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ORIGINAL PAPER

Production of cellulases and xylanases under catabolic repression conditions from mutant PR-22 of *Cellulomonas flavigena*

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Abstract Derepressed mutant PR-22 was obtained by *N-methyl-N′-nitro-N-nitrosoguanidine* (MNNG) mutagenic treatment of *Cellulomonas flavigena* PN-120. This mutant improved its xylanolytic activity from 26.9 to 40 U mg^{-1} and cellulolytic activity from 1.9 to 4 U mg^{-1}; this represented rates almost 2 and 1.5 times higher, respectively, compared to its parent strain growing in sugarcane bagasse. Either glucose or cellobiose was added to cultures of *C. flavigena* PN-120 and mutant PR-22 induced with sugarcane bagasse in batch culture. The inhibitory effect of glucose on xylanase activity was more noticeable for parent strain PN-120 than for mutant PR-22. When 20 mM glucose was added, the xylanolytic activity decreased 41% compared to the culture grown without glucose in mutant PR-22, whereas in the PN-120 strain the xylanolytic activity decreased by 49% at the same conditions compared to its own control. Addition of 10 and 15 mM of glucose did not adversely affect CMCase activity in PR-22, but glucose at 20 mM inhibited the enzymatic activity by 28%. The CMCase activity of the PN-120 strain was more sensitive to glucose than PR-22, with a reduction of CMCase activity in the range of 20–32%. Cellobiose had a more significant effect on xylanase and CMCase activities than glucose did in the mutant PR-22 and parent strain. Nevertheless, the activities under both conditions were always higher in the mutant PR-22 than in the PN-120 strain. Enzymatic saccharification experiments showed that it is possible to accumulate up to 10 g l^{-1} of total soluble sugars from pretreated sugarcane bagasse with the concentrated enzymatic crude extract from mutant PR-22.

Keywords *Cellulomonas flavigena* · Mutant · Derepressed · Saccharification · Sugarcane bagasse · Cellulase · Xylanase

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Introduction

Lignocellulosic biomass is the most abundant organic material on our planet and represents the major source of renewable energy. It is attractive because of its low cost and great potential to be converted into biofuels, antibiotics and organic acids [10]. Cellulases and xylanases have the potential to achieve the complete saccharification of lignocellulosic biomass for ethanol production as an alternative fuel. Also they have been the subject of increasing research in the last 20 years [18]. The cost of enzymes is one of the factors that determines the viability of the bioprocess; thus, the quest for highly efficient enzymes and

hyper-producing mutants could reduce these limitations, increasing the economic feasibility [15]. Cellulases and xylanases are regulated by complex mechanisms usually controlled by the carbon source in induction and repression phenomena [24, 29]. The presence of a readily metabolizable carbon source like glucose, cellobiose, xylobiose or xylose represses the synthesis of cellulase and xylanase enzymes destined to polysaccharide catabolism, thus increasing the cost of enzyme production [11, 14]. The main technological challenge is to avoid such regulatory mechanisms. There have been reports that concentrations higher than 1 mM reduce the expression of such catabolic enzymes [11]. There are several ways to decrease the cost of production by constructing efficient enzymes, isolating hyper-producer mutants, finding optimum culture conditions and using genetic engineering. Mutant isolation has shown extensive results, and using different screening methods and selection strategies allows obtaining a wide range of mutants with the desired improvement. These strategies are the key to cellulase and xylanase improvement [28]. Sugars like glucose and non-metabolizable analogs like 2-deoxyglucose (2DG) have been used as a selection strategy in mutagenesis, increasing enzyme activity and feedback resistance [3, 17]. However, many successful cases have been reported with mutants of *Trichoderma reesei* like Rut-C30 [3, 27]. Gram-positive bacteria are effective secretors of cellulolytic enzymes and present several advantages compared to fungi, such as a higher specific growth rate, higher productivities and easier manipulation [10, 17]. *Cellulomonas flavigena* is a gram (+) facultative bacteria extensively studied in our research group, and it is a well-known xylanase and cellulase producer. The enzymes produced can be recovered from the supernatant and then used in a wide range of industrial processes. The main purpose of this study was to obtain mutants of *C. flavigena* with enhanced and feedback-resistant enzyme activity for potential application in a saccharification process of sugarcane bagasse. The mutant was characterized for xylanase and CMCase production, and studies of repression by glucose and cellobiose were conducted.

Materials and methods

Microorganisms and growth conditions

Cellulomonas flavigena PN-120 strain is an improved mutant of *C. flavigena* wild-type [17] CDBB-531, and PR-22 is an improved mutant derived from the PN-120 strain. Cells were grown in 250-ml Erlenmeyer flasks that contained 50 ml of mineral medium [4, 15], 200 μl l^{-1} of organic non-silicon antifoam (Mazu DF-7911, Mazer, México), 0.02% (w/v) yeast extract and 1% (w/v) alkali-pretreated sugarcane bagasse as the carbon source [4] at 37° C and 150 rpm in an orbital shaker (New Brunswick Science, Edison, NJ). Cultures that were 24 h old (1.8 g cell protein ml^{-1}) in exponential growth phase were used to inoculate the bioreactors in a 10% (v/v) ratio.

Mutagenesis

Induction of mutants was carried out with *N-methyl-N′-nitro-N-nitrosoguanidine* (MNNG; Aldrich). A bacterial culture at the middle of the exponential growth phase was exposed to 150 μg ml^{-1} of MNNG for 60 min. The isolation of catabolite repression-resistant and hypercellulolytic mutants was based on the size of the hydrolysis zone around bacterial colonies. Selection agar plates contained 1% (w/v) carboxymethylcellulose (CMC; Sigma) plus 0.5% (w/v) 2DG (Sigma) and were revealed with Congo red staining [25]. Further experiments were carried out in 250-ml Erlenmeyer flasks containing sugarcane bagasse as sole carbon source to screen for hypercellulolytic mutants.

Repression and inhibition studies in batch cultures

Cells were grown in 500-ml stirred tank bioreactors (Sixfors, Infors) that contained 400 ml of mineral medium [17, 18], 0.02% yeast extract and 1% sugarcane bagasse (mesh 20) at 37°C, pH 7.2 and 0.5 vvm air flow. Glucose or cellobiose (10, 15 and 20 mM) was added at the beginning of the exponential growth phase (i.e., 8 and 12 h for PR-22 and PN-120 strains, respectively). Cultures without glucose or cellobiose were carried out in parallel as control. Samples were taken at different times until cultures reached 48 h and were stored at 4°C until further analysis.

Growth determination

Growth was measured as total cell protein by Lowry's method (1951) using bovine serum albumin (Sigma) as standard (0–200 μg protein ml^{-1}). At first the residual substrate was removed by filtration through a GD120 glass fiber filter disk (MFS, Dublin, CA). Afterwards, filtrate was centrifuged at 5,000 g × 10 min at 4°C. The cell pellet was washed twice and used to determine protein [9]. The specific growth rate (μ) was determined from the slope of the regression of a semi-logarithmic plot of protein biomass (g l^{-1}) versus time. Correlation coefficients of such regressions were higher than 0.98. Supernatants containing crude enzyme were concentrated ten-fold through a polyether-sulphone PM-30 membrane (Amicon, Beverly, MA) and used to determine soluble protein [9].

Enzyme assays

Carboxymethyl cellulase (CMCase) and xylanase activities were determined in supernatant samples according to previous reports [17]. Activities were assayed by measuring the released reducing sugars as cellobiose for CMCase and xylose for xylanase using the dinitrosalicylic acid (DNS) reagent [16]. For CMCase activity, a reaction mixture consisting of 0.5 ml of CMC (Sigma, 1% w/v, in Tris/HCl buffer pH 7.2) and 0.5 ml of the appropriately diluted crude enzyme was incubated for 3 min at 50°C. The xylanase activity reaction mixture consisting of 1 ml of citrate/phosphate buffer (50 mM, pH 7), 1 ml Birchwood xylan (Sigma, 1% w/v, in distilled water) and 1 ml of appropriately diluted crude enzyme was incubated for 5 min at 40°C. In both cases 3 ml of DNS reagent was added to stop the reaction. One unit of activity (U) is defined as the amount (μmol) of product obtained per minute under assay standard conditions. The maximum difference among the three values was less than 5% of the mean.

Enzyme kinetics

The linear regions of CMCase and xylanase activities were determined at different enzyme concentrations. CMC or xylan was used as substrate at different concentrations (0.2–3%), and K_m, V_{max} and K_i values were calculated from the slopes and intercepts of regression lines of Lineweaver-Burk plots [23]. Glucose or cellobiose was used as inhibitor at concentrations of 5, 10 and 15 mM. The assays were performed by triplicate. A model of mixed type competitive inhibition case C1 where $\alpha > 1$ and $\beta = 0$ (Eq. 1), non-competitive (Eq. 2) and competitive (Eq. 3) were used to adjust data obtained in inhibition experiments.

$$\frac{V}{V_{max}} = \frac{[S]}{K_S\left(1+\frac{[I]}{K_i}\right)+[S]\left(1+\frac{[I]}{\alpha K_i}\right)} \quad (1)$$

$$\frac{V}{V_{max}} = \frac{[S]}{K_S\left(1+\frac{[I]}{K_i}\right)+[S]\left(1+\frac{[I]}{K_i}\right)} \quad (2)$$

$$\frac{V}{V_{max}} = \frac{[S]}{K_S\left(1+\frac{[I]}{K_i}\right)+[S]} \quad (3)$$

Saccharification studies

Sugarcane bagasse was alkali-pretreated with 3% (w/v) NaOH at 90°C for 30 min. Afterwards, bagasse was filtered, pressed and washed several times with cold distilled water until bagasse pH was neutral [4]. Saccharification of alkali-pretreated sugarcane bagasse (mesh 60) [4] was carried out in Erlenmeyer flasks with concentrated crude enzyme extract of *C. flavigena* PR-22 in 50 mM citrate-phosphate buffer (pH 7). A general factorial experimental design, with two factors (substrate and enzyme activity) at four levels each, was proposed. The substrate was 0.5, 1.0, 2.0 and 3.0% (w/v). The concentrated enzyme extract used in saccharification studies was adjusted with citrate-phosphate 50 mM buffer (pH = 7) in order to obtain 50, 300, 600 and 1,000 U in 25 ml total reaction mixture. The process was carried out at room temperature or 55°C and 150 rpm. Fractional conversion is defined as the grams of actual total soluble sugar produced per practical grams of total sugar contained in alkali-pretreated sugarcane bagasse mesh 60.

Analysis of data

Several variables were evaluated in terms of a relative index with a form given by Eq. 4: production of soluble protein and volumetric or specific enzymatic activities. Positive values of the index mean an improvement of the results of the treatment or effect (glucose, cellobiose or strain) over that of the control, whereas negative values are interpreted as a reduction or inhibition of the outcome in a given treatment or effect compared to that of the control. The index of behavior (ξ) was defined as:

$$\xi = \left[\frac{\int_0^{t_f} f_p(t) - \int_0^{t_f} f_c(t)}{\int_0^{t_f} f_c(t)}\mathrm{d}t\right] \cdot 100 \quad (4)$$

where ξ represents either the protein index (PI) or the activity index (AI), $f_p(t)$ is either the time course of soluble protein or enzyme activity (CMCase or xylanase) of a given sample or "treatment," $f_c(t)$ ibidem for the control, *and* t_f is the end time of the incubation. The experimental curves represented by $f_p(t)$ and $f_c(t)$ were integrated by a numerical method based on Newton-Cotes integration formulas using Scientific WorkPlace 3.0 (Poulsbo, WA) [8]. The resistance factor (λ) that indicates the tolerance of a strain to the different treatments or effects is given by Eq. (5):

$$\lambda = 100 + \xi \quad (5)$$

Results

Mutant isolation

From the mutagenic treatment of *C. flavigena* PN-120, ten colonies were isolated by presenting higher hydrolysis zones in CMC-2DG plates than in the parent strain. These mutants were proved in submerged culture in 250-ml conical flasks in order to determine growth, CMCase and xylanase activities. Only one strain, called mutant PR-22,

improved its cellulolytic activity compared with the parent strain mutant PN-120. This mutant was used for further experiments in batch culture.

Kinetic parameters of PN-120 and PR-22 strains

Strains PR-22 and PN-120 were grown in batch culture under the same conditions in order to compare their kinetic parameters. The specific growth rates (μ) were 0.16 and 0.15 h^{-1} for PN-120 and PR-22, respectively, without a visible phase lag. Both strains reached similar cell density 1.9 g l^{-1} at 48 h. The extracellular protein production in the PR-22 strain was 0.65 g l^{-1}, and this represents a PI of 39% compared to that of the parent strain PN-120 (Table 1).

CMCase and xylanase activities

Regarding to enzyme activities, the maximum specific activity of xylanases reached in batch culture was 40 and 27 U mg^{-1} for PR-22 and PN-120 strains, respectively, while the maximum specific activity of CMCases was 4 and 1.9 U mg^{-1}, respectively (Table 1). The volumetric productivity also had an increase with an AI of 36% for xylanase (Q_{xyl}) and 230% for CMCase (Q_{cmc}) related to the parent strain, respectively (Table 1). The screening and selection strategy using 2-deoxyglucose allowed obtaining several putative mutants; however, only one of them, mutant PR-22, showed high CMCase production.

Effect of glucose addition

The addition of glucose to cultures growing in sugarcane bagasse increased the cell densities because of an additional carbon source (data not shown). The production of extracellular protein was slightly affected in both strains. When 10 and 15 mM of glucose was added, no significant PI was observed in mutant PR-22 compared to its own control (without glucose), whereas in its parent strain the PIs were −7 and −10% at the same conditions compared to its own control (Table 2). Mutant PR-22 at 20 mM glucose had a PI of −8% compared to its own control, whereas in PN-120 strain the PI was −12%. The xylanase activity of mutant PR-22 at 10 and 15 mM glucose did not have a significant AI, whereas at 20 mM glucose this was −41% compared to its own control. The PN-120 strain had an AI of −9, −21 and −49 at 10, 15 and 20 mM glucose in reference to its own control (Table 2). Xylanolytic activity of mutant PR-22 was more resistant to glucose than the parent strain PN-120 (Fig. 1b). On the other hand, the CMCase activity in the PR-22 strain was not significantly affected at 10 and 15 mM glucose, but 20 mM caused an AI of −28% compared to its own control. The PN-120 strain showed AIs of −20% at 10 and 15 mM glucose; this index increased to −32% when glucose was 20 mM respectively to its own control. CMCase activity of the PR-22 strain was more resistant than that observed in PN-120 (Fig. 1b).

Effect of cellobiose addition

The addition of 10 and 15 mM cellobiose to *C. flavigena* PR-22 cultures growing on sugarcane bagasse did not have a significant effect on their growth; however, when 20 mM of cellobiose was added, it reached a cell density of 2.6 g l^{-1} at 48 h. The growth curve presented a slightly diauxic behavior when cellobiose was added (data not shown). In these conditions, the soluble protein production in mutant PR-22 had PIs of −11, −17 and −17% when 10, 15 and 20 mM cellobiose was added to cultures. Its parent strain had PIs of −15, −19 and −25%, respectively, in the same conditions (Table 2). The volumetric xylanase activity was significantly affected in both mutants (Fig. 1a). When 10 mM cellobiose was added AIs were −26 and −36% for PR-22 and PN-120 strains, respectively. At 15 and 20 mM cellobiose, higher AIs were observed in both strains (Table 2). It is worth mentioning that the AI observed in all cellobiose concentrations assayed for the PN-120 strain were higher than those observed for mutant PR-22 (Fig. 1a). The specific xylanolytic activity of mutant PN-120 decreased considerably, and this was not reestablished. However, the specific xylanolytic activity in PR-22 cultures was slightly recovered 12 h after the addition of any of the cellobiose

Table 1 Kinetic parameters of *Cellulomonas flavigena* strains PR-22 and PN-120

Strain	Growth rate (h^{-1})	Protein (g l^{-1})		Specific enzyme activities (U mg^{-1})		Volumetric enzyme productivity (U l^{-1} h^{-1})	
	μ	X	E	Xyl	CMC	Xyl	CMC
PN-120	0.16 ± 0.001	1.90 ± 0.18	0.50 ± 0.01	26.9 ± 0.17	1.9 ± 0.02	337 ± 1.9	11.51 ± 0.90
PR-22	0.15 ± 0.002	1.89 ± 0.16	0.65 ± 0.04	40.0 ± 0.57	4.0 ± 0.05	458 ± 3.1	38.00 ± 0.01
(ξ)*	−6.2	−0.5	39	36	190	36	230

* The generic index (ξ) represents improvement when the value is positive and repression or inhibition when it is negative for enzyme activities, whereas the production index (PI) is used in the case of secretion of soluble protein

Table 2 Behavioral index in soluble protein (PI), xylanase activity (AI) and CMCase activity (AI)

Mutant	Soluble protein			Xylanase			CMCase		
	10 mM	15 mM	20 mM	10 mM	15 mM	20 mM	10 mM	15 mM	20 mM
PR-22									
Glucose	0	0	−8	0	0	−41	0	−3	−28
Cellobiose	−11	−17	−17	−26	−36	−45	−35	−34	−31
PN-120									
Glucose	−7	−10	−12	−9	−21	−49	−20	−20	−32
Cellobiose	−15	−19	−25	−36	−45	−61	−27	−36	−45

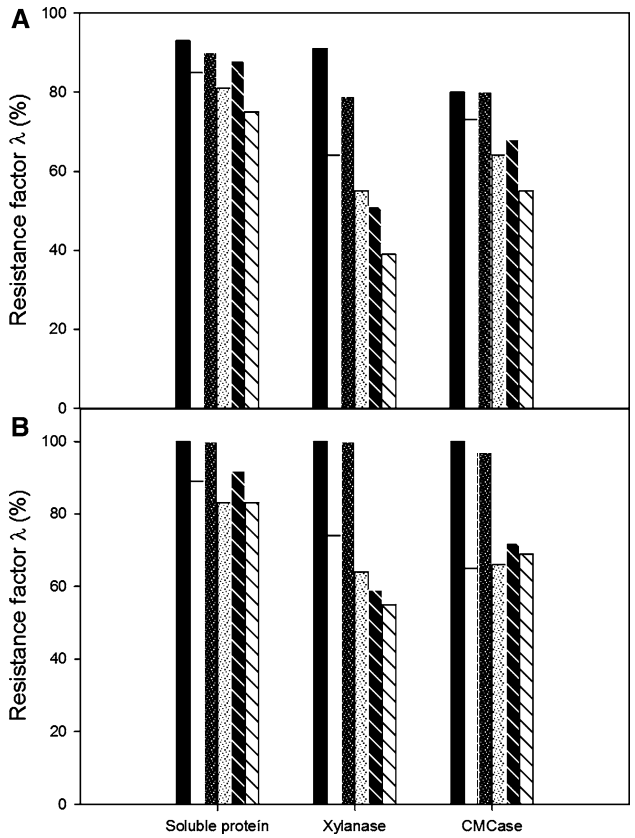

Fig. 1 Resistance factor of soluble protein, CMCase and xylanase production of PR-22 (*black*) and PN-120 (*white*) strains of *Cellulomonas flavigena* growing on sugarcane bagasse added to cellobiose **a** or glucose **b**; *flat pattern* (10 mM), *dotted pattern* (15 mM) and *dash pattern* (20 mM)

concentrations. Regarding the CMCase activity, the AIs in mutant PR-22 were very similar (−35 to −31%) for all the concentrations of cellobiose assayed (10, 15 and 20 mM) (Table 2). In contrast, the parent strain PN-120 exhibited increasing inhibition of CMCase activity with increasing cellobiose concentration, i.e., the AI from −27 to −45% when the cellobiose concentration increased from 10 to 20 mM. Despite the low activities presented in PR-22 cultures under repression conditions, these were always higher than those observed in its parent strain PN-120. When specific CMCase activities were analyzed, the resistance to feedback inhibition in mutant PR-22 was evident (Fig. 1a).

Inhibition studies with the PR-22 enzymatic complex

The effect of glucose and cellobiose on the activity of CMCase and xylanase from mutant PR-22 was studied. Either glucose or cellobiose was added at 5, 10 and 15 mM. The linear region of xylanases and CMCases was made with concentrations of substrate from 0.2 to 3%. When xylanases were assayed, glucose caused a mixed inhibition, whereas cellobiose caused a non-competitive inhibition. In CMCase activity glucose and cellobiose showed a competitive inhibition (Table 3).

Saccharification studies

During the enzymatic saccharification of alkali-pretreated sugarcane bagasse, different enzyme and substrate concentration ratios were assayed. Saccharification at room temperature could not accumulate more than 4 g l^{-1} of total soluble sugars when 1,000 U and 3% (w/v) of substrate were assayed; however, the process did not require additional power besides the agitation (Fig. 2a). When the hydrolysis temperature increased, the best fractional conversion (0.89 g g^{-1}) was obtained at 1,000 U and 0.5% (w/v) of sugarcane bagasse, whereas the highest concentration of total soluble sugars (12 g l^{-1}) was obtained with 1,000 U and 3% (w/v) of sugarcane bagasse at 55°C (Fig. 2b).

Discussion

One of the limitations of the process of producing cellulases and xylanases with microorganisms is the catabolite repression phenomena presented when soluble sugars are accumulated in the culture media [11, 14, 17]. In the saccharification process of lignocellulosic residues, the inhibition of cellulase and xylanase activities reduces the

Table 3 Kinetic parameters of the concentrated crude enzyme

Parameter	Xylanase							CMCase						
	Control	Glucose (mM)			Cellobiose (mM)			Control	Glucose (mM)			Cellobiose (mM)		
		5	10	15	5	10	15		5	10	15	5	10	15
K_m (mg ml^{-1})	5.3	6.7	10.1	12.8	9.1	5.2	5.2	1.5	2.7	3.9	5.0	3.6	4.8	11.6
V_{max} (U mg^{-1})	52.6	51.8	48.3	42.2	52.1	34.7	25.8	3.8	3.7	3.6	3.7	3.9	3.8	4.7
Ki (mg ml^{-1})	–		6			4.7		–		0.7			0.21	
α	–		10.2			–		–		–			–	

The experiments were performed in triplicate in independent experiments; standard deviations are less than 5%

(–) Not applicable

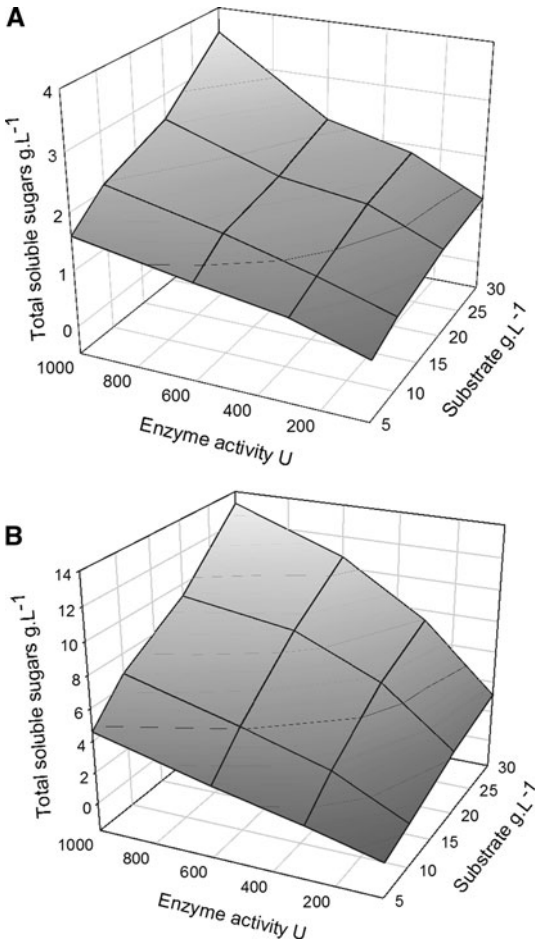

Fig. 2 Effect of different enzyme concentrations, substrate loads and temperature on the saccharification process of sugarcane bagasse. **a** Room temperature; **b** 55°C

soluble sugar accumulation, thus increasing the need for high enzyme loads and production costs, and reducing the economic viability. Derepressed mutants with high enzymatic activity can help to reduce the limitations of cellulose hydrolysis and costs in the process of bioconversion of plant biomass to ethanol. The mutagenic treatment of *Cellulomonas flavigena* has been successfully applied to finding different types of mutants in our research group [15, 17, 18]. It has been found that 0.1% of glucose and cellobiose significantly increases catabolic inhibition and repression in *C. flavigena* wt [18]. Partial derepressed mutants of *Cellulomonas flavigena* PN-120 were sought for high production of xylanase, cellulase and β-glucosidase activities even in the presence of glucose or cellobiose (1%). Then the screening selection strategy using 2-deoxyglucose allowed obtaining several putative mutants; however, mutant PR-22 showed higher CMCase production in CMC agar plates. Mutant PR-22 significantly increased cellulolytic activity and resistance to inhibition phenomena and catabolic repression after mutagenic treatment of the PN-120 parent strain.

Cellulolytic and xylanolytic activities have been improved by mutagenesis in several microorganisms. *Clostridium thermocellum* enhanced CMCase activity from 0.25 to 1 U ml^{-1} [29]. The xylanolytic activity of *Cellulomonas biazotea* was slightly increased from 8 to 10 U ml^{-1} when mutated with gamma rays in a Co-60 irradiator [19]. Mutant PR-22 increased the specific xylanolytic activity almost twice compared to that in *C. flavigena* wt when grown in sugarcane bagasse [17]. Mutant PR-22 increased the CMCase volumetric productivity by 3.3 compared to its parent strain (PN-120), and this volumetric productivity was 1.7 times higher than that observed in *Cellulomonas biazotea* [19]. The partial derepressed characteristic of mutant PR-22 was demonstrated when even at 20 mM glucose added to cells growing on sugarcane bagasse could produce xylanolytic and cellulolytic enzymes. In *Cellulomonas flavigena* wt, 10 mM glucose represses the synthesis of xylanases and CMCase completely [18]. The CMCase activity of *Fibrobacter succinogenes* subsp. *succinogenes* S85 reached 0.07 U ml^{-1} when 16 mM glucose was added to cultures growing in cellulose [6]. This activity was 95% less than that reached by strain PR-22 under similar conditions. On the other hand, the effect of cellobiose was evaluated over the biosynthesis of CMCase and xylanase enzymes in both strains PN-120 and PR-22 grown in sugarcane bagasse.

Concentrations of cellobiose higher than 50 mM repressed the biosynthesis of soluble protein in both strains. However, this repression was more evident in PN-120 strain. In *Aspergillus terreus* the synthesis of cellulases and xylanases was completely repressed after addition of 20 mM cellobiose [1]. Cellobiose in low concentrations stimulates the production of cellulases and in some cases xylanases [12, 20]. In *Agaricus bisporus* growing on CMC, 5.5 mM of glucose or cellobiose repressed its enzymatic system [13]. The CMCase activity in *Clostridium thermocellum* decreased 61.5% at 11.1 mM cellobiose [7].

Our derepressed mutant PR-22 held 65% of its xylanolytic activity at 20 mM cellobiose, whereas in microorganisms such as *Clostridium stercorarium*, 25 mM of this sugar inhibited the cellulase activity by 96% [2]; in *Coniophora puteana* it decreased by 63% at 6 mM cellobiose [22], and in *C. flavigena* wt the same activity decreased 92% at 10 mM cellobiose [18]. Cellobiose has been reported to be an inhibitor of cellulase activity in several cellulolytic microorganisms [10]. The CMCase activity in mutant PR-22 had an AI of −31% at 20 mM cellobiose, whereas in *Trichoderma koningii* 29 mM cellobiose inhibited this activity by 79% [5].

The xylanolytic activity showed a mixed-type and noncompetitive inhibition when glucose and cellobiose were added to enzymatic crude extract of mutant PR-22 (Table 3), whereas CMCase activity was inhibited by glucose and cellobiose in a competitive way (Table 3). Glucose and cellobiose have been reported to be moderated inhibitors of cellulolytic and xylanolytic activities [13, 18]. In *Clostridium thermocellum* glucose showed slight inhibition of its cellulolytic activity. In contrast, glucose and particularly cellobiose at high concentrations significantly inhibited the cellulase of *Trichoderma reesei* [26].

Saccharification of alkali-pretreated sugarcane bagasse was performed using the cellulolytic and xylanolytic complex of PR-22 strain. The fractional conversion obtained in our research is below those reported (0.95) in roller bottle reactors (RBRs) with high substrate and enzyme loads, novel pretreatment techniques and commercial enzyme mixtures [21]. Despite low fractional conversion in high substrate loads, using inhibition-tolerant enzymes in a process where the substrates are mildly pretreated is an approach [4]. It is worthy of mention that the process of saccharification was made by a mixture of enzymes containing cellulases and hemicellulases without purification. Purification costs can be reduced, increasing the economic feasibility of the saccharification process. The use of high inhibition-tolerant enzymes in the saccharification of lignocellulosic materials allows greater accumulation of soluble sugars, thus allowing viable novel bioprocesses.

In conclusion, *C. flavigena* PR-22 proved to be a hyperproducer and partially derepressed mutant for cellulase and xylanase enzymes growing in sugarcane bagasse. It could increase the production and activity of enzymes (xylanase and CMCase) even under catabolic repression conditions. Due to these characteristics, this enzyme complex may be used in saccharification of lignocellulosic biomass in order to obtain bioethanol as an immediate substitute for the exhausted oil.

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