Construction of Engineered Bifunctional Enzymes and Their Overproduction in *Aspergillus niger* for Improved Enzymatic Tools To Degrade Agricultural By-Products

Anthony Levasseur,^{1*} David Navarro,¹ Peter J. Punt,² Jean-Pierre Belaïch,^{3,4}
Marcel Asther,¹ and Eric Record¹

UMR 1163 INRA de Biotechnologie des Champignons Filamenteux, IFR86-BAIM, Universités de Provence et de la Méditerranée, ESIL, 163 Ave. de Luminy, Case Postale 925, 13288 Marseille Cedex 09, France¹; Department of Microbiology, Quality of Life, Zeist, The Netherlands²; Bioénergétique et Ingénierie des Protéines, Centre National de la Recherche Scientifique, IBSM, 13402 Marseille, France³; and Université de Provence, 3 Place Victor Hugo, Marseille, France⁴

Received 18 May 2005/Accepted 2 August 2005

Two chimeric enzymes, FLX and FLXLC, were designed and successfully overproduced in *Aspergillus niger*. FLX construct is composed of the sequences encoding the feruloyl esterase A (FAEA) fused to the endoxylanase B (XYNB) of *A. niger*. A C-terminal carbohydrate-binding module (CBM family 1) was grafted to FLX, generating the second hybrid enzyme, FLXLC. Between each partner, a hyperglycosylated linker was included to stabilize the constructs. Hybrid proteins were purified to homogeneity, and molecular masses were estimated to be 72 and 97 kDa for FLX and FLXLC, respectively. Integrity of hybrid enzymes was checked by immunodetection that showed a single form by using antibodies raised against FAEA and polyhistidine tag. Physicochemical properties of each catalytic module of the bifunctional enzymes corresponded to those of the free enzymes. In addition, we verified that FLXLC exhibited an affinity for microcrystalline cellulose (Avicel) with binding parameters corresponding to a K_d of 9.9×10^{-8} M for the dissociation constant and $0.98 \mu\text{mol/g}$ Avicel for the binding capacity. Both bifunctional enzymes were investigated for their capacity to release ferulic acid from natural substrates: corn and wheat brans. Compared to free enzymes FAEA and XYNB, a higher synergistic effect was obtained by using FLX and FLXLC for both substrates. Moreover, the release of ferulic acid from corn bran was increased by using FLXLC rather than FLX. This result confirms a positive role of the CBM. In conclusion, these results demonstrated that the fusion of naturally free cell wall hydrolases and an *A. niger*-derived CBM onto bifunctional enzymes enables the increase of the synergistic effect on the degradation of complex substrates.

Agricultural residues represent large renewable resources for lignocellulose bioconversion. Daily, the food industry generates significant amounts of by-products that are considered as polluting wastes to be eliminated. A great deal of research has been conducted on the utilization of these by-products in the biotechnology sector. Among these components, ferulic acid (4-hydroxy-3-methoxy-cinnamic acid), one of the most abundant hydroxycinnamic acid in the plant world, is a very attractive phenolic compound. For instance, ferulic acid can be used as an antioxidant (23) or can be transformed by microbial conversion into “natural” vanillin, an expensive flavor for food, cosmetic, and pharmaceutical industries (4, 26). Among agricultural by-products, corn and wheat brans are potential substrates according to their high amounts of ferulic acid in the cell wall, i.e., 3 and 1% (wt/wt), respectively (42). In plant physiology, ferulic acid is a key structural component of plant cell walls, with important physical and chemical properties. Indeed, it may act as a cross-linking agent between lignin and carbohydrates or between carbohydrates themselves (16), in-

fluencing cell wall integrity, and therefore reducing biodegradability by microbial enzymes (6).

Microorganisms evolved enzymes, such as feruloyl esterases (EC 3.1.1.73), that are able to hydrolyze the ester bonds linking ferulic acid to plant cell wall polysaccharides. These enzymes allow an easier access to the polysaccharide backbone for other lignocellulolytic enzymes (for a review, see reference 12). Previous studies demonstrate that feruloyl esterases act in synergy with main-chain-degrading enzymes, such as β -(1,4)-endoxylanases, to increase the release of ferulic acid from plant cell wall (2, 15, 49). Filamentous fungi, such as *Aspergillus niger*, are well-known producers of plant cell-wall-degrading enzymes. Two different genes from *A. niger*, encoding feruloyl esterases, were already cloned (10, 11), and their corresponding recombinant proteins had been overproduced in *Pichia pastoris* and *A. niger* (25, 30, 40). Several fungal feruloyl esterases were purified and characterized (18, 46), but the corresponding genes have not been investigated. Previous work reported the isolation of the first fungal (*Penicillium funiculosum*) cinnamoyl esterase (type B), with a C-terminal domain closely similar to the family 1 carbohydrate-binding module (CBM) (27). Many glycosyl hydrolases from anaerobic and aerobic microorganisms have a modular structure. In addition to a catalytic domain, one or more noncatalytic CBMs can be located either at the N- or the C-terminal regions or at both. CBMs have been classified into families with similar amino-

* Corresponding author. Mailing address: UMR 1163 INRA/Université de Provence de Biotechnologie des Champignons Filamenteux, IFR-IBAIM, Universités de Provence et de la Méditerranée, ESIL, 163 Avenue de Luminy, Case Postale 925, 13288 Marseille Cedex 09, France. Phone: 33 4 91 82 86 07. Fax: 33 4 91 82 86 01. E-mail: anthony.levasseur@esil.univ-mrs.fr.

A/ FLX

B/ FLXLC

FIG. 1. Expression cassettes used in the present study. (A) In order to design the *FLX* insert, the *A. niger* sequences coding for FAEA, a linker region from CBHB and XYNB, were fused together. (B) In the second construct, the *FLX* template was fused to the *cbhb* sequence encoding the linker sequence and CBM generating the *FLXLC* insert. Expression cassettes are under the control of the *gpdA* promoter and *trpC* terminator. Both constructs contained a six histidine-encoding sequence at the 3' end. Superscript "(1)," linker-encoding sequence GSTYSSGSSSGSGSSSSS STTTKATSTTLKTTSTTSSGSSSTAA.

acid sequences and three-dimensional structures (<http://afmb.cnrs-mrs.fr/CAZY/index.html>). CBMs play a major role on insoluble substrate degradation (44). For instance, they are responsible for maintaining the catalytic domain close to the substrate, increasing the efficiency of contact. Moreover, in some cases, CBMs can also alter the cellulose microfibril structure by weakening the hydrogen bonds of the gathered cellulose chains (13, 14, 33).

In a recent study based on engineered bacterial cellulosome, physical proximity of two catalytic components demonstrated an enhanced synergy on recalcitrant substrates (17). On this basis, we designed a chimeric protein associating a fungal feruloyl esterase and a clostridial dockerin domain to be grafted with a second enzyme onto a bacterial CBM-containing scaffolding protein (31). However, the production yield of the recombinant protein was not large enough for test applications on an industrial scale. In the present study, we fused two fungal enzymes and a CBM from *A. niger* to obtain two bifunctional enzymes (FLX and FLXLC). Both hybrid enzymes were successfully produced in *A. niger* and fully characterized considering biochemical and kinetics aspects and finally used to release ferulic acid from natural substrates: corn and wheat brans. The efficiency of ferulic acid release was compared by using free or bifunctional enzymes in order to study enzymatic synergy generated by the physical proximity or the substrate targeting of two fungal enzymes.

MATERIALS AND METHODS

Strains and culture media. *Escherichia coli* JM109 (Promega) was used for construction and propagation of vectors, and *A. niger* strain D15#26 (lacking the *pyrG* gene) (22) was employed for production of the recombinant proteins. After cotransformation with vectors containing, respectively, the *pyrG* gene (PyrG) and the expression cassette FLX (*faeA-xynB*) or FLXLC (*faeA-xynB-CBM*) (Fig. 1), *A. niger* was grown for selection on solid minimal medium (without uridine) containing 70 mM NaNO₃, 7 mM KCl, 11 mM KH₂HPO₄, 2 mM MgSO₄, glucose 1% (wt/vol), and trace elements (1,000× stock; 76 mM ZnSO₄, 25 mM MnCl₂, 18 mM FeSO₄, 7.1 mM CoCl₂, 6.4 mM CuSO₄, 6.2 mM Na₂MoO₄, 174 mM EDTA). In order to screen the transformants for the production of each recombinant protein, 100 ml of culture medium containing 70 mM NaNO₃, 7 mM KCl, 200 mM Na₂HPO₄, 2 mM MgSO₄, glucose 6% (wt/vol), and trace elements were inoculated with 10⁶ spores ml⁻¹ in a 500-ml baffled flask. *A. niger* BRFM131 (Banque de Ressources Fongiques de Marseille) was used for genomic

DNA extraction, and the resulting DNA was used as the template for PCR amplification strategy of the sequence coding for the linker-CBM region of CBHB (AF156269).

Expression vectors construction and fungal transformation. The fusion of the sequences coding for feruloyl esterase A (FAEA; Y09330), XYNB (AY126481), and CBM (AF156269) from *A. niger* were performed by the overlap extension PCR method (24). The *A. niger* FAEA-encoding region was amplified from cDNA (40) by using the forward primer 5'-GGACTCATGAAGCAATTCTCTGCAAAATAC-3' (BspHI) and the reverse primer 5'-ACTGGAGTAAGTCGAGCCCAAGTACAAGCTCCGCT-3'. Genomic DNA coding for the linker region of CBHB (AF156269), was amplified from *A. niger* BRFM131 by using the forward primer 5'-AGCGGAGCTTGACTTGGGGCTCGACTTACTCCAGT-3' and the reverse primer 5'-GGTCGAGCTCGGGGTCGACGCCGCGATGTCGAAC-3'. Finally, the *xynB* gene was amplified from cDNA (29) with a forward primer 5'-AGTTCGACATCGGCGCGCTCGACCCGAGCTCGACC-3' and a reverse primer 5'-GGCTAAGCTTTTGTGGTGGTGGTGGTGAACAGTGATGGACGAAG-3' (HindIII) (the His tag is in italics in all sequences). Resulting overlapping fragments were mixed, and a fused sequence was synthesized in a one-step reaction by using both external primers. The construct was cloned in the pGEM-T vector (Promega), and the cloned PCR product was checked by sequencing. The fused fragment was cloned into NcoI-HindIII-linearized and dephosphorylated pAN52.3 to obtain pFLX plasmid (Fig. 1A). FLXLC plasmid was constructed by using pFLX as the template for amplification of the fragment coding for the recombinant FAEA-linker-XYNB sequence with the forward primer 5'-GGACTCATGAAGCAATTCTCTGCAAAATAC-3' (BspHI) and the reverse primer 5'-ACTGGAGTAAGTCGAGCCCTGAACAGTGATGGACGA-3'. Genomic DNA from *A. niger* BRFM131 was used as the template for PCR amplification of the linker-CBM sequence with two specific primers designed from the available *cbhb* sequence (AF156269): a forward primer, 5'-TCGTCCATCACTGTTTCAGGGCTCGACTTACTCCAGT-3', and a reverse primer, 5'-ATGCAAGCTTTTGTGGTGGTGGTGGTGGTGGTCAAACACTGCGAGTAGTAC-3' (HindIII). The fused fragment was synthesized by the overlap extension PCR method by using both external primers, controlled by sequencing, and cloned into pAN52.3 to obtain the pFLXLC vector (Fig. 1B). In both expression vectors, the *A. nidulans* glyceraldehyde-3-phosphate dehydrogenase gene (*gpdA*) promoter, the 5' untranslated region of the *gpdA* mRNA, and the *A. nidulans* *trpC* terminator were used to drive the expression of recombinant sequences. In addition, the signal peptide (21 amino acids) of the FAEA was used to target the secretion of both recombinant proteins.

Both fungal cotransformations were carried out as described by Punt and van den Hondel (38) by using the pFLX or the pFLXLC expression vectors, respectively, and pAB4-1 (47) containing the *pyrG* selection marker, in a 10:1 ratio. In addition, *A. niger* D15#26 was transformed with the *pyrG* gene without the expression vector for control experiment. Cotransformants were selected for uridine prototrophy on selective minimal medium plates (without uridine) and incubated for 8 days at 30°C. In order to screen transformants, 40 individual clones for each construct were cultivated and checked daily.

Screening of feruloyl esterase and xylanase activities. Cultures were monitored for 14 days at 30°C in a shaker incubator (130 rpm), and the pH was adjusted to 5.5 daily with 1 M citric acid. Each culture condition was performed in duplicate. From liquid culture medium, aliquots (1 ml) were collected daily, and mycelia were removed by filtration. Esterase activity was assayed as previously described by using methyl ferulate (MFA) as the substrate (39), and xylanase activity was calculated by measuring the amount of xylose released from 1% (wt/vol) birchwood xylan based on the method of Bailey et al. (1). The enzymes were incubated with a xylan solution (1% [wt/vol] xylan from birchwood, 50 mM sodium citrate buffer [pH 5.5]) at 45°C for 5 min. The released reducing sugars were determined by the DNS (3,5-dinitrosalicylic acid) method with xylose as the standard (34). All assays were performed by using blanks to correct any backgrounds in enzyme and substrate samples.

Activities were expressed in nanokatals (nkat), with 1 nkat being defined as the amount of enzyme that catalyzes the release of 1 nmol of ferulic acids or of 1 nmol of reducing sugars per s under established conditions. Each experiment was done in duplicate, and measurements were made in triplicate. The standard deviation was recorded to <2% for the mean for esterase activity and <5% for xylanase activity.

Purification of recombinant proteins. The best isolate for each construct was inoculated in the same conditions as the screening procedure. Culture was harvested after 8 days of growth, filtered (0.7- μ m pore size), and concentrated by ultrafiltration through a polyethersulfone membrane (molecular mass cutoff of 30 kDa) (Millipore). Concentrated fractions were dialyzed against a 30 mM Tris-HCl (pH 7.0), binding buffer, and the purification of His-tagged proteins was performed on a Chelating Sepharose Fast Flow column (13 by 15 cm; Amersham Biosciences) (37). Concerning free proteins, recombinant xylanase B (XYNB) containing a His tag sequence was also purified on a Chelating Sepharose Fast Flow column as already described (29). Finally, the recombinant FAEA was purified in a one-step procedure using a phenyl-Sepharose column as already described (40).

Characterization of recombinant proteins. (i) Protein analysis, glycosylation, and N-terminal amino acid sequence determination. Protein concentration was determined with bovine serum albumin as the standard. Protein production and purification were checked by using Coomassie blue-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 11% polyacrylamide slab gels. The level of glycosylation generated by the linker addition was estimated by comparing the observed molecular masses (on SDS-PAGE) for bifunctional enzymes and the observed molecular masses (on SDS-PAGE) for free enzymes and theoretical molecular mass for linker $\pm$ CBM. The N-terminal sequences were determined according to Edman degradation from an electroblotted FLX and FLXLC samples (100 μ g) onto a polyvinylidene difluoride membrane (Millipore). Analyses were carried out on an Applied Biosystems 470A.

(ii) Western blot analysis. Total and purified proteins were electrophoresed in 11% SDS-polyacrylamide gel and electroblotted onto BA85 nitrocellulose membranes (Schleicher & Schuell SARL) at room temperature for 45 min. Membranes were incubated in blocking solution (50 mM Tris, 150 mM NaCl, 2% [vol/vol] milk [pH 7.5]) overnight at 4°C. The membranes were then washed with TBS-0.2% Tween and treated with blocking solution containing anti-FAEA serum at a dilution of 1/8,000 or containing anti-polyhistidine-peroxidase serum (Sigma). For anti-FAEA antibodies, membranes were subsequently incubated with goat anti-rabbit immunoglobulin G conjugated with horseradish peroxidase (Promega). Signals were detected with chemiluminescence Western blotting kit (Roche) according to the manufacturer's procedure.

(iii) Temperature and pH stability of recombinant proteins. Thermostability of the purified recombinant proteins was tested in the range of 30 to 70°C. Aliquots were preincubated at the designated temperature and after cooling at 0°C, esterase and xylanase activities were then assayed as previously indicated in standard conditions. Samples were analyzed by SDS-PAGE after incubation in order to verify integrity of the bifunctional proteins. Effect of the pH on protein stability was studied by incubating the purified recombinant proteins in citrate-phosphate buffer (pH 2.5 to 7.0) and sodium phosphate (pH 7.0 to 8.0). All incubations were performed for 90 min, and then aliquots were transferred in standard reaction mixture to determine the amount of remaining activity. The activity determined prior to the preincubations was taken as 100%.

(iv) Effect of temperature and pH on esterase and xylanase activities. To determine optimal temperature under the conditions used, aliquots of purified recombinant proteins were incubated at various temperatures (30 to 70°C), and esterase and xylanase activities were assayed. Optimal pH was determined by using citrate-phosphate buffer (pH 2.5 to 7.0) and sodium phosphate buffer (pH 7.0 to 8.0) using standard conditions.

(v) Determination of cellulose-binding capacity and dissociation constants. Samples of purified FLX and FLXLC were added to 2-ml microcentrifuge tubes containing cellulose in 25 mM potassium phosphate buffer (pH 7) in a final volume of 1 ml. The capacity of FLX (control) and FLXLC to bind to the Avicel PH101 cellulose (Fluka) was determined by using various amounts of recombinant proteins (between 30 and 170 μ g) and a constant amount of cellulose (2 mg). Both recombinant proteins were incubated with cellulose for 1 h at 4°C with gentle agitation. After centrifugation (4,000 $\times$ g for 10 min), the amount of residual proteins in the supernatant fluid (free enzyme) was determined. The amount of enzyme bound to cellulose was calculated by subtracting the amount of free FLX or free FLXLC from the total amount added. The data were analyzed by drawing double-reciprocal plots of 1/bound enzyme versus 1/free enzyme. The dissociation constant K_d is defined as $1/B = (K_d/B_{max} \times 1/F) + 1/B_{max}$, where B (μ mol of protein per g of Avicel) is the bound enzyme concentration, and F is the free enzyme concentration (21, 36).

Application tests. (i) Enzymatic hydrolysis. Wheat bran (WB) and corn bran (CB) were destarched and provided by ARD (Agro-Industrie Recherche et Développement, Pomacle, France). These substrates were subjected to heat treatment at 130°C for 10 min. Enzymatic hydrolyses were performed in 0.1 M 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer containing 0.01% sodium azide at pH 6.0 in a thermostatically controlled shaking incubator at 40°C. WB or CB (200 mg) were incubated with purified FAEA, XYNB, FAEA+XYNB, FLX, or FLXLC, independently, in a final volume of 5 ml. The purified enzyme concentrations for free and bifunctional enzymes were: 11 nkat of esterase activity and 6,500 nkat of xylanase activity per 200 mg of dry bran for each assay. This ratio corresponds to the molar-to-molar condition found in the purified bifunctional enzyme. Each assay was done in duplicate, and the standard deviation was <5% from the mean of the value for WB and CB.

(ii) Preparation of the alkali-extractable hydroxycinnamic acid. Total alkali-extractable hydroxycinnamic acid content was determined by adding 20 mg of WB or CB in 2 N NaOH and incubated for 30 min at 35°C in the darkness. The pH was adjusted to 2 with 2N HCl. Phenolic acids were extracted three times with 3 ml of ether. The organic phase was transferred to a test tube and dried at 40°C. One milliliter of methanol-H₂O (50:50 [vol/vol]) was added to dry extract, and samples were injected on an high-pressure liquid chromatography (HPLC) system as described below. The total alkali-extractable ferulic acid content was considered to be 100% for the enzymatic hydrolysis.

(iii) Ferulic acid determination. Enzymatic hydrolysates were diluted to one-half with methanol 100% and centrifuged at 12,000 $\times$ g for 5 min, and supernatants were filtered through a 0.2- μ m-pore-size nylon filter (Gelman Sciences, Acrodise 13; Ann Arbor, MI). Filtrates were analyzed by HPLC (25 μ l injected). HPLC analyses were performed at 280 nm and 30°C on a HP1100 model (Hewlett-Packard, Rockville, MD) equipped with a variable UV/VIS detector, a 100-position autosampler-autoinjector. Separations were achieved on a Merck RP-18 reversed-phase column (Chromolith 3.5 μ m, 4.6 by 100 mm; Merck). The flow rate was 1.4 ml/min. The mobile phase used was 1% acetic acid and 10% acetonitrile in water (solvent A) versus acetonitrile 100% (solvent B) for a total running time of 20 min, and the gradient changed as follows: solvent B was started at 0% for 2 min, increased to 50% in 10 min, and then increased to 100% in 3 min until the end of the running period. The data were processed by a HP 3365 ChemStation, and quantification was performed by external standard calibration.

RESULTS

Design and study of the bifunctional enzymes production. The *A. niger* sequences encoding the FAEA and the XYNB were genetically fused by adding, between both partners, a sequence from the cellobiohydrolase B gene (*cbhB*) coding for a hyperglycosylated linker (Fig. 1A). For the second construct FLXLC, the corresponding fusion *FLX* was fused to a partial sequence of *A. niger cbhB* gene coding for the linker-CBM (Fig. 1B). Both translational fusions were placed under the control of the strong and constitutive *gpdA* (glyceraldehyde-3-phosphate dehydrogenase) promoter and the *tpc* terminator with the endogenous *faeA* signal sequence to target the secretion. *A. niger* D15#26 protoplasts were cotransformed with a mixture of one expression vector pFLX or pFLXLC and the plasmid pAB4-1 containing the *pyrG* gene. Transformants were selected for their ability to grow on a minimum medium

FIG. 2. Time course of extracellular feruloyl esterase and xylanase production in *A. niger*. Feruloyl esterase (A) and xylanase (B) activities were measured for the best FLX (◆) and FLXLC (■) transformant. Activities were determined by using MFA and birchwood xylan as substrates for esterase and xylanase activities, respectively.

plate without uridine. Forty transformants for each construct were inoculated into a glucose-minimal medium repressing the endogenous *faeA* and *xynB* gene expression. For both constructs, esterase and xylanase activities were detected in the extracellular media of transformants after 2 days of growth (Fig. 2). Within the control strain transformed with pAB4-1, no esterase or xylanase activity was detected. Feruloyl esterase activity increased simultaneously with xylanase activity during the cultivation. Activities increased until day 10 and day 11 for the best FLXLC and FLX transformant, respectively, to reach a level that was more or less stable until day 14. Maximal esterase activity reached 13.0 nkat/ml for FLX and 9.8 nkat/ml for FLXLC transformant. Concerning xylanase activity, a max-

FIG. 3. SDS-PAGE of extracellular proteins produced by FLX and FLXLC transformants. Total and purified proteins from FLX (lanes 1 and 2, respectively) and FLXLC (lanes 3 and 4, respectively) were loaded onto a SDS-PAGE (11% polyacrylamide). Gel was stained with Coomassie blue. SD, molecular mass standards.

imum of 2870 nkat/ml for FLX and 2038 nkat/ml for FLXLC transformant were obtained. Considering specific activity of the FAEA partner in bifunctional enzymes, production yields were estimated at 1.4 g/liter and 1.5 g/liter for FLX and FLXLC transformants, respectively.

Biochemical and kinetic characterization of bifunctional enzymes. (i) SDS-PAGE and Western blot analysis. In both cases, the produced proteins were checked by electrophoresis on an SDS-polyacrylamide gel (Fig. 3, lanes 1 and 3). Predominant bands at ca. 72 and 97 kDa were observed in the total extracellular proteins from FLX and FLXLC transformants, respectively. The glycosylation generated by the addition of the linker was around 12% (wt/wt) and 26% (wt/wt) for FLX and FLXLC, respectively. Recombinant enzymes were purified on a Chelating Sepharose Fast Flow column, and the homogeneity of fractions was controlled by SDS-PAGE (lanes 2 and 4).

Both chimeric enzymes from total protein supernatants and purified fractions were immunodetected using antibodies raised against FAEA (Fig. 4A). A single band was revealed from the total protein extracts (lanes 1 and 3) and from the purified samples (lanes 2 and 4) for FLX and FLXLC transformants, respectively. Antibodies raised against the C-terminal histidine tag were also used for immunodetection in order to control the integrity of recombinant proteins, since antibodies raised against xylanase or CBM were not available (Fig. 4B). One single band was detected for FLX and FLXLC confirming that the bifunctional enzymes were produced as intact proteins without any degradation form (lanes 5 to 8). A negative control (C) confirmed that both antibodies were epitope

FIG. 4. Western blot analysis of total and purified proteins produced by FLX and FLXLC transformants. Antibodies raised against FAEA (A) or His tag (B) were used for immunodetection of the total extracellular and purified proteins from FLX and FLXLC transformants. Lanes 1 and 5, total extracellular proteins from FLX transformant; lanes 2 and 6, purified FLX; lanes 3 and 7, total extracellular proteins from FLXLC transformant; lanes 4 and 8, purified FLXLC. C, control strain D15#26 transformed with pAB4-1. Detection was performed by chemiluminescence.

specific and that the endogenous FAEA was not produced in these culture conditions.

(ii) **N-terminal sequencing.** The first five amino acids of FLX and FLXLC were sequenced (ASTQG) and aligned with the native FAEA. Alignments were performed and reveal 100% identity between recombinant proteins and native FAEA, confirming that FLX and FLXLC were correctly processed.

(iii) **Analysis of the bifunctional enzyme-cellulose affinity and binding capacity.** In contrast to FLX, FLXLC contains at the C terminus end, the CBM from *A. niger* cellobiohydrolase B. Cellulose binding affinity and binding capacity of FLXLC were determined toward the microcrystalline cellulose, Avicel PH101. Measured values were of 9.9×10^{-8} M and 0.98 $\mu\text{mol/g}$ Avicel for the dissociation constant (K_d) and the binding capacity, respectively. As expected, no interaction was found for the chimeric enzyme FLX (without CBM).

(iv) **Biochemical and kinetic parameters.** Biochemical and kinetic parameters of FLX and FLXLC were compared to the free recombinant FAEA and XYNB according to the esterase and xylanase activities (Table 1). Concerning pH optimum and stability, no significant difference was found between both bifunctional enzymes and free FAEA or XYNB. For the temperature optimum and stability, the only distinction concerns a slight shift measured for the xylanase activities. In addition, the integrity of FLX and FLXLC was controlled by SDS-PAGE after incubation at different temperatures, and both bifunc-

tional enzymes were fully stable up to 45°C and were partly cleaved at 50°C. The first amino acids of the cleaved form were sequenced and identified as GSGSS. Alignment of this sequence with FLX and FLXLC reveals 100% identity with a sequence found in the linker. These results showed that the hyperglycosylated linker is stable up to 45°C and a cleavage appears at 50°C before the GSGSS sequence. The chimeric FLXLC protein, containing two linker sequences, was cleaved

TABLE 1. Physicochemical and kinetic parameters for the feruloyl esterase and xylanase partners

Parameter ^e	Feruloyl esterase activity			Xylanase activity		
	FAEA ^a	FLX	FLXLC	XYNB ^b	FLX	FLXLC
Tp optimum (°C)	60	55–60	55–60	50	45	45
Tp stability (°C)		45	45	50	45	45
pH optimum	5	5	5	5.5	6	6
pH stability	5–6	5–6	5–6	4–7	4–7	4–7
K_m ^c	0.75	0.80	0.78	6.6	7.5	7.5
Sp act ^d	0.72	0.66	0.63	386	394	368

^a Record et al. (40).

^b Levasseur et al. (29).

^c K_m are expressed in millimolar amounts for feruloyl esterase activity and in milligrams per milliliter for xylanase activity.

^d Specific activities are expressed in nkat per nmol of protein in order to facilitate comparison between free and bifunctional proteins.

^e For the pH and temperature (Tp) stabilities, incubations were performed for 90 min.

FIG. 5. Comparison of the ferulic acid release efficiency by action of free or bifunctional enzymes. WB (A) and CB (B) were used for the FA hydrolysis by free or bifunctional enzymes. FA release was determined by HPLC after 4 h (white bars) and 16 h (black bars). Activities were expressed as the percentage of the total amount of FA present in the substrate. The standard deviation was less than 5% from the mean of the value for WB and CB.

only at the C-terminal linker (between XYNB and CBM). Potential cleavage site for proteases were checked on the amino acids sequences of both hybrid proteins by using a peptide cutter tool (19), and no cleavage site was found in the neighborhood of GSGSS.

Concerning kinetic properties, the Michaelis constants for FLX and FLXLC were measured from a Lineweaver-Burk plot by using MFA and birchwood xylan as substrates. The values found for FLX and FLXLC were in agreement with those found for the free recombinant FAEA and XYNB (Table 1). Specific activities of bifunctional enzymes were determined based on feruloyl esterase and xylanase activities and compared to the free FAEA and XYNB (Table 1). Values found for bifunctional enzymes were close to those found for the free FAE and XYNB.

In conclusion, biochemical and kinetic parameters of both bifunctional proteins, FLX and FLXLC, were in the same range compared to those of the free enzymes (FAEA and XYNB).

Enzymatic release of ferulic acid from wheat and corn brans. In order to study the synergistic effect generated by the physical proximity of two enzymes into the bifunctional proteins and the influence of the CBM addition, FLX and FLXLC were compared to the free enzymes FAEA and XYNB for the ferulic acid (FA) release efficiency. All enzymes were purified to homogeneity and incubated with WB and CB because of their naturally high amount of FA contained in the cell wall. By using free FAEA, 41 and 51% of the total alkali-extractable FA from WB was released after 4 and 16 h, respectively (Fig. 5A). These percentages were slightly increased to 51% (4 h) and 54% (16 h) with the addition of free XYNB. As control for FLXLC, a free CBM should be added to the free FAEA+XYNB. However, our assays of production in *A. niger*

of a free CBM were unsuccessful, and the effect of free CBM addition could not be determined. Considering experiments in which FLX or FLXLC were used, a total release of FA was observed after only 4 h of incubation. Using CB as the substrate (Fig. 5,B), free FAEA released 4.2 and 4.8% of FA after 4 h and 16 h, respectively, and the addition of XYNB did not increase this percentage. However, FLX and FLXLC were able to release 6.2 and 7.2% after 4 h of hydrolysis, respectively, while a 16-h treatment, leads to an increase to 6.3 and 7.9% for, respectively, FLX and FLXLC. The synergy factors were determined and compared between free (FAEA+XYNB) and fused enzymes (FLX and FLXLC). As calculated in Table 2, the calculated ratio was higher than 1, demonstrating for both substrates that the synergistic effect is clearly better for the bifunctional enzymes compared to experiment using the corresponding free enzymes. Concerning the FA release from CB, the synergy is higher for FLXLC (1.80 and 1.62) than for FLX (1.53 and 1.30) after 4 and 16 h, respectively.

In conclusion, these results showed that, for both substrates,

TABLE 2. Comparison of synergistic effect on ferulic acid release between free and fused enzymes

Enzyme	Synergy factor ^a for:			
	WB		CB	
	4 h	16 h	4 h	16 h
FLX	1.95	1.85	1.53	1.30
FLXLC	1.95	1.85	1.80	1.62

^a The synergy factor is calculated as follows: (released FA by the bifunctional enzymes FLX or FLXLC)/(released FA by free enzymes FAEA + XYNB). The standard deviation was <5% from the mean of the value for WB and CB.

bifunctional enzymes FLX and FLXLC were more efficient for the FA release compared to the corresponding free enzymes (FAEA+XYNB). Moreover, these results seem to show that FLXLC is more adapted for the FA release from CB as the substrate, suggesting a positive effect generated by the CBM.

DISCUSSION

A wide range of enzymes is required for biodegradation of plant cell wall due to the walls' heterogeneity in composition and structure. Some combinations between different main-chain (endo-xylanases and β -xylosidases) and accessory hydrolytic enzymes (α -arabinofuranosidase and feruloyl esterase) demonstrated an important synergistic effect leading to an efficient degradation of the cell wall (9, 12). In the present study, two plant cell-wall-degrading enzymes and a carbohydrate-binding module of *A. niger* were fused to study the synergistic effect on degradation of natural substrates. The construction of such hybrid proteins is an original aspect of protein engineering opening a wide range of potential applications. Here, the concept lies in the recruiting of two functional units to create improved bifunctional proteins (3, 35). Such multimodular organizations are commonly found in nature, leading to enzyme production with more than one enzymatic activity or protein function. In the biotechnology sector, some bifunctional proteins had already been investigated, including, for instance, hybrids of β -galactosidase and galactose dehydrogenase (28) or α -amylase and glucoamylase (43). The results of the latter study demonstrated an increase of enzyme efficiency compared to the free enzymes for the digestion of raw starch. In another study, a chimeric xylanase/endoglucanase (XynCenA) with an internal CBM was constructed, but results showed that the hybrid enzyme did not significantly affect the hydrolysis on homogeneous xylans or cellulosic substrates compared to the free enzyme; however, no application test was investigated on a natural substrate (45).

In order to evaluate the effect generated by the physical proximity of two cell-wall hydrolases, FAEA was fused to XYNB (construct FLX). A fungal CBM from the *A. niger* CBHB was fused at the C terminus extremity in the second construct (FLXLC) to target the bifunctional enzyme on cellulose. For both constructs, a hyperglycosylated linker peptide was fused between each module (FAEA, XYNB, or CBM) for three main reasons. First, the linker is known to retain the capacity of modules to fold independently and to conserve a conformational freedom relative to one another (35). In our case, both feruloyl esterase and xylanase were able to adopt this conformation, and the engineered bifunctional proteins were active, with biochemical and kinetic properties corresponding to free enzymes. Second, the high degree of glycosylation of the linker allows an increase of the stability of protein sequence by protecting the linker from protease activities and, finally, by avoiding the frequent problem of cleavage between fused modules (8, 35). This effect was observed because both bifunctional enzymes were stable, as shown by SDS-PAGE and Western blot analysis. However, the stability of hybrid enzymes was shown to have some limits with thermal treatment. Indeed, the influence of the heat treatment on the FLX and FLXLC integrity showed that they were stable up to 45°C and then cleaved in the linker sequence at 50°C. Lastly, the

hyperglycosylated linker could have a positive role in secretion, increasing the production yield as demonstrated for the hyperglycosylated linker from the *A. niger* glucoamylase (32). Indeed, glycosylation sites due to the presence of one or two linkers for FLX and FLXLC, respectively, could extend retention of recombinant proteins in the secretion pathway, thereby providing additional time for correct processing and resulting in an increase of production (41). This latter hypothesis could explain why the production yields for both bifunctional enzymes were higher than those obtained for the corresponding free recombinant enzymes (29, 40). However, this hypothesis should be carefully checked for each best transformant by comparing the number of copies and the location of integrated expression cassettes.

In order to study the synergistic effect generated by the proximity of both enzymatic modules, the biochemical and kinetic characteristics of each module were carefully controlled. All of the main biochemical and kinetic properties of both bifunctional proteins FLX and FLXLC, i.e., temperature and pH stabilities, optimal temperature and pH, K_m , and specific activities, were in the same range compared to those of the free enzymes. Therefore, it could be proposed that spatial orientation of active sites is not perturbed between fused modules. Concerning the CBM originating from the *A. niger* CBHB, binding assays were performed on cellulose, since this was not characterized in the past (20). Avicel cellulose has an important degree of polymerization of 100 to 250 glucopyranose units and 50 to 60% of crystalline form with a crystalline phase essentially composed of type I_B characteristic of higher plants (48). The results showed that FLXLC possess affinity for Avicel, confirming that the structure of CBM is not perturbed and that CBM conserved its function in the hybrid enzyme.

Both bifunctional proteins FLX and FLXLC were finally tested to study the effect of the physical proximity of two complementary fungal enzymes and the influence of the CBM addition on the enzymatic synergy. Our application test was based on the FA release from two natural and model substrates, WB and CB, known for their high amounts of FA in the plant cell wall of approximately 1 and 3% (wt/wt), respectively (42). Both substrates are generated from agriculture, and FA could be used in agro-food, cosmetic, and pharmaceutical sectors (4, 26). Thus far, previous results found for the FA release from WB were obtained with a *Trichoderma viride* xylanase and the FAEA from *A. niger*, in which a maximum of 95% (wt/wt) total ferulic acid was released (15). Concerning CB, an important amount of FA was released (up to 13.6%), by using the commercial preparation Novozym 342 from *Humicola insolens* (5). However, we should consider that this commercial preparation contained different kinds of enzymatic activities. In the present assay, the totality of FA from WB was released by the bifunctional enzyme treatment, and less than 8% was obtained with CB. Although ferulic acid content in corn bran is higher than that found in the WB, CB xylan is more often substituted by xylose, arabinose, and galactose residues (7, 15). Thus, the difference in ferulic acid release could be explained by the number of substitutions on the heteroxylan backbone in corn, the presence of highly branched xylose in the side chain, and the presence of a linkage between arabinose and xylose at the proximity of the FA group, which seriously restrict enzyme accessibility. Finally, if we consider the hydrolysis of CB by

FLXLC, CBM showed a positive effect on the FA release, probably because of (i) the cellulose targeting that increases the enzyme concentration close to the substrate and/or (ii) the destabilization of the cellulose structure making the substrate more accessible. As a conclusion of application tests, by using FLX or FLXLC, a better synergistic effect on both substrates was obtained for the FA release compared to the free enzymes FAEA and XYNB. The general enhanced synergy was suggested to be due to the physical proximity of each enzymatic partner in the bifunctional enzymes or the substrate targeting generating by the C-terminal CBM addition for FLXLC. Future works will be performed to describe and explain the observed synergy.

As a general conclusion, construction of new enzymatic tools for plant cell wall degradation associating complementary cell wall hydrolases such as an accessory enzyme (FAEA) and a main-chain cleaving enzyme (XYNB) was shown to be a strategy of interest to increase the synergistic effect of enzymatic partners. For biotechnological applications, utilization of such hybrid proteins could be an alternative to expensive and polluting chemical treatments or to improve already existing enzymatic processes for utilization of vegetal by-products in the pulp-and-paper, agro-industries, and biofuel production sectors. Future studies will be performed to elucidate questions such as: (i) what are the combination and the number of enzymatic modules (according to the glycosyl hydrolases families) to be grafted together in order to obtain the best synergistic effect and (ii) what is the influence of the number of CBM added or the CBM position in the hybrid enzymes?

ACKNOWLEDGMENTS

This research was supported by the ADEME (Agrice program) and European Integrated Project no. 019882 (New Improvements for Lignocellulosic Ethanol) and by grants from MENRT (Ministère de l'Éducation Nationale, de la Recherche et de la Technologie, Paris, France).

We thank Craig Daniels for his helpful comments on the manuscript.

REFERENCES

- Bailey, M. J., P. Biely, and K. Poutanen. 1992. Interlaboratory testing of methods for assay of xylanase activity. *J. Biotechnol.* **23**:257–270.
- Bartolome, B., C. B. Faulds, P. A. Kroon, K. Waldron, H. J. Gilbert, G. Hazlewood, and G. Williamson. 1997. An *Aspergillus niger* esterase (ferulic acid esterase III) and a recombinant *Pseudomonas fluorescens* subsp. *cellulosa* esterase (Xyl1D) release a 5–5' ferulic dehydrotomer (diferulic acid) from barley and wheat cell walls. *Appl. Environ. Microbiol.* **63**:208–212.
- Béguin, P. 1999. Hybrid enzymes. *Curr. Opin. Biotechnol.* **10**:336–340.
- Bonnin, E., L. Lesage-Meesen, C. Stentelaire, M. Asther, and J. F. Thibault. 1999. Method for obtaining *A. niger* cultures and their uses for producing ferulic acid and vanillic acid. Patent 99/04644.
- Bonnin, E., L. Saulnier, M. Brunel, C. Marot, L. Lesage-Meesen, M. Asther, and J. F. Thibault. 2002. Release of ferulic acid from agroindustrial by-products by the cell wall-degrading enzymes produced by *Aspergillus niger* I-1472. *Enzyme Microb. Technol.* **31**:1000–1005.
- Bunzel, M., J. Ralph, J. M. Marita, R. D. Hatfield, and H. Steinhart. 2001. Diferulates as structural components in soluble and insoluble cereal dietary fibre. *J. Sci. Food Agric.* **81**:653–660.
- Chanliaud, E., L. Saulnier, and J. F. Thibault. 1995. Alkaline extraction and characterisation of heteroxylans from maize bran. *J. Cereal Sci.* **21**:195–203.
- Conesa, A., P. J. Punt, N. van Lwijk, and C. A. M. J. J. van den Hondel. 2001. The secretion pathway in filamentous fungi: a biotechnological view. *Fungal Genet. Biol.* **33**:155–171.
- de Vries, R., H. C. M. Kester, C. H. Poulsen, J. A. E. Benen, and J. Visser. 2000. Synergy between enzymes from *Aspergillus* involved in the degradation of plant cell wall polysaccharides. *Carbohydr. Res.* **327**:401–410.
- de Vries, R. P., B. Michelsen, C. H. Poulsen, P. A. Kroon, R. H. H. van den Heuvel, C. B. Faulds, G. Williamson, J. P. T. W. van den Hombergh, and J. Visser. 1997. The *faeA* genes from *Aspergillus niger* and *Aspergillus tubingensis* encode ferulic acid esterases involved in degradation of complex cell wall polysaccharides. *Appl. Environ. Microbiol.* **63**:4638–4644.
- de Vries, R. P., P. A. van Kuyk, H. C. Kester, and J. Visser. 2002. The *Aspergillus niger faeB* gene encodes a second feruloyl esterase involved in pectin and xylan degradation and is specifically induced in the presence of aromatic compounds. *Biochem. J.* **363**:377–386.
- de Vries, R. P., and J. Visser. 2001. *Aspergillus* enzymes involved in degradation of plant cell wall polysaccharides. *Microbiol. Mol. Biol. Rev.* **65**:497–522.
- Din, N., Damude, H. G., N. R. Gilkes, R. C. Miller, Jr., R. A. Warren, and D. G. Kilburn. 1994. C1-Cx revisited: intramolecular synergism in a cellulase. *Proc. Natl. Acad. Sci. USA* **91**:11383–11387.
- Din, N., N. R. Gilkes, B. Tekant, R. C., Jr. Miller, R. A. J. Warren, and D. G. Kilburn. 1991. Non-hydrolytic disruption of cellulose fibers by the binding domain of a bacterial cellulase. *Biotechnology* **9**:1096–1099.
- Faulds, C. B., and G. Williamson. 1995. Release of ferulic acid from wheat bran by a ferulic acid esterase (FAE-III) from *Aspergillus niger*. *Appl. Microbiol. Biotechnol.* **46**:1082–1087.
- Faulds, C. B., and G. Williamson. 1999. Effect of hydroxycinnamates and benzoates on the production of feruloyl esterases by *Aspergillus niger*. *J. Sci. Food Agric.* **79**:450–452.
- Fierobe, H. P., F. Mingardon, A. Mechaly, A. Belaich, M. T. Rincon, S. Pages, R. Lamed, C. Tardif, J. P. Belaich, and E. A. Bayer. 2005. Action of designer cellulosomes on homogeneous versus complex substrates: controlled incorporation of three distinct enzymes into a defined trifunctional scaffoldin. *J. Biol. Chem.* **280**:16325–16334.
- Garcia-Conesa, M. T., V. F. Crepin, A. J. Goldson, G. Williamson, N. J. Cummings, I. F. Connerton, C. B. Faulds, and P. A. Kroon. 2004. The feruloyl esterase system of *Talaromyces stipitatus*: production of three discrete feruloyl esterases, including a novel enzyme, TsFaeC, with a broad substrate specificity. *J. Biotechnol.* **108**:227–241.
- Gasteiger, E., C. Hoogland, A. Gattiker, S. Duvaud, M. R. Wilkins, R. D. Appel, and A. Bairoch. 2005. Protein identification and analysis tools on the ExPASy server, p. 571–607. In J. M. Walker (ed.), *The proteomics protocols handbook*. Humana Press, Inc., Totowa, N.J.
- Gielkens, M. M., E. Dekkers, J. Visser, and L. H. de Graaff. 1999. Two cellobiohydrolase-encoding genes from *Aspergillus niger* require D-xylose and the xylanolytic transcriptional activator XlnR for their expression. *Appl. Environ. Microbiol.* **65**:4340–4345.
- Goldstein, M. A., M. Takagi, S. Hashida, O. Shoseyov, R. H. Doi, and I. H. Segel. 1993. Characterization of the cellulose-binding domain of the *Clostridium cellulovorans* cellulose-binding protein A. *J. Bacteriol.* **175**:5762–5768.
- Gordon, C. L., D. B. Archer, D. J. Jeenes, J. H. Doonan, B. Wells, A. P. J. Trinci, and G. D. Robson. 2000. A glucanase::GFP gene fusion to study protein secretion by individual hyphae of *Aspergillus niger*. *J. Microbiol. Methods* **42**:39–48.
- Graf, E. 1992. Antioxidant potential of ferulic acid. *Free Radic. Biol. Med.* **13**:435–448.
- Ho, S. N., H. D. Hunt, R. M. Horton, J. K. Pullen, and L. R. Pease. 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* **77**:51–59.
- Juge, N., G. Williamson, A. Puigserver, N. J. Cummings, I. F. Connerton, and C. B. Faulds. 2001. High-level production of recombinant *Aspergillus niger* cinnamoyl esterase (FAEA) in the methylotrophic yeast *Pichia pastoris*. *FEMS Yeast Res.* **1**:127–132.
- Kikuzaki, H., M. Hisamoto, K. Hirose, K. Akiyama, and H. Taniguchi. 2002. Antioxidant properties of ferulic acid and its related compounds. *J. Agric. Food Chem.* **50**:2161–2168.
- Kroon, P. A., G. Williamson, N. M. Fish, D. B. Archer, and N. J. Belshaw. 2000. A modular esterase from *Penicillium funiculosum* which releases ferulic acid from plant cell walls and binds crystalline cellulose contains a carbohydrate binding module. *Eur. J. Biochem.* **267**:6740–6752.
- Ljungcrantz, P., H. Carlsson, M. O. Mansson, P. Buckel, K. Mosbach, and L. Bulow. 1989. Construction of an artificial bifunctional enzyme, beta-galactosidase/galactose dehydrogenase, exhibiting efficient galactose channeling. *Biochemistry* **28**:8786–8792.
- Levasseur, A., M. Asther, and E. Record. 2005. Overproduction and characterization of xylanase B from *Aspergillus niger*. *Can. J. Microbiol.* **51**:177–183.
- Levasseur, A., I. Benoit, M. Asther, M. Asther, and E. Record. 2004. Homologous expression of the feruloyl esterase B gene from *Aspergillus niger* and characterization of the recombinant enzyme. *Protein Expr. Purif.* **37**:126–133.
- Levasseur, A., S. Pagès, H. P. Fierobe, D. Navarro, P. J. Punt, J. P. Belaich, M. Asther, and E. Record. 2004. Design and production in *Aspergillus niger* of a chimeric protein associating a fungal feruloyl esterase and a clostridial dockerin domain. *Appl. Environ. Microbiol.* **70**:6984–6991.
- Libby, C. B., C. A. Cornett, P. J. Reilly, and C. Ford. 1994. Effect of amino acid deletions in the O-glycosylated region of *Aspergillus awamori* glucanase. *Protein Eng.* **7**:1109–1114.

33. Linder, M., and T. T. Teeri. 1997. The roles and function of cellulose-binding domains. *J. Biotechnol.* **57**:15–28.
34. Miller, G. L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* **31**:426–428.
35. Nixon, A. E., M. Ostermeier, and S. J. Benkovic. 1998. Hybrid enzymes: manipulating enzyme design. *Trends Microbiol.* **16**:258–264.
36. Pagès, S., L. Gal, A. Belaich, C. Gaudin, C. Tardif, and J. P. Belaich. 1997. Role of scaffolding protein CipC of *Clostridium cellulolyticum* in cellulose degradation. *J. Bacteriol.* **179**:2810–2816.
37. Porath, J., J. Carlsson, I. Olsson, and G. Belfrage. 1975. Metal chelate affinity chromatography, a new approach to protein fractionation. *Nature* **258**:598–599.
38. Punt, P. J., and C. A. van den Hondel. 1992. Transformation of filamentous fungi based on hygromycin B and phleomycine resistance markers. *Methods Enzymol.* **216**:447–457.
39. Ralet, M. C., C. B. Faulds, G. Williamson, and J. F. Thibault. 1994. Degradation of feruloylated oligosaccharides from sugar-beet pulp and wheat bran by ferulic acid esterases from *Aspergillus niger*. *Carbohydr. Res.* **263**:257–269.
40. Record, E., M. Asther, C. Sigoillot, S. Pagès, P. J. Punt, M. Delattre, M. Haon, C. A. van den Hondel, J. C. Sigoillot, L. Lesage-Meessen, and M. Asther. 2003. Overproduction of the *Aspergillus niger* feruloyl esterase for pulp bleaching application. *Appl. Microbiol. Biotechnol.* **62**:349–355.
41. Sagt, C. M., B. Kleizen, R. Verwaal, M. D. deJong, W. H. Muller, A. Smits, C. Visser, J. Boonstra, A. J. Verkleij, and C. T. Verrips. 2000. Introduction of N glycosylation site increases secretion of heterologous proteins in yeasts. *Appl. Environ. Microbiol.* **66**:4940–4944.
42. Saulnier, L., and J. F. Thibault. 1999. Ferulic acid and diferulic acids as components of sugar-beet pectins and maize bran heteroxylans. *J. Sci. Food Agric.* **79**:396–402.
43. Shibuya, I., G. Tamura, H. Shima, T. Ishikawa, and S. Hara. 1992. Construction of an α -amylase/glucoamylase fusion gene and its expression in *Saccharomyces cerevisiae*. *Biosci. Biotech. Biochem.* **56**:884–889.
44. Srisodsuk, M., J. Lehtio, M. Linder, E. Margolles-Clark, and T. Reinikainen, and T. T. Teeri. 1997. *Trichoderma reesei* cellobiohydrolase I with an endoglucanase cellulose-binding domain: action on bacterial microcrystalline cellulose. *J. Biotechnol.* **57**:49–57.
45. Tomme, P. N., R. Gilkes, C. M. Miller, Jr., A. J. Warren, and D. G. Kilburn. 1994. An internal cellulose-binding domain mediates adsorption of an engineered bifunctional xylanase/cellulase. *Protein Eng.* **7**:117–123.
46. Topakas, E., H. Stamatis, P. Biely, and P. Christakopoulos. 2004. Purification and characterization of a type B feruloyl esterase (StFAE-A) from the thermophilic fungus *Sporotrichum thermophile*. *Appl. Microbiol. Biotechnol.* **63**:686–690.
47. van Hartingsveldt, W., I. E. Mattern, C. M. van Zeijl, P. H. Pouwells, and C. A. van den Hondel. 1987. Development of a homologous transformation system for *Aspergillus niger* based on the *pyrG* gene. *Mol. Gen. Genet.* **206**:71–75.
48. Wood, T. M. 1988. Preparation of crystalline, amorphous, and dyed cellulase substrates. *Methods Enzymol.* **160**:19–25.
49. Yu, P., D. D. Maenz, J. J. McKinnon, V. J. Racz, and D. A. Christensen. 2002. Release of ferulic acid from oat hulls by *Aspergillus* ferulic acid esterase and *Trichoderma* xylanase. *J. Agric. Food Chem.* **50**:1625–1630.