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Production of thermophilic and acidophilic endoglucanases by mutant *Trichoderma atroviride* 102C1 using agro-industrial by-products

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Many traditional mutagenic strategies have been used to improve cellulase production by micro-organisms, especially fungi species. *Trichoderma* species are among cellulolytic fungi, those that have been most extensively studied, due to their efficient production of these enzymes. In the present study, N-methyl-N´-nitro-N-nitrosoguanidine (NTG) was used as mutagenic agent to obtain cellulolytic mutant from wild strain *T. atroviride* 676. After mutagenic procedures, two strains (102C1 and 104C2) were selected as promising cellulase-producing mutant. The effect of the carbon (sugarcane bagasse: SCB) and nitrogen (corn steep liquor: CSL) sources on endoglucanase production by the mutants 102C1 and 104C2 was studied using submerged cultivations at 28°C. Different concentrations of SCB and CSL were used and nine different media were generated. Mutant 102C1 showed the best results when using 2.5% SCB and 0.7% CSL. A central composite rotational design (CCRD) was performed to estimate optimal conditions of pH and temperature for endoglucanase activity of strain 102C1, which were pH 3.6 and temperature 66°C. The characterization of this acidophilic and thermophilic endoglucanase activity produced by the mutant strain 102C1 allows its use in biotechnological applications, particularly in the hydrolysis of agro industrial residues, such as SCB, for bioethanol production.

Key words: *Trichoderma atroviride*, CMCase, nitrosoguanidine, sugarcane bagasse, corn steep liquor.

INTRODUCTION

Cellulose, a glucose polymer linked by β-1,4 bonds, is considered to be the primary product of photosynthesis and carbon renewable resources that is highly abundant in nature (Gottschalk et al., 2010; Deswal et al., 2014). Lignocellulosic material can be used to produce ethanol after cellulose hydrolysis with cellulases. The production

cost of cellulase is still the major obstacle associated to hydrolysis of cellulosic materials into fermentable sugars. Therefore, improvements towards a cost effective cellulase production is essential to make this process economically viable (Adsul et al., 2007; Soccol et al., 2010; Borges et al., 2014). The cellulase complex secreted by filamentous fungi consists of three main components, endo-1,4-β-glucanase (EC 3.2.1.4), β-1,4-glucan cellobiohydrolase (EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21). These three enzymes act in synergy during the conversion of cellulose to glucose (Wilson, 2011).

Significant research efforts have been invested in evaluating and understanding the enzymatic hydrolysis of lignocellulosic substrates by cellulases produced by *Trichoderma* species (van Wyk and Mohulatsi, 2003; Palonen et al., 2004), which are several hundred times more active than those produced by bacteria (Adsul et al., 2007; Martins et al., 2008). Various *Trichoderma* strains have been extensively evaluated and implemented in processes for bioethanol production (Rosgaard et al., 2006), including studies on low cost production of cellulases using lignocellulosic residues (Kovàcz et al., 2008; Grigorevski-Lima et al., 2013).

Trichoderma reesei is the most studied species of *Trichoderma* concerning cellulase production. Many traditional mutagenic strategies have been used to improve this characteristic, nevertheless, these attempts have not been totally successful yet (Chand et al., 2005; Kovàcz et al., 2008; Kovàcz et al., 2009; Jiang et al., 2011), since current enzyme production involves high costs and the production process is not yet fully defined (Adsul et al., 2007).

The identification of new cellulase high producing mutants will contribute to lower ethanol production costs especially when using sugarcane bagasse and corn steep liquor as the sole organic substrates. The use of these residues proves an efficient utilization of crops, where not only primary products, but also their by-products are use, which turns the process economically sound.

In the present study we used a mutagenic strategy to obtain a mutant library from *T. atroviride* 676, previously described as a good cellulase producer (Grigorevski-Lima et al., 2013). This library was screened to identify the most promising cellulase producer mutant and best levels of sugarcane bagasse, as carbon source, and corn steep liquor, as nitrogen source, in the production of CMCase by the selected mutant. Finally, a central composite rotational design (CCRD) experiment was performed to estimate the optimal conditions of pH and temperature for best endoglucanase activity of the selected strain.

MATERIALS AND METHODS

Microorganisms

T. atroviride 676 was isolated from the Amazon rainforest soil, and was obtained from the culture collection of Centro de Pesquisa Leonidas e Maria Deane, FIOCRUZ, Manaus, Brazil. Earlier, this strain proved promising for cellulase production (Grigorevski-Lima et al., 2013). During the present research mutants were obtained from this wild strain, using two subsequent mutations, and these used for cellulase production. Spore suspensions of the fungi were prepared according to Hopwood et al. (1985) after cultivation (28°C/15 days) in yeast extract-malt extract-agar medium (Shirling and Gottlieb, 1966) and maintained as stock cultures in 20% (v/v) glycerol at -20°C. Spore concentration was determined using Neubauer counting chamber.

Mutants strains

These were obtained by using nitrosoguanidine (NTG) and were based on Kovàcz et al. (2008). In a first experiment, the system was prepared using 100 µl of a spore suspension (10^7 spores ml^{-1}) of *T. atroviride* 676 and 2.0 ml of a sterile solution of 1.0% NTG and incubated for 8 min at room temperature. The suspension thus obtained was submitted to decimal dilutions and 0.1 ml of each one spread-plate inoculated in carboxymethylcellulose (CMC) medium based on Kovàcz et al. (2008), however a Congo red solution was added (Montenecourt and Eveleigh, 1977) and also, yeast extract was replaced by corn steep liquor (CSL) (SIGMA®, presented as corn steep solids, a spray-dried corn soluble). After 7 days incubation at 28°C, the grown colonies were isolated as pure cultures. Each strain was point inoculated into CMC-Congo red medium in Petri dishes, and after incubation for 7 days at 28°C, the strain presenting the wider halo (NTG21) was selected. Another mutation with NTG 1.0% was performed, as described earlier, using two strains for the experiments, strain 676 and strain NTG21, however incubation time was for 12 and 15 min, respectively. Cellulase production of the pure cultures thus obtained was confirmed by using cellulose-Congo red medium (César and Mrsa, 1996) and then cellulose-azure medium (Plant et al., 1988). As a preliminary fermentation study, the positive ones were then cultivated in 250 ml Erlemeyer flasks with 1/5 of its volume filled with a liquid medium (Mandels and Weber, 1966) containing sugarcane bagasse (SCB) (3.0%) and CSL (SIGMA®, as above) (0.3%) as C and N sources, respectively, at pH 4.8 and inoculated with 3.0 ml of a dense spore suspension. After 3-days of incubation at 28°C under agitation (200 rpm), supernatants were filtrated on fiber glass filter and used to measure the endoglucanase (CMCase) activities. The mutants showing a higher CMCase activity, at least two times the one observed by the original strain, were selected for further experiments.

Endoglucanase production

The enzyme production was performed in submerged fermentation using two selected mutants, in 250 ml Erlenmeyer flasks filled 1/5 of its volume with a culture medium based on the salt solution plus urea described by Mandels and Weber (1966) and added with different concentrations of sugarcane bagasse *in natura* (SCB – main carbon source) and corn steep liquor (CSL – main nitrogen

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source). A combination of different concentrations of carbon and nitrogen sources was performed in order to determine the good conditions for endoglucanase production by *Trichoderma atroviride* 102C1. Five concentration values were tested for SCB and CSL which were 1.1, 1.5, 2.5, 3.5 and 3.9% for SCB and 0.15, 0.3, 0.7, 1.1 and 1.25% for CSL, generating, in total, nine different media (Table 2). The initial pH of all media was adjusted to 5.0. Each set of flasks was inoculated with 25 µl of a spore suspension (10^8 ml^{-1}) of each studied strain and incubation was performed at 28°C in orbital shaking at 200 rev min^{-1} for 3 days. The supernatants, which corresponded to crude enzyme extracts, were used to determine endoglucanase activities.

Enzyme assays

Endoglucanase activity (CMCase) was estimated by reaction mixture containing 500 µl of a solution of 2.0% (w/v) carboxymethylcellulose low viscosity (CMC, SIGMA®) in 50 mM sodium citrate buffer (pH 4.8) plus 500 µl of the supernatant (Ghose 1987). This system was incubated for 6 min at 50°C. The reducing sugars concentration in the reaction mixture was determined by the dinitrosalicylic acid (DNS) method (Miller, 1959). 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 [SCB 2.5% (*w/v*) and CSL 0.7% (*w/v*)] from *T. atroviride* 102C1 was used to investigate the temperature and pH effect on CMCase activity. The enzyme characterization was carried out by employing a response surface methodology having CMCase activity (U ml^{-1}) as the independent variable and pH (between 3.0 and 7.0) and temperature (range of 40 to 70°C) as the dependent variables. A 2^2 full factorial central composite rotational design (CCRD) was used in order to generate 11 run combinations as described in Table 3. This design is represented by a second-order polynomial regression model (as Equation 1, where Y is the predicted response CMCase 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 1. Buffer solutions at 50 mM, (sodium citrate buffer for pH 3.0, 3.6 and 5.0, and phosphate buffer for pH 6.4 and 7.0) and was used at the optimal temperature previously determined. Data analysis was performed using the Statistica 7.0.

$$Y = b_0 + b_1X_1 + b_2X_2 + b_{12}X_1X_2 + b_{11}X_1^2 + b_{22}X_2^2 \quad (1)$$

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

Zymogram

The culture supernatant from cells grown on the best conditions was analyzed by electrophoresis on denaturing 10% sodium dodecyl sulfate-polyacrylamide gel, copolymerized with 0.1% (w/v) CMC (SIGMA®) as substrate. Electrophoresis was performed at constant voltage (90 V) for 3 h at 4°C. 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 sodium citrate buffer at optimum pH and temperature for 6 minutes. For detection of the enzyme activity, the gel was submerged in 0.1 % Congo red solution for 10 min and then washed with NaCl 1 M until visualization of enzyme bands (César and Mrsa, 1996). Molecular masses were calculated from mobility of standards ranging from 14 and 225 kDa (Amersham).

RESULTS

The *T. atroviride* 676 wild strain was previously identified as promising producer of enzymes of the lignocellulolytic complex (Grigorevski-Lima et al., 2013). In the present study, mutants obtained using NTG as mutagenic agent, were screened to identify those displaying increased production of endoglucanases. In a first mutation using strain 676, 15 strains were obtained and NTG21 was selected based on a qualitative test in CMC-Congo red solid medium. In a second mutation, strain 676 and strain NTG21 were used, and then 27 strains were obtained, 24 from strain NTG21 and 3 from strain 676. When these strains were tested for cellulase production in CMC-Congo red and cellulose-azure media, they were all positive. In a subsequent preliminary test, CMCase activity was measured for each strain after three days of cultivation in medium containing SCB (3.0%) and CSL (0.3%). Out of the 27 mutants tested, 14 (52%) showed CMCase activities greater than the wild type and, among these, two (102C1, mutant of NTG21 and 104C2, mutant of 676) were especially interesting, presenting values more than 2.2 times higher than the original strain (Table 1).

The promising selected strains, 102C1 and 104C2, were cultivated for 3 days in different concentrations of SCB and CSL under submerged fermentation conditions. Maximal values of CMCase obtained from submerged fermentation are presented in Table 2. CMCase activity produced by strain 102C1 ranged from 1.01 to 2.93 U ml^{-1}, whereas values obtained for strain 104C2 where lower (from 0.29 to 1.77 U ml^{-1}). The highest CMCase production (2.93 U ml^{-1}) was detected in medium 9, when SCB 2.5% (w/v) and CSL 0.7% (w/v) were used, being 84.2% higher than strain *T. atroviride* NTG21. The pH and temperature profiles for CMCase activity produced by strain 102C1 were determined using crude extract of the strain grown in SCB and CSL at 2.5 and 0.7% concentrations, respectively, corresponding to the optimal conditions identified earlier. The maximum enzyme activity was 3.37 U ml^{-1} which was observed at 66°C and pH 3.6 (Figure 1).

The regression equation obtained after analysis of variance (ANOVA) (Table 4) showed CMCase production in coded values of sugarcane bagasse and corn steep liquor. The F-value of 111.22 and P < 0.1 value indicate the importance and relevance of the model. The obtained coefficient of regression (R^2=0.9911) indicated that 99.1% of the variability shown in the responses might be explained by the model. The equation that represents the model for the production of this enzyme (Y) under these conditions is given as follows:

EG= 2.77 - 0.76*SCB - 0.55*SCB^2 + 0.22*CSL - 0.25*CSL^2 - 0.39*SCB*SCL + 0.00262

Crude enzyme extract obtained on the 3rd day of

Table 1. CMCase activity of mutant strains obtained after treatment of *T. atroviride* NTG21 and *T. atroviride* 676 with NTG 1.0% for 15 and 12 min respectively. Values of CMCase were obtained after 3-days cultivation in a liquid medium containing SCB (3.0%) and CSL (0.3%). CMCase values obtained for the wild strains are presented for comparison.

Strains	Treatment with NTG 1.0% (Contact time, min)	CMCase (U.ml^{-1})
***T. atroviride* NTG21**	**-**	**1.59**
102 A3		3.08
102 B1		0.54
102 C1		**3.62**
102 C2		3.22
102 C3		0.37
102 C4		2.97
103 A2		3.41
104 A1		3.09
104 A2		1.93
104 A3		2.95
104 A4		1.65
104 A5	15	1.8
103 A1		0.94
103 B1		1.05
104 A7		1.29
104 C1		0.16
104 C2		0.57
101 A2		1.35
102 C5		0.42
104 A6		1.57
104 B1		1.19
102 A2		1.59
102 C6		1.73
101 B4		1.48
***T. atroviride* 676**	**-**	**1.37**
104 A1		1.69
104 C1	12	1.85
104 C2		**3.5**

Table 2. Media composition used in the different submerged fermentation conditions for CMCase production by *Trichoderma atroviride* 102C1 and 104C2.

Medium	Raw-material source		CMCase activity (U ml^{-1})	
	Sugarcane bagasse % (*w/v*)	Corn steep liquor % (*w/v*)	Strain 102C1	Strain 104C2
1	1.50	0.30	1.22	0.29
2	3.50	0.30	1.23	0.46
3	1.50	1.10	1.01	0.37
4	3.50	1.10	1.17	0.90
5	1.10	0.70	1.80	0.35
6	3.90	0.70	2.21	**1.77**
7	2.50	0.15	2.04	0.34
8	2.50	1.25	2.54	0.50
9	2.50	0.70	**2.93**	1.06

All media were supplemented with a salt mineral solution (see Material and Methods).

Table 3. Values of independent variables (pH and temperature), used in CCRD, showing the values observed and predicted by the mathematical model for CMCase activity characterization for strain 102C1 .

Run	Coded setting levels		Actual levels		CMCase activity ($U.ml^{-1}$)	
	pH	Temperature	pH	Temperature	O	P
1	– 1	– 1	3.6	44	2.27	2.12
2	+ 1	– 1	6.4	44	1.48	1.38
3	– 1	+ 1	3.6	66	**3.37**	3.36
4	+ 1	+ 1	6.4	66	1.02	1.05
5	– 1.41	0	3.0	55	2.68	2.76
6	+ 1.41	0	7.0	55	0.58	0.61
7	0	– 1.41	5.0	40	1.81	1.95
8	0	+ 1.41	5.0	70	2.62	2.59
9	0	0	5.0	55	2.72	2.78
10	0	0	5.0	55	2.78	2.78
11	0	0	5.0	55	2.83	2.78

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

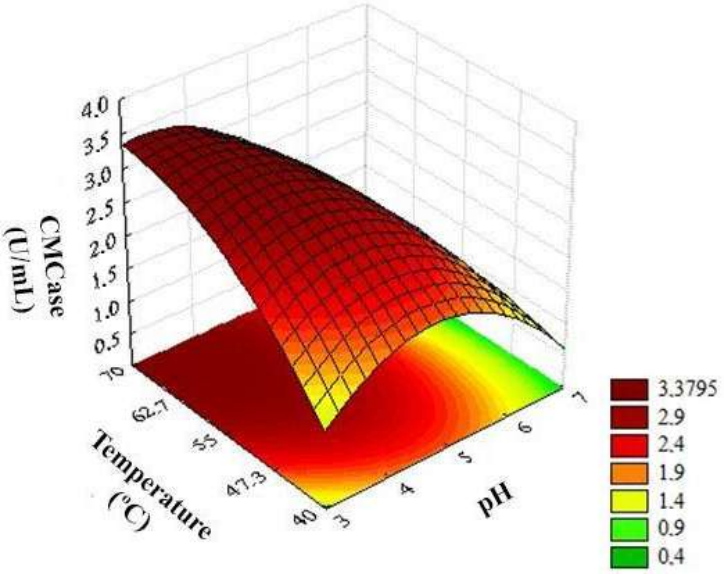

Figure 1. Response surface on partial CMCase characterization from *T. atroviride* 102C1 using pH and temperature as independent variables. The full factorial central composite design (2^2) used response surface methodology to predict the best point for CMCase activity. The values are shown in Table 2.

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

Sources of variations	Sum of squares	Degrees of freedom	Mean square	F-value	p-value (prob>F)[a]
Model	7.40	5	1.48	111.22	<0.0001
Residual	0.067	5	0.013	-	-
Lack of fit	0.061	3	0.020	7.81	0.1156
Pure error	0.005234	2	0.002617	-	-
Total	7.47	10	-	-	-

[a]Statistically significant at 90% of confidence level; R^2= 0.9911.

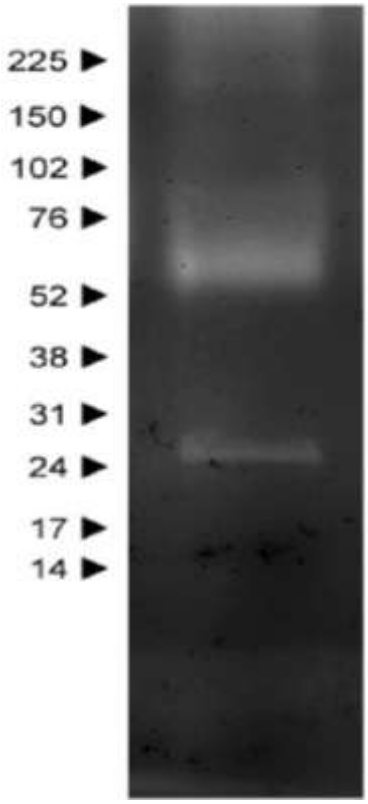

Figure 2. Zymogram analysis of CMCase activity in the supernatant of *T. atroviride* 102C1 cultures grown on best condition. The amounts loaded in the gel contained 500 mU of CMCase activity. The gel containing the MW markers was stained for proteins using the silver staining method and values for the MW markers are shown on the left side of the Figure. On the right side are shown the apparent molecular mass of CMCase bands.

fermentation in culture medium with 2.5% of sugarcane bagasse and 0.7% of corn steep liquor, which were the optimal conditions for the production of CMCase, was used to perform the zymogram experiments. Two bands with approximate molecular weights of 60.6 and 24.8 kDa were observed for CMCase activity (Figure 2).

DISCUSSION

In the present study, two mutants were selected, *T. atroviride* 102C1 and *T. atroviride* 104C2, obtained from the mutant strain *T. atroviride* NTG-21 and the wild type *T. atroviride* 676, respectively. These promising strains were tested over 3-days fermentation for endoglucanase (CMCase) production. Different combinations of SCB and CSL were tested, generating nine different mediums. In this case, the highest production was 2.93 U ml^{-1}, observed, for strain 102C1 when the concentration of C and N sources, were 2.5% for SCB, and 0.7% for CSL, respectively. When an experimental dosing was used to determine best conditions of temperature and pH for detection of enzymatic activity produced by strain 102C1, it was shown that the CMCase activity increased to 3.37 U ml^{-1} when temperature of detection was 66°C and pH 3.6.

T. atroviride 676 wild type has shown, earlier, ability to produce CMCase in lower amounts (1.37 U ml^{-1}) using the same substrates but with different concentrations of SCB (3.0%) and CSL (0.3%) as C and N sources, respectively, and also different conditions of temperature and pH for enzyme detection (Grigorevski-Lima et al., 2013). Our results using mutant *T. atroviride* 102C1 showed a 113.8% increase in enzyme activity compared to the results reported then. It is interesting to note that in our experiments, and also Grigorevski-Lima et al. (2013) research, the sugarcane bagasse used was not submitted to any treatment, as usually occurs in several studies, since, as it is well known, this would be more efficient. So, in our study, good results were also obtained using sugarcane bagasse *in natura* (untreated), which represents an economical cost-wise advantage, considering the elimination of time and efforts necessary for material processing.

The endoglucanase, referred as CMCase, is the enzyme most commonly found in cellulolytic microorganisms. Several studies have reported the production of CMCase using low cost materials as C and N sources, and mutant or wild type strains. Li et al. (2010) increased the production of CMCase up to 7% using *T. viride* mutants compared to the wild type strain. Chandra et al. (2009) observed around 3.0 U ml^{-1} in CMCase activity in *Trichoderma citrinoviride* mutant strains, which was three times higher than the wild type strain. Jiang et al. (2011) observed CMCase activity in *T. viride* mutants reaching 18 U ml^{-1}, which was also three times higher than in the wild type strain. Chand et al. (2005) measured about 0.415 and 0.60 U ml^{-1} in endoglucanase activity in *Aspergillus* mutants compared to 0.280 U ml^{-1} in the wild type strain. Another study tested *Penicillium echinulatum* using various cellulosic substrates and detected the maximum CMCase activity as 1.53 U ml^{-1} (Martins et al., 2008). Kovàcs et al. (2008) obtained 10 best *T. atroviride* cellulolytic mutants from wild strain TUB F-1505 using UV irradiation and NTG (0.1% *w/v*). The best endoglucanase activity observed in mutant strain (TUB F-1724) achieved 143.6 to 160.6 U ml^{-1}, while in the wild strain was 103 to 106 U ml^{-1}, corresponding to an increase of 50%, approximately. Hence, the 102C1 mutant strain is a good candidate for the industrial production of CMCase from untreated sugarcane bagasse and corn steep liquor since it is able to produce up to 2.93 U ml^{-1}, which is a high activity value when compared with some of those previously known.

The temperature and pH are important variables which affects the initial fermentation stage and hydrolysis rate. These profiles for optimal CMCase activity in 102C1 mutant strain supernatant were achieved at pH 3.6 and at 66°C. In a study with *Aspergillus aculeatus*, the optimal temperature for endoglucanase activity was 40°C (Naika et al., 2007). Kaur et al. (2007) observed two endoglucanases produced by thermophilic *Melanocarpus* sp. MTCC 3922 presented optimal enzyme activity at 50 and 70°C, respectively. Studies involving endoglucanases from *Trichoderma* strains described optimal activity at pH and temperature between 3.0 to 5.5 and 50 to 65°C (Gashe, 1992; Sul et al., 2004; Andrade et al., 2011). Our results show that the optimal CMCase activity occurred in the 102C1 mutant strain at more acidic pH (3.6), and at a high temperature (66°C).

The zymogram detected two intense CMCase bands with apparent molecular mass of 60.6 and 24.8 kDa (Figure 2). Javed et al. (2009) also detected an endoglucanase band produced by *Aspergillus oryzae* CMC-1 with apparent molecular mass of 25 kDa. Other studies have shown CMCase bands with different molecular masses, 51 kDa for *Trichoderma* sp. C-4 (Sul et al., 2004) and of 45 kDa for *A. aculeatus* (Naika et al., 2007), for instance. Two endoglucanase bands have also been identified in *T. atroviride* 676, the wild type of strain 102C1 (Grigorevski-Lima et al., 2013), but the reported molecular masses were 200 and 104 kDa, which are considered higher than fungal CMCase values commonly described in the literature. However, it is possible that their results represent enzyme complexes or aggregates of enzymes, which could explain the difference in values between those and the present study (Grigorevski-Lima et al., 2013).

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 capabilities of cellulosic biomass play an important role enhancing the utilization of such residues. Thus, the selection of new fungal strains producing high levels of cellulases might contribute in advancing the use of cellulosic residues towards a variety of goals. Our study used untreated sugarcane bagasse as the carbon source to cultivate a fungus mutant strain producing high cellulase activity, which is an abundant material with low commercial value.

The conversion of biomass to biofuels has been the subject of intense research efforts and gained significant scientific and political force due to concerns about the shortage of fossil fuels and emission of greenhouse gases (Antoni et al., 2007; Service, 2007; Omer, 2014). The need for global energy is projected to double in the next two decades and thus, production of biofuels could become a source of carbon sustainable energy that is compatible with current and future engine technologies (Chu and Majumdar, 2012). Lignocellulosic biomass is, by far, the most abundant source of renewable sugars that can be fermented into biofuels such as ethanol. While the fermentation of corn starch or sugarcane juice by *S. cerevisiae* is a well-established technology, the hydrolysis of lignocellulosic residues is still challenging (Menon and Rao, 2012). Therefore, the development of new organisms with lignocellulolytic capacities is crucial to make this process economically viable. Recently, Oliveira et al. (2014) has characterized this same 102C1 mutant strain as an excellent xylanase producer in comparison to wild strain and others *Trichoderma* species. The authors are convinced that our fermentation results prove that our mutant might be suitable strains for practical applications, and the selection of a new mutant-type *T. atroviride* 102C1 as a good cellulase producer, allows its use in biotechnological applications, particularly in the hydrolysis of agro-industrial by-products, such as sugarcane bagasse and straw, for bioethanol production.

Conflict of Interests

The authors have not declared any conflict of interests.

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