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BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Differential expression of cellulases and xylanases by *Cellulomonas flavigena* grown on different carbon sources

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Abstract The diversity of cellulases and xylanases secreted by *Cellulomonas flavigena* cultured on sugar cane bagasse, Solka-floc, xylan, or glucose was explored by two-dimensional gel electrophoresis. *C. flavigena* produced the largest variety of cellulases and xylanases on sugar cane bagasse. Multiple extracellular proteins were expressed with these growth substrates, and a limited set of them coincided in all substrates. Thirteen proteins with carboxymethyl cellulase or xylanase activity were liquid chromatography/mass spectrometry sequenced. Proteins SP4 and SP18 were identified as products of *celA* and *celB* genes, respectively, while SP20 and SP33 were isoforms of the bifunctional cellulase/xylanase Cxo recently sequenced and characterized in *C. flavigena.* The rest of the detected proteins were unknown enzymes with either carboxymethyl cellulase or xylanase activities. All proteins aligned with glycosyl hydrolases listed in National Center for Biotechnology Information database, mainly with cellulase and xylanase enzymes. One of these unknown enzymes, protein SP6, was cross-induced by sugar cane bagasse, Solka-floc, and xylan. The differences in the expression maps of the presently induced cultures revealed that *C. flavigena* produces and secretes multiple enzymes to use a wide range of lignocellulosic substrates as carbon sources. The expression of these proteins depends on the nature of the cellulosic substrate.

Keywords Cellulases · Xylanases · Two-dimensional gel electrophoresis · Protein pattern

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Introduction

Microbial cellulases and xylanases are used currently in the textile, pulp and paper, and food processing industries and are also attracting great attention for potential application in the production of biofuels (Lynd et al. 2002; Subramaniyan and Prema 2002). These enzymes degrade the plant biomass contributing to carbon recycling in nature. A spectrum of enzymes is involved in the hydrolysis of each lignocellulosic component, and it has become apparent that to achieve this, more than one xylanase or cellulase is usually produced by individual microorganisms (Wong et al. 1988). The occurrence of multiple enzymes with xylanolytic and/or cellulolytic activity arises from the regulation, the substrate cross-specificity, and the post-translational modifications of these enzymes.

Cellulomonas flavigena secretes a complex of several hydrolytic enzymes, which synergistically hydrolyze cellulose and hemicellulose (Rajoka 2005). The biosynthesis of these enzymes is induced by different substrates and repressed by readily metabolized carbon sources, but sugar cane bagasse, a complex substrate, is by far the best inducer of cellulases and xylanases (Pérez-Avalos et al. 1996; Ponce-Noyola and de la Torre 2001).

In spite of significant progress during the last years, many fundamental questions still remain, such as whether the expression of the different cellulases and xylanases is coordinately regulated by a shared mechanism and how different the enzymatic complex is when the bacterium is cultured on diverse substrates.

To gain further understanding of the multiple cellulolytic and xylanolytic complexes produced by *C. flavigena*, the expression levels of its extracellular proteins under different growth conditions were monitored in parallel using proteomic technology.

Material and methods

Bacterial strain and culture conditions

C. flavigena CDBB-531 (Microbial Collection, Department of Biotechnology and Bioengineering, CINVESTAV) was used in all experiments. Cells were grown in 250-ml Erlenmeyer flasks containing 50 ml of mineral medium supplied with biotin (10 μg/l), thiamine (1 mg/l), and 1% glucose (Ponce-Noyola and de la Torre 2001). Cells grown on glucose were washed twice with 0.85% NaCl before switching to growth on mineral medium containing 1% of one of the following substrates: glucose, Solka-floc, xylan, or sugar cane bagasse, until the culture reached the midexponential growth phase at 37°C and 150 rpm in an orbital shaker (New Brunswick Scientific, Edison, NJ). The inoculum ratio was 10% (v/v). The culture supernatants were recovered by centrifugation at 10,000×*g* for 15 min at 4°C. To remove the residual insoluble carbon source, samples were previously filtered through GD-120 glass-fiber filter discs (Whatman). These supernatants were used for enzyme activity assays and for the recovery of the proteins.

Enzyme activity assay

Supernatant samples were used to determine carboxymethyl cellulase (CMCase), filter paper (FPase), and xylanase activity. These were assayed by measuring the released reducing sugars as cellobiose for the first two cases and xylose for xylanase (Ponce-Noyola and de la Torre 1995), using 3 ml of dinitrosalicylic acid reagent (Miller 1959). One unit of activity (U) is defined as the amount (μmol) of product formed per minute under standard assay conditions. All determinations were made in triplicate. The maximum difference among the three values was less than 5% of the mean.

Sample preparation for two-dimensional gel electrophoresis

Extracellular proteins were precipitated by mixing 10 ml of supernatant with 1 ml of 100% trichloroacetic acid (TCA; Merck) and incubated at 4°C overnight. The precipitate was collected by centrifugation at 10,000×*g* for 15 min at 4°C and washed three times with ice-cold acetone to remove traces of TCA followed by cold ethanol washes.

Protein resolubilization

TCA-precipitated protein pellets were resolubilized in 75 μl of ReadyPrep rehydration/sample buffer (8 M urea, 2% CHAPS, 100 mM dithiothreitol [DTT], 0.2% ampholytes [Bio-Rad]) and applied to Micro Bio-Spin columns (P-30 Tris–HCl; Bio-Rad) for cleanup and purification of proteins using microcentrifugation. Protein concentration was determined by 2-D Quant kit (Amersham Biosciences). The final volume was adjusted to 185 μl with ReadyPrep rehydration/sample buffer and used for two-dimensional gel electrophoresis (2-DE).

Two-dimensional gel electrophoresis

The protein solution was loaded onto 11-cm immobilized pH gradient (IPG) strips with pH gradient 4–7 (Bio-Rad) by in-gel rehydration overnight. Isoelectric focusing (IEF) was performed at 4°C by applying a voltage gradient from 0 to 250 V in 30 min, 250 to 8,000 V for 2.5 h, and from 8,000 to 20,000 V for 2 h, on a Protean IEF cell apparatus (Bio-Rad). The protein–IPG strips were equilibrated before sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) in equilibration buffer I (50 mM Tris–HCl [pH 8], 8 M urea, 30% glycerol, 2% SDS, and 0.3% DTT) for 10 min, followed by soaking in equilibration buffer II (50 mM Tris–HCl [pH 8], 8 M urea, 30% glycerol, 2% SDS, and 4.5% iodoacetamide) for 10 min. SDS-PAGE was performed using polyacrylamide gradient gels (8–16%) or 10% acrylamide gels (Bio-Rad). Electrophoresis was performed at 150 V and 10 mA (Criterion camera; Bio-Rad). Protein samples were visualized by staining with Coomassie Blue Imperial protein stain (Pierce Biotechnology). At least three independent 2-DE experiments for each growth condition were performed.

Zymograms and gel analysis

Zymograms were prepared by renaturing the protein after running 2-DE. The enzymes were renatured by treating the gel slabs with 50 mM Tris–HCl, pH 8.0, 1 mM DTT, and 0.1 mM ethylenediamine tetraacetic acid overnight, followed by soaking in 2.5% Triton X-100 in the same buffer; finally, the gels were washed with 50 mM Tris–HCl, pH 6.0. Agarose substrate gels of 0.5% CMCase or 0.15% Remazol Brilliant Blue xylan were used for CMCase or xylanase activity, respectively. These substrate gels were coincided to 2-DE gel slabs and incubated at 40°C for 30 h, then stained with 0.1% Congo red and distained with 1 M NaCl to make spots of hydrolytic activity visible against a red background (Teather and Wood 1982).

Protein identification by liquid chromatography/mass spectrometry

The 2-DE gels, after being used for the zymograms, were stained with Coomassie Blue Imperial protein stain. The protein spots that showed a high hydrolysis zone were excised from gels, tryptic digested, and then subjected to mass spectrometry analyses (W.M. Keck Biomedical Mass Spectrometry Laboratory, Biomolecular Research Facility, University of Virginia).

To identify the proteins, all of the tandem mass spectrometry spectra recorded on the tryptic peptides derived from the spots were searched against protein sequences contained in the National Center for Biotechnology Information (NCBI) database, through the SEQUEST search algorithm.

Results

Growth and enzyme activities on different carbon sources

C. flavigena cultured on sugar cane bagasse, Solka-floc, xylan, or glucose achieved the midexponential growth phase after 48, 72, 38, and 36 h, respectively (55×10^8 CFU/ml; data not shown). The supernatant from cultures grown on sugar cane bagasse had the highest specific activities of CMCase, FPase, and xylanase. The supernatant from the Solka-floc culture had a similar FPase-specific activity but only 56% of CMCase and 80% of xylanase. In the supernatant from cultures grown on xylan, both CMCase- and xylanase-specific activities were 50% of that in Solka-floc, and FPase was not detected. When *C. flavigena* was grown in glucose, none of these enzyme activities were detected in the supernatant (Table 1).

Secreted proteome profile analysis

A proteomic approach was used to examine the differences among the expression patterns of the secreted proteins produced by *C. flavigena* grown on sugar cane bagasse, Solka-floc, xylan, or glucose. At least 95% of the protein spots were consistently detected in independent experiments; thus, protein separation was highly reproducible (Fig. 1).

Most of the proteins were in the acid region (pH 4–6) and had molecular weight between 45 and 140 kDa. Sugar cane bagasse induced at least 54 proteins; 12 were overexpressed (Fig. 1a). On the other hand, 40 proteins were detected in Solka-floc, and ten were overexpressed (Fig. 1b). When the gel from the Solka-floc supernatant was coincided to that of sugar cane bagasse, these 40 proteins matched exactly. During growth on xylan, only 22 proteins were identified (Fig. 1c), and ten of them were not detected in sugar cane bagasse nor in Solka-floc. Eleven spots corresponded to those observed in sugar cane bagasse and Solka-floc, and only one protein was common to the xylan, sugar cane bagasses, and glucose cultures. In the supernatants from bacterial cultures grown on glucose, 37 proteins were observed; 27 of them were differentially expressed in this substrate, while the remaining ten coincided with the proteins observed in the other three substrates (Fig. 1d). A Venn diagram of all the protein patterns showed that only six proteins were expressed in all substrates, and five were common to sugar cane bagasse, Solka-floc, and xylan (Fig. 2).

Table 1 Cellulase and xylanase specific activities in *C. flavigena* supernatants

Supernatant	Enzyme activity[a] (IU/mg protein)		
	CMCase	Fpase	Xylanase
Sugar-cane bagasse	10.70±0.5	0.45±0.02	12.8±0.5
Solka-floc	6.05±0.2	0.38±0.02	10.2±0.4
Xylan	5.30±0.2	ND[b]	5.9±0.3
Glucose	ND	ND	ND

[a] Values are means of three independent experiments.
[b] *ND* Not detected under the assay conditions

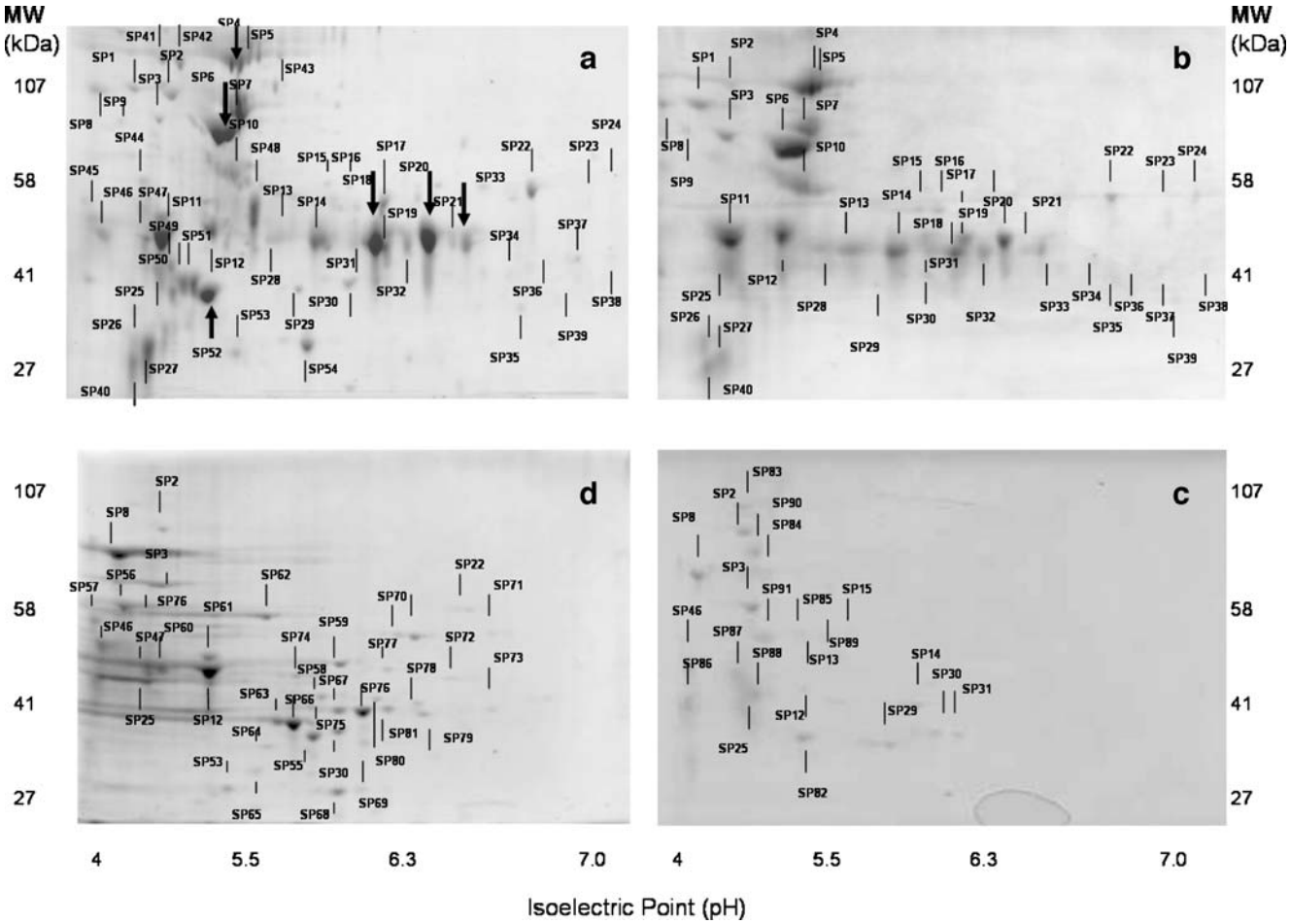

Fig. 1 Secreted proteome of *C. flavigena* grown on: **a** sugar cane bagasse, **b** Solka-floc, **c** xylan, and **d** glucose

Two-dimensional electrophoresis and zymography

We used 2-DE combined with zymograms to determine CMCase or xylanase activity in the extracellular proteome of cells grown on the different substrates. Eight proteins (SP4, SP6, SP7, SP14, SP18, SP20, SP31, and SP33) in the gel slabs from sugar cane bagasse supernatant had CMCase activity, and four of them showed the largest hydrolysis zones (SP4, SP6, SP18, and SP20). In the Solka-floc supernatant, eight proteins were CMCases; these spots matched with the eight CMCases found in the sugar cane bagasse supernatant. CMCase activity was not detected in supernatants from xylan or glucose cultures.

Xylanase activity assays of the 2-DE gel slabs showed only three hydrolysis zones in sugar cane bagasse supernatant (SP6, SP44, and SP52), two of them (SP6 and SP52) larger than the other. In Solka-floc, 2-DE gels only the protein SP6 was xylanase; notably, this protein also had CMCase activity. Proteins SP88, SP90, and SP91 from the xylan cultures were xylanases, but xylanases were absent in glucose supernatants (data not shown).

Identification of the cellulase and xylanase components

The partial amino acid sequence of 13 major protein spots with cellulase or xylanase activity was determined by liquid

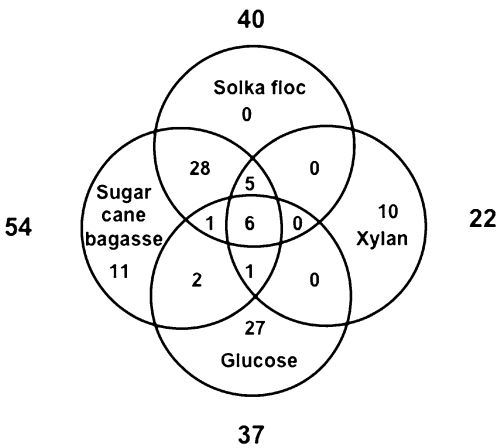

Fig. 2 Venn diagram of the proteins secreted by *C. flavigena* grown on sugar cane bagasse, Solka-floc, xylan, or glucose. *Numbers* indicate the amount of proteins in the specific or coinciding pattern

Table 2 Partial amino acid sequence of the proteins from *C. flavigena* determined by LC/MS

Protein spot	Molecular weight (KDa)	p*I*	Partial sequence[a]	Glycosyl hydrolase	Zymogram activity[b]
SP4 (*CelA*)	102	5.0	MCDPTFVSPK FAEVAK DAAGNVSSASSAXAVTTK GPQESVWETVPQPSCEEFK	Family 6	CMCase
SP6	76	4.9	TSTDVDTYVDESR MCDPTFVSPK FHXDNAVQQQK TGNDWXEAAFR	Family 6	CMCase and xylanase
SP7	90	5.0	EFNMXTAENEMK YKNNDTXVAMDXK AAVSSGVNTYVNPEXVGK GPQESVWETVPQPSCEEFK	Family 6	CMCase
SP8	94	4.1	FHXDNAVQQQK VFHGXWAVNXEDVTR AAVSSGVNTYVNPEXVGK GPQESVWETVPQPSCEEFK	Family 6	ND
SP11	39	4.5	TQGVYNMVK TGNDWXEAAFR XSAXTGNADGK EFNMXTAENEMK	Family 10	ND
SP12	41	4.7	MCDPTFVSPK WDSSTDQDNFK FHXDNAVQQQK AAVSSGVNTYVNPEXVGK	Family 5	ND
SP14	45	5.7	TQGVYNMVK TGNDWXEAAFR AAVSSGVNTYVNPEXVGK XCYNDYNTDNWSHAK	Family 6	CMCase
SP18=(*CelB*)	45	6.1	AAVSSGVNTYVNPEXVGK YQTTSATQQY MCDPTFVSPK AEQFAYNVVER	Family 5	CMCase
SP20=SP33(*Cxo*)	45	6.3 and 6.6	QAAASESGR NDSTYSSXANR VCGVNXCNQYGK NAMXNHVTQVATHYK	Family 10	CMCase
SP44	70	4.3	WDSSTDQDNFK NNDTXVAMDXK YKNNDTXVAMDXK AAVSSGVNTYVNPEXVGK	Family 10	Xylanase
SP48	70	5.5	MCDPTFVSPK WDSSTDQDNFK NNDTXVAMDXK FHXDNAVQQQK	Family 5	ND
SP52	35	4.8	VWAYDXR TPTSEXFR DXGSGTVVSR VGXTDGSVER	Family 6	Xylanase

[a] Partial sequence of four peptides are shown. The proteins listed share CMCase or xylanase activity detected by zymogram analysis after 2D electrophoresis or were subjected to peptide sequencing by LC/MS.
[b] *ND* Not detected

chromatography/mass spectrometry. A Basic Local Alignment Search Tool search against the sequences deposited in the NCBI database revealed identity with glycosyl hydrolase (GH) enzymes (Table 2).

The protein spots SP4, SP18, SP20, and SP33 were identified as proteins previously reported in *C. flavigena* (Gutiérrez-Nava et al. 2003; Pérez-Avalos et al. 2007). Although protein spots SP20 and SP33 shared 15 peptides

derived from their sequencing, they showed different p*I* (Table 2). The peptides derived from these protein spots shared high homology with the sequence of xylanase AAZ76373.1 of *C. fimi*. They also matched with the recently sequenced and characterized bifunctional cellulase/xylanase Cxo (DQ441404) of *C. flavigena* and displayed 70% homology with xylanase A of *S. coelicolor* (CAA16188.1). Indeed, some of these sequences were found in proteins belonging to family GH10.

Discussion

The extracellular proteins of *C. flavigena* grown on different substrates were analyzed at crude enzyme activity level and by analysis of the protein expression pattern and zymograms to provide direct assessment of protein function. The major protein production including cellulases and xylanases was when cells grew on sugar cane bagasse, a heterogeneous substrate that contains both cellulose and xylan. These results correlate with the greater intensity and number of protein spots displayed in the gels after 2-DE. The fact that sugar cane bagasse induces the largest number of proteins must be due to the presence of cellulose and hemicellulose, which have a synergic effect over cellulase and xylanase biosynthesis (Pérez-Avalos et al. 1996). These results indicate that growth conditions markedly affect the profile of secreted proteins including cellulases and xylanases. Previous studies have demonstrated that cellulosomal enzymes in *Clostridium thermocellum* are regulated by the growth conditions that influence the activity of the entire complex (Nochur et al. 1993). Han et al. (2004) found that expression of plant cell wall-degrading enzymes in *C. cellulovorans* is highly influenced by the available carbon source.

We found different protein expression induced by growth on cellulose or xylan, although a set of common proteins was present with both substrates. Cross-induction of xylanase and cellulase activities by cellulosic and hemicellulosic substrates has been documented for *C. fimi* (Kim and Wimpenny 1981), *Thermomonospora fusca* (Spiridonov and Wilson 1988), and *Trichoderma reesei* (Foreman et al. 2003) among others. Although it is not clear how cellulose induces xylanases, a coordinate system for the regulation of genes encoding these enzymes may exist (Foreman et al. 2003). In *C. flavigena*, two genes are closely linked: *celA*, encoding cellobiohydrolase A, and *celB*, encoding endoglucanase B (Gutiérrez-Nava et al. 2003). A similar arrangement has been observed in *Myxobacter* sp. AL-1 (Avitia et al. 2000) and *C. thermocellum* (Demain et al. 2005).

The protein spots SP20 and SP33 identified in this work could be isoforms of a bifunctional cellulase/xylanase (Cxo, DQ441404) recently characterized in our laboratory (Pérez-Avalos et al. 2007). These proteins could represent post-translationally modified versions of the same protein, as many cellulases and xylanases are apparently glycosylated (Stals et al. 2004). We have evidence of the presence of glycosylated xylanases and cellulases in *C. flavigena* (Ponce-Noyola and de la Torre 2001). In addition, we do not discard that at least some of the multiple xylanases from microorganisms are distinct gene products (Wong et al. 1988). At present, we are identifying the genes that code for these bifunctional enzymes.

Finally, the evident differences among the extracellular proteomes of *C. flavigena* that grow on different substrates clearly show the enzyme diversity that can be incorporated into its hemicellulolytic system. Furthermore, these results suggest that *C. flavigena* produces and secretes multiple enzymes to use a wide range of lignocellulosic substrates as carbon sources.

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