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FUNGAL HOME-MADE ENZYMATIC COCKTAILS FOR APPLE JUICE CLARIFICATION

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ABSTRACT

Sugarcane bagasse and cassava bagasse are lignocellulosic industrial residues that can be used as cheap substrates for organisms' growth. These lignocellulosic residues are also suitable for inducing enzyme secretion that can be applied in different bioprocesses such as juices clarification. The goal of our work was to use *Aspergillus niger* LBM 134 to produce xylanases, characterize them and employ these enzymes in the clarification of apple juice and pulp. *A. niger* LBM 134 was isolated from a natural environment and grown on sugarcane bagasse and cassava bagasse. The highest endoxylanase and β-xylosidase activity were 144 ± 5,65 and 0,74 ± 0,05 U mL^{-1}, respectively ($p<0.05$). The optimum activity and high stability at acidic pH values make these enzymes suitable for biotechnological applications in juice industries. The enzymatic cocktails produced by the fungus grown on cassava bagasse reached the major clarification of apple juice (36,66 ± 4,01%) and pulp (60,15 ± 5,63%) ($p<0.05$). These clarification percentages were due to the hydrolysis of hemicellulosic material carried out by the xylanases from *A. niger* LBM 134.

1.Introduction

The high demand for natural fruit juice during the last years, caused by growing health and nutrition consciousness, the development of modern processing technologies for improvement of juice quality in a cost-effective manner is inevitable (Kharazmi et al. 2020). The main cause of quality loss of fresh fruit juices is the presence of pectin, starch, cellulose, and hemicellulose as colloidal dispersion which form a cloudy, viscous, and turbid suspension (Lee et al. 2006). The cloudy juices contain fewer yields, are difficult to pasteurize and concentrate, and shorten the longevity of the membrane used in industrial scales. Therefore, these polysaccharides must be removed before commercialization to produce clarified fruit juices with high-level quality (Lee et al. 2006; Kharazmi et al. 2020).

The utilization of enzymes has been significantly extended in juice processing industries. Treatment of fruit juices with enzymes promotes the degradation of polysaccharides, for maximum yield, juice clarity, safety, shelf life, and storage stability (Rosmine, et al 2017). Xylanases are broadly used in the clarification of fruit and vegetable juices (Rosmine et al. 2017). Endo-1,4-β-xylanases (EXs, EC 3.2.1.8) are one of the most important xylanases; they hydrolyze the xylan, the main hemicellulosic polysaccharide, to small molecules such as xylooligosaccharides and xyloses (Lee et al. 2006; Salupi & Meryandini). In the food industry, particularly, in juice clarification, the main desirable biochemical

properties for xylanases are optimum activity and high stability at acidic pH values (Polizeli et al., 2005). Moreover, the use of enzymatic cocktails including these enzymes and other enzymes such as pectinases, cellulases and amylases, improves the juice clarification.

On the other side, the high cost of available commercial enzymatic cocktails urges bioprospecting and manipulation of microbes for higher productivity and enhancement of existing commercial enzymes (Kumar et al. 2014; Khusro et al. 2016). Therefore, numerous investigations on varying culture conditions for optimal xylanase production include fungal strains of *Trichoderma, Penicillium,* and *Aspergillus* (Rosmine et al. 2017). In this context, the aim of this study was to biochemically characterize the EXs present in the enzymatic cocktails from the fungus *Aspergillus niger* LBM 134 grown on agroindustrial wastes and to clarify the apple pulp and juice employing these home-made enzymatic cocktails.

2. Materials and methods

2.1. Fungal culture and enzyme production

A. niger LBM 134 is deposited in the Collection of the Laboratory of Molecular Biotechnology (LBM, from Spanish *Laboratorio de Biotecnología Molecular*) of the Instituto de Biotecnología Misiones. The fungus was cultivated in potato dextrose agar (PDA, 39 gL^{-1}) and incubated at 28 ± 2 °C for 5 days. A spore suspension of 10^7 spores mL^{-1} was obtained from the fungal culture and 1 mL of this suspension was used for inoculating Erlenmeyer flasks containing previously optimized media (Díaz *et al*., 2019). Then, media were filtrated and centrifugated at 10.000 g, 4 ºC for 15 min to obtain supernatants for enzyme xylanase characterization and juice and pulp clarification.

2.2. Determination of EX activity

EX activity was determined according to Bailey et al. (1992) through the quantification of released reducing sugars using beechwood xylan (Sigma-Aldrich, USA. Reducing sugars were measured by 1,3-dinitrosalicylic acid (DNS) assay (Miller, 1959) using xylose as the standard curve. Absorbance was measured at 540 nm. EX activity was expressed as international units (U), defined as the amount of enzyme needed to produce 1 µmol of xylose per min at 50 °C.

2.3 Effect of temperature and pH on EX activity

To study the effect of the temperature on EX activity, both crude enzymatic cocktails of *A. niger* LBM 134 grown on SCB and CB were incubated at pH 4.8 at 4, 10, 20, 30, 40, 50, 55, 60, 65, 70 and 80 °C. The effect of pH on EX activity in both crude extracts was studied at different pH values (3.0, 4.0, 4.8, 5.0, 6.0, 7.0, 8.0, 9.0 and 10) at 50 °C. EX activity was determined and expressed as described previously.

2.4. Thermostability and pH stability of EX activity

The thermostability of EX activity was evaluated by incubating both crude enzymatic cocktails at different temperatures (4, 30, 40, and 50 °C) during different intervals (6, 12, 24, 48, 72, and 96 h). To determine the pH stability of EX activity, the crude enzymatic cocktails were incubated at pH 5.0, 6.0, and 7.0 at 50 °C during different periods (6, 12, 24, 48, 72, and 96 h). The buffer solutions used were: 0.05 M citrate buffer for pH 3.0; 0.05 M sodium acetate buffer for pH 4.0, 4.8 and 5.0; 0.05 M sodium phosphate buffer for pH 6.0, 7.0 and 8.0; and 0.05 M Tris-glycine buffer for pH 9.0 and 10.0. EX activity was determined as described previously. Thermostability and pH stability was expressed as residual activity in percentage, taking the initial enzymatic activity as 100%.

2.5. Zymography

Previous to the zymography, cocktail supernatants were clarified by Chromafil Xtra PET-20/25 (0.20 µm) filters (Macherey Nagel; Düren, Germany) to obtain the cell-free enzymatic cocktails. Also, supernatants were clarified with a 0.1% Tween 80 aqueous solution, in a 2:1 ratio, to precipitate polysaccharides. Polysaccharides quantification

was determined by the phenol-sulphury technique (DuBois et al. 1956). Electrophoresis was performed in gels containing 7.5% (w/v) acrylamide; 2% (w/v) of beechwood xylan (Sigma-Aldrich, USA) was added into the separating gel. About 20 µg of proteins were applied to the gel. For that, proteins were determined following the Bradford method (Bradford 1976) employing Bradford Protein Assay (Bio-Rad, Hercules, California). Electrophoresis was conducted at 100 V for 2 h. A molecular weight marker (Phage Ruler pre-stained protein ladder (Fermentas, Thermo Scientific, USA) was added to the same gel and separated under the same electrophoresis conditions. Then, the gel was divided in half. One-half of the gel, containing the samples, was fixed with solution methanol:acetic acid: water (4:1:5), immersed in 0.05 mM sodium acetate buffer pH 4.8 and incubated at 50 °C for 60 min. The gel was then stained with 0.1% (w/v) Congo red solution and washed with distilled water and 1 M NaCl. Light yellowish activity bands were visible on deep red background. The other half of the gel, containing the molecular weight marker was stained with 2 g L^{-1} $AgNO_3$ to detect the proteins profile. The native molecular mass of the endoxylanases was estimated by using the molecular weight marker.

2.6.Apple juice and pulp obtention

Apple was washed and macerated using a blender to obtain the pulp. Apple fruit was peeled; separated from the seeds and macerated using a hand blender. A minimum amount of water was added to facilitate the maceration process as well as to help extract more juice from the pulp. The maceration process was repeated four times to get a smooth-textured puree.

2.7. Apple juice and pulp clarification by the supernatants from *A. niger* LBM 134

Apple juice and pulp were clarified by supernatants from *A. niger* LBM 134 grown on SCB and CB. The ratio used was supernatant:pulp or juice, 1:1. Two incubation conditions were assayed: 45 ° for 120 min and 50 °C and 60 min. After this period the samples were boiled for 5 min for enzyme inactivation and centrifuged at 21,000 g for 15 min.

The supernatant (juice) was used for determining juice clarity by recording transmittance at 650 nm, taking distilled water as the blank. Controls were carried out using apple juice and distilled water (1:1) and pulp:destilled water (1:1). Clarification was calculated according to Rosmine et al. 2017:

$$\% \text{ Clarification} = Tt - Tc/Tc \times 100 \quad (1)$$

whereas, Tt is the transmittance of test; Tc is the transmittance of control.

2.8.Statistical analysis

The experimental results were analyzed and graphed with the software GraphPad Prism 5.01 for Windows (GraphPad Software, San Diego, CA, USA).

3.Results and discussions

3.1. Biochemical characterization of proteins with EX activity in supernatants from *A. niger* LBM 134

Different enzymes have been employed for fruit juice clarification with the main purpose to generate a final product that is clear and visually attractive. The use of xylanases leads to an increase in the juice extraction yield and production efficiency and quality (Bajaj and Manhas 2012; Ahmed et al. 2016). The major factors influencing the enzyme activity are temperature, incubation period, pH, and concentration (Dhiman *et al.*, 2008). The EXs presents in both homemade enzymatic cocktails from *A. niger* LBM 134 showed a mesophile behavior; the optimal EX activity occurred at middle temperature and pH values. The effect of temperature and pH on the stability of the enzyme activities was studied in the crude enzymatic extracts of *A. niger* LBM 134 (Fig. 1). The maximal EX activity ($p<0.05$) was yielded when both extracts were incubated at 50 °C, reaching 108 ± 7.58 and 176 ± 1.79 U mL^{-1} in extracts from fungus grown on SCB (Fig. 1a) and CB (Fig. 1b), respectively. Regarding the effect of pH, the highest EX activity ($p<0.05$) of *A. niger* LBM 134 was reached when both crude

enzymatic extracts were incubated at pH 4.8 and 5 without a statistical difference being 119.5 ± 5.79 U mL^{-1} of EXs in the case the extract from the fungus grown on SCB (Fig. 1c) and 177.96 ± 1.32 U mL^{-1} in the case of CB (Fig. 1d). The EX activity was optimal at 50 °C and at higher temperatures, the enzyme activity dramatically decayed. This occurred since most of the globular enzymes such as xylanases are denaturalized under temperatures higher than 60 – 70 °C (Lehninger *et al*., 1976). Also, the enzyme activity depends on the pH of the media. In this case, the optimal pH EX activity was in the range of 4 to 5. At minor and higher pHs, the EX activity was very poor probably due to conformational changes in the enzymes or variation at the ionization states of their active sites, leading to no functional isoforms (Lehninger *et al*., 2006). Our findings agreed with other studies reported for fungal EXs with optimal catalysis at 40 - 60 °C and pH 4 to 7 (Wong *et al.*, 1988; Kulkarni *et al.*, 1999; Cuyvers *et al.*, 2011; Díaz *et al*., 2015; Cayetano-Cruz *et al*., 2016; Barchuk, 2017; Dhiman & Mukherjee, 2018).

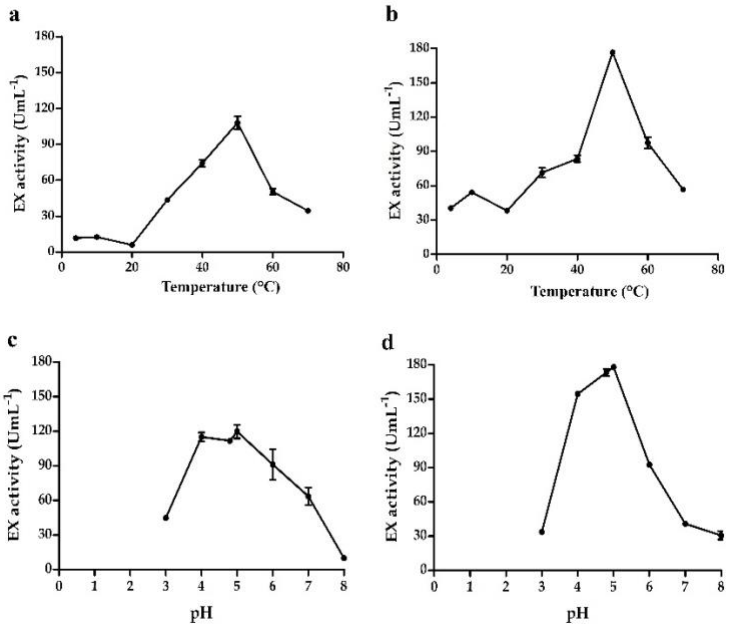

Figure 1. Effect of the temperature and pH on EX activity in supernatants from *A. niger* LBM 134 grown on SCB and CB. Optimal temperature of the EX activity in supernatants from the fungus grown on SCB (a) and CB (b). Optimal pH of the EX activity in supernatants from the fungus grown in SCB (c) and (d).

Also, pH and temperature significantly affected the enzyme stability. For that, we evaluated the effect of these physical parameters on the EX stability of the homemade enzymatic cocktails of *A. niger* LBM 134. Thermal and pH stability curves showed the effect of temperature and pH, respectively, on EX enzyme stability in the crude enzymatic extracts of *A. niger* LBM 134 grown on SCB and CB (Fig. 2). At 4 and 30 °C, the EX activity was above 50% during the studied period in both extracts. At 40 °C, the EX activity was above 50% until 12 h and 48 h in extracts from SCB (Fig. 2a) and CB (Fig. 2b), respectively; and at 50 °C, the enzyme activity decreased under 50% before the 12 h in both extracts. Respect on pH stability, the EX activity remained above 50% at pH 5, 6 and 7 until 96 h although the EX stability was higher at pH 5, remaining above 80% the enzyme activity in crude extracts of the fungus grown on SCB (Fig. 2c) and CB (Fig. 2d). Nevertheless, we evaluated the stability of the enzyme activity in the non-purified cocktails; this could lead to the action of different enzymatic inhibitors such as proteases affecting the EXs.

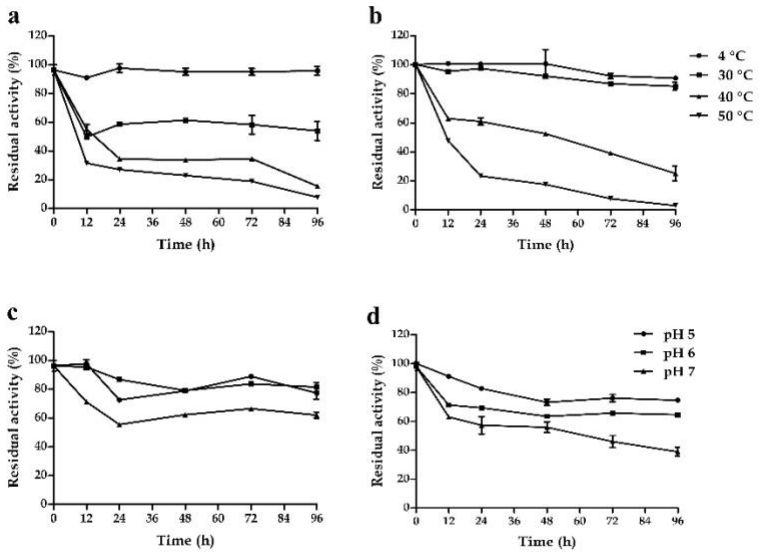

Figure 2. Thermal and pH stabilities of the EX activity in supernatants from *A. niger* LBM 134. Thermostability of EX activity in supernatants from the fungus grown on SCB (**a**) and CB (**b**). pH stability of EX activity in supernatants from the fungus grown on CB (**c**) and CB (**d**). The 100% corresponded to 108 ± 7.58 U mL^{-1} and 176 ± 1.79 U mL^{-1} in the case of the extracts from SCB and CB, respectively. EX, endoxylanase.

Figure 3. Determination of EXs profile by ND-PAGE (the gel was divided in half). **a.** a half gel stained with $AgNO_3$; molecular weight marker. **b.** the other half ND-PAGE using Congo Red staining for detecting EXs in supernatants from *A. niger* LBM 134 grown on SCB and CB and control media. Lanes correspond to culture supernatant of 1, 2 and 3 correspond to 20 µg of proteins in supernatants from the fungus grown on SCB; 4 and 5, 20 µg of proteins in supernatants from the fungus grown on the control medium of SCB; 6 and 7, 20 µg of proteins in supernatants from the fungus grown on the control medium of CB; 8, 9 and 10, 20 µg of proteins in supernatants from the fungus grown on CB.

Table 1. Apple juice and pulp clarification by enzymatic cocktails from *A. niger* LBM 134. Clarification values are expressed as percentages (%) and represent the means of the triplicates ± standard deviation.

Sample	Temperature (C°)	Time (min)	Clarification (%)	Significance
Juice + CB supernatant	45	120	22.82 ± 5.63	**b**
Pulp + CB supernatant	45	120	60.15 ± 4.74	**d**
Juice + SCB supernatant	45	120	ND	
Pulp + SCB supernatant	45	120	ND	
Control 1: juice + water	45	120	ND	
Control 2: pulp + water	45	120	ND	
Control 3: only juice	45	120	ND	
Control 4: only Pulp	45	120	ND	
Control 5: only SCB supernatant	45	120	ND	
Control 6: juice + commercial enzyme	45	120	19.67 ± 3.41	**ab**
Control 7: pulp + commercial enzyme	45	120	45.83 ± 4.88	**c**
Juice + CB supernatant	50	90	36.66 ± 4.01	**c**
Pulp + CB supernatant	50	90	11.57 ± 0.82	**a**
Juice + SCB supernatant	50	90	22.72 ± 2.80	**b**
Pulp + SCB supernatant	50	90	ND	
Control 1: juice + water	50	90	ND	
Control 2: pulp + water	50	90	ND	
Control 3: only juice	50	90	ND	
Control 4: only Pulp	50	90	ND	
Control 5: only SCB supernatant	50	90	ND	
Control 6: juice + commercial enzyme	50	90	35.95 ± 5.32	**c**
Control 7: pulp + commercial enzyme	50	90	11.83 ± 3.28	**a**

3.2. Zymogram analysis of supernatants

To verify the possible presence of EX isoforms, no denaturing-polyacrylamide gel electrophoresis (ND-PAGE) analysis were carried out. Figure 3 shows non-denaturing gel corresponding to the supernatants from *A. niger* LBM 134 grown on SCB and CB and their respective controls. Controls showed no bands on polyacrylamide gel due to the low enzymatic activity of this strain, while supernatants from the fungus grown on the bagasses showed degradation zones on the polyacrylamide gels. The EXs profile for supernatants from the fungus grown on both SCB and CB were similar showing two isoenzymes with electrophoretic mobility of approximately 25 and 35 KDa. We detected the presence of isoenzymes with less electrophoretic mobility in the cocktail from the fungus grown on SCB which can be isoforms with glycosylation events. These isoenzymes belong to EX B (family 11) and EX C (family 10), respectively (Díaz et al. 2020). These phenomena are recurring in EXs and can notably modify the molecular mass of the enzymes (Mura Escorche, 2016).

3.3. Clarification of apple juice and pulp by home-made cocktails rich in EX activity from A. niger LBM 134

The use of the homemade cocktail from *A. niger* LBM 134 grown on CB demonstrated the most clarification power on apple pulp 60.15 ± 4.74% at 45 °C and 120 min (P<0.00) (Table 1). High juice clarification, 36.66 ± 4.01% reached using the same home-made cocktail at 50 °C during 90 min (P<0.00); commercial enzyme on pulp clarification 45.83 ± 4.88% at 45 °C, 120 min and juice (P<0.00) and commercial enzyme on juice clarification 35.95 ± 5.32% at 50 °C, 90 min and juice (P<0.00). Similar observations were made by Kumar et al. (2014) and Rosmine et al. (2017) studying the clarification in other fruit juices. The clarification power is mainly due to disruption of hemicellulosic material and was higher under the action of the homemade cocktails from *A. niger* LBM 134 than the action of commercial enzymes evaluated in this study. In this context, the crude cocktails have the advantage of presenting more enzymes than purified endoxylanases leading to the degradation of other different polysaccharides instead of purified cocktails being used. This fact affects the total process of the clarification of the apple juice and pulp resulting in a visually more attractive product with better quality.

4. Conclusions

The present study features a promising approach for the production of two homemade enzymatic cocktails rich in hydrolytic enzymes, particularly in EXs, from *A. niger* LBM 134 grown on two agroindustrial wastes, SCB and CB, and the application of both cocktails on a biotechnological process, the clarification of apple pulp and juice. The fact of using agroindustrial wastes for obtaining the cocktails reduces the cost of the enzymes and for the biotechnological application.

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