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Trichoderma atroviride 102C1: A promising mutant strain for the production of a β-glucosidase, β-xylosidase and α-L-arabinofuranosidase activities using agroindustrial by-products

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Holocellulolytic accessory enzymes are very important in assisting hydrolysis of biomass. The use of these enzymes in the enzymatic hydrolysis of plant biomass is very important for obtaining building blocks in the concept of biorefinery. In previous studies, the mutant strain *Trichoderma atroviride* 102C1 was tested for production of endoglucanases, FPases and endoxylanases. This study aimed at evaluating the efficiency in holocellulolytic accessories enzymes production (β-glucosidase, β-xylosidase and α-L-arabinofuranosidase) by *Trichoderma atroviride* 102C1 using different lignocelluloses biomass as substrates. Accessory enzymes production was carried out in Erlenmeyer flasks containing Mandels salt medium, supplemented with different concentrations of sugarcane bagasse (SCB) and corn steep liquor (CSL), according to a Central Composite Rotational Design (CCRD). The fermentation system was incubated under agitation for 2 days / 28°C. For pH and temperature profile studies, a new CCRD was carried out. The best condition common to all enzymes, 55.4 U.mL^{-1} (β-glucosidase), 10.8 U.mL^{-1} (β-xylosidase) and 143.23 U.mL^{-1} (α-L-arabinofuranosidase), was observed when 2.5% (w/v) sugarcane bagasse (SCB) and 1.26% (w/v) of corn steep liquor (CSL) were used. All enzymes presented acidophilic characteristic in two different temperatures (44 and 55°C). The optimal profile characteristic for β-glucosidase and β-xylosidase activities were pH 5.0 and 3.0, respectively, both at 55°C, while for α-L-arabinofuranosidase it was pH 3.6 at 44°C. This study demonstrated the potential of *T. atroviride* 102C1 to produce three important holocellulolytic accessory enzymes in the presence of SCB and CSL, suggesting its use for enzymatic hydrolysis of lignocellulosic biomass.

Key words: *Trichoderma atroviride* 102C1, holocellulolytic enzymes, sugarcane bagasse, corn steep liquor.

INTRODUCTION

Plant cell walls are a source of renewable carbon present in nature as cellulose, hemicelluloses and lignin. Cellulose is an abundant linear polymer worldwide and is composed of glucose residues linked by β-1,4-glucosidic bonds. Hemicellulose is a branched heteropolymer of pentoses and/or hexoses and various types of uronic acids residues linked by β-1,4 / β-1,3 glucosidic bonds. The term hollocellulose comprises the cellulose and hemicelluloses of plant cell wall linked together (Gottschalk et al., 2010; Zampieri et al., 2013).

Worldwide attention has focused on the major biotechnological uses of the carbohydrates in agro-industrial by-products, as biomass syrups that have sugars with five or six carbons (derived from xylan and glucan). They can be used as carbon sources in industrial fermentations producing antibiotics, industrial enzymes, and bulk chemicals, including ethanol. For these purposes, however, the polysaccharides in the biomass must first be hydrolyzed (Gottschalk et al., 2010; Shinozaki et al., 2015).

The complete hydrolysis of polysaccharide fraction of lignocellulose biomass requires the cooperative action of several enzymes. Endo-1,4-β-D-glucanases (EC 3.2.1.4) which hydrolyze the cellulose polymer internally, exposing reducing and non-reducing ends, and exo-1,4-β-D-glucanases (3.2.1.91) which act on the reducing and non-reducing ends, releasing cellobiose and cellooligosaccharides. The complete hydrolysis of cellulose is finalized through the action of β-glucosidase (E.C. 3.2.1.21), which cleaves cellooligosaccharides and cellobiose to glucose (Zampieri et al., 2013; Singhania et al., 2013). Considering the hemicellulose fraction, endo-1,4-β-D-endoxylanases (EC 3.2.1.8) can degrade randomly the xylan portion of the polymer, releasing xylooligosaccharides. Synergistically, β-xylosidase (E.C. 3.2.1.37) release D-xylose residues from xylooligosaccharides and xylobiose (Terrasan et al., 2010; Kirikyali and Connerton, 2014). Due to the structural complexity of the hemicelluloses, other enzymes are also required for the hydrolysis of that polymer, as acetyl-xylan esterase (E.C. 3.1.1.72) and a-L-arabinofuranosidase (E.C. 3.2.1.55), which are able to release α-L-1,2-, α-L-1,3- and α-L-1,5-arabinofuranosyl side chains of arabinoxylans, arabinans and arabinogalactans (Temer et al., 2014; Shinozaki et al., 2015).

Trichoderma atroviride 676, isolated from Amazon forest soil, was capable to produce cellulases and endoxylanases when agro-industrial by-products were used as substrates (Grigorevski-Lima et al., 2013). After mutation procedures in this strain, a new mutant, *T. atroviride* 102C1, producer of high titers of endoglucanase, exoglucanase and endoxylanase was obtained (Oliveira et al., 2014). The objective of the present study was to investigate the production of some hollocellulolytic accessory enzymes of *T. atroviride* 102C1, including β-glucosidase, β-xylosidase and α-L-arabinofuranosidase, using submerged fermentation and, as main substrates, sugarcane bagasse *in natura* and corn steep liquor.

MATERIALS AND METHODS

Maintenance of microorganism

Mutant strain *T. atroviride* 102C1 was obtained from the wild strain *T. atroviride* 676 (Grigorevski-Lima et al., 2013), after successive exposures to U.V. radiation and nitrosoguanidine, according to Oliveira et al. (2014). Spore suspensions were prepared according to Hopwood et al. (Hopwood et al., 1985), after cultivation at 28°C for 7 days in potato dextrose agar medium (Hanada et al., 2002). Spores were maintained in 20% (v/v) glycerol at −20°C. Concentration of the spore suspension was determined in a Neubauer chamber.

Enzyme production

Enzyme production was performed in submerged fermentation in Erlenmeyer flasks (125 ml) containing 25 ml of a modified culture medium (Mandels and Weber, 1969), in $g.L^{-1}$: 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; and $ZnSO_4.7H_2O$, 0.014 supplemented with sugarcane bagasse *in natura* (SCB) and corn steep liquor (CSL) at different concentrations, according to experimental design 2^2 central composite rotational design (CCRD). Response surface methodology (RSM) was used as a tool for the optimization of SCB and CSL concentrations (independent variables) in the range indicated in Table 1. Enzymes activities, β-glucosidase, β-xylosidase and α-L-arabinofuranosidase ($U \cdot mL^{-1}$) were the dependent variables. Medium start pH was adjusted to 4.8. The growth media were inoculated with 25 µl of a spore suspension (10^{11} spores$\cdot mL^{-1}$) and incubated at 28°C, under agitation (200 rpm). Based on preliminary tests, a two days period was chosen. After this, the whole content of a shake flask was filtered through a glass microfiber filter (Whatman GF/A) in duplicate, and the culture supernatants obtained were used to determine the all enzymatic assays.

Enzymatic assays

β-Glucosidase (BGU), β-xylosidase (BXU) and α-L-arabinofuranosidase (ARF) activities were determined using standard IUPAC procedures and expressed in international units(IU), by release of *p*-nitrophenol obtained by hydrolysis of the were substrates (Rasmussen et al., 2001). BGU and BXU activities

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Table 1. Values of the independent variables (X_1 = sugarcane bagasse *in natura*; X_2 = corn steep liquor) of the CCRD 2², showing the statistically values for producing β-glucosidase, β-xylosidase and α-L-arabinofuranosidase by *T. atroviride* 102C1

Assay	Coded setting levels		Actual levels (%) (w/v)		β-glucosidase ($U.mL^{-1}$)*	β-xylosidase ($U.mL^{-1}$)*	α-L-arabinofuranosidase ($U.mL^{-1}$)*
	X_1	X_2	X_1	X_2			
1	-1	-1	1.5	0.3	28.32	1.01	70.30
2	+1	-1	3.5	0.3	33.60	1.79	127.23
3	-1	+1	1.5	1.1	38.25	10.92	114.36
4	+1	+1	3.5	1.1	54.59	2.21	159.24
5	-1.41	0	1.09	0.7	42.05	3.18	99.18
6	+1.41	0	3.91	0.7	41.01	2.18	157.43
7	0	-1.41	2.5	0.15	26.11	1.69	86.30
8	0	+1.41	2.5	1.26	55.38	10.75	143.23
9	0	0	2.5	0.7	46.60	2.36	126.73
10	0	0	2.5	0.7	45.74	2.08	130.20
11	0	0	2.5	0.7	49.11	1.97	130.69

* Values are based on Mean ± SD of 2 individual observations.

determined by mixing 50 µl of the enzyme preparation to 100 µl of *p*-nitrophenyl-β-D-glucopyranoside or *p*-nitrophenyl-β-D-xilopyranoside (10 mM) in 200 µl of 100 mM sodium acetate buffer, pH 5.0 at 50°C for 10 min, respectively, supplemented with 650 µl of distilled water. ARF activity was determined by mixing 100 µl of the enzyme preparation to 100 µl of *p*-nitrophenyl-α-L-arabinofuranoside (10 mM) in 200 µl of 100 mM sodium acetate buffer, pH 5.0 at 50°C for 10 min, supplemented with 600 µl of distilled water. After incubation time, the enzyme reaction was stopped by addition of 500 µl of Na_2CO_3 (1.0 M), pH 10.0 and reading in spectrophotometer at 420 nm. One unit of BGU, BXU and ARF (IU) corresponded to formation of 1.0 µmol of *p*-nitrophenol at 50°C per minute. The results were analyzed using Statistica Statsoft 7.0®.

Determination of physico-chemical properties of enzymes: pH, temperature, thermal stability and metal ions effect on enzymatic activities

Temperature and pH effect on BGU, BXU and ARF activities was investigated using standard assay methods at various temperatures and pH range, according to CCRD 2². In the 12 experiments which were carried out, the temperature ranged from 40 to 70°C and the pH values from 3.0 to 7.0 as shown in Table 3. Citrate buffer (50 mM) was used for pH 3.0, 3.6 and 5.0 and citrate-phosphate (50 mM) for pH 6.4 and 7.0. Statistical analysis was performed using the software Statistica Statsoft 7.0®. Temperature stability range was determined by incubating the crude enzyme at 50 and 70°C. Residual enzyme activity was determined at different time interval up to 8 h. Influence of sodium, calcium, potassium, manganese, and barium ions in the chloride form, and copper, magnesium, zinc, and iron in the sulfate form, on the BGU, BXU and ARF activities were performed by the addition of the relevant salts at 2 mM final concentration in the enzyme activity assay using the previously determined optimal conditions for pH and temperature.

RESULTS

Enzyme production

In this study the mutant strain *T. atroviride* 102C1 have produced some holocellulolytic accessory enzymes when using sugarcane bagasse (SCB) and corn steep liquor (CSL) as substrates in submerged fermentation. The use of RSM and CCRD tools for the optimization resulted in enzyme activities accumulation in the range of 26.11 to 55.38 $U.mL^{-1}$ for β-glucosidase, 1.01 to 10.75 $U.mL^{-1}$ for β-xylosidase and 70.30 to 159.24 $U.mL^{-1}$ for α-L-arabinofuranosidase after two days fermentation (Table 1). The fitted response surface for the production of the three enzymes is given in Figure 1 and, as can be seen, best conditions for each one, were different. For β-glucosidase, the best enzyme production (55.38 $U.mL^{-1}$) was observed when 2.5% (w/v) SCB and 1.26% (w/v) CSL were used. For β-xylosidase (10.92 $U.mL^{-1}$) best concentrations were 1.5% (w/v) SCB and 1.1% (w/v) CSL, and for α-L-arabinofuranosidase (159.24 $U.mL^{-1}$), 3.5% (w/v) SCB and 0.3% (w/v) CSL. However, for studying the BGU, BXU and ARF concomitantly, the run 8 [2.5% (w/v) SCB and 1.26% (w/v) CSL] was considered as the best for further analysis. Under these conditions the enzyme activities were 55.38 $U.mL^{-1}$ (BGU), 10.75 $U.mL^{-1}$ (BXU) and 143.23 $U.mL^{-1}$ (ARF). The relevant regression equations, resulting from the analysis of variance (ANOVA) (Table 2) have shown β-glucosidase, β-xylosidase and α-L-arabinofuranosidase production as a function of the codified values of SCB and CSL. The equations that represented a suitable model for β-glucosidase (Y_{BGU}), β-xylosidase (Y_{BXU}) and α-L-arabinofuranosidase (Y_{ARF}) production are given in:

$$Y_{BGU} = 47.15 + 9.04*CSL + 2.7*SCB*CSL - 3.42*SCB^2 - 3.82*CSL^2 + 5.4*SCB*CSL^2 \quad (1)$$

$$Y_{BXU} = 2.25 + 2.89*CSL - 2.37*SCB*CSL + 1.9*CSL^2 - 1.98*SCB*CSL^2 \quad (2)$$

$$Y_{ARF} = 127.9 + 23.02*SCB + 19.57*CSL - 7.75*CSL^2 \quad (3)$$

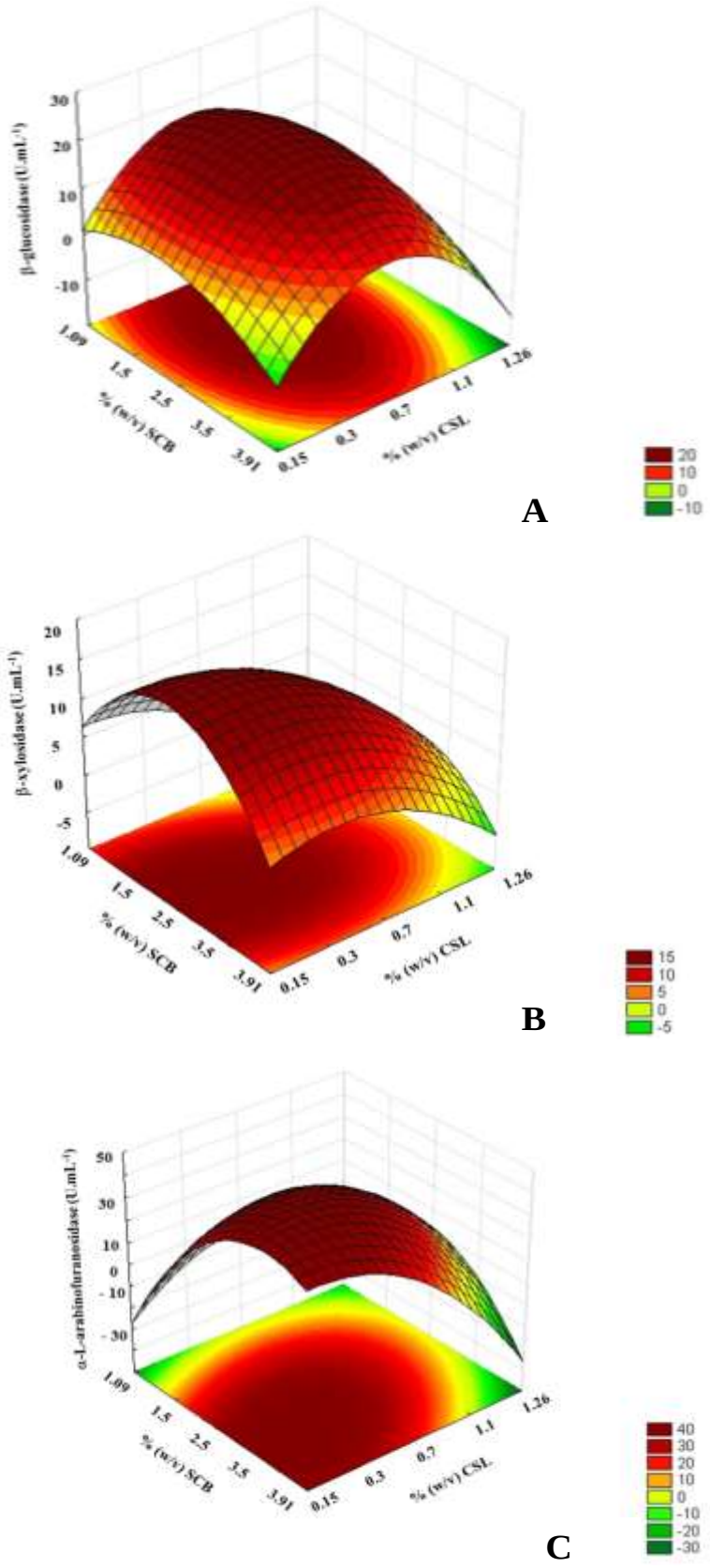

Figure 1. Response surface on β-glucosidase (A), β-xylosidase (B) and α-L-arabinofuranosidase (C) production by *T. atroviride* 102C1 using SCB and CSL concentrations as the independent variables.

Table 2. Statistical ANOVA for the model of β-glucosidase, β-xylosidase and α-L-arabinofuranosidase activities by *T. atroviride* 102C1 at different concentrations of SCB and CSL values.

Enzyme	Source of variation	Sum of squares	Degrees of freedom	Mean squares	F value	Value (prob >)[b]
β-glucosidase	Model	916.14	5	183.23	28.32	0.0011
	Residual	32.37	5	6.47		
	Lack of Fit	26.25	3	8.75	2.86	0.2697
	Pure Error	6.12	2	3.06		
	Total	948.51	10			
β-xylosidase	Model	127.61	4	31.90	93.82	<0.0001
	Residual	2.03	6	0.34		
	Lack of Fit	1.90	4	0.48	7.41	0.1224
	Pure Error	0.13	2	0.064		
	Total	129.64	10			
α-L-arabinofuranosidase	Model	7,676.96	3	2,558.99	133.28	<0.0001
	Residual	134.41	7	19.20		
	Lack of Fit	125.10	5	25.02	5.37	0.1643
	Pure Error	9.31	2	4.66		
	Total	7,811.37	10			

[b] Statistically significant at 95% of confidence level; R^2= 0.967 (β-glucosidase); R^2= 0.984 (β-xylosidase); R^2= 0.982 (α-L-arabinofuranosidase).

The model F values of 28.32, 93.82 and 133.28 imply that the models are significant at a high confidence level. The probability value was also very low (<0.05) indicating the significance of the model.

Determination of physico-chemical properties of enzymes

With respect to pH and temperature effects, the maximum enzyme accumulation were, as expected, influenced by pH and temperature. According to CCRD, the analysis of resulting surface response plots revealed that maximal β-glucosidase relative activity (Figure 2A) occurred in pH range of 4.5-5.5 and temperature of 50-60°C, while the maximal β-xylosidase (Figure 2B) and α-L-arabinofuranosidase (Figure 2C) relative activities occurred in pH range of 3.0-4.0 and temperature of 50-60°C / 40-50°C, respectively. So, characterization of the crude enzyme showed that the best values for enzyme production from *T. atroviride* 102C1 were: 55°C and pH 5.0 for BGU, 55°C and pH 3.0 for BXU, and 44°C and 3.6 for ARF (Table 3). These results indicate an acidic condition favoring all enzymes activities, whereas best temperature varied between 44 and 55°C (Table 3). Considering the concomitant production of the enzymes, one should choose pH 5.0 and temperature of 55°C, for best results (assay number 10, Table 3).

The model was tested for adequacy by ANOVA (Table 4). The model F values of 11.64 (BGU), 45.21 (BXU) and 14.68 (ARF) indicates that the model is significant at a high confidence level. The probability P value was also very low (<0.05) indicating the significance of the model. The coefficient of determination obtained for β-glucosidase (R^2=0.8845), β-xylosidase (R^2=0.9540) and α-L-arabinofuranosidase (R^2=0.9070) indicates that 88.45% (BGU), 95.40% (BXU) and 90.70% (ARF) of the variability of the responses can be explained by the model. The regression equations, obtained after the ANOVA, demonstrated enzymes activities as a function of the codified values of pH and temperature. The equation that represented a suitable model for β-glucosidase relative activity (Y) is given in:

$$Y_{BGU} = 95.80 - 12.01*pH + 2.56*T - 45.38*pH^2 - 17.82*T^2 - 8.25*pH*T \quad (4)$$

$$Y_{BXU} = 86.18 - 30.46*pH - 16.15*pH^2 - 33.83*T^2 - 7.37*pH*T \quad (5)$$

$$Y_{ARF} = 45.87 - 25.03*pH - 15.28*T - 18.22*T^2 + 21.83*pH*T \quad (6)$$

Thermal stability constitutes also an important property when studying the industrial importance of an enzyme. The results of β-glucosidase, β-xylosidase and α-L-arabinofuranosidase thermal stability are shown in Figure 3. When the enzyme crude extract was incubated at 60°C, the relative activities of β-glucosidase (Figure 3A) and β-xylosidase (Figure 3B) decreased around 50% within 30 min, however, a strong decreased (95%) was

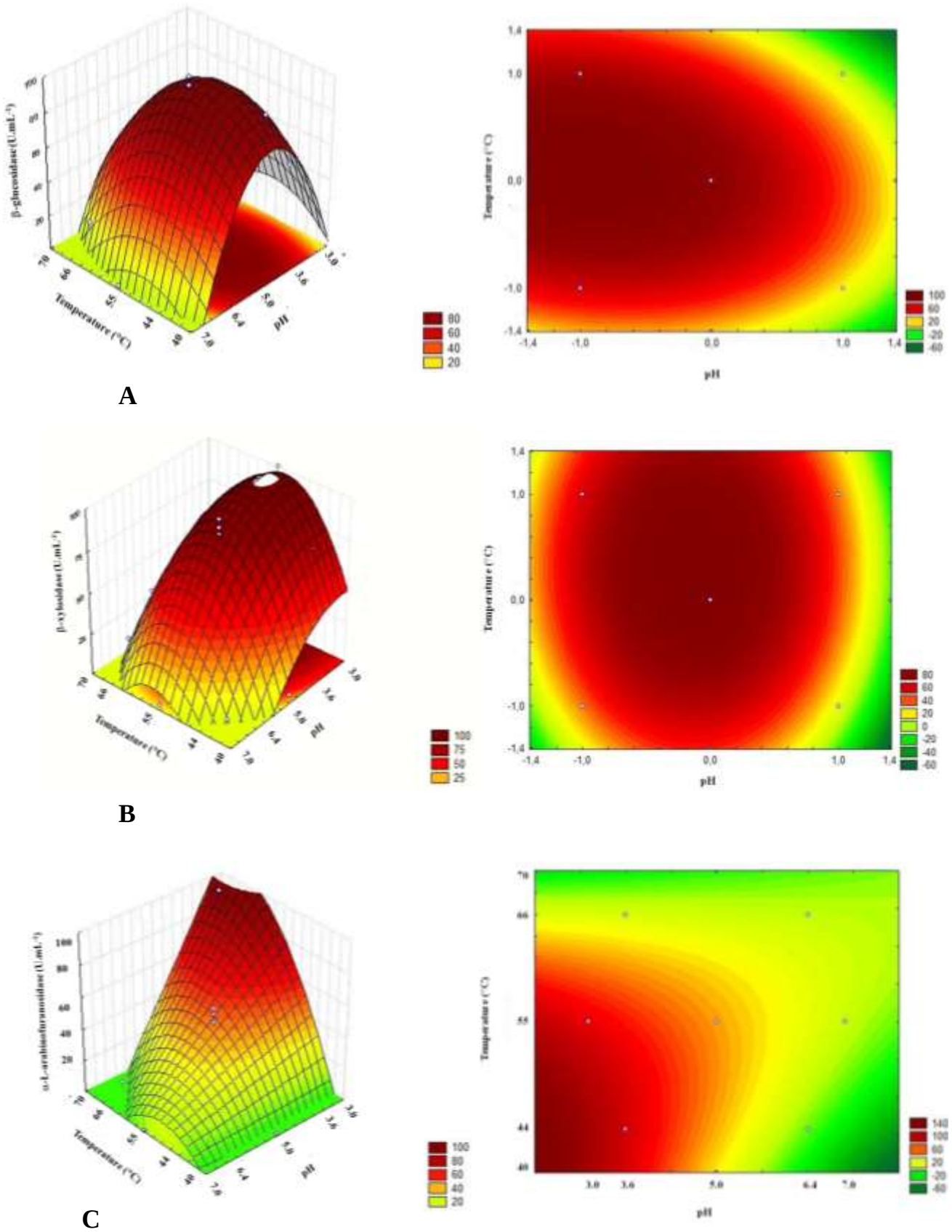

Figure 2. Response surface for *T. atroviride* 102C1 β-glucosidase (A), β-xylosidase (B) and α-L-arabinofuranosidase (C) activities by using pH and temperature values as the independent variables.

Table 3. Values of the independent variables [X_1 = pH; X_2 = temperature (°C)] of the CCRD 2^2, showing the statistically values for producing β-glucosidase, β-xylosidase and α-L-arabinofuranosidase by *T. atroviride* 102C1

Assay	Coded setting levels		Actual levels (%) (w/v)		β-glucosidase (Relative Activity %)*	β-xylosidase (Relative Activity %)*	α-L-arabinofuranosidase (Relative Activity %)*
	X_1	X_2	X_1	X_2			
1	-1	-1	3.6	44	29.61	59.73	100
2	+1	-1	6.4	44	15.52	21.28	7.05
3	-1	+1	3.6	66	49.20	70.89	6.13
4	+1	+1	6.4	66	2.00	3.01	0.49
5	-1.41	0	3.0	55	26.39	100	72.12
6	+1.41	0	7.0	55	1.89	3.12	0.44
7	0	-1.41	5.0	40	38.33	27.58	16.49
8	0	+1.41	5.0	70	99.61	5.19	1.25
9	0	0	5.0	55	92.49	82.01	52.27
10	0	0	5.0	55	100.0	90.73	47.22
11	0	0	5.0	55	94.72	85.91	55.77

* Values are based on Mean ± SD of 2 individual observations.

Table 4. Statistical ANOVA for the model of β-glucosidase, β-xylosidase and α-L-arabinofuranosidase relative activities at different levels of pH and temperature values.

Enzyme	Source of variation	Sum of squares	Degrees of freedom	Mean squares	F value	Value (prob >)[b]
β-glucosidase	Model	13,892.1	4	3,473.03	11.64	0.0054
	Residual	1,790.90	6	298.48		
	Lack of Fit	1,761.17	4	440.29	29.62	0.0329
	Pure Error	29.73	2			
	Total	15,683.0	10			
β-xylosidase	Model	14,267.89	4	3,566.97	45.21	0.0001
	Residual	473.32	6	78.89		
	Lack of Fit	435.34	4	108.84	5.73	0.1540
	Pure Error	37.98	2	18.99		
	Total	14,741.21	10			
α-L-arabinofuranosidase	Model	10,799.6	4	2,699.9	14.68	0.0029
	Residual	1,103.59	6	183.93		
	Lack of Fit	1,066.64	4	266.66	14.43	0.0658
	Pure Error	36.95	2	18.47		
	Total	11,903.19	10			

[b] Statistically significant at 95% of confidence level; R^2= 0.8858 (β-glucosidase); R^2= 0.9806 (β-xylosidase); R^2= 0.9073 (α-L-arabinofuranosidase).

observed for α-L-arabinofuranosidase. When tests were performed at 50°C the β-glucosidase relative activity decreased 35% after 30 min and remained stable up to 8 h incubation, whereas for α-L-arabinofuranosidase a decrease of more than 80% after 1 h of incubation was observed (Figure 3C). Inversely β-xylosidase relative activity was practically not influenced by this temperature, being completely stable even after 8 h incubation.

The effect of metal ions on enzymes activities from *T. atroviride* 102C1 was also studied (Table 5). In general the tested ions have caused a marked inhibition on enzyme relative activities. The presence of Ba^{2+}, Fe^{2+}, Cu^{2+}, Ca^{2+} Zn^{2+} and Mn^{2+} have totally inhibited the three enzymes. The exceptions were for ion K^+, Na^+ and Mg^{+2}. Ion K^+ had no effect on β-xylosidase, but caused a decrease of 40 and 82% on β-glucosidase and α-L-

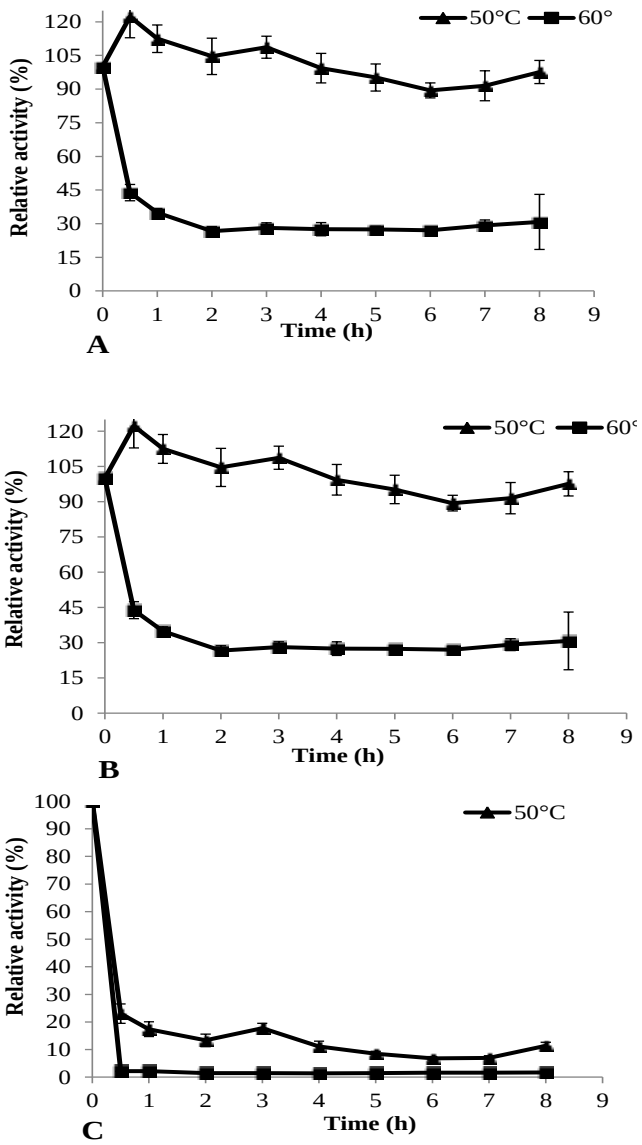

Figure 3. Thermal stability of *T. atroviride* 102C1 β-glucosidase (A), β-xylosidase (B) and α-L-arabinofuranosidase (C) activities at 60°C (filled square) and 50°C (filled triangle). Residual activity is expressed as a percentage of the original activity. Error bars represent one standard deviation of each experimental point (n = 3).

arabinofuranosidase relative activities, respectively. Ion Na^+ has caused the inhibition of only 9% on β-xylosidase, but of 54% on β-glucosidase, and 81% in α-L-arabinofuranosidase. Also effect of Mg^{2+} on β-xylosidase and β-glucosidase relative activities was very strong, but on α-L-arabinofuranosidase a decreased of only 30% was observed.

DISCUSSION

β-glucosidase, β-xylosidase and α-L-arabinofuranosidases are important accessory enzymes in the process of complete degradation of lignocellulosic materials. The α-L-arabinofuranosidases, in particular, participate in the initiation of the degradation process releasing specific side chains of hemicellulose polymers, whereas the β-glucosidases and β-xylosidases have important functions in the later stages of the degradation process releasing the fermentable sugars, glucose and xylose, respectively.

Data concerning β-glucosidase and β-xylosidase accumulation by *Trichoderma* species are scarce in literature. Most studies were performed using

Table 5. Effect of different metallic ions on β-glucosidase, β-xylosidase and α-L-arabinofuranosidase activities of the supernatant of *T. atroviride* 102C1.

Ion [a]	Relative activity (%) [b]		
	β-glucosidase	β-xylosidase	α-L-arabinofuranosidase
Control	100	100	100
Cu^{2+}	0.0	0.0	0.0
Mg^{2+}	0.1	0.4	72.0
Fe^{2+}	0.0	0.0	0.0
Ca^{2+}	0.0	0.0	0.0
Mn^{2+}	0.0	0.0	0.0
Zn^{2+}	0.0	0.0	0.0
K^{+}	60.0	103.0	18.1
Na^{+}	56.0	91.0	19.0
Ba^{2+}	0.0	0.0	0.0

[a] The final concentration in the reaction mixture was 2 mM.
[b] Relative activity is expressed as a percentage of Control.

Trichoderma reesei and *T. atroviride* however, some studies using other fungal species have been described. Also, in general, differently from our research, the enzymes production has been studied in separate. Considering the *Trichoderma* genus, a mutant strain of *T. reesei* RUT-C30 (ATCC 56765) was studied by Gottschalk et al. (2010). They described a very low titer of β-glucosidase (0.15 $U.mL^{-1}$) when the strain was grown in the presence of lactose, after 5 days fermentation. An interesting result was obtained when an heterologous expression of a β-glucosidase gene from *Penicillium decumbens* was inserted in *T. reesei* RUT-C30, the maximal activity observed being improved from 4.4 to 34.3 $U.mL^{-1}$, when using 2% (w/v) wheat bran and 3% (w/v) microcrystalline cellulose, after 7 days fermentation (Ma et al., 2011). As to *T. atroviride,* Kovács et al. (2008), developed a mutants strain, F-1505, which was considered the best mutant for β-glucosidase activity, endoglucanase activity and filter paper cellulase activity (FPase). The maximal activity for β-glucosidase (11.7 $U.mL^{-1}$) was observed when pretreated sugarcane bagasse was used, after 3 days fermentation. Also, in previous studies, our group verified a very low β-glucosidase production (0.17 $U.mL^{-1}$) in sugarcane bagasse, after 4 days fermentation, by *T. atroviride* 676, the wild strain of mutant *T. atroviride* 102C1 (Grigorevski-Lima et al., 2013). The results here obtained, of 55.4 $U.mL^{-1}$ of β-glucosidase for *T. atroviride* 102C1 is favorable.

Other fungal species have also been studied for β-glucosidase production, some with very high titres, such as *Aspergillus niger* strain NII 08121 which was cultivate using 1.0% (w/v) wheat bran for 4 days, a maximal β-glucosidase activity of 1,400.0 $U.mL^{-1}$ being observed (Singhania et al., 2011). Also Aliyah et al. (2017) observed a maximal β-glucosidase activity (91.67 and 85.01 $U.mL^{-1}$) when *A. niger* was cultivate using sugarcane bagasse and corn cob for 6 days, respectively. However, *Aspergillus* and *Penicillium* species, are considered greater β-glucosidase producers (Zampieri et al. 2013).

Studies on β-xylosidase production by *Trichoderma* strains such as *Aspergillus*, *Penicillium* and *Talaromyces* have shown very low results, compared to those here described, were values of 10.8 $U.mL^{-1}$ were obtained. *Trichoderma reesei* RUT C30, for instance, when grown on cellulose 1% (w/v) for 7 days, have shown maximal enzyme activity of 0.25 $U.mL^{-1}$ (Jiang et al., 2011). *Trichoderma virens* CTGxAviL, also grown on α-cellulose + xylan beechwood, after 3 days, have produced activities of 0.38 $U.mL^{-1}$ (Tarayre et al., 2015). Other fungal strains, as *Aspergillus awamori* (Paredes et al., 2015), for instance, have also shown very low results when grown on agro-industrial residues.

However, some studies are comparable with ours, such as those using *Lichtheimia ramose* by Garcia et al. (2015) which have shown a maximal β-xylosidase activity (11.57 $U.mL^{-1}$) in the presence of wheat bran, after 4 days fermentation. *Ceratocystis fimbriata* RM 35 when grown on wheat bran for 7 days was also able to produce 14.40 $U.mL^{-1}$ of β-xylosidase (Martins et al., 2018). Even though, our results were obtained after 48 hrs, which can be considered a great advantage.

Concerning AFR, as far as we are concerned, there are no studies in literature describing this enzyme activity for *Trichoderma* strains. However, some studies have shown a low production by other fungi. *Penicillium janczewskii*, for instance, was tested first with oat spelt xylan, during 7 days fermentation, producing 0.8 $U.mL^{-1}$, and then, using 1% (w/v) brewer's spent grain and 1% (w/v) orange waste after 10 days, with maximal values of 0.7 $U.mL^{-1}$ (Temer et al., 2014). In the same way, production of ARF by *A. awamori* 2B.361 U2/1 was low, of 0.7 $U.mL^{-1}$, when using media containing wheat bran, after 5 fermentation

days (Paredes et al., 2015). Other fungal strains, such as *Acremonium zeae* EA0802 and *Talaromyces thermophilus*, gave also very low values of α-L-arabinofuranosidase, when grown in oat spelts xylan (0.045 $U.mL^{-1}$) after 18 days (Almeida et al., 2011), and wheat bran (0.85 $U.mL^{-1}$) after 100 h (Guerfali et al., 2011). Our strain, *T. atroviride* 102C1, was able to produce 143.23 $U.mL^{-1}$, which, in comparison, is a very high titre.

Considering the production of the three enzymes concomitantly, the results obtained in the present research are quite promising. The mutant *T. atroviride* 102C1 have produced high titers of β-glucosidase (55.38 $U.mL^{-1}$), considering a *Trichoderma* species, good titres of β-xylosidase (10.75 $U.mL^{-1}$) and also high titres of α-L-arabinofuranosidase (143.23 $U.mL^{-1}$), at the same time, using very low cost residues of agro-industry, namely sugarcane bagasse and corn steep liquor, after only 2 days fermentation. There are few reports in the literature which study the production of the three enzymes concomitantly by fungi strain, as was done in the present work. Paredes et al. (2015), observed the production of the three enzymes by *A. awamori* 2B.361 U2/1 in the presence of 5 $g.L^{-1}$ xylan in growth medium at initial pH 5.0. In these conditions, the fungus produced 46 $U.mL^{-1}$ of xylanase (EC 3.2.1.8), 3.3 $U.mL^{-1}$ of β-glucosidase EC 3.2.1.21), 0.24 $U.mL^{-1}$ of β-xylosidase (EC 3.2.1.37), and 0.6 $U.mL^{-1}$ of α-L-arabinofuranosidase (EC 3.2.1.55) in the supernatant.

Data from literature have shown that, in general, BGU activities produced by different fungi were also acidic with optimal temperatures from 54 to 70°C. *Trichoderma harzianum* IOC-4038 showed as optimal conditions for enzyme relative activity pH 5.0 and 54°C (Castro et al., 2010). Bonfa et al. (2018) observed a maximal relative activity at pH 5.0 and 60ºC from thermophilic fungus *Myceliophthora thermophila* M.7.7. *A. niger* NII-08121 showed an acidophilic and thermophilic profile, with pH 4.8 and 70°C (Singhania et al., 2011).

Concerning BXU, it is common to observed in literature an acidophilic and thermophilic profile, with optima of pH and temperature around 4.0-5.0, and between 65-70°C, as observed for *Aspergillus* (Díaz-Malváez et al., 2013; Wakiyama et al., 2008) and *Ceratocystis* strains (Martins et al., 2018). Terrasan et al. (2011) observed the best condition for β-xylosidase when the enzymatic extract was incubated at 75°C and pH 5.0 from *P. janczewskii*. These results concerning optimal temperature are compatible with those showed by *T. atroviride* 102C1, which showed a maximal enzyme relative activity at 55°C. However, concerning pH, the best pH was more acidic (3.0), close to that observed by Knob and Carmona (2009) for *Penicillium sclerotiorum*, which have shown an even lower optimum pH, 2.5, and an optimum of temperature at 60°C. As for ARF data on literature are scarce. Enzyme relative activity from *P. janczewskii* was considered acidophilic and thermophilic, with best production at pH 4.0 and 60°C (Temer et al. 2014) whereas Guerfali et al. (2011) have found optimal ARF activity from *Talaromyces thermophilus* on more mesophilic conditions, pH, 6.0-7.0 at 55°C. In the present study, ARF from *T. atroviride* 102C1, was acidophilic for best pH, 3.6, and mesophilic for best temperature, 44°C.

There are some studies in literature which report inhibitory effects of some metal ions on β-glucosidases activities, mostly decreasing the enzyme relative activity by several degrees. For instance, Bonfa et al. (2018) observed a decreased of 30% for Na^{2+}, 50% for Mn^{2+}, 100% for Fe^{2+} and 37% for Zn^{2+} on BGU enzymatic relative activity from *My. thermophila* M.7.7, but a different result was observed for Ba^{2+} and Mg^{2+}, where no inhibition was detected. Some studies have reported the apparent activation of fungal BXU by Mn^{2+} and Ca^{2+}, suggesting that these ions activate and protect the active site (Yang et al., 2014; Pereira et al., 2015; Martins et al., 2018). Terrasan et al. (2011) observed a slight inhibition of β-xylosidase from *P. janczewskii* by Mn^{2+} (17%) and Ca^{2+} (21%), whereas Bonfa et al. (2018), have shown an inhibition of 50% by Mn^{2+} for the β-xylosidase from crude extract from *M. thermophila* M.7.7. Concerning ARF, Pereira et al. (2015) showed the metal ions Zn^{2+} and Co^{2+} as potential inhibitors of the α-L-arabinofuranosidase from *Penicillium chrysogenum* while Yang et al. (2015) observed that Fe^{2+}, Na^{2+}, Zn^{2+} and Mg^{2+} had no effect, but Ca^{2+} (19%), K^{+} (42%) and Co^{2+} (75%) inhibited the enzyme relative activity from *Alicyclobacillus* spp. A4. As one can seen, all these results from literature are compatible to the results obtained for *T. atroviride* 102C1.

Conclusion

As a conclusion of our study, we can say that the accessory enzymes here studied, produced by the mutant fungus *T. atroviride* 102C1, can be interesting for various industrial applications. It is important to stress that the three enzymes were obtained concomitantly, in only two days, in expressive amounts. Furthermore, the use of sugarcane bagasse and corn steep liquor, the main energy sources for microbial growth and enzymes production, allows for optimum use of these low cost agriculture residues. Activity at high temperatures is interesting, considering the fungus is mesophilic and can be very important for a future application in processes that are carried out at high temperatures. By this way, the environment will be highly favored due to the use of this system by the bioenergy plants for biofuel production, especially the production of bioethanol in Brazil, increasing sustainability and generating less environmental pollutants.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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