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Trichoderma atroviride 102C1 Mutant: A High Endoxylanase Producer for Assisting Lignocellulosic Material Degradation

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Abstract

Endoxylanases have played an important role in many industrial processes as bleachers to kraft pulp, animal feeds and baked goods. Also, nowadays, a special attention has been devoted to the role of these enzymes in saccharification of lignocellulose biomass for biofuels production. Trichoderma species are among fungi those that have been most extensively studied, due to their efficient production of these enzymes. Among the different strategies for improving the production and biochemical aspects of enzymes of commercial interest, mutations induced using chemical agents and/or physical devices can be cited. In the present strain T. atroviride 102C1 was obtained by using UV light and nitrosoguanidine as mutagenic agents. A factorial design (central composite rotational design, CCRD) was performed to estimate the optimal levels of C (sugarcane bagasse) and N (corn steep liquor) sources for best xylanase production. After the CCRD, the 102C1 mutant strain showed increased activity of 340% for xylanase production when compared to the wild type. The enzyme was partially characterized according to its pH and temperature profile, also using CCRD. The characterization of 102C1 mutant strain as a high endoxylanase producer allows its use in biotechnological applications, particularly in the hydrolysis of lignocellulosic biomass for biorefinary proposes.

Keywords: *Trichoderma atroviride* mutant; Endoxylanase; Submerged fermentation; Sugarcane bagasse

Introduction

Xylanases are used in industrial applications such as in pulp pre-bleaching processes, as food and feed additives, in bakery processing, and in xylitol production, among others [1]. However, a great attention has been devoted to its utilization in hemicellulosic biomass degradation, aiming at production of biofuels.

Lignocellulosic biomass consists of cellulose, hemicellulose and lignin and these materials account for approximately 50% of the biomass in the world. The polysaccharides (cellulose and hemicellulose), on average, accounts for 55-75% on dry weight basis in plant cell wall and can be deconstructed into simple sugars, which further can be fermented to alcohols, such as ethanol and butanol, organic acids, acetone and glycerol [2]. Degradation and conversion of lignocellulosic biomass are attracting attention because of its potential for the development of a sustainable and environmentally friendly bioenergy, biorefining and biomaterials industry [3]. The lignocellulosic residues, which don't compete with food demand, provide a low cost feedstock for production of fuels and commodity chemicals, and thereby can offer economic, environmental and strategic advantages. Sugarcane bagasse (SB) is one of the abundantly available low-cost plant residues, which could be used for production of biofuels [2,4]. Moreover, corn steep liquor, a by-product of the large-scale corn milling industries is also a substrate that is inexpensive and widely available. This substrate has also being used as an efficient substitute for yeast extract and a rich source of nutrients such as organic nitrogen and vitamins for microbial culturing [5,6].

Hemicelluloses consist of heteropolymers such as xylan, glucomannan, galactoglucomannan and arabinogalactan. Xylan, the second most abundant polysaccharide in plants, is a linear polymer composed of D-xylose units linked by glycosidic bonds and is the major hemicellulose present in plants [3]. The enzymatic hydrolysis of xylan requires the cumulative actions of endo-beta-1,4-xylanase (EC 3.2.1.8), beta-xylosidase (EC 3.2.1.37), and a series of enzymes that degrade side chain groups. Among these, endo-beta-1,4-xylanase is the most crucial enzyme that cleaves glycosidic bonds to produce short chain xylooligosaccharides of various lengths [7]. Synergic effects of alfa-arabinofuranosidase (EC 3.2.1.55), beta-D-glucuronidase (EC 3.2.1.1), and acetyl xylan esterase (EC 3.2.11.6) are required to release the side chains of xylan. However, two enzymes, namely beta-1,4-endoxylanases (EC 3.2.1.8) and beta-xylosidase (EC 3.2.1.37), are sufficient to degrade the xylan back-bone [1,7].

T. atroviride is, among microorganisms, a species that shows excellent potential for secretion of an enzyme complex able to perform an efficient enzymatic conversion of biomass into ethanol [8,9]. Among the different strategies for improving the production and biochemical

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aspects of enzymes of commercial interest, mutations induced using chemical agents and/or physical devices have been cited in literature [8]. It is well known that mutants of *T. atroviride* are able to secrete high cellulase titers in both submerged and solid state fermentation [8,9]. However, as far as we are concerned, there is no report concerning the use of mutagenic agents to obtain endoxylanolytic fungi mutants. In the present study a mutant strain of *T. atroviride* 676 (strain 102C1, previously obtained in our laboratory) was studied for endoxylanase production. Being the use of agroindustrial residues an excellent strategy for a low-cost enzyme production, a central composite rotational design (CCRD) experiment was performed to estimate the optimal levels of sugarcane bagasse as C source and corn steep liquor as N source in endoxylanase production.

Materials and Methods

Microorganisms

Trichoderma atroviride 102C1 was obtained from the wild strain *T. atroviride* 676, aiming at, originally, the selection of cellulolytic strains (data not shown). The procedure to obtain the mutant strain was carried out according to Kovàcz et al. [8], with some modifications. The mutation involved two subsequent exposures to nitrosoguanidine as the mutant agent. In the first one 2.0 mL of a sterile solution of nitrosoguanidine 1% were added to 100 µL of a spore suspension of *T. atroviride* 676 and the system incubated for 8 minutes. The spore suspension thus obtained was streaked in a malt extract-yeast extract-agar plate. After incubation at 28°C for 7 days, the growing colonies were tested for cellulase activity and strain NTG21 selected. Then, for selection of 102C1, the same procedure was performed using spores from NTG21, however the time of exposure was 15 minutes. Although this mutation and selection was towards cellulase activity, the wild strain has shown to be, also, a good endoxylanase producer [10]

Spore suspensions were prepared according to Hopwood et al. [11] after cultivation (28°C/15 days) in yeast extract-malt extract-agar medium [12] and maintained as stock cultures in 20% (v/v) glycerol at -20°C. Spore concentration was determined using Neubauer counting chamber.

Production of endoxylanases

The enzyme production was performed in submerged fermentation in Erlenmeyer flasks with 1/5 of its volume of a culture medium based on the salt solution plus urea described by Mandels and Weber [13] and added of sugarcane bagasse (SB-main carbon source) and corn steep liquor (CSL-main nitrogen source). The optimization of SB and CSL concentration was carried out by employing a response surface methodology having endoxylanase activity ($U.mL^{-1}$) as the dependent variable and C source (SB) and N source (CSL) concentrations as the independent variables. A 22 full factorial central composite rotational design (CCRD) was used in order to generate 11 run combinations as described in Table 1 [14]. This design is represented by a second-order polynomial regression model, Eq. (1), to generate contour plots:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_{12}X_1X_2 + b_{11}X_1^2 + b_2^2X_2^2 \quad (1)$$

where, Y is the predicted response (endoxylanase activity); X_1 and X_2 the coded forms of the input variables (SB and CSL, respectively); b_0 a constant; b_1 and b_2 the linear coefficients; b_{12} a cross-product coefficient; b_{11} and b_{22} the quadratic coefficients. The test factors were coded according to the following regression equation:

$$x_i = (X_i - X_0)/\Delta X_i \quad (2)$$

Run	Coded setting levels		Actual levels %(w/v)		Endoxylanase activity ($U.mL^{-1}$)	
	X_1	X_2	X_1	X_2	O	P
1	– 1	– 1	1.5	0.3	99.70	109.84
2	+ 1	– 1	3.5	0.3	50.80	69.80
3	– 1	+ 1	1.5	1.1	147.28	132.94
4	+ 1	+ 1	3.5	1.1	266.30	260.83
5	– 1.41	0	1.09	0.7	105.41	109.35
6	+ 1.41	0	3.91	0.7	179.93	171.29
7	0	– 1.41	2.5	0.15	91.79	72.08
8	0	+ 1.41	2.5	1.26	208.03	223.04
9	0	0	2.5	0.7	241.41	241.67
10	0	0	2.5	0.7	242.58	241.67
11	0	0	2.5	0.7	240.97	241.67

Results are the mean of two experiments; O observed, P predict

Table 1: Values of independent variables (SB concentration X1, and CSL concentration X2), used in CCRD, showing the values observed and predicted by the mathematical model for endoxylanase production by *Trichoderma atroviride* 102C1.

where, x_i is the coded value and X_i the actual value of the independent variable, X_0 the actual value at the center point and ΔX_i is the step change value. Data analysis was performed using the Statistica 7.0.

The initial pH of all media was adjusted to 4.8. The incubation was performed at 28°C in orbital shaking at 200 rpm for 5 days. The supernatants, which correspond to crude enzyme extracts, were used to determine the activities of endoxylanases. Assays were performed varying the C and N sources according to the matrix shown in Table 1.

Enzyme assays

Endoxylanase (EC 3.2.1.8.) activity was estimated by reaction mixture containing 750 µL of a solution of 1.0% (w/v) oat spelts xylan (SIGMA®) in 50 mM sodium citrate buffer (pH 5.3) plus 0.25 µL of the supernatant. This system was incubated for 10 min at 50°C. The reducing sugars concentration in the reaction mixture was determined by the dinitrosalicylic acid (DNS) method [15]. One unit (U) of enzymatic activity corresponded to 1 µmol of xylose equivalents released per minute, under the assay conditions [16].

All assays were performed in duplicates, and results were expressed as average values. Variations in the multiple assays were <10%.

Crude enzyme partial characterization

A culture supernatant of 3-days fermentation [SB 3.5% (w/v) and CSL 1.1% (w/v)] was used to investigate the temperature and pH effect on endoxylanase activity. The enzyme characterization was carried out by employing a response surface methodology having endoxylanase activity ($U.mL^{-1}$) as the independent variable and pH (between 3.0 and 7.0) and temperature (range of 40-70°C) as the dependent variables. A 22 full factorial central composite rotational design (CCRD) was used in order to generate 11 run combinations as described in Table 2 [14]. This design is represented by a second-order polynomial regression model (as Equation 1, where Y is the predicted response endoxylanase activity; and X_1 and X_2 the coded forms of the input variables, pH and temperature, respectively) and the test factors coded according to Equation 2. Buffer solutions at 50 mM, where: sodium citrate buffer for pH 3.0, 3.6 and 5.0, and phosphate buffer for pH 6.4 and 7.0 and were used at the optimal temperature previously determined. Data analysis was performed using the Statistica 7.0.

Zymogram

The culture supernatant from cells grown on the best conditions

Run	Coded setting levels		Actual levels		Endoxylanase activity (U.mL^{-1})	
	X_1	X_2	X_1	X_2	O	P
1	– 1	– 1	3.6	44	158.90	122.36
2	+ 1	– 1	6.4	44	144.54	108.42
3	– 1	+ 1	3.6	66	17.79	29.54
4	+ 1	+ 1	6.4	66	52.79	64.98
5	– 1.41	0	3.0	55	19.82	32.38
6	+ 1.41	0	7.0	55	35.59	47.53
7	0	– 1.41	5.0	40	125.70	172.21
8	0	+ 1.41	5.0	70	98.15	76.14
9	0	0	5.0	55	205.88	205.20
10	0	0	5.0	55	204.24	205.20
11	0	0	5.0	55	205.78	205.20

Results are the mean of two experiments; O observed, P predict

Table 2: Values of independent variables (pH X1 and temperature X2), used in CCRD, showing the values observed and predicted by the mathematical model for endoxylanase activity characterization.

Sources of variations	Sum of squares	Degrees of freedom	Mean square	F value	P value (prob>F)[a]
Model	54746.65	5	10949.33	38.29	0.0005
Residual	1429.97	5	285.99		
Lack of fit	1428.58	3	476.19	687.71	0.0015
Pure error	1.38	2	0.69		
Total	56176.62	10			

[a]Statistically significant at 90% of confidence level; R2=0.9745

Table 3: Statistical ANOVA for the model of endoxylanase production at different levels of concentration of SB and CSxL.

was analyzed by electrophoresis on denaturing 10% sodium dodecyl sulfate (SDS)–polyacrylamide gel, copolymerized with 0.1% (w/v) oat spelts xylan (Sigma) as substrate. Electrophoresis was performed at constant voltage (90 V) at 4°C for 3 hours. After electrophoresis, gel was incubated with Triton X-100 sodium acetate 1.0% for 60 min in ice-bath for SDS removal, and then incubated with 50 mM sodium citrate buffer pH 5.0 for 10 minutes at 55°C. For endoxylanase activity detection, the gel strip was submerged in 0.1% (w/v) Congo Red solution for 10 min., and then washed with 1M NaCl solution until visualization of enzyme bands [17]. Molecular masses were calculated from mobility of standards ranging from 24 and 225 kDa (HMW electrophoresis calibration kit–Pharmacia Biotech).

Results

Enzyme production in agroindustrial residues

The kinetic profile of enzymatic production of *T. atroviride* 102C1 in liquid media, containing the combination of different concentrations of SB and CSL was obtained after cultivation under agitation, at 28°C for 5 days (data not show). The maximal values of endoxylanase activity were obtained on the third day fermentation process and analyzed statistically. Endoxylanase activity varied from 50.8 to 266.3 U.mL^{-1}. The best result was obtained on run number 4, where SB and CSL concentrations were 3.5% (w/v) and 1.1% (w/v), respectively.

The model was tested for adequacy by the ANOVA (Table 3). For SB+CSL combination (Table 1), the computed F value (38.29) indicates that the model was significant at a high confidence level. The probability P value was also very low (<0.1) indicating the significance of the model. The coefficient of variation (R^2=0.97) also indicates a very good correlation between the experimentally observed and predicted values. The mathematical model representing the endoxylanase activity (Y) for the combination SB + CSL in the experimental region studied can be expressed by Equation (3).

$$Y=241.67+21.96^{*}X_1-50.98^{*}X_1^{2}+53.53^{*}X_2-47.34^{*}X_2^{2}+41.98^{*}X_1X_2+0.69243 \quad (3)$$

The independent variable SB concentration had a significant effect on the interactions. The regression analysis for the experiment using the combination SB + CSL, Equation 3, show the significant coefficients of the full second-order polynomial model of endoxylanase production, determined by Student's T test and P values.

The resulting surface response plots showing the effect of substrate concentration on the endoxylanase production by *T. atroviride* 102C1 is presented in Figure 1. Besides maximum endoxylanase activity, observed at 3.5% SB and 1.1% CSL, the surface response suggests a second good level of enzyme activity (242.58 U.mL^{-1}) in central area (2.5% SB and 0.7% CSL).

Optimal pH and temperature for enzymatic activity

The pH and temperature profiles for endoxylanase activity were obtained from crude enzymatic extract of *T. atroviride* 102C1 grown in the optimal conditions: 3 days incubation, 3.5% (w/v) SB and 1.1% (w/v) CSL. According to the data presented in Table 2, the maximum endoxylanase activity of 205.9 U.mL^{-1} was observed at 55°C and pH 5.0 (Figure 2). The analysis of the resulting surface response plots revealed that the maximal endoxylanase activity occurred in acidic conditions as well as higher temperatures.

The model was tested for adequacy by the ANOVA (Table 4). The model F-value of 12.46 indicates that the model was significant at a high confidence level. The probability P value was also very low

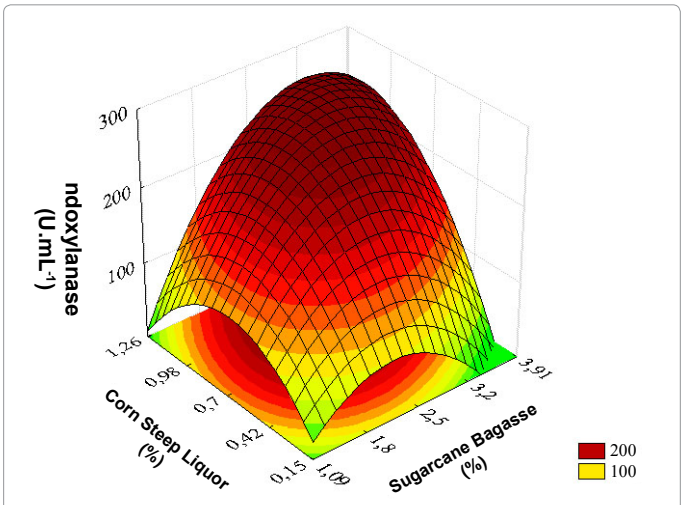

Figure 1: Response surface for endoxylanase production by *T. atroviride* 102C1 using SB and CSL concentration as dependent variables. The full factorial central composite design (2^2) used response surface methodology to predict the best point for endoxylanase production. The values are shown in Table 1.

Sources of variations	Sum of squares	Degrees of freedom	Mean square	F-value	p-value (prob>F)[a]
Model	50292.00	4	12573.00	12.46	0.0046
Residual	6054.65	6	1009.11		
Lack of fit	6052.97	4	1513.24	1793.64	0.0006
Pure error	1.69	2	.84		
Total	56346.65	10			

[a]Statistically significant at 90% of confidence level; R^2=0.8925

Table 4: Statistical ANOVA for the model of endoxylanase activity at different levels of pH and temperature.

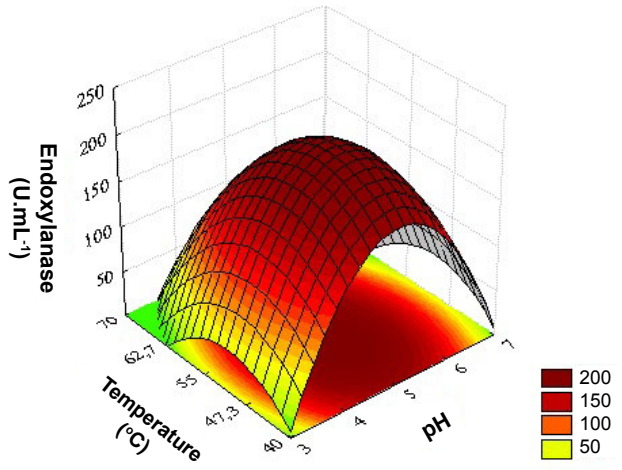

Figure 2: Response surface on partial endoxylanase characterization from *T. atroviride* 102C1 using pH and temperature as independent variables. The full factorial central composite design (2^2) used response surface methodology to predict the best point for endoxylanase activity. The values are shown in Table 2.

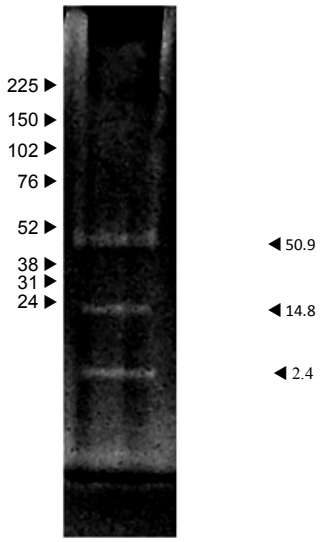

Figure 3: Zymogram analysis of endoxylanase activity in the supernatant of *T. atroviride* 102C1 cultures grown on best condition. The amounts loaded in the gel contained 150 mU of endoxylanase activity. The gel containing the MW markers was stained for proteins using the silver staining method and values for the MW markers are shown on the left side of the Figure. On the right side are shown the apparent molecular mass of endoxylanases bands.

(<0.1) indicating the significance of the model. The coefficient of determination adjusted obtained (R_2=0.89) indicates that 88.9% of the variability of the responses can be explained by the model.

The regression equations, obtained after the ANOVA, demonstrated endoxylanase activity as a function of the codified values of pH and temperature. The mathematical model representing the endoxylanase activity (Y) under these conditions can be expressed by Equation (4).

$$Y=205.20-83.12X_1^2-34.06X_2-40.76X_2^2 + 0.84367 \quad (4)$$

The graphic of response surface (Figure 2) suggests that central area was the best condition. Based on these results, it can be stated that *T. atroviride* 102C1 produces a thermoacidophilic endoxylanase.

Detection of xylanase activities through zymograms

The zymogram analysis of the culture supernatant of *T. atroviride* 102C1 obtained in culture medium after growth in the optimal conditions (3 days fermentation, 3.5% SB and 1.1% CSL) was shown in Figure 3. Three protein bands with endoxylanase activity and estimated molecular masses of 50.9, 14.8 and 2.4 kDa are clearly shown.

Discussion

Endoxylanases represent an enzymatic alternative strategy for access to cellulosic material, which is protected by hemicellulose and lignin in biomass. The activity of endoxylanases culminates in the generation of pentoses/hexoses, as well as other chemical compounds (acetyl and methyl groups, glucuronic acid, etc) which can be applied in various chemical industries, including bioenergy. In the present study *T. atroviride* 102C1 was tested over 5 days of fermentation for endoxylanase production. The highest production was observed on the 3rd day, where values of 266.3 U.mL^{-1} of endoxylanase activity were obtained and C and N concentrations were SB 3.5% (w/v), and CSL 1.1% (w/v), respectively.

Endoxylanase activity is generally reported in much lower values in literature for other Trichoderma strains, especially when low-cost residues are used as substrates. For instance, Silva and Carmona [18], working with *T. inhamatum* used various lignocellulosic residues in culture medium composition which resulted in enzyme production between 0.3 and 11.37 U.mL^{-1}. When SB 1.0% (w/v) was the main carbon source a production of 3.38 U.mL^{-1} of xylanase was obtained. Pretreated willow, corn stover and pine, were tested as C sources for cellulases and endoxylanases production by *T. reesei* RUT C30, showing endoxylanase activity between 1.0 and 2.3 U.mL^{-1} [19]. In a similar study also using *T. reesei* RUT. Olsson et al. [20] observed 0.07 U.mL^{-1} of endoxylanase activity using beet pulp for production of cellulases and endoxylanases. Delabona et al. [21] described levels of 36.96 U.mL^{-1} of xylanase from Trichoderma harzianum P49P11 grown in steam-pretreated bagasse, pH 6.0, after 96 hours. Liu et al. [22] obtained higher values, between 245.0 and 300.0 U.mL^{-1} for endoxylanase activity, but this was obtained after molecular vectors from *Trichoderma reesei* QM9414 were express in *E. coli*.

As far as we are concerned, there are no other citation in literature dealing with endoxylanase production when using *T. atroviride*, except in an earlier study by our group, where *T. atroviride* 676 wild type has shown ability to produce endoxylanase in amounts of 61.3 U.mL^{-1}, using SB 3.0% and CSL 0.3% as C and N sources, respectively [10]. In the present research, when mutant 102C1 was used, a 430% increase in enzyme activity was observed, compared to the results thus reported. In addition, the high production of endoxylanase by mutant 201C1 occurred in the presence of sugarcane bagasse "in natura" (untreated), as observed earlier by us with the wild type 676 [10] which represents, as already stated, an economical cost-wise advantage, considering the elimination of time and efforts needed for material processing. The results here obtained for *T. atroviride* 102C1 are better than, or similar to, those observed in literature for other fungi, as *Penicillium* sp. [23], *Aspergillus* sp. [24] and *Achaetomium* sp. [25].

According to the results of endoxylanase produced by *T. atroviride* 102C1, the enzymatic activity can be characterized as thermoacidophilic. The pH and temperature profile results thus obtained, with optimum pH and temperature of 5.0 and 55°C, respectively, are quite similar to those observed for other thermoacidophilic endoxylanases as those described for *Fusarium proliferatum* grown on xylan, where optimal

activity was between pH 4.5 and 6.0, and temperature at 55°C [26]. Another study concerning *Aspergillus fumigatus* SK1, growing on oil palm trunk, observed the optimal temperature and pH for endoxylanase activity as being 60°C and pH 4.0, respectively [24]. Anthony et al. [27] reported higher optimal values of temperature and pH for endoxylanases produced by *Aspergillus fumigatus* AR1, using xylose or xylan as substrate: optimal temperature between 60 and 65°C and the optimal pH between 5.5 and 6.5. Lower optimal temperature (between 45 and 50°C) and higher optimal pH (between 5 and 6) was observed for *T. inhamatum* when xylan was the substrate [18]. Trevizano et al. [28] obtained xylanolytic mutant strains from Orpinomyces sp with optimal temperature for enzymatic production between 60 and 70°C and pH range 5.0 to 8.0. Zhao et al. [25] observed, for an endoxylanase produced by *Achaetomium* sp. Xz-8, an optimal temperature between 70 and 80°C and pH between 5.0 and 6.0. So, as can be seen, results from different endoxylanase produced by different fungi grown on different substrates have different ranges of optimal temperature and pH being endoxylanase activity from mutant *T. atroviride* 102C1 grown in SB and CSL considered, in a broad range, similar to some of those already reported. The mild thermoacidophilic characteristic observed in endoxylanase activity from *T. atroviride* 102C1 could be very interesting when concerning biotechnological process.

The three bands observed in the zymogram for endoxylanase activity presented relatively low apparent molecular masses (50.9, 14.8, and 2.4 kDa), which is in accordance with most fungal endoxylanases described in the literature [10,22,24,25]. Studies with *T. reesei* have reported two bands with apparent molecular masses of 19 and 20 kDa [29], and 23 and 32 kDa [30]. According to Chen et al. [31] only one endoxylanase (37.7 kDa) was observed for T. longibrachiatum and Ang et al. [24] detected three endoxylanase (45.7 kDa, 39.8 kDa and 18.2 kDa) produced by *Aspergillus fumigatus* SK1. Considering the wild strain, *T. atroviride* 676, three different bands were found earlier by our group, with apparent values of 55.7, 36.0, and 23.0 kDa [10], which suggests that different enzymes were expressed in the mutant strain. Zhao et al. [25] detected two bands between 43 and 55 kDa produced by *Achaetomium* sp. Xz-8.

It is interesting to note that although three different enzymes were detected for endoxylanase activity produced by *T. atroviride* 102C1, it is not possible, with the data here obtained, to predict which of them is being responsible to each enzymatic activity produced. It could be all three together, as well as just one, favorable to the set of conditions of a given experiment.

Residue waste with biomass high-energy value is constantly generated by a variety of activities such as processing of agricultural products and by the paper and timber industries. However, many of these residues are difficult to be degraded and become an environmental problem. Hydrolysis of lignocellulosic biomass may play an important role enhancing the utilization of such residues. Thus, the identification of fungal strains producing high levels of endoxylanases might contribute for the use of lignocellulosic residues towards a variety of goals. In our study we used sugarcane bagasse, an abundant material, especially in Brazil, with low commercial value, as the main carbon source, and corn steep liquor, also a low-cost residue, as the main nitrogen source, to cultivate a mutant fungus strain that is a high endoxylanase producer.

While the fermentation of corn starch or sugar cane juice by *S. cerevisiae* is a well-established technology, the hydrolysis of lignocellulosic residues is still challenging. Therefore, the development of new organisms with lignocellulolytic capacities is crucial to make this process economically viable. We have concluded that *T. atroviride* 102C1 was capable of growing and producing endoxylanases in culture medium containing SB or CSL. The best result obtained for endoxylanase production (266.3 $U.mL^{-1}$) was better in medium containing SB 3.5% (w/v) and CSL 1.1% (w/v). The identified 102C1 mutant strain and its characterization as a high endoxylanase producer allows its use in biotechnological applications, particularly in the hydrolysis of agro industrial residues, such as sugar cane bagasse, for bioethanol production.

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