ISSN 1672-9145 **Acta Biochimica et Biophysica Sinica** 2007, **39**(11): 811–828 **CN** 31-1940/Q

Minireview

Feruloyl Esterases as Biotechnological Tools: Current and Future Perspectives

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Abstract Feruloyl esterases represent a diverse group of hydrolases catalyzing the cleavage and formation of ester bonds between plant cell wall polysaccharide and phenolic acid. They are widely distributed in plants and microorganisms. Besides lipases, a considerable number of microbial feruloyl esterases have also been discovered and overexpressed. This review summarizes the latest research on their classification, production, and biophysicochemical properties. Special emphasis is given to the importance of that type of enzyme and their related phenolic ferulic acid compound in biotechnological processes, and industrial and medicinal applications.

Keywords microbial feruloyl esterase; classification; production; biophysicochemical property; application

In the past decade, microbial feruloyl esterases (FAEs) have become important materials with considerable roles in biotechnological processes for many industrial and medicinal applications. Thus, discovery of new FAEs with novel properties continues to be an important research area. The amount of research effort directed towards FAEs has increased dramatically since 1990. For example, over the periods 1990–2000 and 2001–2006, the average number of refereed publications describing research involving FAEs as a major component (within abstract, title, and keywords; estimated using Elsevier SDOS search engine) was 24 and 44, respectively. It is interesting to note that the dramatic increase in publications concerned with FAEs observed between 2001 (four documents) and 2006 (11 documents) coincides with the most recent discoveries on isolation, purification, and characterization of FAEs including fungal and bacterial FAEs. There were also striking increases in the number of publications concerned with ferulic acid (the related compound to FAEs). The average number of documents was 242 for the period (1990–2000), while for the period 2001–2006, the average number was 329 using the same search engine (Elsevier SDOS).

To date and to the best of our knowledge, the following is a concise and complete review of the previous relevant studies conducted in nature, chemistry, and biochemistry of ferulic acid, and FAEs about carbohydrate esterase type, and their classification, crystal structure, microbial production, biophysicochemical properties, and finally their biotechnological applications in life.

Nature, Chemistry and Biochemistry of Ferulic Acid

Ferulic acid ($C_{10}H_{10}O_4$) is the most abundant, ubiquitous hydroxycinnamic acid derived from phytochemical phenolic compounds [1], distributed widely throughout the plant kingdom (spices, vegetables, grains, pulses, legumes, cereals, and fruits), their by-products (tea, cider, oil, and beverages) and medicinal plants [1–10]. It is a renewable resource for the biocatalytic or chemical conversion to other useful aromatic chemicals from agricultural by-products in nature [11–15].

Ferulic acid is a phenylpropenoid derived from the cinnamic acid 3-(4-hydroxy-3-methoxyphenyl)-2-propenoic acid, 4-hydroxy-3-methoxycinnamic acid, or coniferic acid (**Fig. 1**). It shows two isomers: *cis* (a yellow oily liquid) and *trans* (crystalline) [3].

Its nomenclature comes from Umbelliferae *Ferula*

Received: April 18, 2007 Accepted: June 16, 2007

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DOI: 10.1111/j.1745-7270.2007.00348.x

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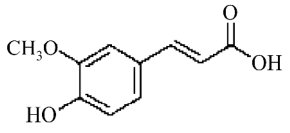

Fig. 1 Ferulic acid structure [3]

foetida from which this active compound was isolated for the first time in 1866. Its functions within living bodies include seed bud break suppression, indoleacetate enzyme inhibition, dopa deoxidant enzyme inhibition, and protection against microorganisms and pests [11–15].

It is said that ferulic acid supplies hydrogens to free radicals with phenolic-OH groups to provide the antioxidation effect. Ferulic acid also has an active oxygen erasing function and the effect has been reported to be similar to superoxide dismutase, known as the enzyme that protects living bodies from the toxicity of active oxygen. Ferulic acid is believed to suppress melanin generation by antagonizing tyrosine because their chemical structures are similar. It is also believed to powerfully absorb the harmful long wave ultraviolet (UV) band. It is listed as an "oxidation inhibitor" in the "Food additive list" and expected to be used as an anti-oxidant or anti-discoloration agent with many patents. Thus, ferulic acid has a wide variety of applications because it has radical and active oxygen erasing effects, absorbs UV causing active oxygen generation, and is a natural substance. It seems to be particularly effective for cosmetic use as a whitening agent and sunscreen, making use of its powerful long wave UV absorbing function. It has been reported that the ferulic acid ester of vitamin E drastically decreased melanin generation, with an expectation that it can be a promising UV pigmentation depressor [14]. Many *in vivo* and *in vitro* studies in humans, animals, and cell culture [1–17] have provided evidences for the following actions of ferulic acid: (1) inhibition or prevention of cancers of the breast, colon, lung, stomach, and tongue; (2) prevention of brain damage by Alzheimer's proteins; (3) inhibiting prostate growth; (4) strengthening of bone; (5) prevention of diabetes-induced free radical formation; (6) expansion of pancreatic islets; (7) reduction of elevated lipid, triglyceride, and blood glucose levels; (8) lowering cholesterol production; (9) prevention of hot flashes; (10) prevention of free radical damage to cell membranes; (11) protection of skin from aging effects of UV light; (12) stimulation of the immune system; and, finally, (13) stimulation of retinal cell growth in degenerative retinal diseases.

The polysaccharide network can be strengthened by further hydroxycinnamate oligomers, for example, ferulic acid trimers or tetramers. Due to the reactive nature of ferulic acid and the polymerization reactions of cell wall peroxidases, it is most likely that trimers and large polymeric compounds are formed by the radical coupling of plant ferulic acid. Because of the chemical nature of the radical-generated polymerization, the extent of linkage could continue until the reaction is limited either by the exhaustion of substrate or the physical nature of the cell wall controlling movement and flexibility of feruloylated polysaccharides. Fry *et al.* published evidence for higher oligomers in maize suspension culture but did not present defined structures [18]. It has been postulated that soluble feruloylated arabinoxylans in bread dough are prevented from interfering with the formation of the gluten protein network by oxidative coupling with free ferulic acid [10]. It is due to the complexity of these compounds that only with the aid of modern analytical equipment, such as mass spectrometry and 2-D nuclear magnetic resonance spectroscopy, can the structure of such compounds be elucidated. Diferulates and larger products make the major contribution to cross-linking of wall polysaccharides in cultured maize cells [18]. Four different dehydrotrimers of ferulic acid have recently been identified in maize bran [19–22]. The amount of diferulate in maize bran varies from 0.0075% to 0.01% of the dry weight of maize cell walls [22] in comparison to 1.3% of total diferulate levels and 2.6% monomeric ferulic acid [23] in the same cereal bran.

White asparagus cell walls have been shown to contain large amounts of phenolics, including ferulic acid and its dimers and trimers. It has been suggested that these phenolic compounds are mainly responsible for cross-linking the different cell wall polymers related to asparagus hardening [24]. During post-harvest storage, the asparagus texture and the amount of cell wall phenolics increased significantly. The effect of post-harvest storage conditions on the accumulation of ferulic acid and its derivatives were investigated. Three different storage conditions were used: keeping the spears in aerobic conditions at room temperature and at 4 ºC, and in anaerobic conditions at room temperature. A direct relationship between phenol accumulation and increases in texture was observed, and these changes were dependent on temperature and storage atmosphere. An increase in temperature led to a high amount of cell wall phenolics and the absence of oxygen in the storage atmosphere delayed the accumulation [24].

Two new dehydrotriferulic acids and two dehydrotetraferulic acids were isolated from saponified maize bran insoluble fiber using size exclusion chromato-

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graphy on Bio-Beads S-X3 followed by Sephadex LH-20 chromatography and semipreparative phenyl-hexyl reversed phase high performance liquid chromatography (HPLC) [25]. The structures were identified on the basis of UV spectroscopy, mass spectrometry, and 1- and 2-D nuclear magnetic resonance equipment. [25].

There are three kinds of pathways to extract ferulic acid from natural resources: (1) from low-molecular-weight ferulic conjugates; (2) from plant cell walls; and (3) from tissue culture or microbial fermentation. Ferulic acid can also be synthesized chemically by the condensation reaction of vanillin with malonic acid catalyzed by piperidine [26]. However, this method produces ferulic acid as a mixture of *trans*- and *cis*-isomers. The yield is high, but it takes as long as three weeks to complete the reaction. Ferulic acid is one of the most abundant phenolic acids in plants, varying from 5 g/kg in wheat bran and corn kernel, 9 g/kg in sugar beet pulp [22,27], and 15–28 g/kg of rice bran oil [28].

Today, the commercial natural ferulic acid is mainly produced from γ-oryzanol in rice bran oil, although plant cell wall materials contain more ferulic acid. One of the main reasons is that ferulic acid cross-links with polysaccharides, such as arabinoxylans in grasses, pectin in spinach and sugar beet, and xyloglucans in bamboo, and it is not easy to release ferulic acid from polysaccharides and purify it. Fortunately, two methods were developed [29] to break the cross-link and release ferulic acid from plant cell wall materials. One is an enzymatic method using FAEs. Although there was extensive research on the preparation of ferulic acid using FAEs, and in some cases in combination with polysaccharide hydrolases, this is not a practical way to produce commercial ferulic acid because of high cost in the production of the enzyme by microorganisms, and the long reaction time required to hydrolyze the bound ferulic acid. Alkaline hydrolysis is another way to release ferulic acid from polysaccharides and was often used to determine the content of ferulic acid in bran [30], which could totally release the bound ferulic acid in short time at high alkaline concentration and high temperature. However, it is difficult to purify ferulic acid from the hydrolysate because it contains many components and is a deep brown color. Using activated charcoal(carbon) to adsorb ferulic acid could purify it from enzymatic solution, but it is not a feasible way to purify ferulic acid in alkaline hydrolysate, because activated charcoal strongly adsorbed the color substances produced during alkaline hydrolysis and could be also washed out using alcohol or sodium hydroxide as the elute. Previous studies found that anion macroporous resin had high capacity for adsorbing ferulic acid in enzyme-hydrolysate from wheat bran and could be washed out by solutions of ethanol-acetic acid-water, suggesting that it is a choice to purify ferulic acid from alkaline hydrolysate [31]. Sugarcane is one of the most important crops in the tropics, with global production now estimated at 1.25 billion tons a year. It contains 73–76 g liquid/100 g and the remaining 24–27 g/100 g is fiber discarded as bagasse. Sugarcane bagasse contains more cellulose and less hemicellulose compared to wheat bran and maize bran, thus the alkaline hydrolysate solution would not be viscous after treatment by sodium hydroxide or potassium hydroxide [32]. Moreover, sugarcane bagasse contains 1.36%–2.58% ferulic acid [33,34], indicating that it is a suitable resource for the preparation of ferulic acid by alkaline hydrolysis. However, anion macroporous resin exchange chromatography can not be directly used to purify ferulic acid in alkaline hydrolysate from the bagasse, as NaCl formed during neutralization after NaOH hydrolysis would greatly decrease the purification efficiency [35].

The similarity of chemical structures between ferulic acid and vanillin has led to the development of a biotechnological way to transform ferulic acid into vanillin [36–38]. In the process, ferulic acid was released from raw materials by enzymatic treatment, and was biotransformed into vanillin by two different white rot basidiomycetes. *Aspergillus niger* first transformed ferulic acid into vanillic acid, and vanillic acid was then metabolized into vanillin by *Pycnoporus cinnabarinus*. Vanillin obtained in this manner could be considered as "natural" according to European and American legislations. The release of ferulic acid from various raw materials was studied with some commercial enzyme mixtures. For example, SP 584 (Novozymes, Bagsvaerd, Denmark) was shown to be able to solubilize a high percentage of ferulic acid present in sugar beet pulp to give both free and esterified ferulic acid forms [39,40].

Carbohydrate Esterases

Through evolution, enzymes have acquired the ability to attack ester linkages between hydroxycinnamic acids and carbohydrates in the process of biodegradation of plant cell walls [41]. Plant cell walls constitute the largest source of renewable energy on earth. They are composed of an intricate network of polysaccharides that are among the most complex structures known. For the complete breakdown of these polysaccharides, microorganisms require a battery of specific enzymes. These polysaccharides that

complex together with polymers, such as lignin and cellulose, modulate the plant cell wall material [41]. Complex highly-branched polysaccharides (arabinoxylans, pectin), are the main compounds of the cell wall. Cinnamic acid derivatives such as ferulic or coumaric acids are covalently bound to these polysaccharides through ester linkages, increasing the complexity of these polysaccharidic structures [42].

The complete degradation of plant cell wall polymers requires an array of enzymes with different activities. The hydrolysis of hydroxycinnamate esters in plant cell walls is catalyzed by cinnamoyl esterases (CEs; such as cinnamoyl ester hydrolases, feruloyl/p-coumaroyl esterases, and ferulic/p-coumaric acid esterases). CEs are a subclass of the carboxylesterases (EC 3.1.1.1) and characterized by a relatively high activity on various hydroxycinnamate esters, for example, methyl ferulate or [5-O-(*trans*-feruloyl)-α-*L*-arabinofuranosyl]-[(1→3)-β-*D*-xylopyranosyl-(1→4)-dxylopyranose, compared to that of short chain alkyl esters (e.g., p-nitrophenyl/α-naphthyl acetate) [43]. We might consider two classes: those in which sugar plays the role of the "acid", such as pectin methyl esters; and those in which sugar behaves as the alcohol, such as in acetylated xylan [44]. A number of possible reaction mechanisms could be involved. The most common is a Ser-His-Asp catalytic triad catalyzed deacetylation analogous to the action of classical lipase and serine proteases. Other mechanisms such as a Zn^{2+} catalyzed deacetylation might also be considered for some families [45].

Two enzymes, feruloyl and cinnamoyl esterases (FAE-B), purified from *A. niger* strains, have different physicochemical characteristics and catalytic properties against cinnamoyl model substrates, such as methyl derivatives of hydroxycinnamic esters and soluble feruloylated oligosaccharides derived from plant cell wall [46]. These enzymes are exocellular and the corresponding expression is inducible. A new strain of *A. niger* I-1472 was shown to produce numerous polysaccharide-degrading enzymes as well as esterases that released ferulic acid from natural feruloylated oligosaccharides, when grown on sugar beet pulp and maize bran [47].

In 2002, it was reported that ferulic acid had been shown to link hemicellulose and lignin. Cross-linking of ferulic acids within cell wall components influences wall properties such as extensibility, plasticity, and digestibility, and limits the access of polysaccharidaes to their substrates [48]. The degradation of these cell wall polymers requires several hydrolytic enzymes such as hemicellulases, xylanases, pectinases, and esterases [49].

Classification of FAEs Enzymes

FAEs (EC 3.1.1.73), including cinnamoyl esterases and cinnamic acid hydrolases, are a subclass of the carboxylic acid esterases (EC 3.1.1) that play a key physiological role in the degradation of the intricate structure of the plant cell wall by hydrolyzing the ferulate ester groups involved in the cross-linking between hemicelluloses and between hemicellulose and lignin [50,51].

Sugar beet pulp is an important source of ferulic acid with a dry weight content of approximately 1% [52]. FAE occurs as a single catalytic module and also as a part of a multimodular protein structure. Some enzymes contain carbohydrate-binding modules and others are a part of a multimodular complex. The fusion of a carbohydrate binding module to a catalytic domain improves the catalytic efficiency of FAEs [53,54].

The use of multiple alignments of sequences or domains that show FAE activity, as well as related sequences, helped to construct a neighborhood-joining phylogenic tree. The outcome of this genetic comparison supported substrate specificity data and allowed FAEs to be sub-classified into four types, A, B, C, and D [55–62], based on their substrate specificities towards synthetic methyl esters of hydroxycinnamic acids (ferulic acid, diferulic acids, p-coumaric acid, sinapinic acid, and caffeic acid) for substitutions on the phenolic ring, and on their amino acid sequence identity (protein sequence), indicating an evolutionary relationship among FAEs, acetyl xylan esterases, and certain lipases. The nomenclature of FAEs follows both the source of the enzyme and the type of FAE (**Table 1**) [63,64]. The classification of carbohydrate esterases including acetyl xylan esterases and FAEs is available on the Carbohydrate Active Enzymes database (http://www.cazy.org) [65].

FAE Specificity

FAEs from mesophilic and thermophilic sources [66] show substrate specificity in accordance with their classification over a wide range of phenylalkanoate substrates. Within types, however, differences can be observed with respect to the position of certain substitutions, together with the necessity for a specific group to be present at a specific location, for example, the type C enzyme, StFaeC, requires a hydroxyl group to be present at C-4 of the benzoic ring. Thermophilic FAEs in general had a lower catalytic efficiency than the mesophilic

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Table 1 Classification of feruloyl esterases (FAEs)

FAE type	Type A	Type B	Type C	Type D
Microorganism	*Aspergillus niger*	*Penicillium funiculosum*	*Talaromyces stipitatus*	*Pseudomonas fluorescens*
FAE	FAE-A	FAE-B	FAE-C	XYLD
Preferential induction medium	WB, OSX	SBP	SBP-WB	WB
Hydrolysis of methyl esters	MFA, MSA, M*p*CA	MFA	MFA, MSA, M*p*CA, MCA	MFA, MSA, M*p*CA, MCA
Release of free diferulates from plant cell walls	5,5′-diferulic acid only	No	No	5,5′-diferulic acid only
Sequence similarity	Lipase	Cinnamoyl esterase family 1 acetyl xylan esterase	Chlorogenate esterase tannase	Xylanase
References	[55–57]	[54,58]	[59,60]	[59,60]

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

counterparts, but released more ferulic acid from plant cell walls within a short time interval at comparable temperatures.

Studies indicate that type C FAEs show specificity for soluble feruloylated arabinoxylans, whereas type A FAEs act more efficiently with xylanases on water-unextractable wall material [67]. FAEs have enhanced activity with family 11 xylanases for the release of monomeric ferulic acid, but family 10 xylanases show preferential activity on diferulates. This might be linked to the location of these ester-linkages on the arabinoxylan chain. As preliminary results show that type C FAEs are effective bread improvers, and are more specific for water-extractable arabinoxylans, the role of water-extractable arabinoxylans in the process might require re-examination [56]. At present, the lack of highly conserved sequences within the sequenced esterases does not permit further classification of the FAEs, other than that their primary amino acid sequences place the majority of these enzymes in carbohydrate esterase family 1 of the Carbohydrate Active Enzymes database [65].

Crystal Structure of FAE Enzymes

There are only 17 protein database entries in that type of enzyme found in the Comprehensive Enzyme Information System (http://www.brenda-enzymes.info/). One of the most thoroughly studied FAEs is type A (FAE-A) from *A. niger* [42,46,68]. The crystal sequence analysis of FAE-A indicates that the enzyme is α/β-hydrolase with a serine, histidine, and aspartic acid catalytic triad. The protein with the highest sequence similarity, not including the very similar FAEs with more than 93% identity from *A. awamori* and *A. tubingensis*, is the lipase from *Rhizomucor miehei* [69], with an overall sequence identity of 32%. FAE-A contains the characteristic lipase serine active site motif and, given the sequence similarities, it was predicted that FAE-A would have considerable structural homology and a similar catalytic mechanism to fungal lipases. However, FAE-A has been shown not to possess lipase activity [70,71].

The crystal studies show that many FAEs are modular, comprising of a catalytic domain covalently linked to a non-catalytic carbohydrate-binding module [54,65,60, 71–75]. The structures of two bacterial FAEs have been published: FAE_XynZ from *Clostridium thermocellum* [74]; and FAE_Xyn10B [63], also from *C. thermocellum*. Both structures displayed the canonical eight-strand α/β-fold of an esterase with the catalytic triad at the heart of the active site. FAE is a compact globular protein (**Fig. 2**) [64, 68]. The C-terminal polyhistidine purification tag is clearly observed in the density and has been modeled along with five cadmium ions per protein monomer that coordinate the tag [64]. Although these enzymes have similar functionality to FAE-A, there is no apparent sequence homology. Therefore, to examine the structure of the enzyme and its substrate specificity, FAE-A has been crystallized and the crystallographic structures solved for the native enzyme and for the enzyme in complex with ferulic acid. The overall topology of the protein is a classical α/β-hydrolase fold based on an eight-stranded β-sheet surrounded by α-helices. In FAEs, six of the strands are parallel, with a pair of antiparallel strands at the C-terminal side of the fold.

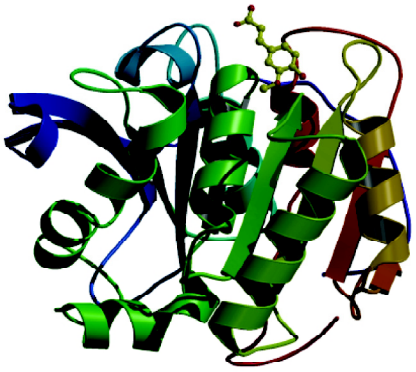

Fig. 2 3-D structure of the Xyn10B ferulate esterase module [64,68]

There have also been reports of FAEs that are part of large multidomain structures, such as cellulosomes [76]. Furthermore, fungal chimeric enzymes composed of the sequences encoding the FAE-A fused to the endoxylanase B of *A. niger* has also been constructed [65]. This fusion of naturally free cell wall hydrolases onto bifunctional enzymes enabled the increase of the synergistic effect on the degradation of complex substrates such as corn and wheat bran compared to the result obtained using the free enzymes for both substrates.

Relatively few studies have been carried out to elucidate the functional relationships between sequence-diverse FAEs. These FAEs have a common α/β hydrolase fold and a catalytic triad (Ser-His-Asp) as shown in lipases [64]. Faulds *et al.* [77] solved the crystal structure of an inactive mutant of the FAE-A, AnFaeA (S133A), in complex with a feruloylated trisaccharide and showed that, in agreement with the work of Schubot *et al.* [74], tight binding of the carbohydrate was not required for catalysis. In contrast to FAEs, the determination of the crystal structure of a family 10 xylanase from *Thermoascus aurantiacus* in complex with xylobiose containing an arabinofuranosyl-ferulate side-chain revealed that the distal glycone subsite of the enzyme makes extensive direct and indirect interactions with the arabinose side-chain, whereas the ferulate moiety is solvent-exposed [78].

Generally, FAEs display broad substrate specificities, although the structural motifs that discriminate the various enzymatic targets remain to be elucidated. The first indication of the molecular determinants controlling the substrate specificity of the cellulosomal FAE module of Xyn10B was achieved by Prates *et al.* [63]. It is suggested that the presence of at least one m-methoxy group in the substrate is required for binding, which explains why the enzyme can not recognize methyl coumarate or caffeic acid. Considering the remarkable impact of FAEs in various industrial and medical applications, it is anticipated that these structural studies will provide an initial framework for the rational design of novel enzymes with improved biotechnological potential [63].

Microbial Production of FAEs

All microbial FAEs are secreted into the culture medium. They are sometimes called hemicellulose accessory enzymes, because they help xylanases and pectinases to break down plant cell wall hemicellulose. Most research on the microbial production of FAEs to date involve the isolation, purification, and characterization of FAEs derived from a wide range of microorganisms (fungi and bacteria), as well as the enzymatic release of the products from cell wall degradation.

Summary experimental observations, with the knowledge of the specific microorganisms and kind of esterase required, suggest that an appropriate fermentation technique is required for the production of a specific esterase. Esterases like FAEs and cinnamic acid esterases are produced from *Aspergillus* sp. using either solid state or submerged fermentations. There are many bioprocesses used for the production of enzymes. Many studies show that solid-state fermentation (SSF) is attractive because it presents many advantages for fungal cultivations [79]. SSF is defined as a fermentation process in which micro-organisms grow on solid materials without the presence of free liquid. Water is present in an absorbed form within the solid matrix of the substrate [80,81]. In SSF, productivity per reactor volume is much higher than that in submerged culture [82]. Operational cost is also lower because simpler plant and machinery are required [83]. Solid culture processes are practical for complex substrates including agricultural, forestry, and food processing residues and wastes, used as inducing carbon sources for the production of FAEs. *A. niger* [84,85], different *Penicillium* sp. [84–87] and the thermophilic fungus *Sporotrichum thermophile* [88] were capable of producing FAEs when grown on agricultural residues such as sugar beet pulp, wheat bran, wheat straw and brewer's spent grain under SSF. *Penicillium brasilianum* [85] was the more effective FAE producer under SSF. However, in all of these fermentations, other hydrolytic enzymes, such as cellulases and hemicellulases, are also produced that incur severe costs in downstream separation.

The choice of the appropriate substrate is of great

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importance for the successful production of esterases. The substrate not only serves as a source of carbon and energy, but also provides the necessary inducing compounds for microorganisms. Monosaccharides and disaccharides such as glucose, xylose, lactose, maltose, and xylitol generally do not support the production of FAEs at all. This might be due to glucose catabolite repression and/or the induction mechanism that has not been completely elucidated. Complex carbon sources that contain high amounts of esterified ferulic acid such as de-starched wheat bran [89–93], maize bran [94–96], brewer's spent grain [85,97], sugar beet pulp [55,60,83,84] and wheat bran [58,59,83, 98–101] have been efficiently used for the microbial production of FAEs (**Table 2**). Several FAE enzymes have been purified and characterized from aerobic and anaerobic microorganisms that use plant cell wall carbohydrates. FAE activities have also been detected in mammalian cells and plants, but these enzymes have yet to be purified and their protein sequences determined for comparison with the microbial enzymes [102,103].

Over the end of the last decade, FAEs and xylanase have also been produced by the lignocellulolytic actinomycete *Streptomyces avermitilis* CECT 3339 [92, 93] during growth on de-starched wheat bran and sugar beet pulp as the carbon sources. Although the production of FAEs from mesophilic microorganisms has been well documented, a limited number of thermophilic microorganisms have been reported to produce FAEs [92,93].

Generally, FAE enzymes are produced (purified and characterized) from several microorganisms (various bacteria and fungi) (**Table 2**), including *Streptomyces* C254, *Streptomyces* C248, *Streptomyces olivochromogenes* NRCC 2258 [89], *S. thermophile* ATCC 34628 [100,104], *Strep. avermitilis* CECT 3339 [92], *Schizophyllum commune* ATCC 38548 [91], *S. avermitilis* UAH 30 [93], *Neocallimastix* MC-2 [105], *P. brasilianum* IBT 20888 [85], *Penicillium pinophilum* CMI 87160ii [86], *Trichoderma reesei* QM 9414 [106], *Streptomyces* sp. S10 [107], *Talaromyces stipitatus* CBS 375.48 [108], *Piromyces* MC-1 [109], *Penicillium funiculosum* IMI-134756 [54], *Piromyces brevicompactum*, *Piromyces expansum* [61], *A. awamori* IFO4033 [99], *A. awamori* VTTD-71025, *A. foetidus* VTTD-71002 [98], *A. niger* [57,83], *A. niger* CBS 120.49 [55], *A. flavipes* [97], *A. niger* CS 180 (CMICC 298302) [110], *A. oryzae* VTTD-85248, *A. niger* VTTD-77050 [98], *A. niger* NRCC 401127 [89], *A. niger* NRRL3 [111], *A. niger* CS 180 (CMICC 298302), *A. niger* 1-1472 [59,60,110], *Aureobasidium pullulans* NRRLY 23311-1 [112], *Bacillus subtilis* ATCC 7661, *B. subtilis* FMCCDL1, *B. subtilis* NCIMB 3610 [113], *Clostridium stercorarium* NCIMB 11754 [112], *Neurospora crassa* ST A(74 A) [58], *C. stercorarium* [112], *C. thermocellum* [114], *Humicola insolens* [115], *Sporo. thermophile* [107, 104,116,117], and *Fusarium oxysporum* [106,108].

Action, Synergism, and Organic Synthesis of FAEs

In 2000, six novel feruloyl esters of triterpene alcohols and sterols, two *trans*-ferulates (cycloeucalenol and 24-methylenecholesterol *trans*-ferulates), and four *cis*-ferulates, (cycloartenol, 24-methyelenecycloartanol, 24-methylcholesterol, and sitosterol *cis*-ferulates), as well as five known *trans*-ferulates (cycloartenol, 24-methylenecycloartanol, 24-methylcholesterol, sitosterol, and stigmastanol *trans*-ferulates), and one known *cis*-ferulate (stigmastanol *cis*-ferulate), were isolated from the methanol extract of edible rice bran [118]. The synthesis of pentylferulate was achieved using a water-in-oil microemulsion system containing an FAE from *A. niger* [83]. Although some reports describe the enzymatic synthesis of alkyl and glyceride ferulates, there are few papers concerning enzymatic preparation of feruloylated carbohydrates [119].

Ferulic acid was also efficiently released from wheat bran by a mixture of *Trichoderma viride* xylanase and *A. niger* ferulate esterase FAE-A [120–122]. In addition, the release of ferulic acid from maize bran by commercial enzymes was low and autoclaving treatment of the bran improved the solubilization of feruloylated oligosaccharides, which are substrates for FAEs [60,65]. However, the use of cell wall-degrading enzymes and FAEs involved either dependence on commercially available mixtures or numerous steps of purification of the enzymes, as in the case of FAE-A.

Recently, FAEs from *Humicola insolens* [123] catalyzed the transesterification of secondary alcohols with excellent enantioselectivity, which are substrates that bear no structural similarity to the natural substrates of this enzyme [124]. The observations of feruloyl and p-coumaroyl esterase activities of the cleavage of ester cross-linkage have led to the purification and partial characterization of FAEs from various sources [55,87,120–122,125,126]. Several studies on the release of ferulic acid from feruloylated polymers by FAE have indicated that the participation of a much more complicated enzymatic system is needed [86,127–129]. However, previous studies on the interactions of FAEs have been mainly focused on enzymes such as xylanase and β-xylosidases

Table 2 Microbial production of feruloyl esterases (FAEs)

No.	Microorganism	Induction conditions (temperature, induction days, carbon source) and assay technique	FAE activity (mU/mg	mU/ml)	Reference
1	*Streptomyces* C254	37 ºC, 3 d, DSWB; DSWB	300.00	80.00	[89]
2	*Streptomyces* C248	37 ºC, 3 d, DSWB; DSWB	380.00	130.00	[89]
3	*Streptomyces olivochromogenes*	37 ºC, 3 d, DSWB; DSWB	2350.00	1200.00	[89]
	NRCC 2258	37 ºC, 3 d, OSX; DSWB	6900.00	2560.00	
		37 ºC, 3 d, SCB; DSWB	1800.00	830.00	
4	*Sporotrichum. thermophile*	50 ºC, 7 d, WS; DSWB	156.00†	–	[88,104]
	ATCC 34628				
5	*Streptomyces avermitilis*	37 ºC, 2 d, DSWB; DSWB	16.80	–	[68]
	CECT 3339	37 ºC, 2 d, OSX; DSWB	11.20	–	
6	*Schizophyllum. commune*	30 ºC, 14 d, DSWB; DSWB	41.20	7.00	[91]
	ATCC 38548	30 ºC, 14 d, OSX; DSWB	9.50	2.00	
		30 ºC, 14 d, cellulose; DSWB	28.00	28.00	
7	*Streptomyces avermitilis* UAH 30	37 ºC, 4 d, DSWB; DSWB	16.82	1.75	[93,132]
		37 ºC, 4 d, OSX; DSWB	11.15	1.55	
		37 ºC, 4 d, SCB; DSWB	2.61	0.44	
8	*Neocallimastix* MC-2	39 ºC, 5 d, cellulose; FAXX	55.00	–	[105]
9	*Penicillium brasilianum* IBT 20888	30 ºC, 8 d, BSG; MFA	1542.00†	–	[85]
10	*Penicillium pinophilum* CMI 87160ii	30 ºC, 12 d, OS+WB; MFA	156.00	–	[86]
11	*Trichoderma reesei* QM 9414	30 ºC, 7 d, MFG+Glu; MFA	–	3000.00	[106]
12	*Streptomyces* sp. S10	30 ºC, 4 d, DSWB; DSWB	15.45	2.00	[107]
13	*Talaromyces stipitatus* CBS 375.48	25 ºC, 7 d, WB; MCA	–	27.00	[108]
14	*Piromyces* MC-1	39 ºC, 5 d, CBG+S; MFA	560.00	–	[109]
15	*Penicillium funiculosum* IMI-134756	25 ºC, 6 d, SBP; M*p*CA	–	120.00	[54]
16	*Piromyces brevicompactum*	26 ºC, 4 d, MFA; MFA	–	32.00	[61]
17	*Piromyces expansum*	26 ºC, 4 d, MFA; MFA	–	45.00	[61]
18	*Aspergillus awamori* IFO4033	30 ºC, 3 d, WB; DSWB-SFO	6900.00	–	[99]
19	*Aspergillus awamori* VTTD-71025	30 ºC, 7 d, SFC; WS	–	10.00	[86]
20	*Aspergillus foetidus*	30 ºC, 7 d, WB; WS	–	12.00	[98]
	VTTD-71002	30 ºC, 7 d, SFC; WS	–	–	
21	*Aspergillus niger*	26 ºC, 4 d, MFA; MFA	–	28.00	[57,83]
22	*Aspergillus niger* CBS 120.49	25 ºC, 4 d, OSX+Glu; MFA	130.00	1250.00	[55]
		25 ºC, 4 d, WB+Glu; MFA	340.00	–	
		24 ºC, 4 d, SBP; MFA	10.00	–	
23	*Aspergillus flavipes*	28 ºC, 5 d, WB; MFA	6980.00	33,180.00	[97]
		28 ºC, 6 d, MB; MFA	10,570.00	6820.00	
24	*Aspergillus niger* CS 180	25 ºC, 5 d, SBP; MCA	413.00	10.30	[59,110]
	(CMICC 298302)				
25	*Aspergillus oryzae* VTTD-85248	30 ºC, 7 d, WB; WS	–	72.00	[98]
26	*Aspergillus niger* VTTD-77050	30 ºC, 7 d, WB; WS	–	96.00	[98]
		30 ºC, 7 d, SFC; WS	–	132.00	
27	*Aspergillus niger* NRCC 401127	37 ºC, 4 d, DSWB; DSWB	13,2250.00	10,580.00	[89]
28	*Aspergillus niger* NRRL3	30 ºC, 5 d CB; MFA	–	13.90	[111]
29	*Aspergillus niger* CS 180	25 ºC, 5 d, MCA; MFA	413.00	10.30	[59,60,110]
	(CMICC 298302)				
30	*Aspergillus niger* 1-1472	30 ºC, 5 d, CB; MFA	–	–	[59,60,110]

(To be continued)

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(Continued)

Table 2 Microbial production of feruloyl esterases (FAEs)

No.	Microorganism	Induction conditions (temperature, induction days, carbon source) and assay technique	FAE activity mU/mg	mU/ml	Reference
31	*Aureobasidium pullulans* NRRLY 23311-1	30 ºC, 2.5 d, BX; NPh-5-Fe-Araf	1600.00	347.50	[112]
32	*Bacillus subtilis* ATCC 7661	30 ºC, 1 d, MFA; MFA	1.30	–	[113]
33	*Bacillus subtilis* FMCCDL1	30 ºC, 1 d, MFA; MFA	19.90	–	[113]
34	*Bacillus subtilis* NCIMB 3610	30 ºC, 1 d, MFA; MFA	3.40	–	[113]
35	*Clostridium stercorarium* NCIMB 11754	30 ºC,3 d CC; DSWB	3.40	–	[126]
36	*Neurospora crassa* STA(74 A)	30 ºC, 3 d,WB; MSA	9000.00	–	[58]
37	*Orpinomyces* PC-1	90 ºC, 5 d, (CBG+S; MFA	220.00	–	[109]
38	*Orpinomyces* PC-2	90 ºC, 5 d, CBG+S; MFA	360.00	–	[109]
39	*Orpinomyces* PC-3	90 ºC, 5 d, CBG+S; MFA	380.00	–	[109]
40	*Fibrobacter succinogenes* S851	39 ºC,2 d, A; FAX	10.00	–	[128]
41	*Fusarium oxysporum* F3	30 ºC,3 d, CC; DSWB	–	98.00	[120]
42	*Fusarium proliferatum* NRRL 26517	30 ºC, 5 d, CB; MFA	–	33.46	[111]
43	*Fusarium verticillioides* NRRL 26517	30 ºC, 5 d, CB; MFA	–	19.60	[111]

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

[120,121,130–134], and there is little information on xylan-debranching enzymes such as α-*L*-arabinofuranosidase or on the effect of the co-existence of xylanase and arabino-furanosidase on FAEs.

It has been documented that FAEs are inducible [89,90, 92,93,125,135,136]. The expression of the FAE-encoding gene from eukaryotic *A. niger* is regulated by xylose, arabinose and ferulic acid. In human, fecal inoculum FAE activity could be induced by fine bran cell wall material, but the induction of FAE activity in intestinal microflora is largely unknown [27]. Using Novozym 435 as catalyst, the syntheses of ethyl ferulate from ferulic acid (4-hydroxy 3-methoxy cinnamic acid) and ethanol, and octyl methoxycinnamate from p-methoxycinnamic acid and 2-ethyl hexanol were successfully achieved recently in our laboratory [137].

In 2001, it was reported that the solubilization of *A. niger* FAE-A in a cetyltrimethylammonium bromide water-in-oil microemulsion offered a number of advantages for the enzymatic synthesis of ferulate esters [138]: (1) a good yield of pentylferulate was obtained (60%); (2) the pentylferulate yield was independent of the water content of the microemulsion system; (3) a high substrate (n-pentanol) concentration was achievable; and (4) an increased stability of FAE-A in cetyltrimethylammonium bromide water-in-oil microemulsion was observed when compared with the correspondent stability in water.

Activity Assays of Microbial FAEs

Several methods have been reported for measuring the activity of the purified microbial FAEs. Most are based on HPLC techniques, using enzymatic hydrolysis of ferulic acid esters [139,140], plant polysaccharides [92,93,109, 130], their fragments [141] and fragment analogs [142], as well as chlorogenic acid or hydroxycinnamic tartrate-containing materials [139]. The assay was based on the measurement of ferulic acid released from ethyl ferulate. One volume of enzyme solution was mixed with 3 volumes of 1.33 mM ethyl ferulate in 0.05 M potassium phosphate buffer, pH 6.5. Both solutions were preheated to 40 ºC before mixing. The final mixture was incubated at the same temperature. At various time intervals, 0.3 ml aliquots of

the reaction mixture were withdrawn and mixed with 0.1 ml of 0.35 M H_2SO_4 to stop the reaction. This was followed by the addition of 0.3 ml of 1.0 mM benzoic acid as the internal standard and 0.1 ml of 0.7 M NaOH. The solution was mixed by vortexing, passed through a 0.45 μm syringe filter, and analyzed by HPLC. The acidic components of the samples were eluted with a mixture of water:acetic acid:1-butanol (350:1:7; *V*/*V*/*V*) with a flow rate of 1 ml/min. A linear gradient of methanol (ramped up to 100% methanol in 5 min) was used to wash off the unreacted ethyl ferulate. All ethyl ferulate, ferulic acid, and benzoic acid were dissolved in minimal volume of ethanol prior to mixing with buffer or water.

Unfortunately, these HPLC methods require expensive equipment, are time-consuming, and not suitable for rapid analysis of large numbers of samples. Some of these methods require the isolation of natural substrates, which adds another laborious step. Capillary zone electrophoresis [87] and gas chromatography [109] have also been applied to FAE assays using natural substrates, their analogs and hydroxycinnamic methyl esters, but these methods possess similar disadvantages.

Spectrophotometric analysis for FAE activity described in published reports relies on the use of differences in spectral properties of free ferulic acid and its natural esters [143,144] or their analogs [142]. Such methods measure relatively low changes of absorbance and have not become generally adopted. A new spectrophotometric method [145] for determining FE activity using 4-nitrophenyl ferulate, a cheap substrate that is easy to prepare [146], was used. The method is simple and is based on the measurement of 4-nitrophenol released upon enzyme action. The main drawback in using such a substrate is its low solubility in aqueous buffer solutions. Inspired by the method for lipase assays, dimethyl sulfoxide and a detergent, Triton X-100, were used to create transparent emulsions stable for several hours, enabling the measurement of FAE activity spectrophotometrically [147]. A variety of natural substrates such as feruloylated oligosaccharides [61,114] and de-starched wheat bran [148] have been used to assay the enzymes. Synthetic esters of various cinnamic acids and short chain alcohols, such as methyl and ethyl ferulates, were also introduced for assaying FAEs [27,131]. In all cases, FAE activity was determined by measuring the rate of hydrolysis of model substrates such as methyl esters of hydroxycinnamic acids, that is, methyl ferulate, methyl sinapinate, methyl p-coumarate, and methyl caffeate. One unit of FAE activity was defined as the amount of enzyme required for releasing 1 μM ferulic acid per min at pH 6.0, 37 ºC.

Biophysical and Biochemical Properties of Microbial FAEs

From 1991 to 2006, more than 40 FAEs were purified and characterized from microorganisms such as *C. stercorarium* [126], *C. thermocellum* [114], *Sporo. thermophile* [121], *S. olivochromogenes* [127], *A. awamori* [149], *F. oxysporum* [122,127], *Fusarium proliferatum* [111], *N. crassa* [58,150], *A. nidulans* [149], *Aureo. pullulans* [112], *A. niger* [151], *Piromyces equi* [61], *Cellvibrio japonicus* [106], and *Tal. stipitatus* [59,108] (**Table 3**).

The biophysical and biochemical characteristics of the purified FAEs show significant variations in chemical characteristics such as molecular weight (27–210 kDa), isoelectric point, and optimum hydrolytic reaction conditions (pH 3.0–9.5) (**Table 3**). There is no correlation between the biochemical characteristics of the FAEs and their optimal reaction conditions found to date. Microbial FAEs have a broad range of pH and temperature dependence, with optimal activities occurring between pH 5.0 and 8.0, and between 30 ºC and 65 ºC.

Results obtained from the biophysico-chemical studies on FAEs will help to advance the use of that type of enzyme in food processing and agricultural industries. In addition, knowledge of their biophysical and biochemical properties should be useful for research workers in the biomedicine field

Applications of Microbial FAEs

There has recently been considerable interest in a large number of potential applications of these enzymes due to their roles in many biotechnological processes, in various industries (chemicals, fuel, animal feed, textile and laundry, pulp and paper, food and agriculture, and pharmaceutical), also in their potential applications in obtaining ferulic acid from agro-industrial waste materials such as those produced by milling, brewing, and sugar industries. The prospect of broad applications of FAEs has fueled much interest in these enzymes, as shown by the increasing number of FAEs discovered in microbial organisms in recent years.

FAEs could be used in pulp and paper processes [152, 153] and as animal feed additives [3,4,120–122] to facilitate nutrient assimilation. In addition, ferulic acid, which is the most abundant cinnamic acid in plant cell walls, is a precursor for vanillin and its access through biotechno-

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Table 3 Biophysicochemical properties of microbial feruloyl esterases (FAEs)

No.	Microorganism	Enzyme	Enzyme type	MW (kDa)	Isoelectric point	pH optimum	Thermostability (^{o}C)	Reference
1	*Clostridium stercorarium*	FAE	C, D	33	6.5	8.0	65	[126]
2	*Clostridium thermocellum*	XynZ, XynY	–	45	5.8	5–8	55	[40,74,114]
3	*Sporotrichum thermophile*	StFAE	A, B, C	33, 23	3.5	6.0	55	[116,117]
4	*Streptomyces olivochromogenes*	FAE	–	29	7.9	5.5	30	[126]
5	*Aspergillus awamori*	AwFAE	A	37	–	7.0	45	[99,170]
6	*Fusarium oxysporum*	FoFAE	A, B	27, 31	9.5	7.0	45–55	[120,123]
7	*Fusarium proliferatum*	FAE	B	31	–	6.5–7.5	56	[112]
8	*Neurospora crassa*	FAE	B, D	35	–	6.0	55	[58,151]
9	*Aspergillus nidulans*	AnFAE	B	56	–	7.0	45	[113]
10	*Aureobasidium pullulans*	FAE	B	210	6.5	6.7	60	[113]
11	*Aspergillus niger*	FAE	A, B	36	3.3, 3.0	5.0	55	[36,50,51,54, 55,57,60,68, 69,71,77,83,84]
12	*Piromyces equi Cellvibrio japonicus*	EstA	D	55	–	6.7	50–60	[106]
13	*Talaromyces stipitatus*	TsFAE	A, B, C	35, 35, 65	5.3, 3.5, 4.6	6.0–7.0	60	[109,159]
14	*Penicillium pinophilum*	FAE	–	57	–	6.0	55	[86]
15	*Penicillium expansum*	FAE	–	65	–	5.6	37	[87]
16	*Aspergillus* sp.	FAE	–	42	–	4.0	50	[152]

logical methods is crucial in the quest for natural vanillin [1–8]. As well as being exploited as a hydrolase, FAE was shown to be a good catalyst in synthesizing sugar-phenolic esters [3,4,120–122], and could also be used to functionalize sugar polymers by adding phenolic derivatives onto the natural biopolymers.

Generally, FAEs benefit microorganisms, industry, and biochemists. Some of the ester-linked substituents on plant cell wall polysaccharides retard or inhibit microbial infection [154]. There are many examples in published reports concerning the antimicrobial nature of the phenolic compounds towards some microorganisms. Phenolic components of the plant cell wall, especially p-coumaric acid, ferulic acid, and p-hydroxybenzaldehyde, inhibit the growth of rumen microorganisms [155,156] and phenolic acids derived from plant cell walls have long been used as food preservatives [69] to inhibit microbial growth. *Magnaporthe grisea*, a rice blast fungus, produces a xylanase and an arabinofuranosidase that act synergistically to release arabinoxylo-oligosaccharides from rice cell walls. These compounds contain esterified ferulic acid, and the release leads to the death (presumably programmed cell death) of surrounding rice cells. *In vitro* removal of the ferulic acid moiety (*i.e.*, to give phenolic and oligosaccharide separately) destroyed more than 95% of this killing ability, which would enhance the chances of the colonization of the rice cell wall by the pathogen in the presence of a cinnamoyl esterase [157].

As phenolic acid sugar esters have clear antitumor activity and the potential to be used to formulate antimicrobial, antiviral, and/or anti-inflammatory agents [1, 108], specific FAEs could be used in the tailored synthesis of such pharmaceuticals [158–160]. The FAE system of *Talaromyces stipitatus* has been studied and three discrete FAEs, including a type C esterase, with broad specificity against hydroxycinnamate esters [161,162] have been found. The re-establishments of efficient use of cheap agricultural waste materials, with their synergistic action with other lignocellulose-degrading enzymes, are promising tools in various agro-industrial processes [163]. Other potential applications include production of important medicinal compounds, improvement of bread quality, pulp treatment, juice clarification, improvement of quality of animal feedstock, production of biofuel, and synthesis of oligosaccharides [156]. Therefore, effective FAEs production is a vital prerequisite for successful applications in various industries. To achieve this goal, it is necessary to use FAEs, as well as defined polysaccharides and oligo-

saccharides from different agricultural raw materials.

Hemicellulases and cellulases offer alternatives to augment chemical and mechanical paper-pulping methods, and there is a large amount of published work on this subject [60,156]. Acetylxylan esterases and FAEs might enhance this process by removing substitutions and linkages between polymers during pulping, thus making the solubilization of lignin-carbohydrate complexes easier [50,51, 56,57,60,61,110,156]. After the removal of acetyl groups, the hemicellulases crystallize and form more cellulose-like structures thus affecting polysaccharide solubility and cohesiveness. Ferulic acid is postulated to form cross-links with proteins in wheat, which is important in the rheology of doughs. Pretreatment of lignocellulosic material by secreted fungal enzymes leads to de-esterification, which increased the rate of *in vitro* digestion by ruminal microorganisms by approximately 80% [50,51,56,57,60, 61,110,156].

FAEs are potential analytical aids in modern carbohydrate chemistry. In combination with other plant cell wall-degrading enzymes, the esterases will provide important tools in understanding the fine structure and linkage patterns that exist in the plant cell wall, but the science is at an early stage and ripe for exploitation [50,51,56,57,60,61, 110,156]. Arabinoxylans and β-glucans in the cell walls of barley have been shown to be associated either together or to a common component through an ester bond, as shown by specific hydrolysis by a pure cinnamoyl esterase [164]. The exact nature of the covalent bond between lignin and carbohydrate polymers in the cell wall matrix of various plants has still to be determined, although evidence is beginning to accrue on these structures. FAEs could provide a useful tool in helping to determine this link [165]. FAEs are secreted by a number of bacterial and fungal organisms that exploit plants either to enter the plant cell or to use the cell wall material as a nutritional resource. The complete degradation of plant cell wall polymers requires multi-enzyme complex systems. Most FAEs have been shown to act synergistically with xylanases, cellulases, and pectinases to break down complex plant cell wall carbohydrates [166,167].

Products of the maize industry are ideal stock materials for biotechnology processes. An example is ferulic acid, an aromatic food anti-oxidant that can be isolated from maize fiber after wet milling and is converted to valuable compounds such as vanillin, an important flavourant used extensively in foodstuffs [168]. Apart from its use in flavoring, vanillin is also required for the synthesis of pharmaceutical drugs and is used extensively in the perfume and metal plating industries. In agriculture it has herbicidal action, and can be used as a ripening agent to increase the yield of sucrose in sugar cane [169].

The demand for ethanol has generated the most significant market, where it is used either as a chemical feedstock or as an octane enhancer or petrol additive. Global crude oil production is predicted to decline from 25 billion barrels to approximately 5 billion barrels in 2050 [164]. In the USA, fuel ethanol has been used in gasohol or oxygenated fuels since the 1980s. These gasoline fuels contain up to 10% ethanol by volume. It is estimated that 4.54 billion liters of ethanol is used by the American transportation sector and that this number will rise phenomenally as the American automobile manufacturers plan to manufacture a significant number of flexi-fueled engines which can use a blend of 85% ethanol and 15% gasoline by volume. The production of fuel ethanol from sugars or starch impacts negatively on the economics of the process, thus making ethanol more expensive compared with fossil fuels. Hence the technology development focus in the production of ethanol has shifted towards the use of residual lignocellulosic materials to lower production cost. An improved use of wheat endosperm by-products in this type of ethanol production would generate a fermentable hydrolysate based on the hemicellulose fraction. Complete enzymatic hydrolysis of arabinoxylan requires both depolymerizing and side-group cleaving enzyme activities such as FAEs. Any hemicellulose containing lignocellulose generates a mixture of sugars upon pretreatment alone or in combination with enzymatic hydrolysis. In Europe, potable alcohol manufacturing plants are based on wheat endosperm processing, with the hemicellulosic by-product remaining after fermentation consisting of approximately 66% (*W*/*W*) arabinoxylan [164]. Fermentable sugars from cellulose and hemicellulose will essentially be glucose and xylose, which can be released from lignocellulosics by single- or two-stage hydrolysis, thereby leading to mixtures of glucose and xylose or separate glucose- and xylose-rich streams. Conventional methods, applied for bioconversion of cellulose and hemicellulose to ethanol, involve acid or enzyme hydrolysis of biopolymers to soluble oligosaccharides followed by fermentation to ethanol. A synergistic effect between cellulases, FAEs and xylanases was proven under a critical enzymatic concentration, in the saccharification of steam-exploded wheat straw [170].

In 2004, for the first time, the homologous over-expression of the FAE-B gene in *A. niger* was evaluated [100,101]. The main characteristics of the recombinant FAE-B were in good agreement with those of the corresponding native protein and new insights were provided in

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order to present a more complete description of this enzyme. Sufficient amount of proteins obtained from the FAE-B overproduction will allow structure-function studies to be carried out. In addition, these promising results of overproduction will permit to envisage the first experiments of application and to check the potential of this enzyme in the pulp and paper industry, or more generally in the biotransformation of phenolic compounds of agricultural byproducts.

The synthetic activity pattern of FAEs for the transesterification of various methyl esters of cinnamic acids is similar to that of their hydrolytic action [120–122, 133,104,116,117]. The active sites of FAEs from mesophilic and thermophilic sources were probed using methyl esters of phenylalkanoic acids. Type B and type A FAEs were found to be appropriate biocatalysts for the synthesis of hydroxylated phenolic compounds and methoxylated phenolic compounds, respectively. StFaeC showed maximum hydrolytic activity towards 4-hydroxy-3-methoxy cinnamate, indicating that it might be the most promising type of FAE as a biocatalyst for the enzymatic feruloylation of aliphatic alcohols and oligosaccharides or polysaccharides. Many reports show that StFaeC catalyzed the transfer of the feruloyl group to *L*-arabinose in a ternary water-organic mixture consisting of n-hexane, t-butanol, and water system, with an approximately 40% conversion of *L*-arabinose to its feruloylated derivative [120–122,133, 104,116,117]. FAEs derived from non-recombinant producing strains might be important for better acceptance by consumer's food related applications. Optimizing FAE production in native producing strains is therefore important. An increase in biomass production in a bioreactor will lead to a significant improvement in overall productivity, which will in turn result in a cost reduction, and thus a more competitive and more accessible FAE-based enzyme technology [153,170].

A potentially important application of FAE is in the use of vastly abundant renewable chemical feedstocks, cellulose/lignocellulose. Ferulic acid bond, which provides the cross-linking between lignin and cellulose, and between polysaccharide chains, is an important factor that makes plant cell wall materials recalcitrant and resistant to enzyme hydrolysis. Although chemical hydrolysis is effective to de-polymerize cellulose and hemicellulose materials, the harsh conditions it entails often lead to the generation of toxic by-products that require additional processing steps. The use of enzymes could avoid such problems but is not currently feasible, as complete hydrolysis has not been achieved and the cost of enzymes is considered to be inhibitive for application on a commercial scale [109,171]. Due to the complex nature of the polymeric material, it is conceivable that its complete hydrolysis will require synergistic use of a suite of enzymes. FAE, being an enzyme that breaks down the cross-linking of the polymers chain, is expected to be particularly important in separating lignin from cellulose, de-polymerizing hemicellulose into fermentable sugars, and in making polymeric materials more accessible to other enzymes [172].

Conclusion

Microbial FAEs acting on plant cell wall polymers represent key tools for degradation of plant cell wall polysaccharides, modification of physical and chemical properties of plant cell walls and components, and elucidation of plant cell wall structures. The field is at an early stage, and there is a lot of work to be done on the enzymology, especially 3-D structures and site-directed mutagenesis combined with rigorous kinetics to enhance understanding of binding sites, substrate recognition, and catalytic mechanisms. Cloning of more enzymes would allow classes and relationships between these esterases to be identified. Further work on the structure of the plant cell wall is required, helped by the existence of highly purified esterases, to improve understanding of the synergistic interactions between enzymes. There are many unanswered questions concerning regulation of expression, including full gene sequences, extent of coordinate regulation, and molecular mechanisms in response to putative inducers. In summary, future work on FAEs should include: (1) elucidating the structural characteristics that determine specificity (structure-function relationships); (2) isolation, characterization and cloning of plant FAEs; (3) extending the use of existing and novel esterases as probes for cell wall structures; and (4) production of tailor-made esterases with novel functionalities.

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