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Breakdown of Corn Fiber by a Metagenomic Ferulolyl Esterase in Combination with Glycosyl Hydrolases

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Abstract

A feruloyl esterase (FAE-C6) gene of 957 bp was isolated from rumen microbial metagenome, subcloned into pET32b vector, and expressed in *Escherichia coli*. The enzyme purified in active form, consisted of 319 amino acid residues, with a molecular weight of 43.7 based on SDS-PAGE. Homology modeling showed that the FAE contained the catalytic triad composed of Ser_{154}-$Asp_{263}$$His_{295}$ and a classical Gly-X-Ser_{154}-X-Gly nucleophile motif commonly found in esterases. The FAE-C6 was characterized using corn fiber as substrate. Its combining action with glycoside hydrolases (C, X, A) individually and in various combinations was studied with focus on the difference in the effects on FA and sugar release. Glycoside hydrolases with endo-xylanase included in the enzyme mixture showed significant impact on increasing the FA yield. For the release of sugar, FAE enhanced the yield in all hydrolase combinations moderately and endo-xylanase was not the key factor in the enzyme formulation.

Keywords

Feruloyl Esterase, Ferulic Acid Esterase, Ferulic Acid, Metagenomics, Corn Fiber

1. Introduction

Corn fiber (CF) is a mixture of coarse fiber from kernel pericarp or hull and fine fiber from endosperm m cellular materials in the corn kernel. The major components consist of hemicellulose (50%), cellulose (20%), starch (11% - 23%), protein (15%), lignin (15%) and ash, and 2% to 3% oil [1]. The structural complexity of CF is due to the large hemicellulose fraction, consists of heteroxylan, which in its native state, has 70% of the β-(1,4)-xylopyranosyl (Xyl*p*) backbone

carrying arabinofuranosyl, acetyl, glucuronyl side groups, and oligosaccharide chains containing galactose, xylose, and arabinose [2] [3]. Arabinofuranosyl side units are often ester-linked with ferulic acid moieties that can form crosslinks. In 2017 [4], 6.7×10^5 tons of corn was wet-milled, producing about 6.4×10^4 tons of corn fiber yearly. Corn fiber from wet-milling process is almost completely insoluble and highly recalcitrant with 50% of the starting material surviving pretreatment, enzymatic hydrolysis and fermentation (as used in corn ethanol production). Recent studies revealed that resistant complex xylan oligomers (remained non-degraded) contain ferulic acid, diferulates, acetic acid, galactose, arabinose, and uronic acid groups [5]. A common structural feature represents a sidechain of *α*-L-galactopyranosyl-(1,2)-*β*-D-xylopyranosyl-(1,2)-5-*O*-*trans*-feruloyl-L-arabinofuranosyl group attached to the *O*-3 position of the *β*-1,4 linked xylosyl residue [6] [7]. The recalcitrance of CF can be ascribed to feruloylation of the side units and/or backbone of the xylan structure.

A variety of bacteria and fungi are known to produce feruloyl esterases (FAE, EC 3.1.1.73) that catalyze the cleavage of feruloyl-arabinose (Araf-FA) ester bonds linking ferulic acids to arabinofuranosyl side groups of the xylan main chain. It has been proposed that enzymes, such as FAE, can be used to augment the hydrolytic efficiency of cellulases and hemicellulases in biomass conversion, thus requiring less severe pretreatment conditions [8] [9]. Direct cloning of metagenomes provides high efficiency exploration of the sequence space of unculturable microbial communities for novel gene discovery and biocatalyst development. Most studies on the enzymology of feruloyl esterases focus on the reaction of hydrolyzing FA from the substrate. In a previous study, combinatorial enzyme approach has been applied for production and screening of libraries of feruloyl oligosaccharides [10]. The objective of this paper is to report the effect of cellulolytic and xylanolytic enzymes individually or in combination acting synergistically with FAE for the hydrolysis of CF with focus on sugar release.

2. Experimental

Cloning of Feruloyl Esterase

Metagenomic DNA was isolated from the microflora of a cow's rumen, and was used to construct a *λ*ZAP library. Library screening for FAE enzyme activity identified the FAE-C6 gene. It was subcloned in pET vector, transformed in BL21, and the protein was purified to homogeneity, using previously reported procedures [11] [12].

Bioinformatics

Sequence analysis was performed using Vector NTI (Informax, Bethesda, MD, USA) and Geneious (Biomatters Ltd., Auckland, New Zealand). The gene sequence was submitted to GenBank with the accession number: MK607950.

Electrophoresis

The purified enzyme was run on a Bis-Tris NuPAGE gradient gel (4% - 12%) using 50 mM 3-morpholinopropane-1-sulfonic acid (MOPS) buffer solution at constant 100 V for 2 h. The developed gel was stained with SimplyBlue Safe stain

(Invitrogen, Carlsbad, CA, USA). The protein and marker bands were analyzed by image analysis software (Alpha Inotech, AlphaImager, San Jose, CA, USA). For pI determination, the enzyme protein was run on an electrofocusing gel (pH 3 to 10, Invitrogen, Carlsbad, CA, USA). Serva IEF markers 3 - 10 mix (Biophoretics, Reno, NV, USA, Heidelberg, Germany) was used as standards.

Enzyme Activity Measurement

A typical enzyme reaction mixture contained 100 mg CF and various nmole concentrations of FAE-C6 in 1 ml volume of 50 mM K_2HPO_4, pH 7.0 buffer, and was incubated for 2 h in a 40˚C shaker bath. For all reactions, the CF was milled 3 × 10 s (Micro-Mill, Technilab Instruments, Vineland, NJ, USA), washed 4X with water, and oven-dried at 50˚C. Activity was expressed as μg FA released from 100 mg substrate per hour measured by high performance liquid chromatography analysis. The HPLC system (Gilson 307 HPLC, Middleton, WI, USA) consisted of a Gilson 307 pump equipped with a Brownlee analytical C18 column (260 × 4.5 mm), using a mobile phase of water/formic acid/acetonitrile (7/1/2 v/v) at a flow rate of 0.2 mL/min at ambient temperature. Ferulic acid peaks were detected at 315 nm.

Enzyme pH and Temperature Optima and Stability

For pH optimum, the reaction mixture of 100 mg CF and 0.3 nmole FAE-C6 in 0.5 mL universal buffer of varying pH was incubated for 2 h in a 37˚C shaker bath. For determining pH stability, the C6 was incubated at various pH at 37˚C for 4 hr, reconstituted to pH 6.0, and the residual activity was determined by adding the CF substrate. For temperature optimum, the enzyme reaction mixture was incubated in 100 mM K_2HPO_4 buffer, at pH 7.0 at temperatures from 20˚C to 70˚C for 2 h.

Alkali Hydrolysis of Corn Fiber

A sample of 200 mg CF in 6 mL 1 N NaOH was incubated for 16 h in a 37˚C shaker bath [13]. Following centrifugation, the supernatant was recovered and adjusted to a pH ≤ 2 with HCl. Ferulic acid products were then extracted with ethyl acetate (3 × 3 mL), dried under N_2, and dissolved in CH_3OH:H_2O (1:1 v/v) for HPLC analysis.

Activity with Added Accessory Enzymes

To 100 mg of CF, 0.5 nmole FAE-C6 was added, supplemented with endo-xylanase (XYN, endo-1,4-*β*-xylanase, GH11), *α*-L-arabinofuranosidase (ABF), and cellulase (CEL, endo-1,4-*β*-D-glucanase) all from *Aspergillus niger* (Megazyme, Bray, Ireland) in various nmole concentrations. The three enzymes were added individually and in various combinations to the C6 reaction. The mixture was incubated for 2 h in a 40˚C shaker bath. These enzymes exhibit optimum pH of 4.5 and temperature at 40˚C.

3. Results and Discussion

Isolation, Cloning, and Bioinformatics of the FAE Gene

The genomic insert isolated from metagenomic library contained an esterase

sequence domain (FAE-C6) of 957 bp (319 residues). The gene was subcloned into pET 32b vector as a fusion protein containing a thioredoxin (Trx) tag, a Met start codon and a 6xHis tag. A BLASTP search reveals the amino acid sequence related to the primary structures of *Prevotella sp.* and *Bacteroidaceae bacterium* α/β fold hydrolases isolated from ruminant gastrointestinal micobiome MBP57-15121 and MBQ9883982 with identity percentages of 96.6% and 75.8%, respectively (Figure 1). Homology modeling showed an α/β hydrolase fold commonly observed in various esterases (Figure 2). The FAE-C6 is closely related to and shows high conservation of the C-terminal region of the *Bacteroides eggershii* bifunctional protein BeGH43/FAE (PDB 6MLY) [14]. Based on sequence and structural comparison, the FAE-C6 contains the catalytic triad composed of $Ser_{154}Asp_{263}His_{295}$, and a classical Gly-X- Ser_{154}X-Gly nucleophile motif commonly found in esterases [15] [16].

FAE-C6 Activity on Corn Fiber

The enzyme FAE-C6 was characterized using CF as the substrate. Enzyme unit is defined as the amount of enzyme that catalyzes the formation of 1 μg of ferulic acid product per 100 mg of substrate per hour reaction. The FAE shows a pH optimum at pH ≥ 7, and a temperature optimum of 50˚C (Figure 3). Under the described reaction conditions (100 mg CF, in 1 mL pH 7.0 buffer, incubated

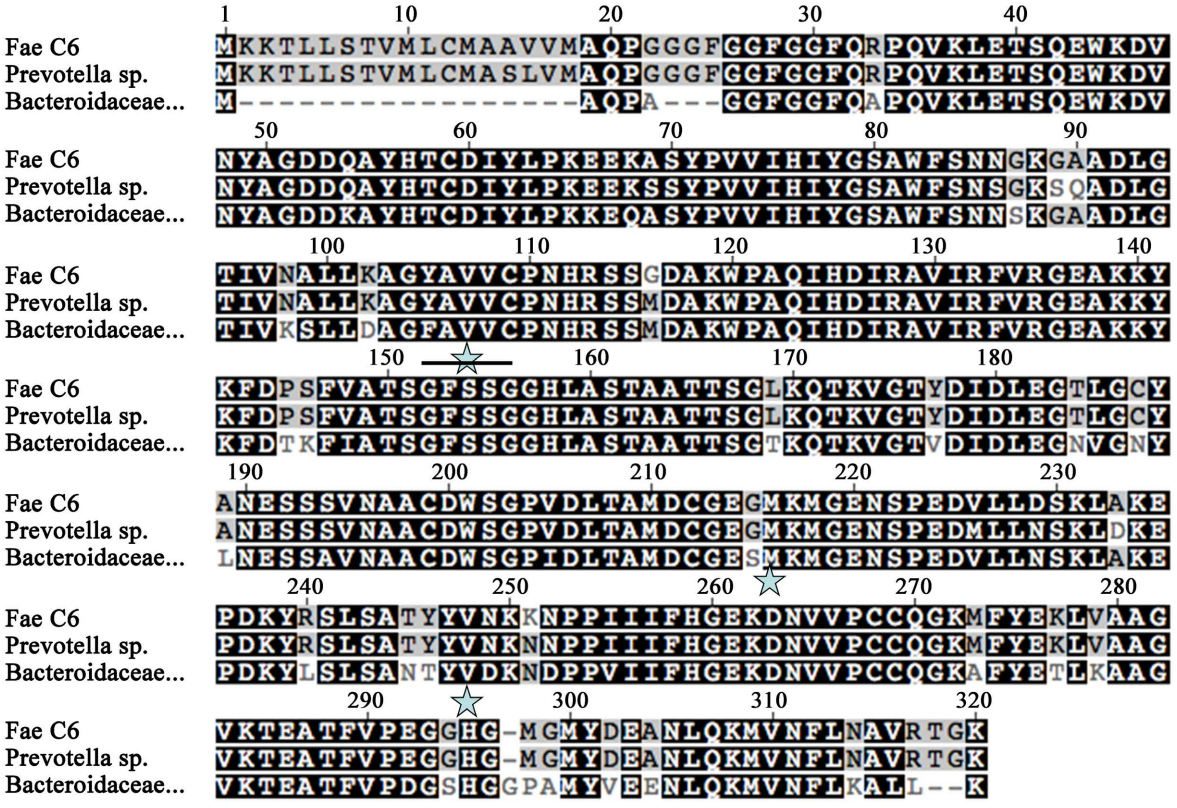

Figure 1. Multiple sequence alignment of FAE-C6. *Prevotella sp.* and *Bacteroidaceae bacterium* α/β fold hydrolases isolated from ruminant gastrointestinal micobiome MBP5715121 and MBQ9883982 show identity percentages of 96.6% and 75.8%, respectively. The Gly-X-Ser_{154}-X-Gly motif sequence is highlighted with a striated bar above the residues. The catalytic triad $Ser_{154}Asp_{263}His_{295}$ is indicated with stars above the residues.

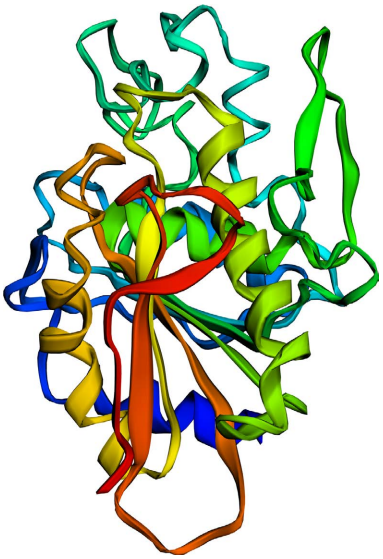

Figure 2. The 3D model of FAE-C6. Homology modeling based on comparison with the high conserved C-terminal region of the *Bacteroides eggershii* bifunctional protein Be-GH43/FAE (PDB 6MLY) [14] as template. All images and data generated by Phyre2 [22].

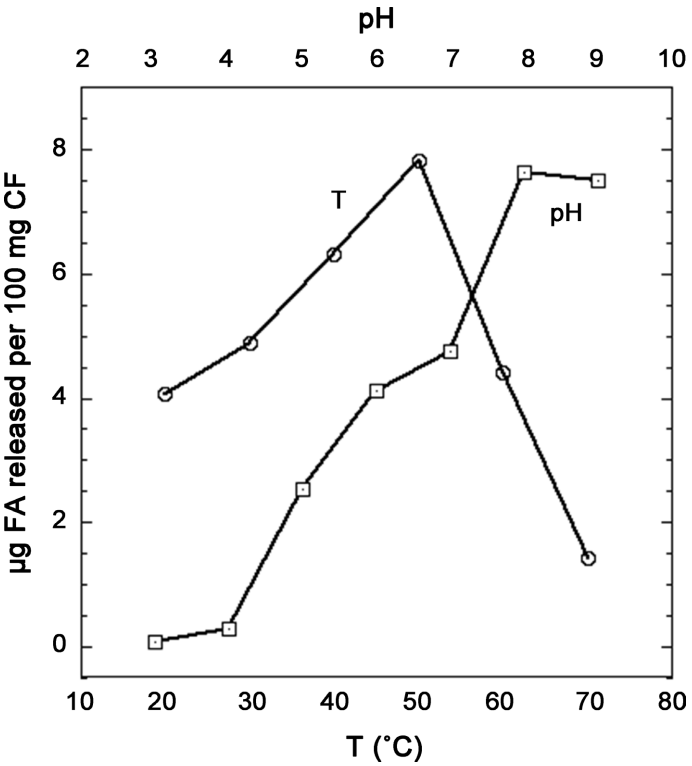

Figure 3. Effects of pH and temperature on FAE-catalyzed release of ferulic acid from CF. Reaction conditions: 100 mg CF, 1 ml universal buffer of varying pH, 1 nmole FAE-C6, varying temperature, incubated for 2 hr.

for 2 h at 50˚C), 1 nmole FAE catalyzed the release of 7.8 µg FA from 100 mg CF/h equivalent to 1.3% of total FA content in the 100 mg CF (as determined by alkaline hydrolysis) used in the reaction mixture.

Glycoside Hydrolases Activities on Sugar Release from CF

The following three glycoside hydrolases were tested individually or in various combinations in catalyzing sugar release from CF: endo-1,4-β-D-glucanase (endo-cellulase, EC 3.2.1.4, GH12), endo-1,4-β-xylanase (EC 3.2.1.8, GH11), α-L-arabinofuranosidase (EC 3.2.1.55, GH51), labeled as C, X and A in the figures, respectively. All enzymes are from *Aspergillus niger*, and exhibit pH and temperature optima of 4.0 - 4.5 and 60˚C, respectively (specifications from Megazyme, Ireland). The enzymes (2U each) were added individually and in combinations to 100 mg CF in pH 5.0 buffer, incubated for 2 hr at 37˚C. Figure 4 shows that combinations of the enzymes substantially increased the sugar yield compared to single enzymes. A combination of CXA increased the sugar yield by 93% over that by cellulase alone. Likewise, combination of XA increased the yield by 167% and 245%, respectively, over that by X or A alone.

Effect of FAE added to Glycoside Hydrolases

FAE alone had little effect on the sugar yield (Figure 5). It is evident that the sugar yield was largely attributed to the actions of glycoside hydrolases. Adding FAE-C6 to CXA increased the yield by 6.5% and 17.6%, respectively at pH 5 and 6. Apparently, it was the non-covalent sugar molecules in the fiber complex that was released by the action of FAE. The sugar released in this case may not represent entirely hydrolytic products. The sugar yield decreased with increasing pH, with a 40% reduction when the reaction was performed at pH 7 instead of pH 5 (Figure 5). This result suggests further evidence that the hydrolase enzymes (CXA) provided key catalytic actions in the release of sugar from the CF substrates.

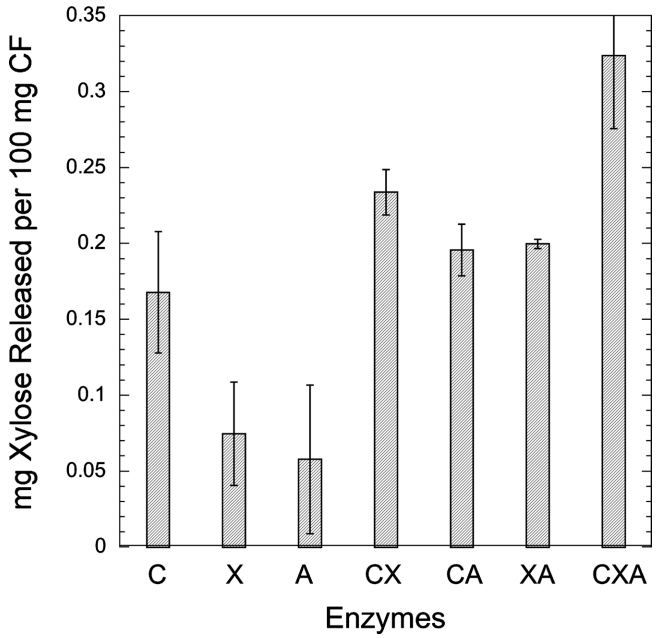

Figure 4. Effects of cellulase, xylanase, and arabinofurnosidase acting individually (C, X, A) or in combinations (CX, CA, XA, CXA) in the release of sugar (as xylose equivalent) from CF. Reaction conditions: 100 mg CF, 2U each enzyme, pH 5.0, 37˚C for 2 hr.

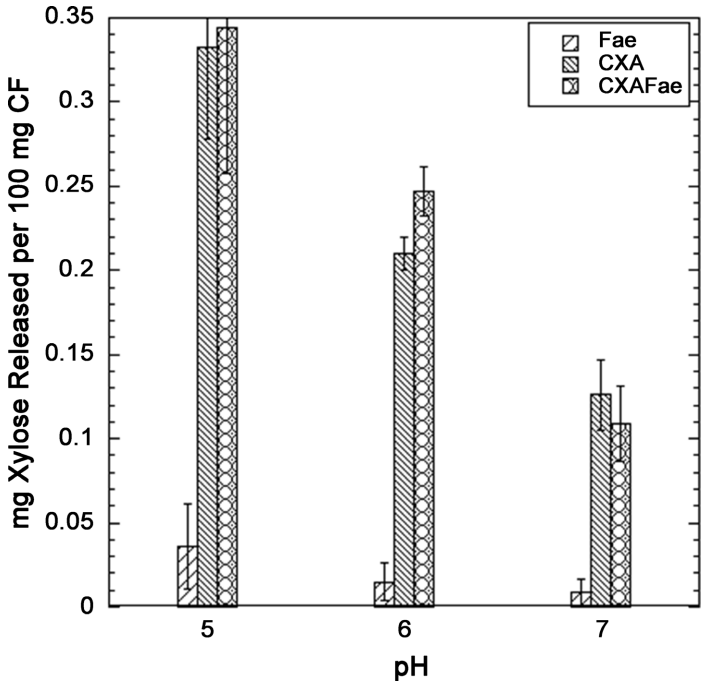

Figure 5. Effects of adding FAE to C+X+A enzymes combination in various pH conidi-tions: 100 mg CF, 0.3 nmole FAE-C6, 2U each of C+X+A combined, buffer at pH 5.0, 6.0 or 7.0, 37˚C, 2 hr.

Effect of FAE-C6 on FA and Sugar Release

Adding FAE-C6 to the glycoside hydrolases (C, X, A) individually and in combinations affects the release of FA and Sugar in different patterns. The release of FA was enhanced in CXF, XAF and CXAF, showed similar increases of, 6.9%, 8.7% and 8.3%, respectively compared to FAE alone (**Figure 6(a)**). All these three enzyme mixtures contained endo-xylanase. It is noted that the treatment with CAF (which contain no endo-xylanese) showed a 21.7% decrease in the FA yield. The synergistic action of endo-xylanase has been extensively investigated in our previous studies. Adding increasing concentrations of endo-xylanase (1.0 nmole to 20 nmole) at a constant concentration (0.5 nmole) of FAE resulted in an average increase of 1.11 ± 0.14 μg of FA for each doubling of the concentration of the xylanase [10]. The release of FA from CF and corn bran by FAE in synergism has been reported to be in a similar range, 1.21 and 1.19 fold, respectively [17]. The enhancement by endo-xylanase is due to the formation of shorter chain xylooligosacchatides, which are more susceptible (hydrolyzed at faster rates by FAE) than long chain substrates [18] [19]. Furthermore, FAE are known to act synergistically with endo-xylanases in the release if FA, because short-chain feruloylated xylo-oligosaccharides are better substrates with a higher rate of Araf-FA cleavage [17]. Other xylanolytic accessory enzymes that act on various side chains may also enhance the hydrolysis of xylan main chain and in turn the ferulic acid linkage [20] [21]. However, our previous studies showed that arabinofuranosidase and acetylxylan esterase showed no synergistic effect on the CF substrate under similar experimental conditions [10].

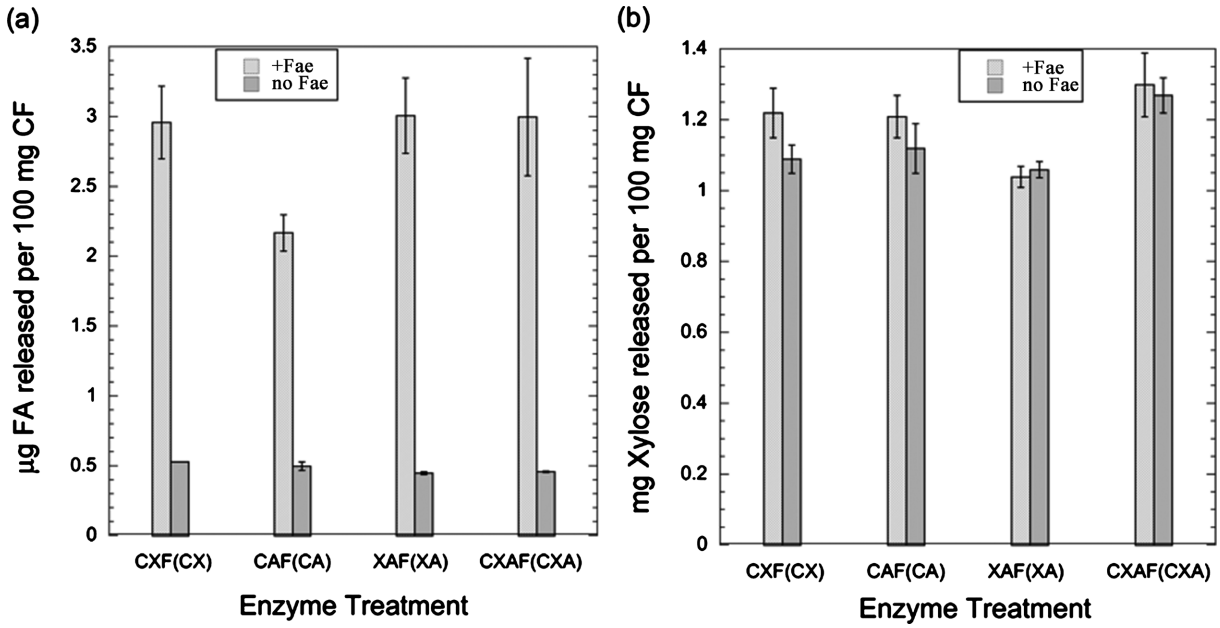

Figure 6. Effect of adding FAE to various combinations of glycoside hyudrolases (CX, CA, XA, CXA) on the release of (a) FA, (b) sugar as xylose equivalent from CF substrate. Reaction conditions: 100 mg CF, 0.3 nmole FAE, 2U each of the hydrolases, pH 5.0, 37˚C, 2 hr.

The effect of adding FAE-C6 to the glycoside hydrolases mixtures showed a different product patterns on sugar release (**Figure 6(b)**). The increase in sugar release was very moderate in all cases (CXF, CAF, XAF, CXAF), showing 8.0% and 11.9% increase in the CXF and CXAF enzyme mixtures, respectively. The results indicate that the influence of endo-xylanase was no different from the other enzymes, (2) addition of FAE enhanced the sugar yield in all enzyme combinations, but was not a key factor for sugar release in the reactions.

4. Conclusion

A feruloyl esterase (FAE) gene was isolated from rumen microbial metagenome, expressed in *E. coli*, and the enzyme protein (fae-C6) was purified in active form. It consists of an esterase domain with an α/β hydrolase fold, composed of a catalytic triad $Ser_{154}Asp_{263}His_{295}$. The FAE-C6 was characterized using corn fiber as substrate. Its combining action with glycoside hydrolases (C, X, A) individually and in various combinations was studied with focus on the difference in the hydrolytic effects on FA and sugar release. Glycoside hydrolases with endo-xylanase included in the enzyme mixture show significant impact on increasing the FA yield. For the release of sugar, FAE enhanced the xylose yield in all hydrolases moderately with 11.9% the highest increase in CXAF. Endo-xylanase was not the key factor in the enzyme formulation.

Acknowledgements

Reference to a company and/or product is only for purposes of information and does not imply approval of recommendation of the product to the exclusion of

others that may also be suitable. All programs and services of the U.S. Department of Agriculture are offered on a nondiscriminatory basis without regard to race, color, national origin, religion, sex, age, marital status, or handicap. The authors declare that there is no conflict of interest regarding the publication of this paper.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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