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ORIGINAL PAPER

Marìa Eugenia Hidalgo-Lara · G. S. Amelia Farrés
María del Carmen Montes-Horcasitas

β-Methyl-xyloside: positive effect on xylanase induction in *Cellulomonas flavigena*

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Abstract Synthesis of extracellular xylanase in *Cellulomonas flavigena* is induced in the presence of xylan and sugarcane bagasse as substrates. The essential factors for efficient production of xylanase are the appropriate medium composition and an inducing substrate. The increase in xylanase production levels in *C. flavigena* were tested with a number of carbon sources and different culture conditions. Xylose, arabinose, glycerol and glucose did not induce xylanase production in this microorganism. β-Methyl-xyloside (β-mx), a structural analog of xylobiose, also did not induce xylanase when used as the sole carbon source, but when xylan or sugar cane bagasse was supplemented with β-mx, extracellular xylanase production increased by 25 or 46%, respectively. The response of *C. flavigena* to xylan plus β-mx was accompanied by a significant accumulation of reducing sugar, an effect not observed with the combination sugarcane bagasse plus β-mx as substrate. To our knowledge, this is the first report on the effect of β-mx on the induction of xylanase in *C. flavigena*.

Keywords *Cellulomonas* · Induction · β-Methyl xyloside · Xylanase · Sugar cane bagasse

Introduction

Xylan, the major hemicellulosic polysaccharide present in plant cell walls, is an abundant and renewable resource in nature. Breakdown of xylan to soluble sugars requires the combined and synergistic action of a family of extracellular enzymes, e.g., xylanases (1,4-β-xylan xylanohydrolase; EC 3.2.1.8), produced by numerous bacteria, yeasts, and filamentous fungi [5]. The use of cellulase-free xylanases is particularly important in the pulp and paper industry, thus the study of factors involved in the induction and improvement of xylanase production by xylanolytic microorganisms remains significant.

In most bacteria, synthesis of xylanases is regulated by induction and catabolite repression. Known xylanase inducers include xylan, sugar cane bagasse, xylose, and xylobiose, whereas glucose acts as a xylanase catabolite repressor [3, 16]. Interestingly, β-methyl-xyloside (β-mx), a non-metabolizable analog of xylobiose, has shown positive effects on xylanase production in *Aureobasidium pullulans* [13], but did not induce xylanase formation in *Schizophyllum commune* or *Clostridium absonum* [10, 18]. This suggests that the mechanism governing induction and regulation of xylanases is complicated, not yet fully understood, and that it differs in different microorganisms [3, 11].

Cellulomonas flavigena is a cellulose- and xylan-degrading bacterium that secretes a number of glycoside hydrolases, mainly cellulases and xylanases, in the presence of cellulose and xylan-rich polysaccharides as the sole carbon source. *C. flavigena* produces a complex of enzymes, including cellulases, xylanases and β-xylosidases, to degrade components of the plant cell wall [2]. In *C. flavigena*, xylanase synthesis is induced by solka floc, avicel, xylan and sugar cane bagasse, of which the latter is the best [16]. In this paper, we report the positive effect of β-mx, in combination with xylan or sugar cane bagasse, on extracellular xylanase production by *C. flavigena*.

Materials and methods

Microorganism and culture conditions

C. flavigena CDBB 531 was obtained from the National Collection of Microbial Cultures CINVESTAV (Mexico)

M. E. Hidalgo-Lara · M. C. Montes-Horcasitas (✉)
Departamento de Biotecnología y Bioingeniería,
Centro de Investigación y de Estudios Avanzados,
Ave. IPN 2508, México D.F.C.P., 07360, Mexico
E-mail: cmontes@mail.cinvestav.mx
Tel.: +52-55-50613800
Fax: +52-55-50613313

G. S. A. Farrés
Departamento de Alimentos y Biotecnología,
Facultad de Química, Universidad Nacional Autónoma de México,
Circuito Cd. Universitaria, México D.F.C.P., 04510, Mexico

and was maintained on xylan slants at 4°C with monthly transfer. An inoculum of *C. flavigena* was obtained by growing the bacteria in 250 mL flasks containing 50 mL mineral medium at pH 7.2 [17] and 1% (v/v) glycerol as a carbon source, on an orbital shaker at 150 rpm at 37°C for 48 h.

Induction experiments

A 5 mL aliquot of the inoculum was added to 250 mL Erlenmeyer flasks containing 50 mL mineral medium supplemented with different carbon sources for induction experiments: 0.5% (w/v) xylan (birchwood), 1% (w/v) alkaline pre-treated sugar cane bagasse [7], 0.5% (w/v) D-xylose, 0.5% (w/v) D-arabinose or 0.5% (w/v) D-glucose. Cultures were further incubated at 150 rpm and 37°C for 24 h. Cells were harvested by centrifugation at 10,000 *g* at 4°C for 10 min. Xylanase activity in the bacterial culture supernatant was measured.

Assay of enzyme activity

Xylanolytic activity was assayed in 0.1 M citrate-phosphate buffer pH 6.5 with 0.25% (w/v) birchwood xylan as substrate at 60°C (modified from [20]). Reducing sugars were measured by the dinitrosalicyclic acid (DNS) method [15]. One unit of enzyme activity released 1 µmol xylose equivalent per milliliter per minute. Specific activity was expressed as IU mg protein^{-1}. All determinations and experimental cultures were performed in triplicate with average values reported.

Analytical methods

Bacterial growth was measured by optical density at 600 nm in a λ3A Spectrophotometer (Perkin-Elmer, Foster City, CA). Protein concentration was measured by the Lowry method [14] using bovine serum albumin as standard.

Results and discussion

Effect of different carbon sources on growth and xylanase induction

The effect of different carbon sources on growth of *C. flavigena* and extracellular xylanase activity was tested. D-Glucose, D-arabinose and D-xylose were used efficiently as carbon sources (Fig. 1a), but they showed no inductive effect on xylanase production (Fig. 1b). Glycerol was able to support *C. flavigena* growth, but only low xylanase activity (basal level) was detected with this substrate (data not shown). The extracellular xylanase activity was repressed by glucose, D-arabinose, and D-xylose. Catabolite repression by glucose and other

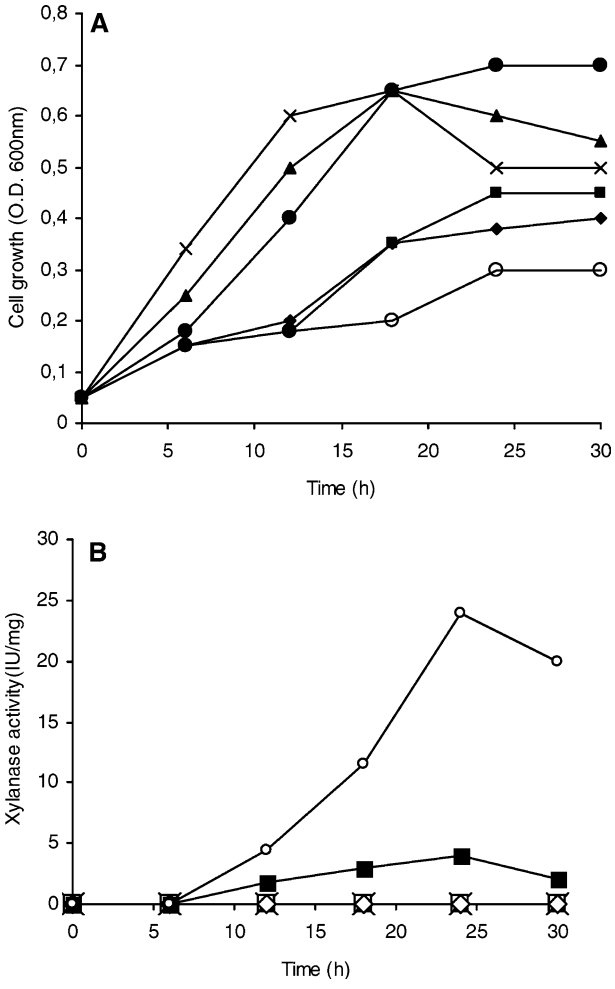

Fig. 1 a *Cellulomonas flavigena* growth on different carbon sources. Cells were grown at 37°C, 150 rpm in shake flasks containing 50 mL mineral medium, 0.5% (w/v) carbon source, pH 7.0. *Filled circles* Sugar cane bagasse, *filled triangles* glycerol, *crosses* glucose, *filled diamonds* D-arabinose, *filled squares* D-xylose, *open circles* xylan. **b** Induction of xylanases with different substrates in *C. flavigena*. Cells were grown on mineral medium supplemented with 1% glycerol. At the onset of the exponential phase of growth, the inducers (0.5%, w/v) were added to the cultures under sterile conditions and incubated for 24 h at 37°C at 150 rpm. Enzyme activity was measured in cell culture supernatants by the DNS method. *Open circles* Sugar cane bagasse, *filled squares* xylan, *open triangles* D-arabinose, *open squares* D-xylose, *open diamonds* D-glucose

easily metabolizable carbon sources is a frequently observed phenomenon in xylanase biosynthesis. In contrast, high extracellular xylanase activity was detected when xylan and sugarcane bagasse were used as growth substrates. Sugarcane bagasse was the best substrate for induction of enzymatic activity (Fig. 1b). These results are consistent with previous reports [16].

Effect of substrate concentration

In *C. flavigena*, xylanase synthesis is induced by sugar cane bagasse, solka floc, avicel and xylan, of which sugar

cane bagasse is the best xylanase inducer [16]. *C. flavigena* was grown on sugar cane bagasse or xylan at concentrations ranging from 0.25% to 1.0% to determine the effect of the concentration of these substrates on induction of extracellular xylanase activity. The highest level of xylanase activity was detected with 1.0% sugar cane bagasse as substrate. The xylanase-inducing effect of sugar cane bagasse was associated with a low accumulation of reducing sugars, ranging from 0.2 to 1.7 µmol/mL. When xylan was used as inducer, the highest xylanase activity was detected with 0.25% xylan. At higher xylan concentrations, the xylanase-inducing effect decreased sharply and was almost absent at 1% xylan. At xylan concentrations higher than 0.25%, an increasing accumulation of reducing sugars, ranging from 0.2 to 2.5 µmol/ml, occurred. The accumulation of reducing sugars with 1% xylan was almost 10-fold higher than that observed with 1% sugar cane bagasse (Table 1). Previous reports have pointed out that the activity of xylanases is often inhibited by the presence of high concentrations of their hydrolysis products [4]. The fact that reducing sugars did not accumulate using sugarcane bagasse might explain why it is better than xylan as an inducer of extracellular xylanase in *C. flavigena*. Xylanase activity on sugarcane bagasse was 40% higher than that observed with xylan under the same experimental conditions. Our data suggest that the xylanase activity induced by xylan was under catabolic repression due to the accumulation of hydrolysis products. Catabolite repression of xylanases has been reported in *Cellulomonas uda* [19], *C. fimi* [12] and *C. thermolacticum* [6].

Effect of β-mx on xylanase induction

β-mx, as a structural analogue of xylobiose, specifically induces xylanase formation and leads to elevated enzyme activity in *Streptomyces* sp. [21] and in yeast of the genera *Cryptococcus* [4]. In *C. flavigena*, β-mx as sole substrate in concentrations up to 0.05% did not induce formation of xylanase. β-mx was not used for growth by *Cellulomonas* presumably because it was not hydrolyzed to provide xylose. However, β-mx did have a positive effect on xylanase formation in *C. flavigena* when combined with xylan or sugarcane bagasse. There was a 46% increase in xylanase activity when bacteria were grown in culture media supplemented with sugarcane bagasse plus β-mx compared to those cultivated in sugarcane bagasse alone (Fig. 2). There was also a 25% increase in extracellular xylanase activity when bacteria were cultured in media supplemented with xylan and β-mx compared to those grown on xylan alone (Fig. 2). The effect of β-mx on xylanase production differs between microorganisms. In *Aureobasidium pullulans, Aspergillus niger*, *Aspergillus sydowii* and *Aspergillus tamarii*, induction of xylanase synthesis was achieved using β-mx alone or in combination with xylan [1, 8, 9, 13]. In *Streptomyces* strains, an increase in xylanase production was observed in the presence of β-mx or some of its derivatives [21]. Thus, our data suggest that there is a common xylanase regulation pathway for the xylanolytic bacterium *C. flavigena* and the xylanolytic fungi *Aureobasidium pullulans, Aspergillus niger*, *Aspergillus sydowii* and *Aspergillus tamarii*, because they all showed increased xylanase production in the presence of xylan and β-mx. To our knowledge, the effect of β-mx on xylanase production by *C. flavigena* has not previously been reported. Further work is required to better understand the mechanism regulating production and secretion of xylanase in *C. flavigena*, the molecules involved in the hydrolysis of xylan and xylan-rich polysaccharides, and, in this specific case, if β-mx acts on a single or multiple enzymes.

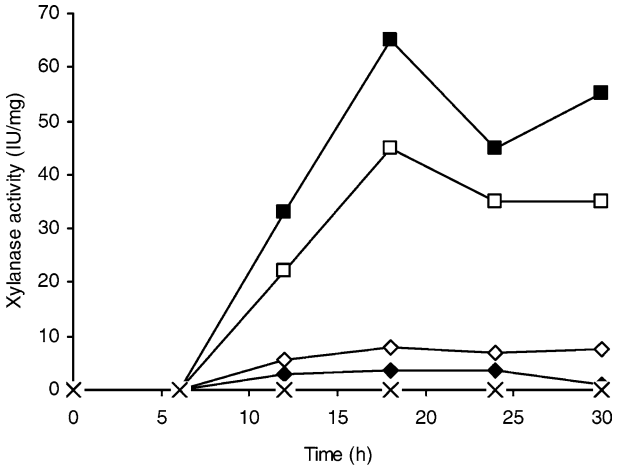

Fig. 2 Effect of β-methyl-xyloside (β-mx) on xylanase induction in *C. flavigena*. Cells were grown on mineral medium supplemented with 1% glycerol. At the onset of the exponential phase of growth, the inducers were added to the cultures under sterile conditions and incubated at 37°C at 150 rpm. Enzyme activity was measured in cell culture supernatants by the DNS method. *Filled squares* Sugar cane bagasse 1% + β-mx 0.05%, *open squares* sugar cane bagasse 1%, *filled circles* xylan 0.5% + β-mx 0.05%, *open circles* xylan 0–5%, *crosses* β-mx 0.05%

Table 1 Effect of increasing sugar concentration on xylanase induction in *Cellulomonas flavigena*. Cells were grown in flasks with 50 mL minimal medium supplemented with xylan or sugar cane bagasse. Growth conditions are in given in Materials and methods

Substrate	Xylan		Sugar cane bagasse	
	IU/mg[a]	µmol/mL[b]	IU/mg[a]	µmol/mL[b]
0.25%	9.0	0.2	7.0	1.7
0.50%	8.0	0.7	9.0	1.0
0.75%	6.0	1.2	18	0.7
1.00%	4.0	2.5	24	0.2

[a]IU/mg protein in supernatant fluid
[b]µmol/mL reducing sugars in supernatant fluid

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