Int.J.Curr.Microbiol.App.Sci (2018) *7*(3): 962-970

International Journal of Current Microbiology and Applied Sciences
***ISSN: 2319-7706* Volume 7 Number 03 (2018)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2018.703.114

β-Glucosidase, β-Xylosidase and α-L-Arabinofuranosidase Production by Mutant *Trichoderma atroviride* 102C1 in Different Lignocellulosic Biomass Sources

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Keywords

Accessory enzymes, Sugarcane bagasse, Sugarcane straw, Sorghum bagasse, Wheat bran

Article Info

Accepted:
10 February 2018
Available Online:
10 March 2018

A B S T R A C T

Fungi are microorganisms considered decomposers of the organic matter, by the action of several enzymes that can present biotechnological potential, emphasizing the holocelulases (endoglucanases, exoglucanases, β-glucosidases, endoxilanases, β-xylosidases, among others). Among holocellulolytic fungi described in literature, *Penicillium*, *Aspergillus* and *Trichoderma* species are the most important. This study aimed to evaluate the efficiency in production of holocellulolytic accessories enzymes (β-glucosidase, β-xylosidase and α-L-arabinofuranosidase) by *Trichoderma atroviride* 102C1, a mutant strain obtained in our laboratory, using different lignocelluloses biomass as substrates. The mutant strain 102C1 was cultivated at 28°C in salt mineral solution supplemented with corn steep liquor as nitrogen source (1.26% *w/v*), and wheat bran, sorghum bagasse, sugarcane straw (*in natura* and pre-treated with steam explosion) or sugarcane bagasse (*in natura* and pre-treated with steam explosion), as carbon source (2.5% *w/v*), 200 rpm, for 5 days. The best results for (β-glucosidase, β-xylosidase and α-L-arabinofuranosidase activities were observed in sorghum bagasse (13.09 $U.ml^{-1}$), sugarcane straw pretreated (5.24 $U.ml^{-1}$) and wheat bran (192.6 $U.ml^{-1}$), respectively, between 3 and 5 days fermentation. These results suggest the use of different agro-industrial by products to obtain holocellulolytic accessories enzymes by the mutant fungi *T. atroviride* 102C1 and the possibility for biotechnology process.

Introduction

Holocellulases are one of the largest industrial enzymes worldwide, by dollar volume, because of their use in cotton processing, paper recycling, as detergent enzymes, in juice extraction, and as animal feed additives. However, holocellulases will become the largest volume industrial enzyme, if ethanol, butanol, or some other fermentation product of sugars, produced from biomass by enzymes, becomes a major transportation fuel (Wilson, 2009). The mainly holocellulases producers in nature are the filamentous fungi, like *Aspergillus*, *Penicillium* and *Trichoderma*. The holocellulase complex from *Trichoderma* sp., one of the most important microorganisms used in industry is consisted mainly of cellobiohydrolases (EC 3.2.1.91), endoglucanases (EC 3.2.1.4), β-glucosidases (EC 3.2.1.21), endoxylanases (EC 3.2.1.8), β-xylosidases (EC 3.2.1.37) and α-arabinofuranosidases (EC 3.2.1.55). These enzymes act synergistically to the hydrolysis of lignocellulose biomass in sugars (pentoses and hexoses) and could be produced by bacteria and filamentous fungi in nature, which a potential for biotechnology purposes, through the use of agro-industrial by-products within the biorefinery concept (Sanchez, 2009).

The Brazilian sugarcane system of agroenergy is considered as the most efficient system (Santos *et al.*, 2012). In 2015, Brazil produced about 593 million tons of sugarcane, producing about 150 million tons of sugarcane bagasse and 150 million tons of sugarcane straw. Therefore, in order to meet wider needs, a significant increase in the production of ethanol would be possible only if the basic knowledge necessary for the development of technologies that will be capable to obtain energy from lignocellulosic materials present in sugarcane is developed (Soccol *et al.*, 2010). The conversion of lignocellulose biomass requires a mixture of enzymatic complex, including cellulases (endo-1,4-glucanase, exo-1,4-cellobiohydrolase and β-1,4-glucosidase) as well as the hemicellulases (endoxylanase, β-1,4-xylosidase, α-$_L$-arabinofuranosidase). Some of these enzymes are considered accessories in bioconversion of lignocellulose biomass (Hansen *et al.*, 2015). There are many studies using β-glucosidase and β-xylosidase enzymes and few reports using α-arabinofuranosidase.

The β-1,4-glucosidases are essential for complete hydrolysis of cellulose fiber. These enzymes cleave cellobiose and cellooigosaccharides liberating molecules of glucose as the end product (Gottschalk *et al.*, 2010). β-1,4-Xylosidases are essential enzymes of the microbial xylanolytic system, and contribute to decrease the inhibition of xylanases by the end-product of xylan hydrolysis. This enzyme is cell-associated in most bacteria and yeast, but it is freely found in the culture media of some fungi (Michelin *et al.*, 2012). α-$_L$-Arabinofuranosidases are accessory enzymes that cleave □-L-arabinofuranosidicα linkages and act synergistically with other hemicellulases and pectic enzymes to promote the complete hydrolysis of hemicelluloses and pectins (Grigorevski-Lima *et al.*, 2013).

In the present work, the production of three enzymes with important accessory role in lignocellulose biodegradation, i.e. β-1,4-glucosidases, β-1,4-xylosidase and α-$_L$-arabinofuranosidase, were studied using different agro-industrial by-products, by the mutant strain *Trichoderma atroviride* 102C1.

As agro-industrial by-products the authors used sugarcane bagasse (SCB) and straw (SCS) *in natura*, sugarcane bagasse (SCSE) and straw (SSSE) pre-treated by steam explosion, sorghum bagasse (SB) and wheat bran (WB).

Materials and Methods

Microorganism

Initially the strain *Trichoderma atroviride* 676 was originally isolated from Amazon forest soil and identified by Dr Maria Ines Sarquis, at Fundação Oswaldo Cruz (Rio de Janeiro, Brazil). After nitrosoguanidine and U.V. radiation exposition, the mutant *T. atroviride* 102C1 was selected as holocellulolytic promising strain (Grigorevski-Lima *et al.*, 2013; Oliveira *et al.*, 2014; Oliveira *et al.*, 2016).

Spore suspensions of the mutant strain were prepared according to Hopwood *et al.*, (1985) after cultivation (28ºC/15 days) in yeast extract-malt extract-agar medium (1966) and maintained as stock cultures in 20% (v/v) glycerol at -20ºC. Spore concentration was determined using Neubauer counting chamber.

Production of Holocellulolytic Enzymes

T. atroviride 102C1 cells were cultured in a growth medium containing either of the six different agro-industrial by-products as carbon source (2.5% *w/v*): (i) SCB, (ii) SCS; (iii) SBSE; (iv) SSSE; (v) SB; (vi) WB and corn steep liquor (CSL) as main nitrogen source (1.26% *w/v*).

Media were always supplemented with modified Mandel solution (Mandels and Weber, 1969) containing ($g.l^{-1}$): urea, 0.3; $(NH_4)_2SO_4$, 1.4; KH_2PO_4, 2.0; $CaCl_2$, 0.3; $MgSO_4.7H_2O$, 0.3; $FeSO_4.7H_2O$, 0.005; $CoCl_2.6H_2O$, 0.02; $MnSO_4.4H_2O$, 0.016; $ZnSO_4.7H_2O$, 0.014. Cultivation was performed in submerged fermentation using *T. atroviride* 102C1, in 125 ml Erlenmeyer flasks filled 1/5 of its volume with a culture medium based (initial pH 5.5), which was inoculated with a spore suspension to a final concentration of 10^6 $spores.ml^{-1}$. Cells were incubated at 28ºC, in an orbital shaker (200 $rev.min^{-1}$), for up to 5 days. At each day, the whole content of a shake flask was filtered through a glass microfiber filter (Whatman GF/A), in triplicate, and the culture supernatants obtained were used in enzymatic assays.

The chemical characterization of six agro-industrial by-products was carried out by Laboratório de Controle Bromatológico e Microscópico (LabC BroM / UFRJ), based on methodology of Mendez *et al.*, (1985) and Van Soest (1963).

Enzyme assays

The β-1,4-glucosidase, β-1,4-xylosidase and α-L-arabinofuranosidase activities were determined by release of *p*-nitrophenol obtained by hydrolysis of the substrates *p*-nitrophenyl-β-D-glucopyranoside (Sigma-Aldrich®), *p*-nitrophenyl-β-D-xilopyranoside (Sigma-Aldrich®) and *p*-nitrophenyl-α-L-arabinofuranoside (Sigma-Aldrich®), respectively, to 10 mM.

Enzyme assays were prepared with 200 µl of 0.5M sodium acetate buffer pH 5.0, 650 µl for β-1,4-glucosidase and β-1,4-xylosidase or 600 µl for α-$_L$-arabinofuranosidase of distilled water and 50 µl for β- glucosidase and β-xylosidase or 100 µl for α-$_L$-arabinofuranosidase of the enzyme extract. Reaction happened for 10 minutes at 50°C with the addition of 100 µl of the substrate corresponding to each enzyme. After this period, the enzyme reaction was stopped with the addition of 500 µl of 1 M Na_2CO_3 pH 10.0. The reading of the amount of *p*-nitrophenol released during the enzyme assays was performed in spectrophotometer at 420nm (Da Silva *et al.*, 2010).

A unit β-1,4-glucosidase, β-1,4-xylosidase and α-$_L$-arabinofuranosidase (U) was defined as

the amount of enzyme that released 1 µmol of *p*-nitrophenol at 50°C in 1 minute. All assays were performed in triplicates, and results were expressed as average values. Variations in the multiple assays were < 10%.

Results and Discussion

β-Glucosidase (BGL), β-xylosidase (BXL) and α-L-arabino-furanosidases (ARF), holocellulolytic enzymes, are very important enzymes in the process of complete biodegradation of lignocellulosic biomass releasing fermentable sugars (glucose, xylose and arabinose).

In this research, the mutant strain *Trichoderma atroviride* 102C1 was studied aiming at the production of holocellulolytic accessories enzymes using different agro-industrial by-products as main carbon source. *T. atroviride* 102C1 is already known as endoglucanase and endoxylanase producer when grown in lignocellulose biomass (Kóvacz *et al.,* 2008; Kóvacz *et al.,* 2009; Oliveira *et al.,* 2014; Oliveira *et al.,* 2016).

For accessories enzymes production from *T. atroviride* 102C1, six different carbon sources (SCB, SCS, SBSE, SSSE, WB and SB) were used as an inducer. Individual evaluation of the carbon sources revealed that use of SB resulted in the highest production of β-glucosidase (13.09 $U.ml^{-1}$), after 4 days, compared with other sources used (Figure 1).

Annually, several researches on β-glucosidase production by microorganisms have been carried out, confirming its relevance in cellulose fiber biodegradation. The production of β-glucosidase (BGL) by *Trichoderma* species using lignocellulosic residues are reported in literature. Grigorevski-Lima *et al.,* (2013) produced a maximal β-glucosidase activity (0.17 $U.ml^{-1}$) in the parental strain of T. *atroviride* 102C1 after 4 fermentation-days using sugarcane bagasse (*in natura*). Rana *et al.,* (2014) have reported β-glucosidase production with *T. reesei* RUT-C30 (ATCC 56765) using corn straw pretreated by alkali explosion as 4.77 $U.ml^{-1}$, after 7 fermentation-days in STR bioreactor. Kóvacs, Szakacs and Zacchi [18] have showed enzyme production using *T. reesei* RUT-C30, *T. atroviride* TUB F-1505 and *T. atroviride* TUB F-1663 (mutant of wild strain TUB F-1505) with β-glucosidase activity of <0.2, 4.9 and 7.1 $U.ml^{-1}$, respectively, using steam pretreated spruce as feedstock.

Delabona *et al.,* (2012) produced a maximal β-glucosidase activity (9.18 $U.ml^{-1}$) with *T. harzianum* P49P11 using sugarcane bagasse pretreated, after 4 fermentation-days. The authors observed that the BGL production by the mutant strain *T. atroviride* 102C1 was very promising using agro industrial by-products, especially SB and WB at 5 days of fermentation.

When β-xylosidase (BXL) production was studied, the maximal activity (5.24 $U.ml^{-1}$) was detected using SSSE after 4 fermentation-days (Figure 2). Jiang *et al.,* (2011) produced a maximal β-xylosidase activity (0.25 $U.ml^{-1}$) with *T. reesei* RUT-C30 (ATCC 56765) using lactose 1% (*w/v*), after 7 fermentation-days. Menezes *et al.,* (2010) observed a maximal β-xylosidase activity (0.09 $U.ml^{-1}$) with *Pleurotus* sp. BCCB068 after 40 fermentation-days, using xylan as substrate.

Guerfali, Maalej-Achouri e Belghith (2013) have showed enzyme production using *Talaromyces thermophilus* with β-xylosidase activity of 1.4 $U.ml^{-1}$, in the presence of wheat bran 2% (*w/v*), after 7 fermentation-days. There are few reports in literature concerning to BXL production by filamentous fungi. Once again, the production of BXL by mutant *T. atroviride* 102C1 was higher than reported in literature (Table 1).

Table.1 The dry matter-based composition of the biomass components (%)

Carbon Source	Cellulose	Hemicellulose	Lignin
SCB	32.78 + 0.72	42.35 + 1.69	10.74 + 0.19
SBSE	45.40 + 0.64	10.53 + 0.64	11.91 + 0.55
SCS	25.63 + 2.66	37.76 + 2.21	14.31 + 0.65
SSSE	15.72 + 3.81	17.34 + 0.69	19.79 + 1.06
WB	7.17 + 0.11	26.96 + 0.45	2.78 + 0.19
SB	44.91 + 0.16	26.22 + 1.34	9.00 + 0.25

Fig.1 Fermentation time-course for β-1,4-glucosidase (BGU), production by *T. atroviride* 102C1, in submerged fermentation, upon the use of different substrates: (◆) SCB; (-◇-) SBSE; (▲) SCS; (-△-) SSSE; (■) WB and (●) SB

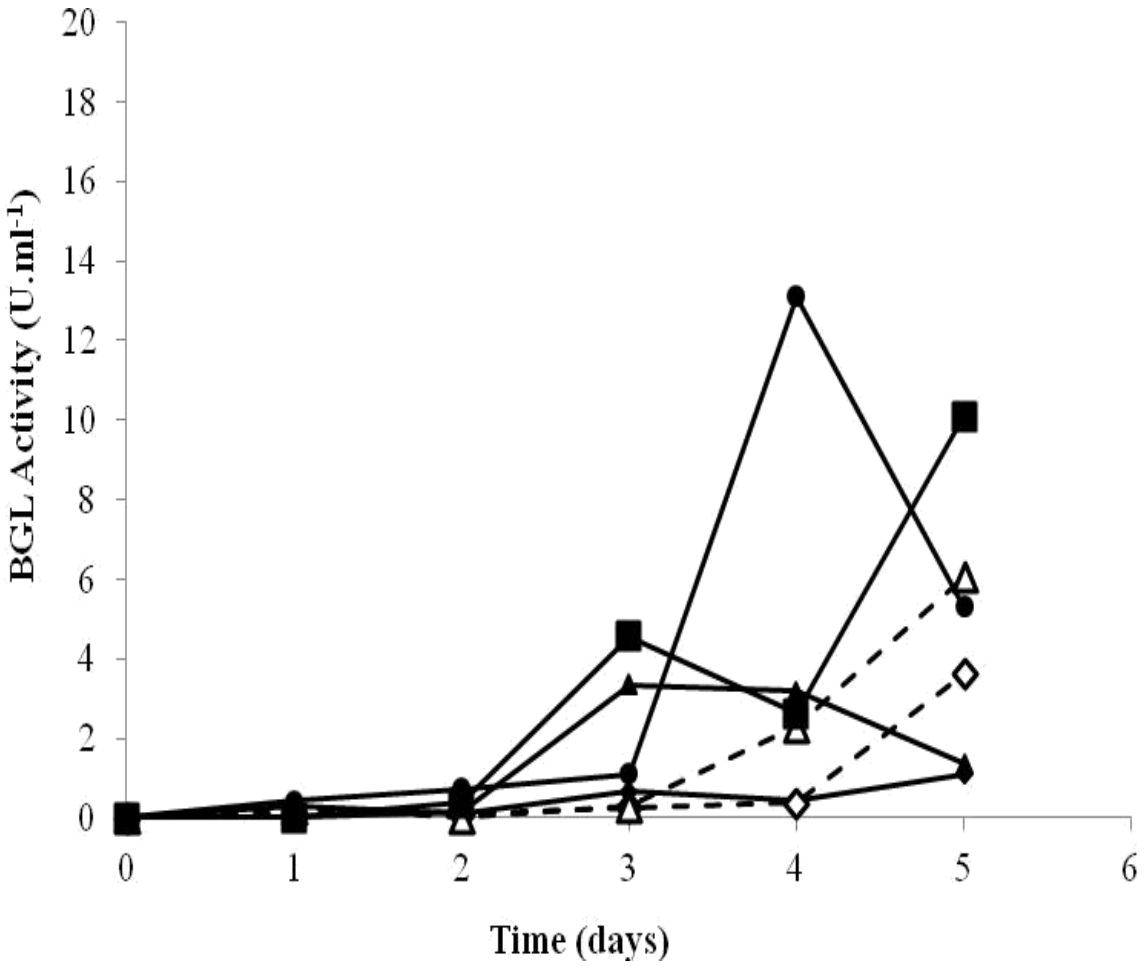


Fig.2 Fermentation time-course for β-1,4-xylosidase (BXU), production by *T. atroviride* 102C1, in submerged fermentation, upon the use of different substrates:
(◆) SCB; (-◇-) SBSE; (◆)SCS; (-△-) SSSE; (■) WB and (●) SB

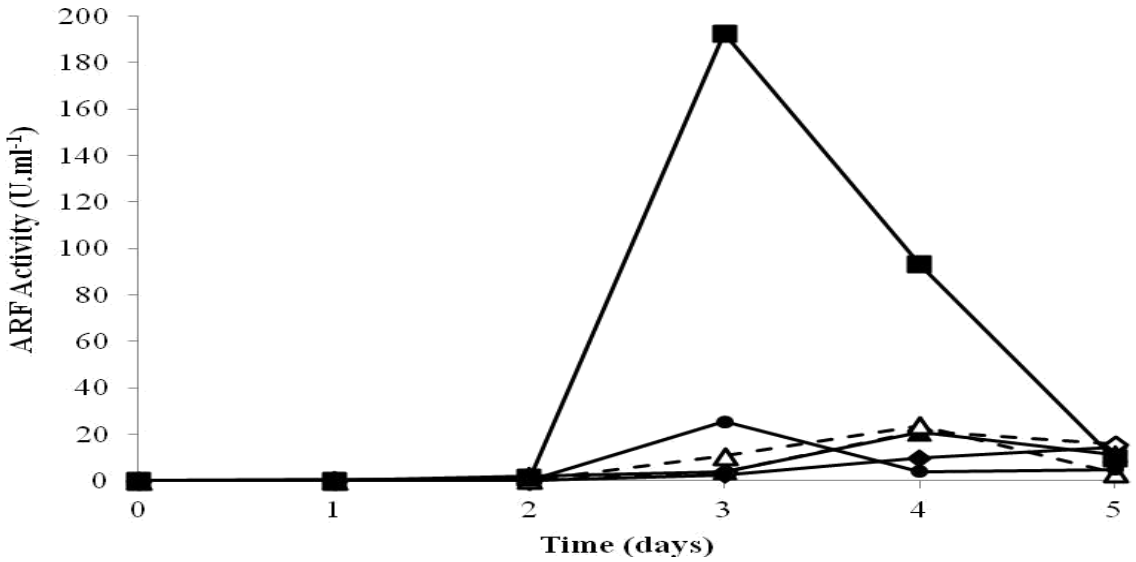
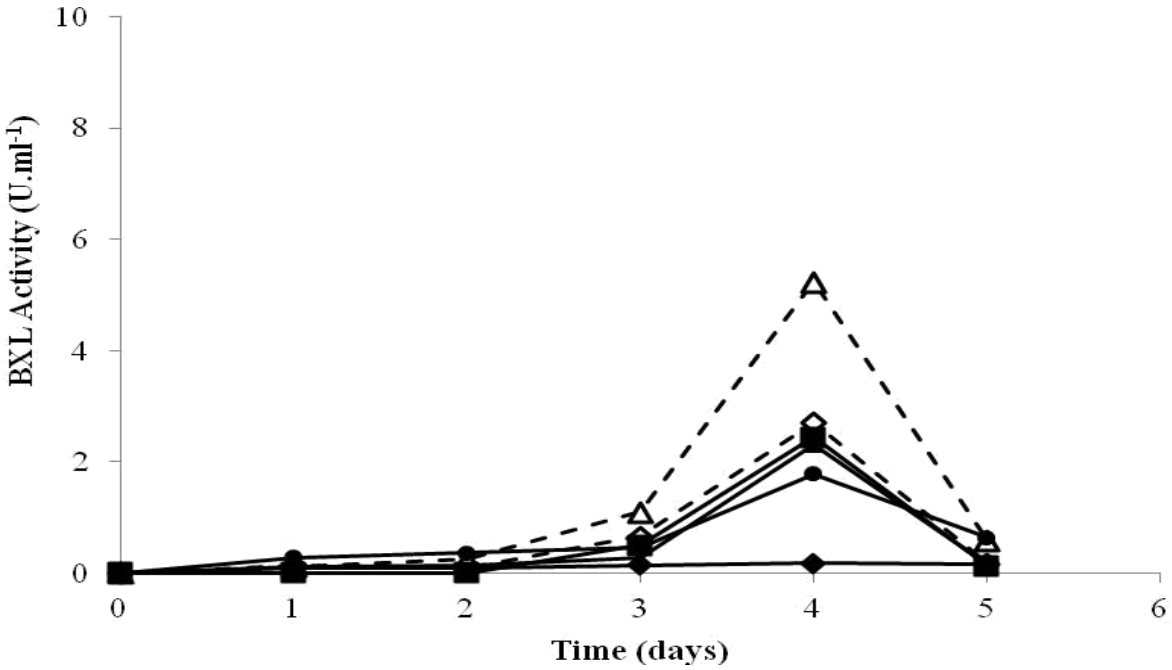


Fig.3 Fermentation time-course for α-$_L$-arabino-furanosidase (ARA), production by *T. atroviride* 102C1, in submerged fermentation, upon the use of different substrates:
(◆) SCB; (-◇-) SBSE; (▲) SCS; (-△-) SSSE; (■) WB and (●) SB



Individual evaluation of the carbon sources revealed that use of WB resulted in the highest production of α-L-arabinofuranosidase (ARF), 192.60 $U.ml^{-1}$, after 3 days of submerged fermentation (Figure 3). Almeida *et al.,* (2011) produced 0.045 $U.ml^{-1}$ of α-L-arabinofuranosidase with *Acremonium zeae* EA0802 using oat spelts xylan, after 18 fermentation-days. Temer, Terrasan and Carmona (2014) have showed enzyme production using *Penicillium janczewskii* with α-L-arabinofuranosidase activity of 0.7 $U.ml^{-1}$, using orange waste mixed brewer's spent grain as feedstock, after 10 days of fermentation. Ioannes *et al.,* (2000) observed α-L-arabinofuranosidase activity (0.7, 0.85 and 1.00 $U.ml^{-1}$) with *P. purpurogenum* using oat spelts xylan, beet pulp and L-arabitol, respectively, after 4 days of fermentation. Visser *et al.,* (2013) have showed enzyme production using *Penicillium pinophilum* and *Chrysoporthe cubensis* with α-L-arabinofuranosidase activity of 0.27 and 0.52 $U.ml^{-1}$, using knife-milled elephant grass 3% (w/v) and wheat bran, respectively, after 7 fermentation-days. Guerfali, Maalej-Achouri and Belghith (2013) produced a maximal α-L-arabinofuranosidase (1.05 $U.ml^{-1}$) with *Talaromyces thermophilus* using wheat bran 2% (w/v), after 8 days. At the current days, there are few reports concerning ARF production. The mutant strain 102C1 was capable to produce a high ARF activity in the presence of WB (192.60 $U.ml^{-1}$), in a short period time (3 days). This enzyme activity value was higher than those reported in the literature.

The use of accessories enzymes in biodegradation of hemicellulose fraction from lignocelluloses biomass could be very important. When combine β-xylosidase and α-L-arabinofuranosidase in an enzymatic blend, almost doubled xylose release from water extractable wheat arabinoxylan compared to β-xylosidase treatment alone, for example. The removal of 1-3 linked arabinose from singly substituted xylopyranosyls near non- reducing ends provided access for β-xylosidase (Rasmussen *et al.,* 2012). The combination of endo-1,4- β -xylanase and β-xylosidase to hydrolyse wheat arabinoxylan, could be increased 2.5-fold when α-L-arabinofuranosidase was added (Rasmussen and Meyer, 2010; Rasmussen *et al.,* 2012; McCleary *et al.,* 2015).

Lignocellulosic biomass is the most abundant source of renewable sugars that can be fermented into biofuels. However, when focus in their hydrolysis, many efforts still need to be coordinated. The study of accessories enzymes such as β-1,4-glucosidase, β-1,4-xylosidase and α-$_L$-arabinofuranosidase, can be of great significance to assist in the complete hydrolysis of plant biomass, allowing greater accessibility of the main enzymes (endoglucanase, cellobiohydrolase and endoxylanase) of the cellulose and hemicellulose fibers. In this work, we could observe different effects of the carbon sources (SCB, SBSE, SCS, SSSE, WB and SB) on enzymes production. The present authors are convinced that our results of the fermentation study of accessories enzymes using different substrates as main carbon source prove that our mutant 102C1 might be suitable strain for practical applications, allowing its use in biotechnological applications, particularly in the hydrolysis of agro-industrial by-products, such as wheat bran (WB) and sorghum bagasse (SB).

Conflict of Interest

The authors declare that there are not conflicts of interest in this study.

Acknowledgements

The authors thank LADEBIO-UFRJ for the supply of sorghum bagasse and the Sugarcane

Technology Center (CTC) for the supply of straw and sugarcane bagasse. This work was financially supported by Conselho Nacional de Desenvolvimento Cientifico e Tecnológico (CNPq), Financiadora de Estudos e Projetos (FINEP) and Coordenação de Aperfeiçoamento de Pessoal do Ensino Superior (CAPES).

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How to cite this article:

Jessica Caroline Araujo Silva, Daniela Sales Alviano, Celuta Sales Alviano, Elba Pinto da Silva Bon and Rodrigo Pires do Nascimento. 2018. β-Glucosidase, β-Xylosidase and α-L-Arabinofuranosidase Production by Mutant *Trichoderma atroviride* 102C1 in Different Lignocellulosic Biomass Sources. *Int.J.Curr.Microbiol.App.Sci.* 7(03): 962-970.
doi: https://doi.org/10.20546/ijcmas.2018.703.114