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MICROBIOLOGY

β-glucosidase from thermophilic fungus *Thermoascus crustaceus:* production and industrial potential

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Abstract: Microbial β-glucosidases can be used in several industrial processes, including production of biofuels, functional foods, juices, and beverages. In the present work, production of β-glucosidase by solid state cultivation of the fungus *Thermoascus crustaceus* in a low-cost cultivation medium (comprising agroindustrial residues) was evaluated. The highest production of β-glucosidase, about 415.1 U/g substrate (or 41.51 U/mL), was obtained by cultivating the fungus in wheat bran with 70% humidity, during 96 h at 40°C. The enzymatic activity was optimum at pH 4.5 and 65°C. β-Glucosidase maintained its catalytic activity when incubated at a pH range of 4.0-8.0 and temperature of 30-55°C. The enzyme was strongly inhibited by glucose; even when the substrate and glucose concentrations were equal, the inhibition was not reversed, suggesting a non-competitive inhibition. In the presence of up to 10% ethanol, β-glucosidase maintained its catalytic activity. In addition to β-glucosidase, the enzymatic extract showed activity of 36 U/g for endoglucanase, 256.2 U/g for xylanase, and 18.2 U/g for β-xylosidase. The results allow to conclude that the fungus *T. crustaceus* has considerable potential for production of β-glucosidase and xylanase when cultivated in agroindustrial residues, thereby reducing the cost of these biocatalysts.

Key words: Cellulases, filamentous fungus, industrial enzymes, solid state cultivation, xylanases.

INTRODUCTION

The economic dependence on fossil fuels and their imminent depletion in the near future has escalated the search for renewable sources of energy. Lignocellulosic biomass, found in abundance in nature, is mainly composed of cellulose, hemicellulose, and lignin, wherein cellulose is of great interest for obtaining fermentable sugars, aiming at ethanol production (Singhania et al. 2010).

Cellulases are enzymes capable of acting on cellulose, promoting their hydrolysis, which can be performed using three enzymes synergistically: endoglucanases (EC 3.2.1.4), which internally cleave the cellulose chains; exoglucanases (EC 3.2.1.91), which act at the ends of chains, releasing mainly cellobiose, and lastly, β-glucosidases (EC 3.2.1.21), which terminate hydrolysis, converting cellobiose to glucose (Bansal et al. 2012). β-Glucosidase is used in various industrial processes, such as the manufacture of functional foods derived from soybeans and also in the juice and beverage industry improving its aromatic quality (Leite et al. 2008). The main industrial sectors that use cellulases are textile, food, detergent, agriculture, and livestock industries. In these

industrial processes, enzymes may be exposed to extreme pH and temperature conditions. Thus, it is essential that biocatalysts used industrially must have high structural stability (Pellegrini et al. 2015).

Despite the wide applicability of β-glucosidases, their large-scale use remains a problem owing to the high cost of production. An alternative to reducing the cost of these enzymes would be the use of hyper-producing fungal strains, associated with the use of low value cultivation media, e.g. agroindustrial residues that are employed as substrates in solid state cultivation. This type of cultivation has several advantages for enzymatic production because it uses less energy, does not require strict control of cultivation parameters, produces less wastewater, has lower processing costs, and is similar to the natural environment of filamentous fungi (Santos et al. 2016, Garcia et al. 2015, Almeida et al. 2018).

Our research group has been working on the description of new biocatalysts from enzyme-producing filamentous fungi isolated from the Midwest region of Brazil, with appreciable biotechnological characteristics such as low production cost, high structural stability, and broad industrial applicability (Cavalheiro et al. 2017, Costa et al. 2019, Garcia et al. 2015, 2018, Morais et al. 2018). Among the strains recently isolated by our team, the thermophilic filamentous fungus *Thermoascus crustaceus* stood out for the production of the enzyme β-glucosidase. Considering the small number of studies that use this microorganism for the production of industrial enzymes, this study aimed to optimize the solid state cultivation of *T. crustaceus* aiming at the production of β-glucosidase and to evaluate the catalytic properties of this enzyme and the enzymatic extract, both obtained under optimal conditions.

MATERIALS AND METHODS

Microorganism

The present work used the thermophilic filamentous fungus *Thermoascus crustaceus*, isolated from sugarcane bagasse from São Fernando Açúcar e Álcool Ltda., Dourados - MS, Brazil. The microorganism was identified by Fungi Ecology and Systematics Laboratory, Institute of Biosciences, São Paulo State University (UNESP), Rio Claro – SP, Brazil. This strain was maintained in Sabouraud Dextrose Agar at 4°C, at the Laboratory of Enzymology and Fermentative Processes - LEPFER, Faculty of Biological and Environmental Sciences - FCBA, Federal University of Grande Dourados – UFGD.

Inoculum

The fungus was grown in 250 mL Erlenmeyer flasks containing 40 mL of inclined Sabouraud Dextrose Agar, maintained for 96 h at 45°C. The microbial suspension was obtained by adding 25 mL of nutrient solution consisting of ammonium sulfate, magnesium heptahydrate, and ammonium nitrate (0.1% w/v, each one), followed by smooth scraping of the medium surface. The microorganism was inoculated by transferring 5 mL of the microbial suspension (10^5 spores/g dry substrate) to flasks containing the agroindustrial residues (Garcia et al. 2015).

β-glucosidase production by solid state cultivation

The enzyme was produced by cultivating the fungus in 250 mL Erlenmeyer flasks containing 5 g of agroindustrial residues, previously washed and dried at 60°C for 24 h. Several residues, namely soybean meal, rice husk, corn husk, corn cob, sugarcane bagasse, orange bagasse, barley, and wheat bran, were used. All materials were previously autoclaved for 20 min at 121°C. The substrate that promoted the best enzyme

production was adopted for the evaluation of other fermentative parameters, such as initial humidity (50–80%), temperature (30–50°C), and cultivation time (24–144 h) in subsequent trials. The optimized parameter in each step was adopted in subsequent cultivation. All assays were performed in triplicate, and the described values represent the respective averages (Morais et al. 2018).

Enzyme extraction

The enzymatic extract was obtained by the addition of 50 mL of distilled water in the Erlenmeyer flasks containing the fermented residues. The flasks were stirred for 1 hour at 150 rpm. The medium was then filtered using a synthetic cloth (nylon) and centrifuged at 3000x*g* for 5 minutes at 10°C. The supernatant was considered the enzymatic extract and used in subsequent assays (Garcia et al. 2015).

Determination of β-glucosidase activity

β-glucosidase activity was determined with 50 µL enzymatic extract, 250 µL sodium acetate buffer (0.1 M, pH 4.5) and 250 µL p-nitrophenyl-pD-glucopyranoside (4 mM, Sigma), and keeping the mixture for 10 minutes at 50°C. The enzymatic reaction was stopped using 2 mL of sodium carbonate (2 M) and the released product was quantified in a spectrophotometer at 410 nm. One unit of enzymatic activity was defined as the amount of enzyme required to release 1 µmol of nitrophenol per minute of reaction (Palma-Fernandez et al. 2002).

Effect of pH and temperature on β-glucosidase activity

Optimal pH was determined by measuring the activity at 50°C at different pH values (3.0-8.0) using McIlvaine 0.1 M buffer. Optimum temperature was determined by measuring the enzymatic activity at different temperatures (30-80°C), at the respective optimum pH. The pH stability was determined by incubating the enzymes for 24h at 25°C at different values of pH, using the following buffer solutions: 0.1 M McIlvaine (3.0-8.0) and 0.1 M Glycine - NaOH (8.5-10.5). Thermostability was determined by incubating the enzyme for 1 h at different temperatures, which ranged from 30 to 80°C. Residual activity was determined under the respective optimum pH and temperature conditions (Martins et al. 2012).

Effect of glucose and ethanol on enzymatic activity

Enzyme activity was quantified by the addition of glucose or ethanol at different concentrations in the reaction mixture (0–100 mM glucose; 0–40% ethanol). The reversibility of the glucose inhibitory effect on enzyme activity was assessed by measuring catalytic activity with substrate addition at concentrations twice more than the inhibitor concentration.

Catalytic potential of crude enzymatic extract

Endoglucanase (CMCase), exoglucanase (avicelase), and xylanase activities were quantified using 3% carboxymethylcellulose (Sigma, C5678), 1% avicel (Sigma) and 1% xylan (Sigma, Beechwood), respectively, as substrates. The reducing sugar released was quantified by the DNS method described by Miller (1959). The activity of β-xylosidase was measured with the synthetic substrate p-nitrophenyl-p-D-xylopyranoside (4 mM, Sigma), following the methodology described previously for β-glucosidase. One unit of enzymatic activity was defined as the amount of enzyme required to release 1 µmol of product per minute of reaction (Garcia et al. 2015).

Statistical analysis

The experiments were performed in triplicates and data were expressed as the mean value of the three independent assays. Statistical analysis of the data was performed by ANOVA with significance level of 1%. Tukey test was applied to all data.

RESULTS AND DISCUSSION

β-glucosidase production by solid state cultivation

T. crustaceus was able to grow on all substrates used (visual evaluation), but the highest production of β-glucosidase (410.93 U/g substrate or 41.09 U/mL) was obtained by cultivating the fungus in wheat bran (Table I). According to El-Shishtawy et al. (2014), cultivations in wheat bran promote higher enzymatic production owing to their nutritional value, large surface area, better air circulation, and efficient penetration of mycelium from filamentous fungi. Previous work confirms wheat bran as an excellent substrate for β-glucosidase production by filamentous fungi (Santos et al. 2016, Garcia et al. 2018, Morais et al. 2018).

Considering the results presented in Table I, wheat bran was selected as substrate for optimization of parameters that affect enzyme production by solid state cultivation, such as: initial moisture, temperature, and cultivation time.

Table I. Production of β-Glucosidase by *T. crustaceus*, by solid state cultivation in agroindustrial residues with 70% of initial moisture, at 40°C for 96 h. Distinct letters indicate significant difference (p<0.01) according to Tukey test.

Substrates	U/g substrate
Wheat bran	410.9±26.9[a]
Sugarcane bagasse	4.3±1.2[c]
Orange Bagasse	8.6±0.4[c]
Barley	74.4±3.6[b]
Soybean meal	59.1±2.4[b]
Corn straw	13.5±0.7[c]
Rice husk	0.6±0.0[c]

Among the evaluated moisture values, the highest enzyme production was obtained in wheat bran containing 70% of initial moisture; however, there was no significant difference in enzyme production between cultivation with 65 and 70% of moisture (Figure 1a). The moisture of 70% was adopted for subsequent assays because it has a higher absolute value of β-glucosidase activity, when compared to the other results. Zimbardi et al. (2013) obtained higher β-glucosidase production by the fungus *Colletotrichum graminicola* with substrate moisture values close to those described in the present work.

Among the factors that influence the enzymatic production by solid state cultivation, water content is recognized as one of the most critical. Ideal moisture varies by microorganism and substrate used, affecting microbial growth and enzyme production. In cultivations containing low moisture content, solubility and nutrient diffusion are reduced, thereby impairing the growth of the microorganism. In contrast, high levels of moisture increase the risk of bacterial contamination and limit gas exchange, reducing enzyme production (Leite et al. 2008, Bansal et al. 2012, Delabona et al. 2013).

Regarding the influence of temperature, the highest β-glucosidase production by *T. crustaceus* (411.6 U/g of substrate or 41.16 U/mL) was obtained at 40°C (Figure 1b). *T. crustaceus* is a thermophilic fungus, adapted to grow at higher temperatures. According to Gomes et al. (2007), the ideal temperature for the cultivation of thermophilic fungi is between 40 and 50°C. This corroborates the results described in the present work.

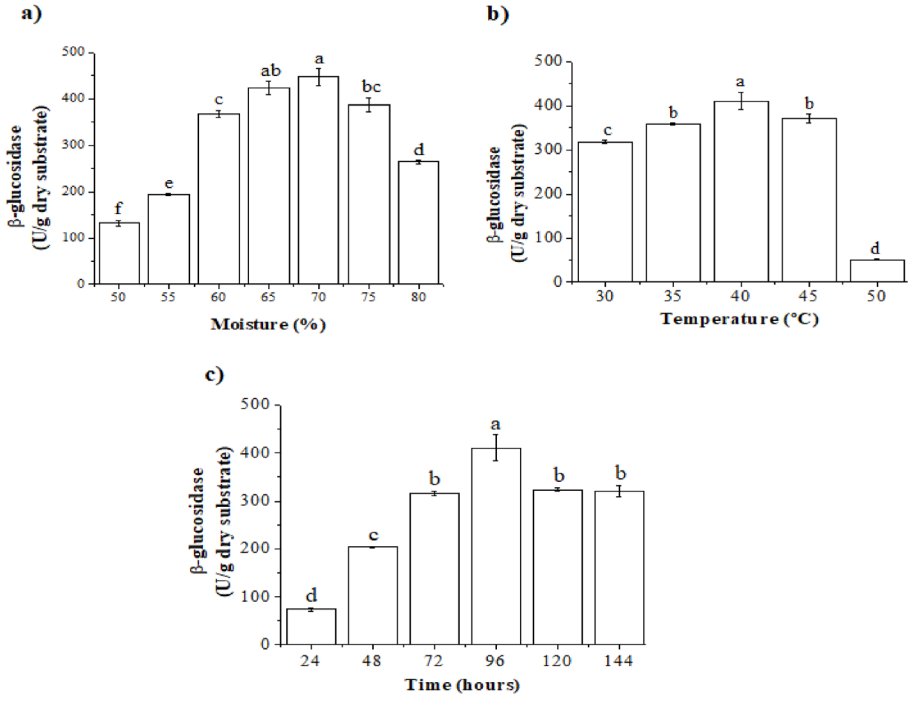

Figure 1. Production of β-glucosidase by solid state cultivation of *T. crustaceus* in wheat bran. a) Effect of moisture; b) Effect of temperature; c) Effect of cultivation time. Distinct letters indicate significant difference (p<0.01) according to Tukey test.

Considerable production of the enzyme of interest could be observed in cultivations maintained at 30 to 45°C; however, when the temperature was raised to 50°C, a drastic reduction in β-glucosidase production was observed. High temperatures may cause degradation of membrane structures as well as denaturation of structural proteins and enzymes (Rajoka et al. 2004).

The last parameter evaluated at this stage of the work was the cultivation time. The highest enzyme production was obtained in 96 h of cultivation at 40°C, using wheat bran as substrate with 70% initial moisture, reaching about 415.1 U/g substrate (or 41.51 U/mL). After this period, enzyme production dropped considerably (Figure 1c).

When compared to other fungal species, the optimal cultivation time for the production of β-glucosidase by *T. crustaceus* was lower (or equal) and the enzyme activity was considerably higher then those obtained by the authors cited (Table II).

Microbial cultivation time is a factor that influences enzymatic production and the cost of obtaining the biocatalyst. Incubation for a short period may not result in maximum enzyme production, as the microorganism may still be adapting to the environmental conditions. On the other hand, prolonging cultivation can lead to nutrient depletion, thereby causing a decline in microorganism growth and enzyme production, increasing the production cost of the enzyme (Haq et al. 2006).

Biochemical characterization of β-glucosidase produced by *T. crustaceus*

The β-glucosidase produced by *T. crustaceus* showed optimum activity at pH 4.5 and 65°C (Figures 2a and 2b). The characteristics of the enzyme produced by *T. crustaceus* are close to those described in the literature for fungal β-glucosidases. According to Santos et al. (2016), β-glucosidase produced by the fungus *Gongronella butleri* showed optimal activity at pH 4.5 and 65°C. Pereira et al. (2015) demonstrated

Table II. Production of β-glucosidase by several fungal species.

Microorganism	Time (Hours)	β-glucosidase (U/g substrate)	Author
Thermoascus crustaceus	96	415.1	This study
Gongronella butleri	96	215.4	Santos et al. (2016)
Colletotrichum graminicola	169	159.3	Zimbardi et al. (2013)
Aspergillus fumigatus	96	105.8	Delabona et al. (2013)
Byssochlamys spectabilis	96	77.0	Morais et al. (2018)
Thermomucor indicae-seudaticae	192	41.8	Pereira et al. (2015)
Lichtheimia corymbifera	144	39.0	Morais et al. (2018)

that *Thermomucor indicae seudaticae* N31 β-glucosidase showed maximum activity at pH 4.5 and temperatures of 65–75°C. The optimal pH and temperature values of β-glucosidase produced by *Lichtheimia corymbifera* described by Morais et al. (2018) resemble the results obtained in this work.

The β-glucosidase produced by the fungus *T. crustaceus* maintained its catalytic activity after 24 h of incubation between pH 4.0 and 9.0, and retained around 50% of the initial activity at pH 10.0 (Figure 2c). As for thermal stability, the enzyme remained stable at 30–55°C after 1 h of incubation, presenting about 60% of residual activity at 60°C (Figure 2d). The results described confirm the structural stability of the β-glucosidase produced by *T. crustaceus*. Delabona et al. (2013) described the stability of β-glucosidase produced by *Aspergillus fumigatus* P40M2; this enzyme maintained its catalytic properties at pH 4.0-5.5 and temperatures of 40-60°C. The results obtained by Fusco et al. (2018) demonstrated that *Dictyoglomus turgidum* β-glucosidase is reasonably stable in the pH range of 5.0-8.0, maintaining over 90% of its activity.

Enzymes produced by thermophilic microorganisms generally have high structural stability, favoring their application in several industrial processes. This fact justifies the constant search for thermophilic strains. Thermostability is an important characteristic for industrial enzymes, since the substrate conversion rate increases at high temperatures and under these conditions the risk of contamination is also reduced (Gomes et al. 2007, Leghlimi et al. 2013, Morais et al. 2018).

Effect of ethanol and glucose on β-glucosidase activity

The study of the effect of ethanol on β-glucosidases activity is very important, because these enzymes are generally exposed to several industrial process containing alcohol (Garcia et al. 2015).

Assays to evaluate the effect of ethanol on enzymatic activity were performed at 50°C to prevent alcoholic evaporation and to ensure that the reaction mixture contains the desired ethanol concentration. At initial ethanol concentrations, the enzyme increased its catalytic activity up to 35%, and the catalytic activity reduced in the assays performed with ethanol concentration equal to or higher than 25% (Figure 3a).

The increased catalytic potential of β-glucosidase may be related to the enzyme glycosyltransferase activity. In this case, ethanol acts as the preferential acceptor of intermediate glycosyl cation during substrate hydrolysis,

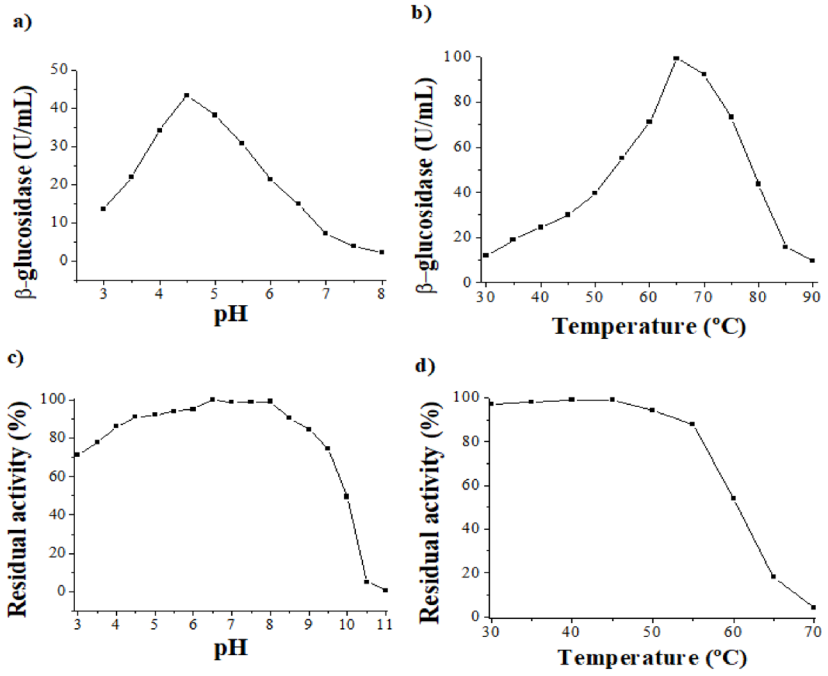

Figure 2. Effect of pH and temperature on the β-glucosidase activity from *T. crustaceus*. a) Optimal pH; b) Optimal temperature; c) pH stability; d) Temperature stability.

thereby increasing the rate of enzymatic reaction (Leite et al. 2008, Krisch et al. 2012, Garcia et al. 2015).

Considering that fermentative processes using *Saccharomyces cerevisiae* are carried out at temperatures below 50°C and that concentrations above 10% of ethanol are harmful to the fermenting microorganism itself (Gu et al. 2001), it can be stated that the β-glucosidase produced by *T. crustaceus* exhibits greater stability than is necessary for use in these processes.

Another important parameter evaluated was the effect of glucose on β-glucosidase activity. Enzymatic activity decreased as glucose concentration increased in the reaction mixture, remaining 30% of the initial activity in the assay performed with 100 mM glucose (Figure 3b).

According to the literature, most fungal β-glucosidases are strongly inhibited by glucose. Garcia et al. (2018) reported that the β-glucosidase produced by *Lichtheimia ramosa* was inhibited by this monosaccharide, and 50% of the initial activity was recovered in solutions containing 50 mM glucose. Santos et al. (2016) reported similar results for the β-glucosidase produced by *Gongronella butleri*. The β-glucosidases of *Lichtheimia corymbifera* and *Byssochlamys spectabilis* were strongly inhibited by glucose, and approximately 50% of their activities were recovered at 50 and 30 mM glucose, respectively (Morais et al. 2018).

The type of inhibition exerted by glucose on enzyme activity was evaluated by adding substrate to the reaction mixture at concentrations twice more than the inhibitor. Despite the high substrate concentration, the reversibility of inhibition was not observed, which is a non-competitive inhibition characteristic (Table III).

In this type of inhibition, the inhibitor and substrate do not compete for the same binding site (active site); the inhibitor binds to another region of the enzyme, decreasing the product generation rate. In this case, increasing the substrate concentration in the reaction mixture does not reverse the inhibition (Leite et al. 2008, Santos et al. 2016).

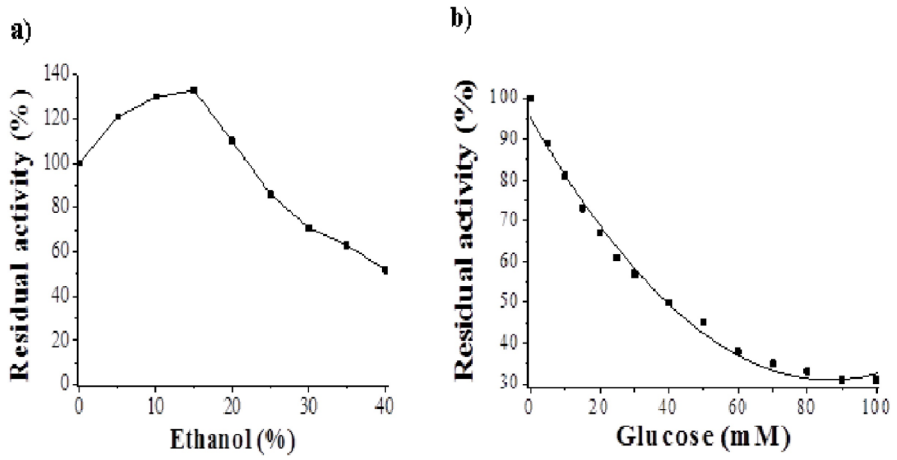

Figure 3. Effect of ethanol and glucose on the β-glucosidase activity from *T. crustaceus*. a) Effect of ethanol; b) Effect of glucose.

Glucose inhibition of β-glucosidase is a serious problem for obtaining fermentable sugars from cellulose hydrolysis present in vegetable biomass (Pereira et al. 2015, Garcia et al. 2018). An alternative to overcome this problem is to perform the process called saccharification and simultaneous fermentation. In this process, the saccharification of cellulose is performed simultaneously with the alcoholic fermentation. Thus, glucose released by cellulose hydrolysis is converted to ethanol by the fermenting microorganism, reducing the inhibitory effect of this monosaccharide on enzyme activity. However, the enzymes employed in the process must be tolerant to the presence of ethanol in the reaction mixture (Singhania et al. 2013, Pereira et al. 2015, Garcia et al. 2018, Morais et al. 2018).

The results described in the present work confirm the ethanol stability of the β-glucosidase produced by *T. crustaceus,* allowing the inference that this enzyme has potential to be applied in saccharification and simultaneous fermentation processes.

Catalytic potential of the enzymatic extract obtained under optimized conditions

The catalytic potential of the enzymatic extract produced by *T. crustaceus* under optimized cultivation conditions was evaluated (Table IV). Enzymatic extract showed no considerable cellulases activities, with 36 U/g for endoglucanase and 3.3 U/g for exoglucanase. The values described are below the values obtained by microorganisms described as producers of these enzymes. Hansen et al. (2015) reported that the cellulase production by solid state cultivation of the fungi *Fusarium chlamydosporum* and *Trichoderma reesei* (QM9414) yielded 281.8 and 94.2 U/g of endoglucanase and 182.4 and 99.7 U/g exoglucanase, respectively.

Regarding hemicellulases, *T. crustaceus* showed potential for production of xylanase (256.2 U/g substrate) and a small amount of β-xylosidase (18.2 U/g substrate), under the optimized culture conditions. The catalytic properties described for the enzymatic extract are favorable for application in the pulp and paper industry. The presence of xylanases and the low activities of endo and exocellulases make the enzymatic extract suitable for application in the pulp and paper industry, in the paper bleaching process, as they ensure that the cellulose fibers of the paper are not degraded by cellulolytic enzymes (Brienzo et al. 2012).

The characteristics described are difficult to observe in enzymatic cocktails obtained by

Table III. Residual activity at equal substrate (pNPβG) and inhibitor concentrations.

Enzyme	Res. Activity (%) PNPG – 2 mM	Res. Activity (%) PNPG – 2 mM Glucose – 25 mM	Res. Activity (%) PNPG – 50 mM Glucose – 25 mM	Inhibition type
T. crustaceus	100	56	58	Non-competitive

microbiological cultivations in complex media, such as wheat bran, thereby motivating the production of xylanolytic enzymes by submerged cultivation containing commercial xylan as exclusive carbon source, which increases the cost of producing these enzymes, making impractical the use of these biocatalysts in the pulp and paper industry (Costa et al. 2016).

CONCLUSIONS

The results show that the use of agroindustrial residues in the solid state cultivation process is viable for production of β-glucosidase by *Thermoascus crustaceus*, especially when cultivated in wheat bran. The β-glucosidase produced has wide industrial applications, justified by its characteristics and high production in low value culture media, and can be used in processes to obtain second-generation ethanol, as well as in the juice and beverage industry. The enzymatic extract has the potential to be applied in pulp and paper bleaching processes owing to its high xylanase activity, low cellulolytic activity, and low production cost.

Table IV. Catalytic potential of enzymatic extract obtained by solid state cultivation of the fungus *T. crustaceus* in wheat bran containing 70% of initial moisture, at 40°C and 96 h.

Enzyme	U/mL	U/g substrate
Endoglucanase	3.6	36.0
Exoglucanase	0.3	3.0
β-glucosidase	41.5	415.0
Xylanase	25.6	256.0
β-xylosidase	1.8	18.0

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** In memoriam*

Author contributions

Maria Alice Silvestre: collected samples, isolated and selected the fungus for β-glucosidase production; André Rodrigues: carried out the taxonomic identification of the microorganism; Andreza de Paula Garbin, Nayara Fernanda Lisboa Garcia and Gabriela Finoto Cavalheiro were responsible for the execution of all laboratory assays; Marcelo Fossa da Paz and Gustavo Graciano Fonseca were responsible for the orientation of the enzymatic production assays (Bioprocesses) and Rodrigo Simões Ribeiro Leite was responsible for the enzymatic analyzes. All authors discussed the results and contributed to the writing of the manuscript.