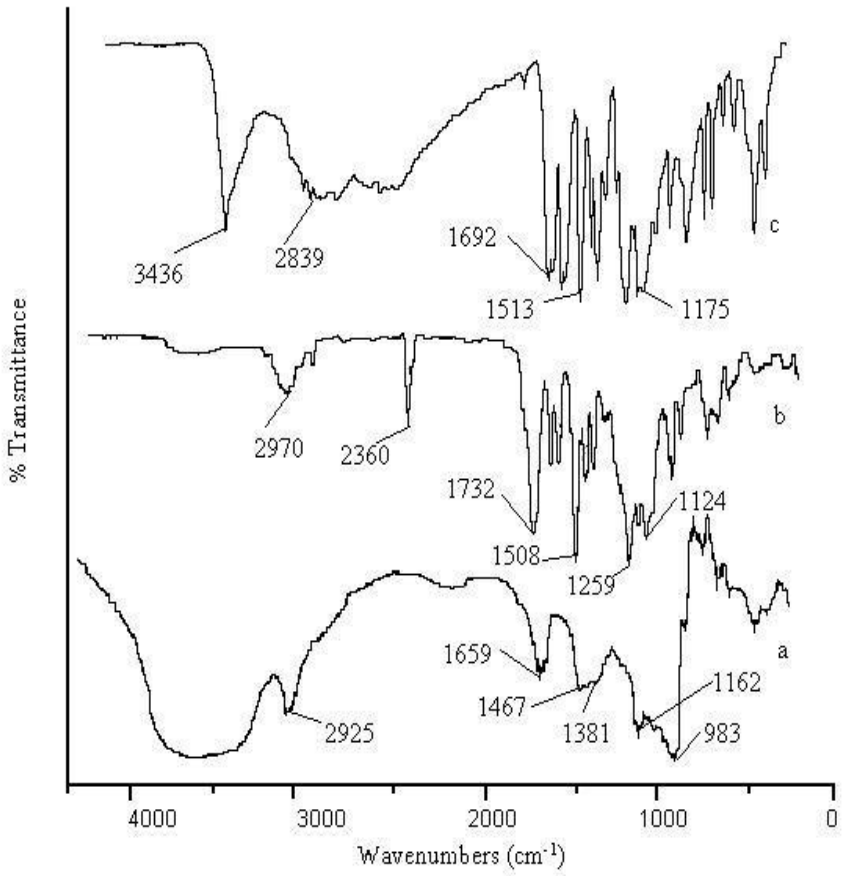


Figure 5.5: IR Spectra of native potato starch (a), ferulic acid chloride (b) and ferulic acid (c).

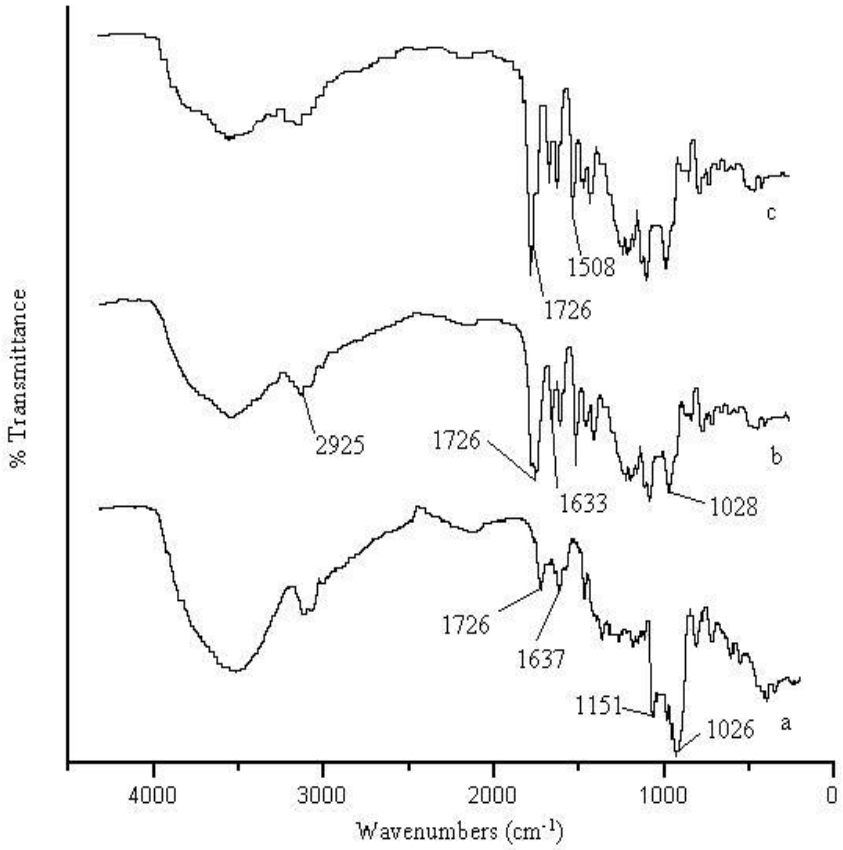


Figure 5.6: IR Spectra of starch ferulates of different degree of substitution - St. fer. 2(a); St. fer. 3(b); St. fer. 4(c).

5.3.6 XRD

The native potato starch powder had a typical B-type crystalline structure (Rindlav et al., 1997) as shown in figure 5.7 with strong diffraction peak at around 17° (2θ) and a few small peaks at around 2θ of 15°, 20°, 22° and 24°. Linear amylose composed of α 1, 4 glucopyranose contributed to amorphous regions while the branched amylopectin composed of α 1, 4 and α 1, 6 glucopyranose contributed to the crystalline region. The intra and intermolecular hydrogen bonds were responsible for the highly ordered crystalline structure (Xu et al., 2004). Ferulic acid has strong crystallinity peaks at 2θ of 9, 10.4, 12.7, 15.6, 17.3, 26.3 and 29.4° and several minor peaks at 21, 24.5, 31.5, 34.6, 36 and 39°. On esterification, the feruloyl groups replaced some of the hydroxyl groups on starch reducing the formation of intermolecular

hydrogen bonds and thereby the destruction of the ordered crystalline structure. The starch ferulate ester had broad peaks at 17.7, 20.2, 21.8, 23.8 and 25.47°.

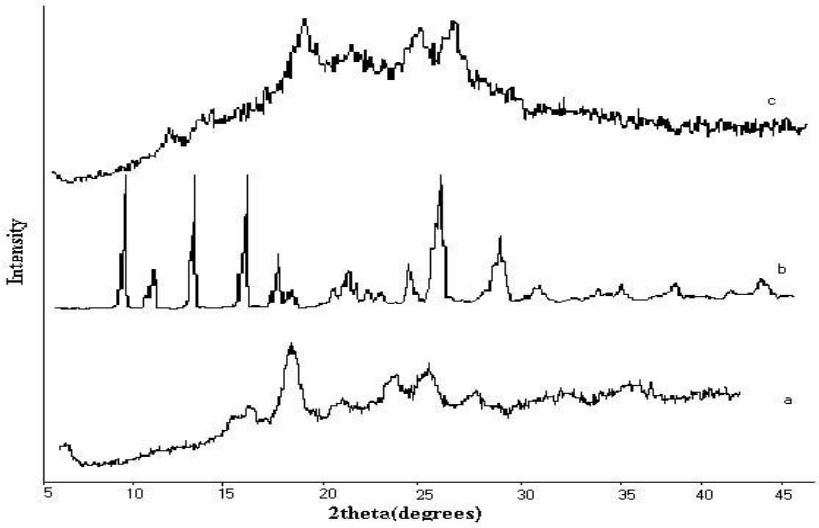
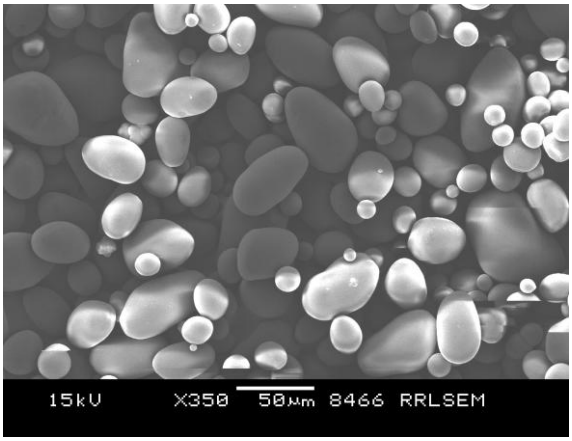


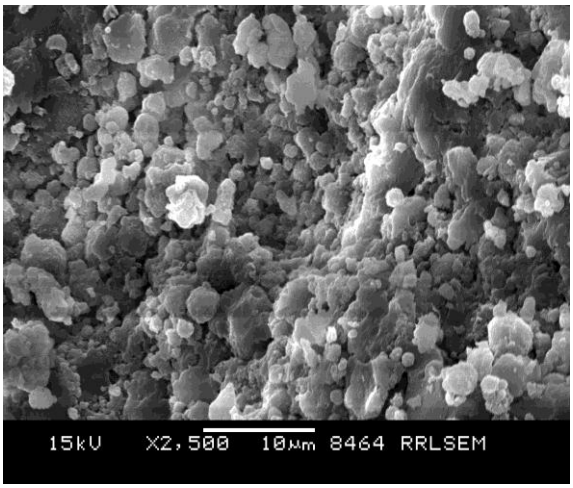
Figure 5.7: XRD profile of native potato starch (a), ferulic acid (b) and starch ferulate (c).

5.3.7 SEM observations

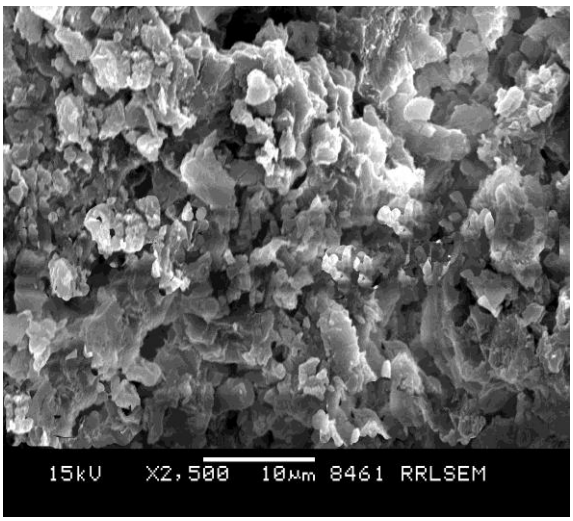
Majority of the potato starch granules were oval to ellipsoid in shape with few spherical ones and ranged in size between 15-60μm. The starch granules lost their individuality and smoothness on esterification as a result of gelatinization and substitution of hydroxyl groups and formed agglomerates. As the DS of the starch ferulates increased there was an enhancement in the net working nature (Figure 5.8) as has been observed earlier by Xu et al. in 2004.



a



b



c

Figure 5.8: SEM photographs of native potato starch granules (a) and starch ferulates of different degree of substitution (b and c).

5.3.8 Solubility

The introduction of feruloyl groups in to the starch might alter the solubility profile of starch which depends on the extent of esterification, nature of the group substituted, type of starch, temperature and solvent employed (Fang et al., 2004).

The starch ferulates were found to be soluble in hot DMSO and partially in pyridine but insoluble in all the other organic solvents employed suggesting that the degree of substitution was not high enough to bring about a significant change in the solubility.

5.3.9 Spectrophometric analyses

Starch did not show any absorbance in the 200-700 nm wavelength range (Figure 5.9). Ferulic acid had absorbance maxima at 320 nm in the non ionized state, while the starch ferulates had two absorption maxima one at 300 and another at 330 corresponding to the absorption of ferulic acid ester linked to the starch. The absorption intensity in the UV range increased with higher degree of substitution.

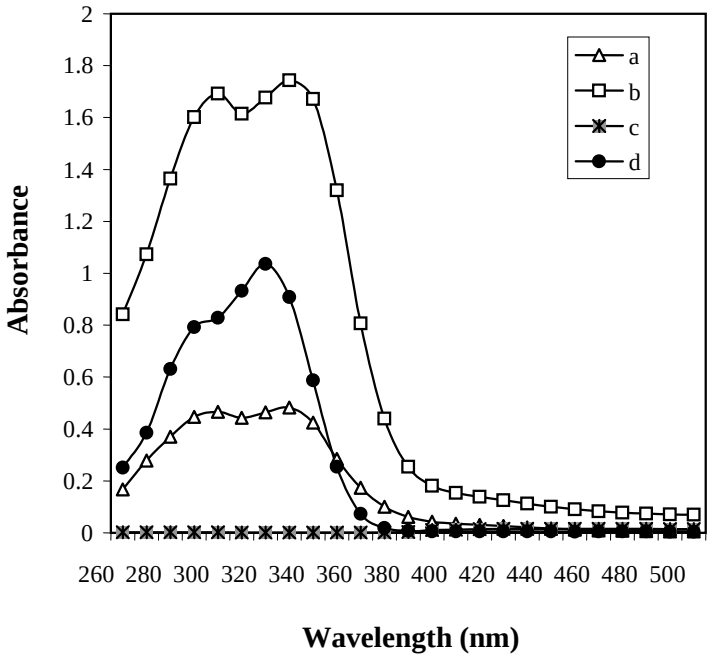


Figure 5.9: UV absorption spectra of starch ferulates of different degree of substitution (a)- St. Fer. 1(Δ), (b)-St. Fer 2 (□), (c)- Starch (*) and (d)-Ferulic acid (●).

5.3.10.1 DPPH radical scavenging activity

DPPH$^{\bullet}$ is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule (Soares et al., 1997). Stained silica layer revealed a purple background with yellow spots at the location of the drops, due to the decrease in the local concentration of DPPH$^{\bullet}$ indicating radical scavenging activity in the case of both ferulic acid and starch ferulate. The intensity of the yellow color depends on the amount and nature of radical scavenger present in the sample. Ferulic acid exhibited a faster reaction rate compared to the starch ferulate (Figure 5.10B). Significant radical scavenging activity was evident at all the tested concentrations and the scavenging activity increased with increasing concentration of starch ferulate and ferulic

acid. The starch ferulates of different DS showed free radical scavenging capacity at all the concentrations tested.

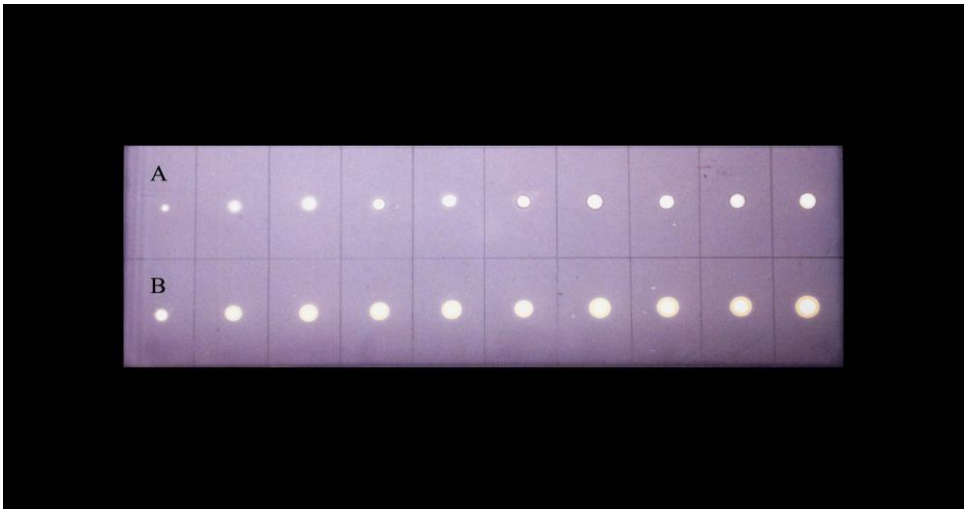


Figure 5.10: Dot blot assay on a silica sheet stained with DPPH solution in methanol for rapid detection of DPPH radical scavenging activity. (A)-St. fer. (100-1000µg/ml); (B)-FA (10-100µg/ml).

5.3.10.2 ABTS radical cation decolorisation assay

This method measures the ability of starch ferulate to scavenge the radical $ABTS^{+\bullet}$ and is an excellent tool for determining the antioxidant activity of hydrogen donating antioxidants and of chain breaking antioxidants.

Figure 5.11 depicts a steady increase in the ABTS radical scavenging capacity of starch ferulates up to a concentration of 750 µg/ml. The starch ferulates of higher degree of substitution exhibited a higher degree of scavenging as expected. When the starch ferulates were added to $ABTS^{+\bullet}$, a biphasic reaction was observed with a comparatively faster reaction rate in the first one minute followed by a relatively slow

scavenging. This reaction pattern implies that for the reaction of $ABTS^{+\bullet}$ with starch ferulates, the reduction in absorbance depends on the time point at which absorbance is read.

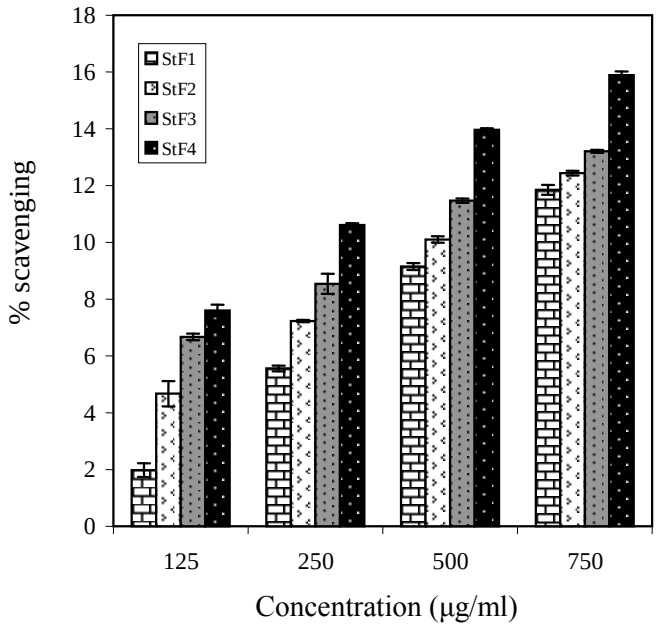


Figure 5.11: ABTS radical scavenging capacity of starch ferulates of different DS.

5.4 Conclusions

Starch ferulates with different degrees of substitution were prepared by acylation of starch with ferulic acid chloride in organic solvent. Starch has the disadvantages of hydrophilicity and poor mechanical properties especially in humid environments and chemical modification is a means to overcome these shortcomings.

Structural modification of starch resulted in significant changes in the physico-chemical properties of starch and an increased hydrophobicity. The DS values of starch ferulate could be controlled by the addition of stoichiometric quantities of the acid chloride. Ingestion

of ferulic acid in the form of starch ferulate facilitates quantitative recovery to the colon and thereby a higher bioavailability at the site of action compared to ferulic acid (Curini et al., 2005). The starch ferulates were found to exhibit free radical scavenging activity which is of significance in the prevention of colon cancer. Non-digestible oligosaccharides have aroused significant interest in recent years, due to their ability to stimulate growth of potentially beneficial bacteria such as *Bifidobacteria* in the gut. Feruloyl oligosaccharides released from wheat bran have been found to possess bifidogenic property on *Bifidobacterium bifidum* (Yuan et al., 2005). Bifidobacteria would not increase by exogenous bifidobacteria intake suggesting that the main pathway to increase probiotic bacteria in the colon is not by exogenous probiotic intake but by prebiotic intake such as dietary fibres and oligosaccharides. More detailed studies are needed to assess the bifidogenic and dietary fiber property of starch ferulates.

Colon health care seems to have become very important in the modern society as more and more products for colon health as active bacilli, active bifidobacteria and prebiotic factors are becoming increasingly popular in the market. Development of dietary compounds as potential cancer chemopreventive agents is highly desirable due to their safety, low toxicity and general acceptance as dietary supplements.

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CHAPTER VI

CHARACTERIZATION OF STARCH-CHITOSAN BLEND FILMS CONTAINING FERULIC ACID

6.1 Introduction

Chitosan (poly [β-(1-4)-2-amino-2-deoxy-D-glucopyranose]) is a non-toxic, biodegradable and biocompatible cationic polysaccharide, produced by the partial deacetylation of chitin, isolated from naturally occurring crustacean shells (Wong et al., 1992). Many attempts have been made to produce new biofunctional materials from chitosan such as films, fibers, sponges and gels (Zheng et al., 2001). High molecular weight chitosans have been reported to have good film forming properties as a result of intra and intermolecular hydrogen bonding (Muzzarelli and Peter, 1997). Chitosan coatings applied to fruits and vegetables reduce water loss and extend shelf life (El Ghaouth, 1991). Chitosan films are compatible with animal tissue (Balassa and Pruden, 1978) including the human eye and are resistant to microbial growth (Allan and Hadwiger, 1979). Although chitosan films are highly impermeable to oxygen, they have relatively poor water vapor barrier characteristics (Butler, 1996). Antimicrobials, antioxidants, nutrients, colorants and flavors can be possibly carried by chitosan based films and released in a controlled manner (Park and Zhao, 2004). The functional properties of chitosan films are improved when chitosan is combined with other film forming materials.

Starch is a naturally occurring polysaccharide consisting of (1-4) and (1-6) linked α-D-glucopyranosil units, and has been widely studied for many years in the field of materials (Ellis, 1998). Native potato starch contains about 20-23% amylose and the amylose is responsible for the film forming capacity of starches (Mali and Grossmann, 2003). It has been used to produce biodegradable films to partially or entirely replace plastic polymers because of its low cost and renewability. However, wide application of starch film is limited by its water solubility and brittleness (Wu and Zhang, 2001).

It was reported that the addition of cross-linking agents like gossypol, formaldehyde, glutaralaldehyde (Marquie et al., 1995), calcium salts, glucono-δ-lactone, carbodiimide, acetylated monoglyceride and transglutaminase (Mariniello et al., 2003; Park et al., 2001) in to film forming solutions might improve the mechanical properties and water vapor permeability of polysaccharide based edible films.

Ferulic acid, a health component of food with multiple phytochemical functions (Ou and Kwok, 2004) can cross-link with protein and polysaccharides by producing a resonance-stabilized free radical intermediate (Hopkins et al., 2003; Oudgenoeg et al., 2001). As the quinone, (oxidized ferulic acid), it has been found to react with amino and thiol groups in protein (Figueroa-Espinoza et al., 1999). Additionally, the free radical formed from ferulic acid can react with itself to form diferulic acid (Oudgenoeg et al., 2001), which act as a bridge between polysaccharide molecules. This suggests that ferulic acid could act as a satisfactory cross-linking agent in the preparation of polysaccharide-based edible films. Liyama et al. in 1990 clearly demonstrated that ferulic acid that was etherified to lignin in plant cell walls was also esterified to arabinoxylans, thus demonstrating that ferulates were indeed cross-linking lignin and polysaccharides. There are only very few reports on the cross-linking properties of ferulic acid in the preparation of edible films, although it is a cross-linking agent in plant cell walls (Liyama et al., 1984).

Here we report a method of producing biocompatible starch-chitosan blend films, the characterization of its mechanical, chemical, thermal and swelling properties and morphological features for various applications such as wound dressings and in drug delivery and incorporation of oxidized ferulic acid in to the blend films for application in edible films and coatings.

6.2 Materials and Methods

Chitosan (84% deacetylated) was obtained from Sigma Chemical Co, St. Louis, Mo, USA and Glycerol from Sisco Research Laboratories, India Ltd. Potato starch was isolated and purified from the fresh tubers of potato (*Solanum tuberosum*) using the modified procedure of Willinger (Willinger, 1964). The ash, protein and fat content of the starch were determined according to the standard AOAC methods (AOAC Official methods of analysis, 1995). The viscosity of gelatinized potato starch (1%w/v) was determined using a Synchro-Lectric Brookfield Viscometer (Model RVT, MA, USA) at 30°C.

6.2.1 Viscosity measurements and molecular weight determination of chitosan

The molecular weight (Mw) of chitosan was determined using the viscometric method (Roberts and Domszy, 1982). The intrinsic viscosity of chitosan in 2% acetic acid/0.2M sodium acetate solution was measured in triplicate using an Ubbelohde capillary viscometer in a constant temperature water bath at 25±0.01°C.

6.2.2 Film preparation

Chitosan solutions (2%, w/v) were prepared, by dispersing 4g of chitosan in 200 ml of acetic acid solution (1%, v/v) and stirring overnight. After the chitosan was dissolved completely, the solutions were filtered with cheese cloth. Aqueous starch solutions of concentrations 1, 2 and 3% (w/v) were prepared by heating under stirring, beyond its gelatinization temperature (90±2°C) for 20 minutes. The solutions were then cooled under stirring at an approximate rate of 2-3°C/minute to 25°C. A series of chitosan-starch composite films were prepared by mixing 40 ml of 2% chitosan solution with 40 ml of 1, 2 and 3% starch solutions respectively and stirring at 80±5 rpm. Glycerol was added at 25% (w/w) of the total solid weight in solution. The mixtures were then cast on to flat, level acrylic plates. After drying the

films at 45°C for 50 hours, they were peeled off from the plates. The dried films were conditioned in a desiccator at ~50% relative humidity and 25°C for 48 hours prior to testing.

6.2.3 Film Thickness

Film thickness was measured using a Screw gauge (Dollar, Ultrascience Aids). Five thickness measurements were taken along the gauge length of each specimen and the mean value was used in calculating the film tensile strength. The precision of the thickness measurements was ± 5%.

6.2.4 Film moisture content

The moisture content of the films were determined gravimetrically by oven drying to constant weight at 105°C with forced air circulation for 24 hours and cooling in a desiccator.

6.2.5 Swelling property of films as a function of pH

The dried films of known weight were allowed to swell in solutions of acidic, neutral and alkaline pH namely 2, 7 and 10 at ambient temperature and at 100 rpm in a shaker (Remi Instruments, India) by preparing 0.1M solutions of KCl-HCl buffer, Phosphate buffer and Glycine-NaOH buffer respectively. The swollen gel films were removed from the solution at regular intervals and dried superficially with filter paper, weighed and replaced in the same bath. The degree of swelling, Sw in the starch/chitosan films was calculated as

Sw =We-Wd/Wd

where We is the weight of starch/chitosan films in the swollen state and Wd is the dry weight of starch/chitosan films.

6.2.6 Mechanical tests

All mechanical tests were performed using a Universal Testing Instrument Model H5KS (Tinius Olsen, Horsham, USA) fitted with a 100N static load cell. The films were cut into strips 10 mm wide and 110mm long and mounted between cardboard grips (250 x 300 mm) using adhesive so that the final area exposed was 10 x 50 mm. A

minimum of six strips were prepared from each film. The tensile properties of the films were measured according to the standard testing method (ASTM, 1995) at a crosshead speed of 50mm/min and extension of 100mm and the initial grip separation was 50mm.

6.2.7 Thermal analyses

6.2.7.1 TGA

Thermogravimetric analyses (TGA) were performed in a Simultaneous DTA-TG Apparatus (DTG-60, Shimadzu, Japan). Samples (2-8 mg) were heated at the rate of 20°C/min from ambient temperature to 500°C. Nitrogen was used as the purge gas at a flow rate of 20 ml/min.

6.2.7.2 Differential scanning calorimetry (DSC) analyses

Thermal properties of the blend films were characterized using a Perkin-Elmer Pyris DSC 6 (Perkin Elmer, Boston, MA). Nitrogen at a rate of 30 ml/min was used as the purge gas. 2-3 mg of film material were taken in aluminum pans, crimped close using the DSC sample press and heated up to 180°C ensuring that the samples did not decompose and cooled to 25°C in the first run followed by a second run in which they were heated to 200°C.

6.2.8 Fourier transform infrared (FTIR) spectroscopy

The FTIR spectra of the films were recorded in an IR spectrometer (Nicolet Magna 4R 560, USA) in the range of 4000cm^{-1} to 400cm^{-1}.

6.2.9 X-ray diffraction

X-ray patterns of chitosan, starch powders, and starch-chitosan composite films were analyzed using an X-ray diffractometer (XPERT, Philips, The Netherlands) in the angular range of 5–40(2θ) with Nickel filtered Cu Kα radiation (λ=0.154 nm) at a voltage of 40 kV and current of 30 mA.

6.2.10 Light Microscopic observations

The film surfaces were observed with a Leica DM RX optical microscope (Leica Microsystems, Wetzlar, Germany) under transmitted

light and the images at 200X magnification were captured using a CCD camera.

6.2.11 Microstructure studies by SEM

The morphological structures of the films were studied by JSM-5600 LV scanning electron microscope of JEOL, Japan. The dried film 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 and 4500-5000X magnification.

6.2.12 Microstructure studies by Atomic Force Microscopy

Atomic force microscopy images were recorded under ambient conditions using a multimode nanoscope IV (Digital Instruments, Santa Barbara, CA) operating in the tapping mode regime. Micro-fabricated silicon cantilever tips (MPP-11100-10) with a resonance frequency of 299 KHz and a spring constant of 20-80 Nm^{-1} were used. The scan rate varied from 0.5 to 1Hz. Samples used for film casting in aqueous solution was transferred to freshly cleaved mica sheet by drop casting.

6.2.13 Preparation of blend films containing ferulic acid

Oxidized ferulic acid was prepared by mixing different concentrations of ferulic acid in ethanol (50, 75 and 100 mg respectively) with hydrogen peroxide (30%v/v) so as to have final concentrations of 1g/L of hydrogen peroxide and was kept at room temperature for one hour under stirring. Chitosan solutions (2%, w/v) were prepared, by dispersing 4g of chitosan in 200 ml of acetic acid solution (1%, v/v) and stirring overnight at 100 rpm. After the chitosan was dissolved completely, the solutions were filtered with cheese cloth. Aqueous potato starch solutions of concentration 1% (w/v) were prepared by heating, beyond its gelatinization temperature (90±2°C) for 20 minutes, under stirring. A series of starch-chitosan films containing oxidized ferulic acid were prepared by mixing 40 ml of 2% chitosan

solution, 40 ml of 1% starch solution and different concentrations of ferulic acid (50, 75 and 100 mg) in hydrogen peroxide and stirring at 80±5 rpm for one hour. Glycerol was added at 25% (w/w) of the total solid weight in solution. The mixtures were then cast on to flat, level acrylic plates. After drying the films at 45°C for 50 hours, they were peeled off from the plates. The dried films were conditioned in a desiccator at ~50% relative humidity and 25°C for 48 hours.

6.2.14 Film Thickness

Film thickness was measured using a Micrometer (Model 549 E, Testing machines Inc, Mineola, LI, NY). Ten thickness measurements were taken along the gauge length of each specimen and the mean value was taken.

6.2.15 Spectrophotometric analyses

The absorption spectra of ferulic acid, hydrogen peroxide and ferulic acid oxidized with hydrogen peroxide were recorded in the wavelength range 200-400 nm.

6.2.16 Determination of water vapor permeability

The water vapor transmission of the films was determined using the ASTM method (ASTM, 1993). Circular aluminum cups with a diameter of 7cm and depth of 3.5cm were used and anhydrous calcium chloride was added to the base of each cup so as to form a layer. The cups were then covered with the prepared blend films containing ferulic acid and sealed using melted paraffin. The cups were weighed and then placed at 92% relative humidity and 37°C in a humidity chamber. The cups were weighed at one hour intervals. The water vapor transferred through the films at different time intervals were determined from the weight gain of the cups. The water vapor transmission rates were calculated according to Kaya and Kaya (2000).

6.2.17 Determination of peroxide value

A 10 ml container was filled with linoleic acid and sealed using the blend films containing different concentrations of oxidized ferulic acid and stored at 30°C for 10 days. The peroxide value of the fatty acid was determined using sodium thiosulfate titration (Aurand and Woods, 1977).

6.2.18 Thermogravimetric analyses of ferulic acid incorporated films

Thermogravimetric analyses were performed in a Simultaneous DTA-TG Apparatus (DTG-60, Shimadzu, Japan). Samples (2-8 mg) were heated at the rate of 20°C/min from ambient temperature to 500°C. Nitrogen was used as the purge gas at a flow rate of 20 ml/min. In the case of starch powder the analysis was performed up to 800°C.

6.2.19 Statistical Analyses

Microsoft Excel 98 (Microsoft Corp., Redmond, WA) was used for all statistical analyses. Differences were considered to be significant at $P<0.05$.

6.3 Results and discussion

6.3.1 Physico-chemical properties of potato starch

The potato starch was found to have an ash, protein and fat content of 0.16±0.01, 0.30±0.01 and 0.27±0.01 respectively on a percentage dry basis. The viscosity of potato starch powder at 1% concentration was determined to be 22.8cP.

6.3.2 Molecular weight of chitosan

The viscosity average molecular weight of chitosan was determined by solution viscometry to be 1351 kDa. For chitosans with a DD value of 84%, the constants $K=1.38x10^{-5}$ and $a=0.85$ have been reported (Gamzazade, 1985).

6.3.3 Film preparation and thickness

The blend compositions of the films are given in Table 6.1. The flexibility of the chitosan film is poor, probably due to the compactness

of the polymer macromolecules and difficulty in their movement (Gutie'rrez-Rocca and McGinity, 1994). In starch-chitosan blend films, the highly branched nature of amylopectin in potato starch may result in the formation of a looser molecular network and relatively free molecular movement. Therefore the concentration ratio of starch and chitosan in the blend film needs to be optimized so as to obtain strong as well as flexible films.

Starch to Chitosan ratio	Composition		
	Chitosan solution (2%w/v) ml	Starch solution (w/v) ml	Glycerol (g)
0	80	0	0.4
0.5:1	40	40 ml of 1%	0.3
1:1	40	40 ml of 2%	0.4
1.5:1	40	40 ml of 2%	0.5
	0	80 ml of 2%	0.4

Table 6.1: Composition of starch-chitosan composite films

The thicknesses of the films were found to range from 65 to 110µm (Table 6.2). In acidic solution, the glucosamine units of chitosan are ionized to the soluble form of $R\text{-}NH_3^+$ and starch being a non ionic polymer, cross links are formed between the hydroxyl and amino groups of chitosan and the hydroxyl group in starch ensuring good compatibility. The moisture content in the blend films were found to decrease with increasing content of starch (Table 6.2). The oven dried chitosan films had a pale yellow color which is probably due to the preferential drying of the surface layers and also could be attributed to maillard reaction products (Srinivasa, 2004). The starch films were however white in color and the blend films exhibited an intermediate color.

Composition of the films	Thickness (µm)	moisture content (%)
Chitosan	98.9±5.1	24.5±0.07
Starch	90.8±3.8	11.6±0.03
0.5:1 starch-chitosan	66.1±4.9	15.6±0.05
1:1 starch-chitosan	84.9±4.6	12.8±0.05
1.5:1 starch-chitosan	107.1±3.9	8.25±0.02

Table 6.2: Physical properties of the chitosan-starch films.

6.3.4 Determination of swelling characteristics

As shown in Figures 1a-c, the swelling of starch-chitosan blend films, increased with increasing starch content. One reason for swelling is the formation of NH_3^+ groups at the acidic pH of 2 which is well below the acid dissociation constant (pKa) of 6.3 for the amino groups of chitosan (Kim, 1992). These cause the migration of counter ions in to the film, thus changing the osmotic pressure of the film and inducing water transfer from the exterior buffer solution, resulting in swelling. Compared to the starch or chitosan films, the blended films exhibited a greater degree of swelling partly due to the swelling capacity of chitosan in acetic acid and partly due to the hydrophilic nature of starch.

At pH 10, the films exhibited considerable swelling due to the ionization of the carboxyl groups in the alkaline medium. The starch film exhibited a higher degree of swelling compared to the blend films since alkaline conditions can cause a certain degree of depolymerisation, which in turn favors the imbibing of water. The glycerol starch interactions are weakened by the presence of alkali. At neutral pH, both amino and carboxyl groups could be ionized but the degree of ionization was very low in the form of some electrostatic ion pairs and the degree of swelling was also low. At neutral and alkaline pH the transition of NH_3^+ to NH_2 reduced the degree of swelling, induced by the acidic conditions in the chitosan. The destruction of crystallization in the chitosan film made it easier for water to permeate in to the chitosan film. The swelling of the film involved water diffusion, ionization of amino or carboxyl groups, dissociation of hydrogen and ionic bonds and polymer relaxation. In the neutral buffer, the swelling involved mainly water diffusion and polymer relaxation. At the alkaline pH, the hydrogen bonding between amino and hydroxyl groups still existed and so the polymer relaxation was lesser. The COOH groups dissociated to form COO^- and this induced the partial dissociation of the hydrogen bonds

concerning the COOH groups. Films with pH sensitive swelling properties are of use in site specific and controlled drug delivery. Their swelling property finds application in wound dressings, as they are capable of absorbing extra liquid from wounds.

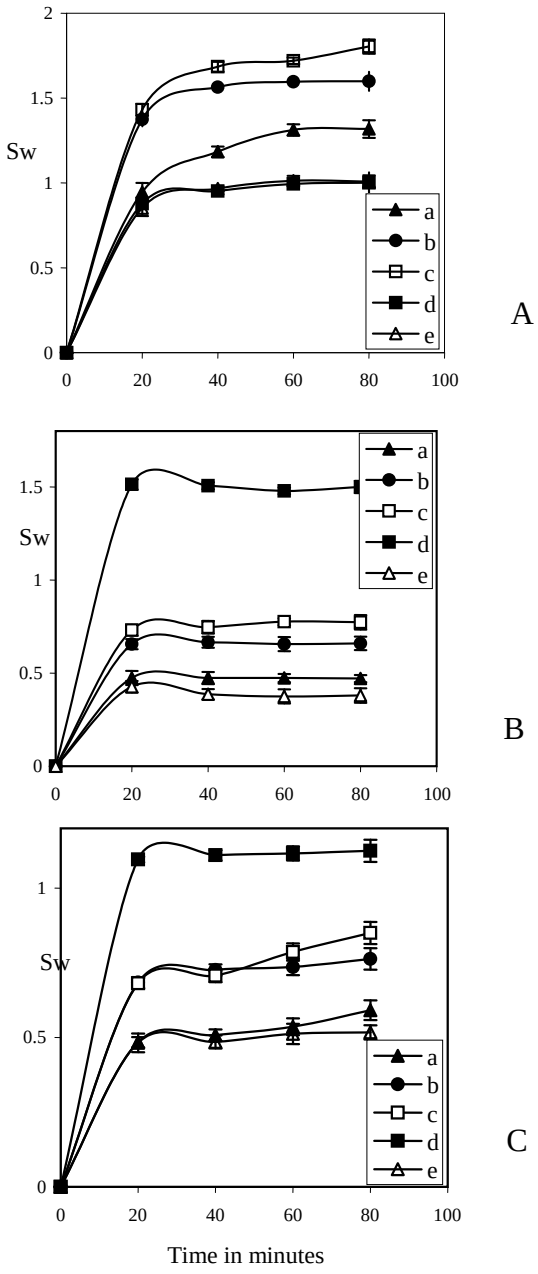


Figure 6.1a-c: Swelling properties of the films at pH 2 (A), 10 (B) and 7 (C) (a) 0.5:1 starch- chitosan film; (b) 1:1 starch- chitosan

film; (c) 1.5:1 starch- chitosan film; (d) Starch film; (e) Chitosan film. Results are mean ±S.D of three parallel measurements.

7.3.5 Mechanical strength

The tensile strength (TS) values of the starch-chitosan composite films with the different starch ratios are shown in fig. 6.2. It was found that, the TS values of the blend films, increased first with the addition of starch, with a maximum of 37.5 MPa at a starch chitosan ratio of 0.5:1 probably due to the formation of intermolecular hydrogen bonds between the NH_3^+ of chitosan and hydroxyl groups of starch. The TS values decreased with further increase in starch to chitosan ratio up to 1.5:1 which may be due to the formation of starch intra-molecular hydrogen bonds rather than inter-molecular hydrogen bonds, resulting in a phase separation between the two main components.

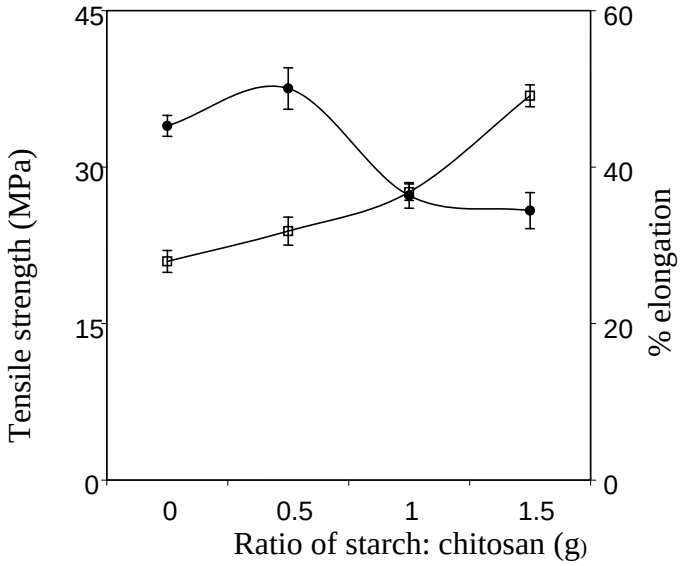


Figure 6.2: Effect of the film composition on the tensile strength and percentage elongation. Results are mean ±S.D of six parallel measurements.

The percentage elongation (*E*) values of the composite films are a measure of the flexibility of the film and were affected by the starch to chitosan ratios. The average *E* values of the films increased from 27.95% for chitosan film to a maximum of 49.12% for the 1.5:1 starch-chitosan composite film (Fig. 6.2). The increase in percentage elongation with increase in starch content is due to the reduction in the number of intermolecular crosslinks and increase in the intermolecular distance. The ternary system obtained by plasticizing starch-chitosan system with water and glycerol behave in a rather complicated way.

The addition of glycerol increased the flexibility of the film but reduces tensile strength as it inserts itself between the polymeric chains and reduces intermolecular forces in the film by establishing hydrogen bonds with the polymeric chains via its hydroxyl groups (Godbillot et al., 2005).

There is a possibility of interaction between starch and chitosan molecules in the starch-chitosan blend films, especially between amylose of starch and chitosan molecules as it is easier for amylose to mix with chitosan than the branched chain amylopectin. The nonionic property of starch makes it compatible with chitosan. Moreover the blend is biodegradable and nontoxic.

6.3.6 Thermal studies

6.3.6.1 Thermogravimetric studies

The TG spectra are used to determine the weight loss of the material as it is heated. The thermogravimetric curves of the starch, chitosan and the blend films are shown in fig. 6.3. The initial weight loss at approximately 100°C was due to evaporation of water, while the second range (200-450°C) corresponds to a complex process including the dehydration of the saccharide rings, depolymerisation and decomposition of the acetylated and deacetylated units of polymer (Peniche-Covas et al., 1993). As the starch content increased, the initial

thermal decomposition temperature of the blend films shifted to a higher temperature. The TG spectra showed that potato starch is stable up to 200°C with maximum rate of decomposition occurring at 300°C. Compared to the corresponding powders, the starch film and chitosan film exhibited a slightly earlier initial decomposition temperature. The processing of chitosan powder in to films resulted in decomposition at a lower temperature probably due to the presence of acetic acid which induces its thermal degradation (Wang et al., 2005). The decomposition temperature of chitosan is molecular weight dependent and it has been shown that chitosan with a molecular weight of 250-500 kDa showed a maximum decomposition temperature of 280°C and low molecular weight chitosan degraded at lower temperatures (Shiriu et al., 2004).

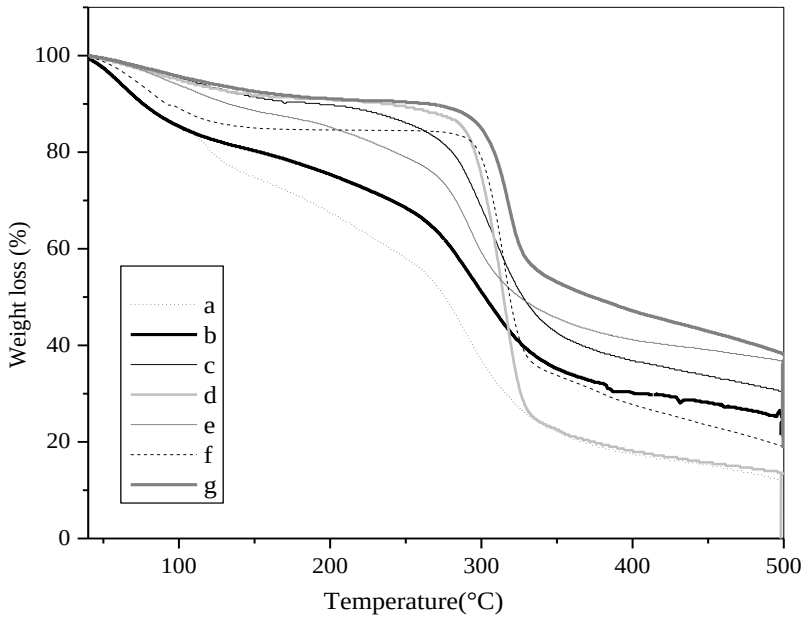


Figure 6.3: TGA spectra of starch and chitosan films and powders (a) 0.5:1 starch-chitosan film; (b) 1:1 starch-chitosan film; (c) 1.5:1 starch- chitosan film; (d) Chitosan film; (e) Starch film; (f) Starch powder; (g) Chitosan powder

The DTG data of the starch and chitosan powders and the films are shown in fig. 4. The peak maximum decomposition of starch powder was observed at 316°C and that of chitosan was close with peak maximum at 317°C. Chitosan showed considerable reduction in thermal stability when it was in the film form with the decomposition peak maximum at 294°C which increased to 308°C in the blend films with increasing starch content, indicating the formation of intermolecular interactions between the two components.

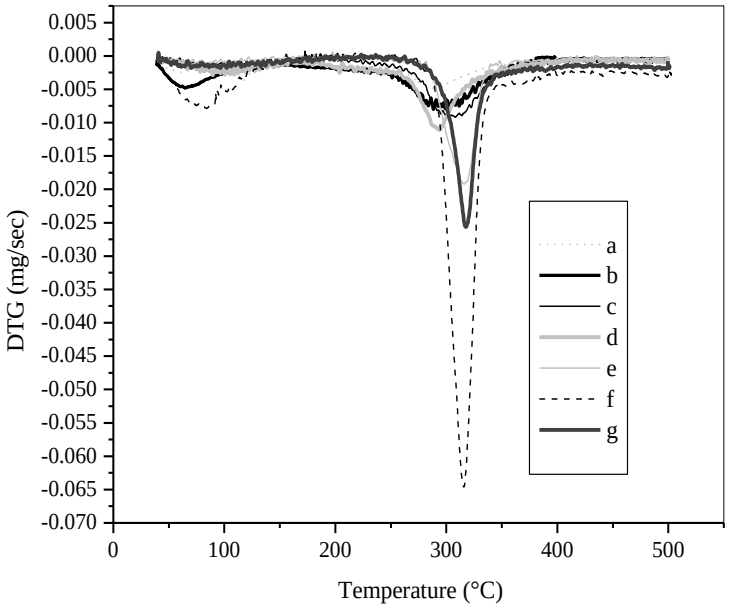


Figure 6.4: DTG spectra of the starch and chitosan films and powders (a) 0.5:1 starch-chitosan film; (b) 1:1 starch-chitosan film; (c) 1.5:1 starch-chitosan film; (d) Chitosan film; (e) Starch film; (f) Starch powder; (g) Chitosan powder

6.3.6.2 Differential Scanning Calorimetry

The DSC spectra revealed that chitosan and starch film and their blend had one distinct reaction zone. The endothermic peak around 100°C in the first run corresponds to the evaporation of water in the films, and appeared in all the film samples as has been reported earlier (Li et al., 2002) (Fig. 6.5).

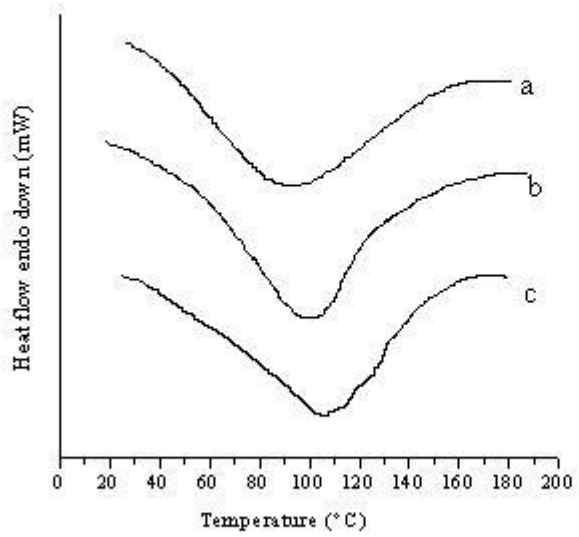


Figure 6.5: DSC thermogram of starch (a), chitosan (b) and blend film (c).

6.3.7 FT-IR Spectroscopy

The FT-IR spectra of the chitosan, starch and starch-chitosan blend films are shown in fig. 6.6 In Potato starch, the finger print region of the spectrum consists of three characteristic peaks between 923 and 1162 cm^{-1} attributed to the C-O bond stretching (Goheen and Wool, 1991). The bands at 1659 cm^{-1} and 1467 cm^{-1} are assigned to the δ (O-H) bendings of water and CH_2 respectively (Mano et al., 2003). The sharp

band at 2926 cm^{-1} is characteristic of C-H stretches associated with the ring methine hydrogen atoms. An extremely broad band occurs at 3400 cm^{-1} due to the hydrogen bonded hydroxyl groups that contribute to the complex vibrational stretches associated with free inter and intra-molecular bound hydroxyl group which make up the gross structure of starch (Fang et al., 2002).

The IR spectrum of the chitosan film was slightly different from that of chitosan powder because of the ionization of the primary amino groups. In chitosan film, the relatively broad band at 3429 cm^{-1} was due to the primary amine (Gupta and Kumar, 2000). The peaks at 2839 and 2925 cm^{-1} are typical C-H stretch vibrations (Wang et al., 2004). The peak at 1739 cm^{-1} suggested the presence of carbonyl group in the film and the one at 1633 cm^{-1} was due to the C=O stretching (amide I) and the peak at 1314 due to amide III peaks (Pearson et al., 1960). The sharp peaks at 1374 cm^{-1} and 1414 cm^{-1} correspond to the CH_3 symmetrical deformation mode (Sannan et al., 1978). The broad peak at 1076 cm^{-1} indicates the C-O stretching vibration in chitosan. The peaks at 850 cm^{-1} and 1157 cm^{-1} correspond to the saccharide structure (Yoshioka et al., 1990).

When two or more substances are mixed, physical blends versus chemical interactions are reflected by changes in the characteristic spectral peaks (Yin et al., 1999). In the typical spectrum of 1:1 starch-chitosan composite film, the characteristic peak of starch at 1659 cm^{-1} and the amide peaks of chitosan at 1633 cm^{-1} and 1580 cm^{-1} shifted to a higher frequency indicating that there was interaction between the NH_3^+ and hydroxyl groups of starch which improved the compatibility between them, while the amide III peak at 1314 cm^{-1} shifted to 1374 cm^{-1}(Meenakshi et al., 2002). Formation of the 1548-1580 cm^{-1} peak is due to the symmetric deformation of NH_3^+ resulting

from the ionization of primary amino groups in the acidic medium (Lee et al., 1999).

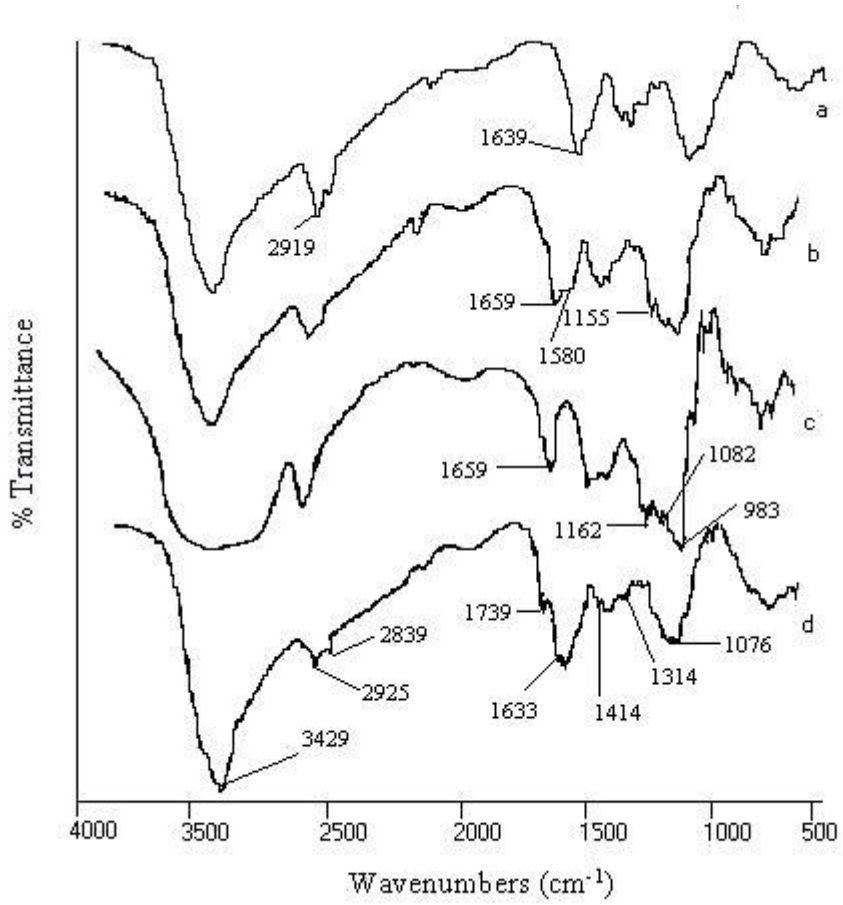


Figure 6.6: IR spectra of the starch and chitosan films and powders (a) Chitosan powder; (b) 1:1 starch- chitosan film; (c) Starch powder; (d) Chitosan film.

6.3.7 WAXD

X-ray diffractograms of chitosan/starch composite films are shown in fig. 6.7. As observed, the chitosan powder was in a crystalline state because two main diffraction peaks (2θ = 10.2 and 20.02) were observed in its X-ray diffraction pattern. The intensity of the hydrated crystal peak at 20° (2θ) was higher than that at 10° (Fernandez Cervera et al., 2004). In the chitosan film, the intramolecular interactions

between the NH_3^+ and hydroxyl groups in chitosan, limited the molecular movement of the chitosan chain and reduced its crystallization. Chitosan films prepared by casting from aqueous acetic acid solution were in amorphous to partially crystalline form due to the presence of the acetic acid solvent residue, which may hinder the formation of inter and intra molecular hydrogen bonds in chitosan and result in less dense packing.

The native potato starch powder had a typical B-type crystalline structure (Rindlav et al., 1997) but the dried potato starch films obtained after gelatinization, had very low crystallinity with diffraction peak at around 17° (2θ) and a few small peaks at around 2θ of 15, 20°, 22° and 24°. The crystallinity in polymeric materials is generally dependent on the crystallisation rate, which follows a bell shaped curve between the temperatures Tg and Tm (Fatou, 1989). Apparently in cases where the temperature is increased to 45°C for drying, the rate of nucleation is decreased and the overall rate of crystallization is low (Rindlav et al., 1997). Upon storage at high relative humidity the E_h type of crystallinity found in glycerol containing starch material is found to rearrange in to a six fold helical crystal structure, V_h (h-hydrated) and this facilitates the starch reorganization, by lowering the Tg of starch materials (Mercier et al., 1980).

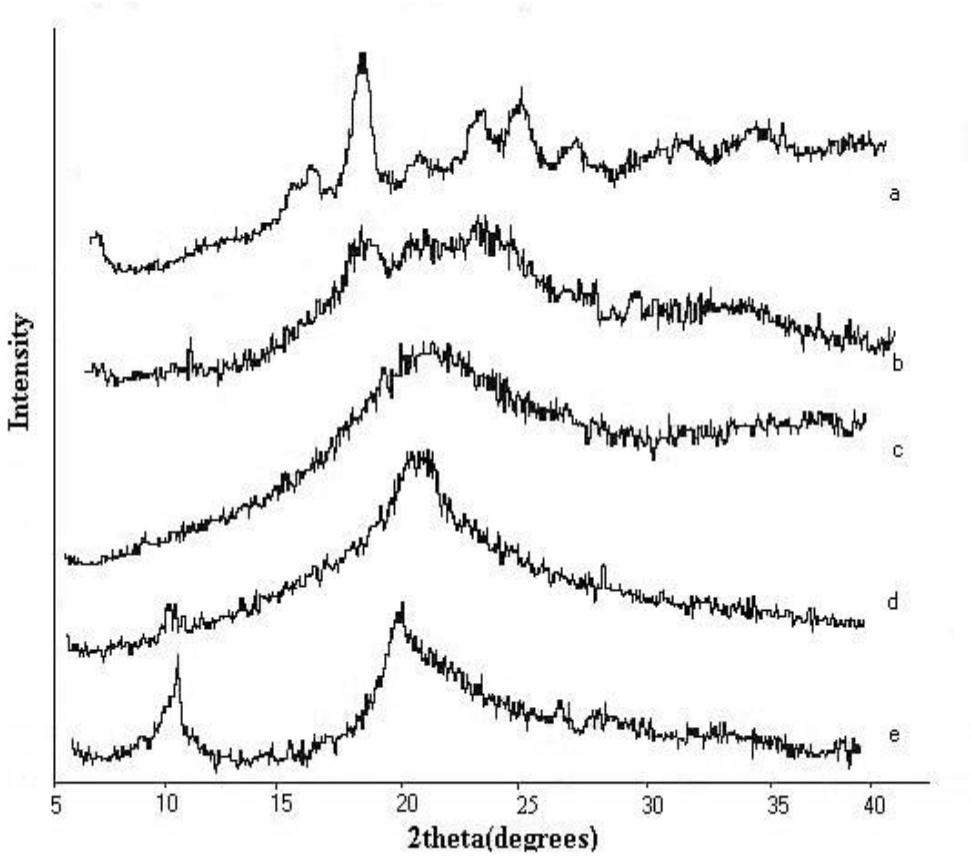
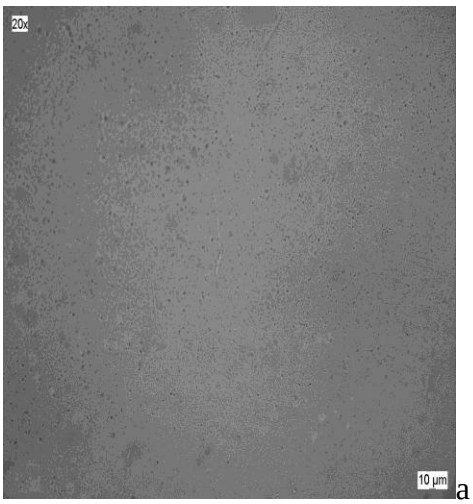


Figure 6.7: XRD profile of the starch and chitosan films and powders (a) Starch powder; (b) Starch film; (c) 1:1 starch-chitosan film; (d) Chitosan film; (e) Chitosan powder

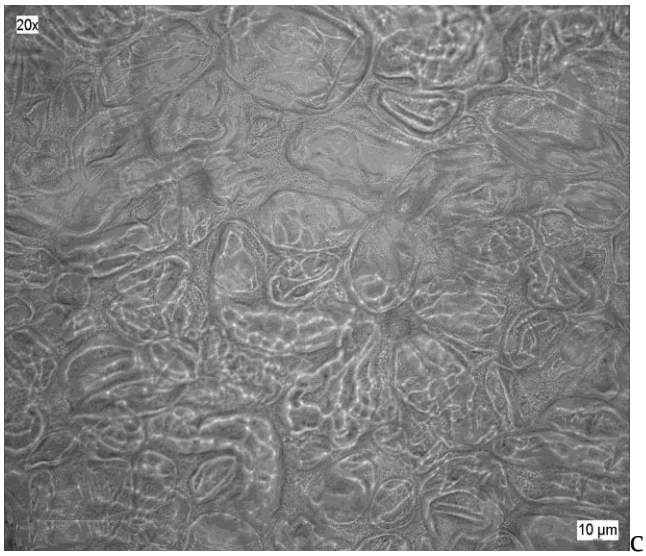
6.3.8 Light Microscopy

The light micrographs showed the surface of all the films to be smooth and compact (Figure 6.8 a, b and c). Interestingly the starch films (Fig. 6.8c), revealed characteristic patterns on the film surface. These patterns represent the withered ghost granules of starch. The blend films (Fig. 6.8b) also exhibited such patterns, the intensity of which reduced with the decreasing concentration of starch. Such features could not be observed by SEM, indicating that light microscopy should be used in combination with SEM for characterizing the film structure.



a

b



c

Figure 6.8: Light microscopic images of the starch and chitosan films (a) Chitosan film; (b) 1:1 starch-chitosan film; (c) Starch film.

6.3.9 SEM observations

Scanning electron microphotographs of the surfaces of starch films and the blend films are shown in figures 6.9 a and b. The

starch films did not exhibit the characteristic features shown by its light microscopic images and were found to be smooth and homogenous. Chitosan microdomains were dispersed within the starch matrix in the blend films with relatively good interfacial adhesion between the two components and were similar to the surface of cellulose/CM–chitosan blend films (Li et al., 2002).

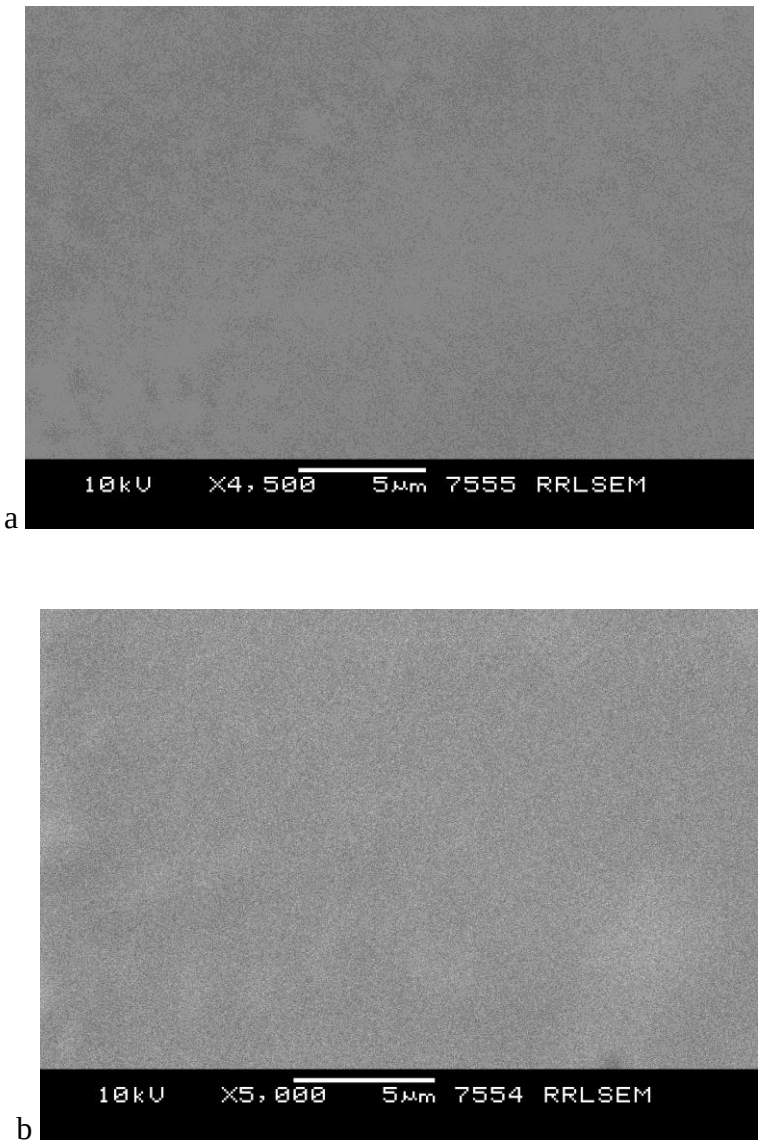
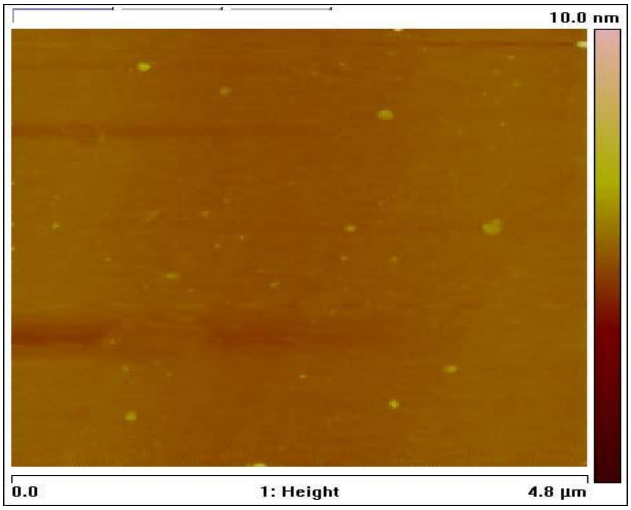


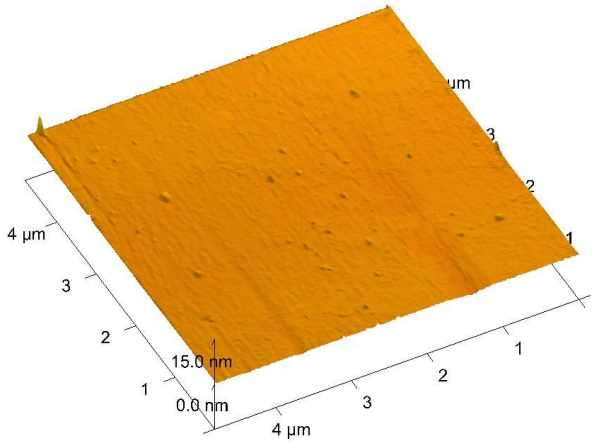
Figure 6.9: SEM photographs of (a) starch film and (b) 1:1 starch-chitosan blend film

6.3.10 AFM

Tapping mode AFM images yielded information about the surface features of the various films. Fig.6.10 shows the surface topography of chitosan films to be relatively smooth, homogenous and as a continuous matrix without any pores or cracks with good structural integrity. They were flat and compact with very sparsely distributed small particles with peak heights of 15-20nm with out any phase separation. The surface of starch film (Fig.6.11) exhibited undulations with irregulary spaced features of various size ranging from 100-300nm in height and few micrometers in width corresponding to the intact and withered granular envelopes or ghost resulting from the dissolution of amylose from the starch granules upon gelatinization (Rosanna et al., 2003). Though film formation involved a drying step known to cause a flattening of the structure of flexible polysaccharide molecules, (Stokke and Elgsaeter, 1991) the water holding capacity of starch molecules has significant effect on the observed images as many features were found to maintain a nearly round shape. The AFM topographic images of the blend films exhibited an intermediate character (Fig. 6.12) to that of the chitosan and starch films with particles of peak heights between 0-75 nm and the uniformity in the distribution of particles reduced with the increasing concentration of starch. Both phase contrast and slight phase separation were observed in the phase images (not shown) of blend films.

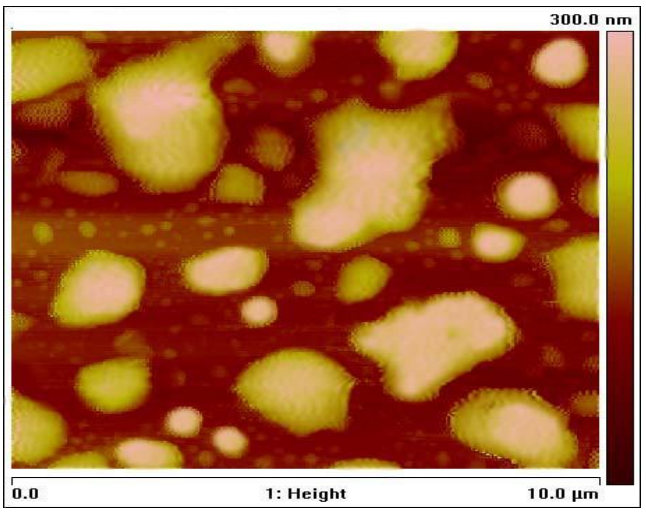


a

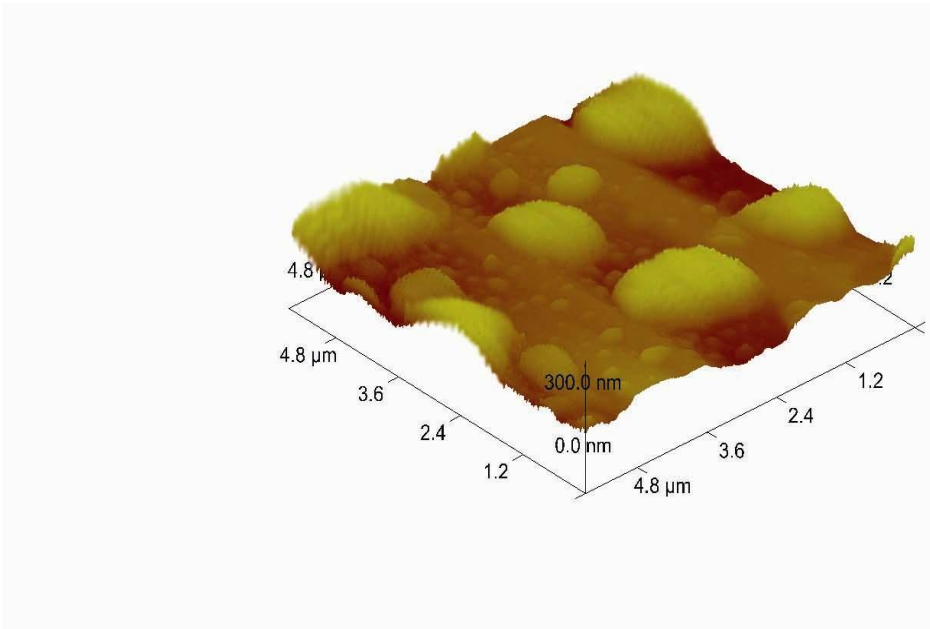


b

Figure 6.10: AFM topographic images of chitosan film (a) and its three dimensional image (b).

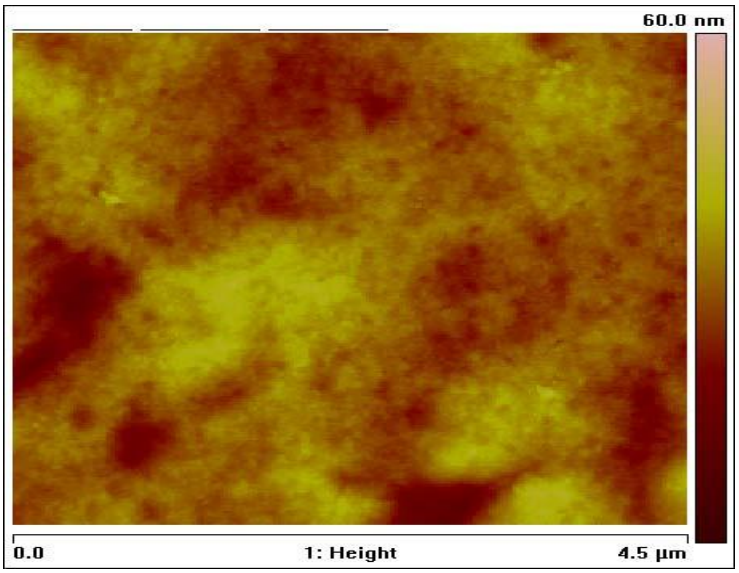


a

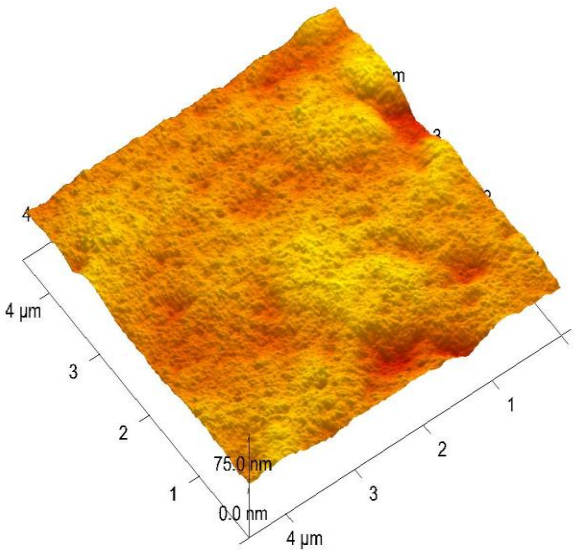


b

Figure 6.11: AFM topographic images of starch film (a) and its three dimensional image (b).



a



b

Figure 6.12: AFM topographic images of starch-chitosan blend film (1:1) (a) and its three dimensional image (b).

6.3.11 Film preparation

There was not much variation in the film thickness of ferulic acid incorporated films which was found to range between 73.5 to 78.5 μm (Table 6.3).

6.3.12 Absorption spectra

The UV-Visible spectrum of ferulic acid in ethanol showed an absorption maximum at 305 nm while hydrogen peroxide showed an absorption peak at 243 nm. Reaction of ferulic acid with hydrogen peroxide is accompanied by its decomposition to more reactive species such as hydroxyl radical and superoxide radical (Dence, 1994) and the radicals initiate hydrogen abstraction from ferulic acid, the phenolic substrate to form a phenoxy radical that can rearrange to a quinone methide radical intermediate (George et al., 1999). On oxidation of ferulic acid with hydrogen peroxide, the absorption peak exhibited a hyperchromic shift to 360 nm which corresponds to the peak maxima of its phenoxy radicals.

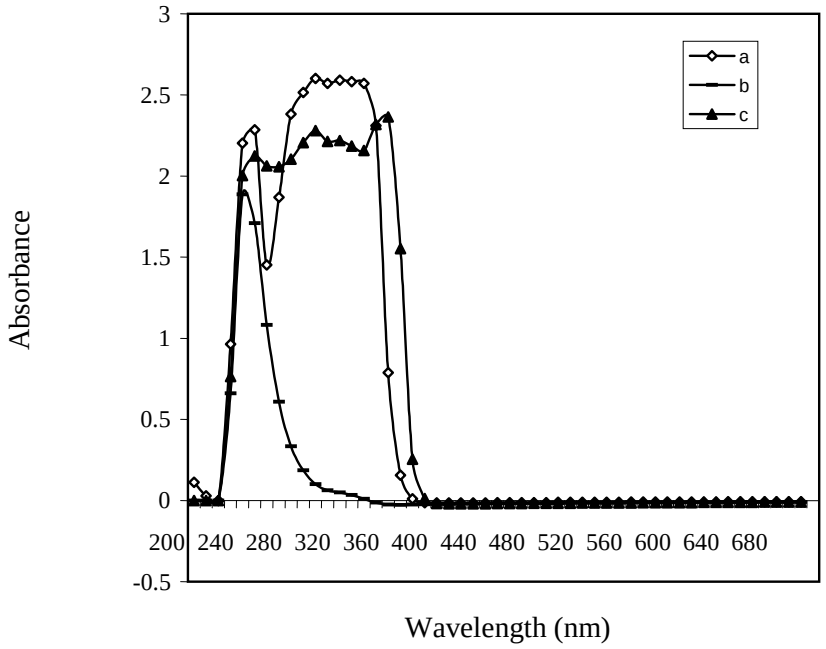


Figure 6.13: Absorption spectra of ferulic acid (a), hydrogen peroxide (b) and oxidized ferulic acid (c).

6.3.13 Effect of oxidized ferulic acid on water vapor permeability

Oxidised ferulic acid incorporated films were found to significantly decrease the water vapor permeability compared to the control blend films, probably due to the higher degree of cross linking that result from the formation of quinones and free radicals which tend to promote the cross linking (Oudgenoeg et al., 2001). However the water vapor permeability of the ferulic acid incorporated film at a concentration of 100 mg increased slightly in comparison to the blend film containing 75 mg of ferulic acid, but was lower than that of the control film (Table 6.3).

Film	WVTR ($g/m^2/day$)	Peroxide value (mg/kg)	OTR (cc/m^2)	Thickness (µm)
Blend film alone	2332	20.5	75.07	73.5
Blend film containing 50mg/100g	1954	14	56.44	78.94
Blend film containing 75mg/100g	1882	11.5	nd	74.77
Blend film containing 100mg/100g	2016	12	nd	78.47

Table 6.3: Effect of ferulic acid incorporation on water vapor transmission, oxygen transmission rate and peroxide value.

6.3.14 Effect of ferulic acid incorporation in film in preventing lipid peroxidation

Edible starch-chitosan films containing ferulic acid were used to cover fresh oil (linoleic acid) filled containers to test their ability to prevent the oxidation of the fatty acid. Studies showed that the addition of oxidized ferulic acid in to the blend films reduced the peroxide value of the fatty acid covered by such films. One possible factor responsible for the reduction in peroxidation is the lowered oxygen permeability of ferulic acid incorporated films as shown in Table 6.4

Ferulic acid can enhance the cross linking between polysaccharides through several mechanisms; through free radical mediated cross linking, by esterification with the hydroxyl groups of chitosan and starch or by quinone mediated reactions (Ou et al., 2005).

6.3.15 TGA and DTG analyses

The thermogravimetric curves of the starch powder, ferulic acid and ferulic acid incorporated blend films are shown in fig. 6.14.

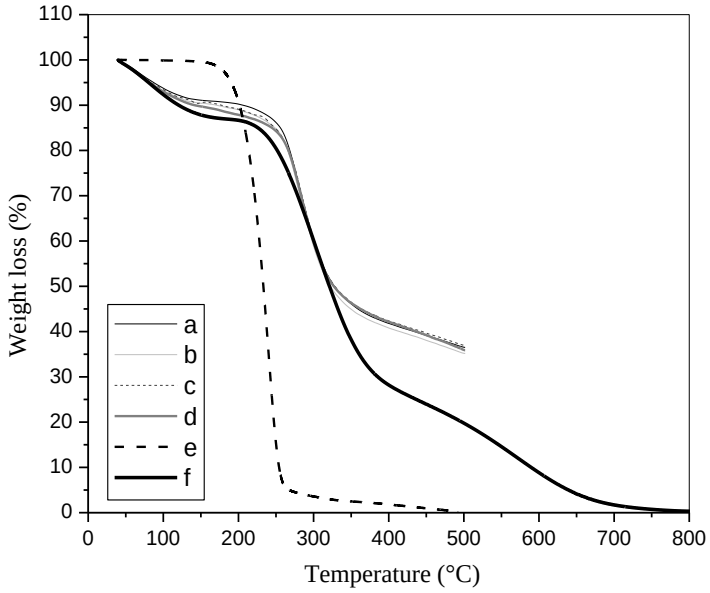


Figure 6:14 Thermogravimetric curves of blend film (a) ferulic acid incorporated films 50-100mg (b, c, d), ferulic acid (e) and potato starch (f).

Ferulic acid showed a single stage weight loss. However the starch powder and blend films containing ferulic acid exhibited decomposition in three stages. The initial weight loss at approximately 100°C was due to evaporation of water, while the second stage (200-350°C) and third stage (400-600°C) corresponds to complex processes including the dehydration of the saccharide rings, depolymerisation and decomposition of the acetylated and deacetylated units of polymer (Peniche-Covas et al., 1993).

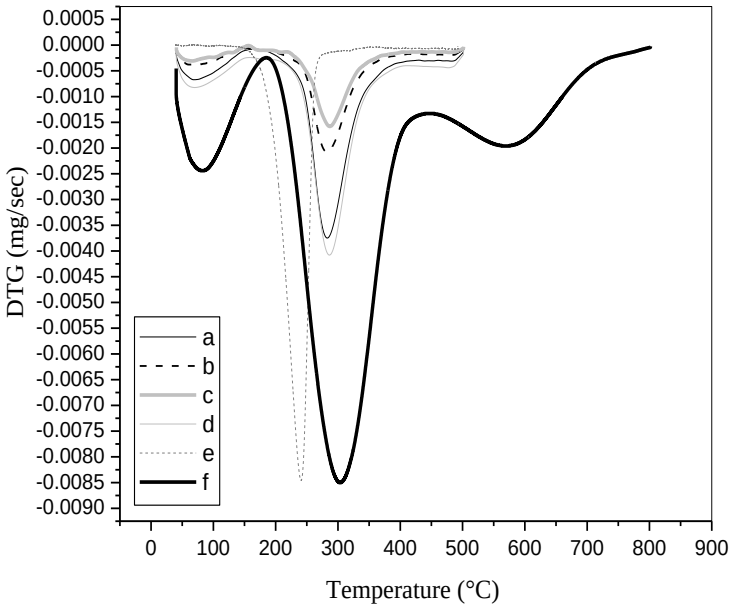


Figure 6:15 Differential thermogravimetric data of blend film (a) ferulic acid incorporated films 50-100mg (b, c, d), ferulic acid (e) and potato starch (f).

The DTG data of the starch and ferulic acid containing films are shown in fig. 6.15. The peak maximum decomposition of ferulic acid was observed at 241°C and that of the blend film was at 283°C. The ferulic acid incorporated films exhibited peak maximum decomposition temperatures in the range 283-288°C.

6.4 Conclusion

The high molecular weight chitosan employed, was found to have good film forming properties, as a result of inter and intra molecular hydrogen bonding and the optimum ratio of blended starch-chitosan film led to strong, stable and flexible films. The various physicochemical analyses performed, indicated interaction between starch and chitosan

molecules and the two film forming components were found to be compatible. These films can be used in multilayered, complex, controlled release systems for oral specific delivery. The water sorption ability indicates the capacity of the film to absorb moisture from the atmosphere to the skin and this property can be made use of in transdermal patches (Viyoch et al., 2003).

Incorporation of ferulic acid in to starch-chitosan blend films was found to significantly enhance the lipid peroxide inhibition. One possible mechanism for the prevention of oxidation in the fatty acid is the reduced oxygen permeability of the film as supported by the data on oxygen transmission rate studies. Such films can be used to extend the food shelf-life.

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SUMMARY AND CONCLUSION

The present study has dealt with the screening and production of feruloyl esterase enzyme by different microbial strains on pretreated cereal brans and sugar cane bagasse. Various other applications of ferulic acid, making use of its crosslinking property and antioxidant activity have also been studied. The biotransformation of ferulic acid to a highly value added product like 4-vinyl guaiacol has also been accomplished.

Among the different microorganisms screened for feruloyl esterase activity, the isolated strain JCS-3 was found to be the best organism to produce feruloyl esterase when grown on pretreated cereal brans and sugar cane bagasse. JCS-3 grown on maize bran supported maximum enzyme activity (7.17 U/ml) as well as specific activity (36.71 U/mg protein). Among the growth substrates employed, feruloyl esterase activity was found to increase in the order: Maize bran>rice bran>wheat bran> sugarcane bagasse indicating the induction of high levels of feruloyl esterase by the higher content of ferulic acid in the growth substrates. *Aspergillus flavipes* was also shown to be a good microbial source for the production of feruloyl esterase. Several million tons of cereal brans are being produced from the cereal processing industries every year and these would provide a good source for the production of feruloyl esterase enzyme.

The rapid transformation of ferulic acid to 4-vinylguaiacol, a highly value added compound used in perfumery, was achieved by using an isolated yeast culture identified as *Debaryomyces hansenii*. Ferulic acid decarboxylase was found to catalyse the non oxidative decarboxylation of the side chain of ferulic acid to yield 4-vinyl guaiacol (molar yield 95.07%). 4-vinyl guaiacol production by *Debaryomyces* reached a maximum of 1470 mg/l at the tenth hour, while vanillin

production reached a maximum of 169 mg/l at the fifth hour. 4-vinyl guaiacol is nearly 40 times more costly than ferulic acid and thereby makes the bioconversion a value added process.

Studies on the free radical scavenging and antioxidant properties of phenolic acids showed the cinnamic acid derivatives in general to be more efficient scavengers than their benzoic acid counterparts as the conjugation in the side chain stabilises the cinnamic acid radicals by resonance (ferulic acid > vanillic acid; caffeic acid > protocatechuic acid). Also the electron withdrawing properties of the carboxylate group in benzoic acid derivatives were found to have a negative influence on hydrogen donating ability which is a key factor responsible for radical scavenging.

Studies on the free radical scavenging properties of *Cinnamomum verum* bark (CBE) and leaf extracts (CLE) rich in phenolic acids showed them to be good scavengers of hydroxyl radical, superoxide radical, DPPH radical and ABTS radical cation. They also exhibited reducing power and metal ion chelating activity. They possess antioxidant properties, which are concentration dependent and could be attributed to the ability of its phenolic constituents to quench reactive oxygen species. CLE at a concentration of 150µg scavenged more than 80% of ABTS and DPPH radical and 95% scavenging of hydroxyl radical at a concentration of 125µg. CLE also exhibited antioxidant activity with 82.4% inhibition of linoleic acid peroxidation at a concentration of 125µg. While CBE exhibited more than 80% scavenging of ABTS and DPPH radical at a much lower concentration of 50µg and exhibited 82.3% inhibition of linoleic acid peroxidation at a concentration of 50µg. The total phenolic content of the *Cinnamomum* bark extract (CBE) was estimated to be 288.96 mg gallic acid equivalents/g of plant extract while that of *Cinnamomum* leaf extract (CLE) was estimated to be 116 mg gallic acid equivalents/g of

plant extract which partly accounts for the lower free radical scavenging property of CLE. The ability of these plant extracts to inhibit lipid peroxidation can be made use of in stabilizing food against oxidative deterioration.

Starch ferulates with different degrees of substitution (DS) ranging from 0.1 to 0.7 could be prepared by esterification of starch with ferulic acid chloride in organic solvent and their DS values could be controlled by the addition of stoichiometric quantities of the acid chloride. FTIR studies confirmed the synthesis of the starch ferulate as evidenced by the formation of a carbonyl signal around 1726 cm^{-1}. Thermogravimetric analyses revealed the starch ferulates to have an earlier initial decomposition temperature than the control starch. Structural modification of starch resulted in significant changes in the physico-chemical properties of starch and an increased hydrophobicity. The starch esters exhibited pseudoplastic nature and shear thinning behaviour. The starch ferulates of different degree of substitution were found to possess scavenging property against DPPH radical and ABTS radical cation.

Investigations on the preparation and characterization of biodegradable and biocompatible starch-chitosan blend films showed that an optimum ratio of starch-chitosan (0.5:1) led to strong, stable and flexible films. The thicknesses of the films were found to range from 65 to 110µm. They exhibited relatively good tensile strength and elongation which varied with the composition. The blend films exhibited good compatibility and intermolecular interactions as revealed by IR. Thermogravimetric analyses showed that in the blend films, the thermal stability increased with the increasing starch content and the stability of starch and chitosan powders reduced when they were converted to film. The swelling properties of the different films studied as a function of pH showed that the sorption ability of the blend films increased with the

increasing content of starch. The water sorption ability indicates the capacity of the film to absorb moisture from the atmosphere to the skin and this property can be made use of in transdermal patches The blend films containing ferulic acid at a concentration of 75mg had a water vapor transmission rate of 1882g/m^2/day and peroxide value of 11.5mg/kg which was much lower than that of the control films. The oxygen transmission rate was also lowered in ferulic acid incorporated films. The starch-chitosan composite films can be used in multilayered, complex, controlled release systems for oral specific drug delivery and in wound dressings and starch-chitosan blend films containing ferulic acid can be used to extend the food shelf-life.

Debaryomyceshansenii and *Aspergillus flavipes* have been found to be efficient producers of feruloyl esterase enzyme and would serve as analytical aids in understanding the detailed carbohydrate chemistry of plant cell walls. In addition to their role in paper pulp processing and feed manufacture, they can also be used to catalyze stereoselective organic synthesis. Succesful attempts have been made to make use of the antioxidant potential of ferulic acid in diverse ways. Recovery of value added products from ferulic acid through biotransformation has also been accomplished.

Detailed electrochemical characterization of ferulic acid as a radical scavenger is needed to obtain information on the physicochemical parameters, which determine its antioxidant potential. In order to be used as a dietary fibre with chemopreventive action, in *vivo* studies on the bifidogenic property and chemopreventive potential of starch ferulates need to be carried out. Derivatives of ferulic acid have been shown to have potent anticancer properties and extensive studies are needed to develop more potent biologically active molecules.

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