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Low-cost extraction of multifaceted biological compounds from citrus waste using enzymes from *Aspergillus Niger* LBM 134

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Abstract

Agricultural industries search for biotechnological alternatives for waste management as they mean a significant concern and environmental challenge. More importantly, within a circular economy concept, such secondary substrates can be used to produce value-added compounds. This work is aimed at obtaining bioactive compounds from citrus waste by using a homemade enzymatic cocktail from *Aspergillus niger* LBM 134. The fungal enzymes were produced using raw sugarcane bagasse as substrate, which increased the ecological sustainability and the cost-effectiveness of the bioprocess. As the most relevant enzyme of this cocktail, a β-glucosidase showed to optimally act at 50 °C, retaining up to 70% of residual activity after 72 h. By means of an optimized enzyme-assisted extraction, the crude enzymatic cocktail produced was efficiently employed to extract the phenolics hesperetin, quinic, *p*-coumaric, and gallic acid, and the bioactive amino acid tryptophan from citrus waste. These assays yielded approximately 112% and 30% of phenolic compounds over alkaline conventional and commercial enzyme extraction methods, respectively.

Keywords Fungal enzymes β-glucosidase, Hesperetin, Gallic acid, Quinic acid, *p*-coumaric acid, Tryptophan

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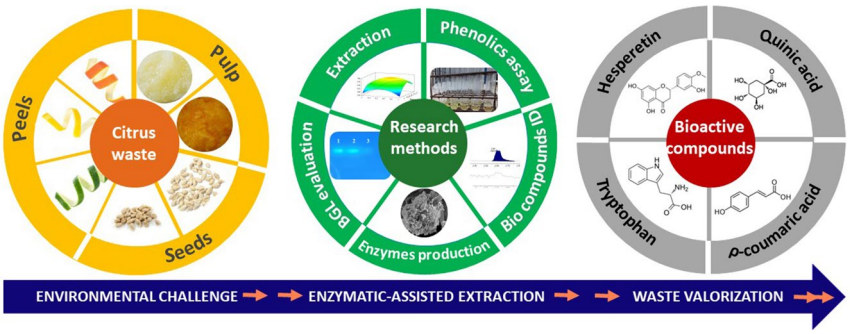
Graphical Abstract



Introduction

The increase in the global population has led to a substantial increase in the demand for food supply, and consequently in agroindustrial waste generation which has become a serious concern (Naik et al., 2023). Citrus waste refers to the by-products generated during the processing of citrus fruits and can come from biorefinery in the form of solid wastes, liquid waste, and distillery effluent, accounting for approximately 55–60% of raw fruit weight. Citrus waste is characterized by its high moisture, making it difficult and expensive for its appropriate disposal which is a serious problem for the food industry (Panwar et al., 2021; Sharma et al., 2017). Still, this by-product is a potential source of phenolic compounds including natural antioxidants polyphenols such as phenolic acids and flavonoids, and bioactive amino acids (Sharma et al., 2017).

Naturally occurring polyphenols are an important group of bioactive compounds in food because they decrease the risk of degenerative diseases through their anti-oxidative stress, anti-apoptosis, and anti-viral mechanisms (Bai et al., 2021). Also, L-Tryptophan is an aromatic essential amino acid of interest for the food industry, due to it is a pleiotropic precursor for many important metabolites that are known to influence cellular pathways and physiological responses required for normal growth (Bellmaine et al., 2020; Gao et al., 2020). The multifaceted biological properties of phenolic compounds and bioactive amino acids such as tryptophan make them good substitutes for antibiotics and chemical preservatives and promising compounds for new drug development. Consequently, in recent years, researchers worldwide have explored different processes to valorize citrus waste and use it as a natural source of added-value products for the food and pharmaceutical industries (Panwar et al., 2021; Sharma et al., 2017; Yadav et al., 2022).

Functional compounds have been extracted from citrus waste by several conventional techniques such as maceration, Soxhlet, and hydro-distillation. These methods present various disadvantages, such as long operation times, use of toxic solvents, high energy consumption, low yield, and purity of end products. Therefore, researchers have now focused on the development of novel techniques that can offer safer, more efficient, energy-saving, and sustainable extraction processes such as enzymatic-assisted extraction (EAE) (Panwar et al., 2021). The main enzymes reported for EAE of bioactive compounds, particularly phenolics, are those that can degrade the carbon components of the cell wall of biomass. These are cellulases (endo-β-1,4-glucanases, EG, EC 3.2.1.4; β-glucosidases, BGL, EC 3.2.1.21; cellobiohydrolases, CBH, EC 3.2.1.91), xylanases (endo-β-1,4-xylanases, EX, EC 3.2.1.8; β-xylosidases, BXL, EC 3.2.1.37), pectinases and amylases (Chávez-González et al., 2020; Coniglio et al., 2022). However, most of the work reports the use of commercial enzymes for carrying out the EAE which considerably increases the cost of the process (Coniglio et al., 2022). In this sense, the employment of a homemade enzymatic cocktail having the required enzymes for degrading the components of the plant matrix can supply commercial enzymes.

Filamentous fungi, such as *A. niger*, possess the enzymatic machinery to synthesize and extracellularly release hydrolytic enzymes, facilitating the degradation of cell wall carbon components (Díaz et al., 2020). Moreover, *A. niger* has been recognized for its involvement in the metabolic transformation of flavonoids, resulting in increased biological efficacy and facilitating the retrieval of bioactive compounds (Chávez-Gonzalez et al., 2020).

For that, this work aimed to (1) release bioactive compounds, particularly phenolics, from citrus waste using a homemade enzymatic cocktail of *A. niger* LBM 134 applying a surface methodology design; (2) characterize

the BGL activity and electro morpho profile in the fungal enzymatic cocktail.

Materials and methods

Fungus and feedstock materials

The fungus used in this work was *A. niger* LBM 134 (Díaz et al., 2021), which is deposited in the culture collection of the Instituto de Biotecnología Misiones "Dra. María Ebe Reca" of the Universidad Nacional de Misiones, Argentina. Stock cultures were maintained on 39 g L^{-1} potato dextrose agar (PDA) plates at 4 °C.

Citric waste was used as a source of bioactive compounds, particularly phenolics. This waste was provided by the Cooperativa Citrícola Agroindustrial de Misiones Ltda, Argentina. This material was dried at 60 °C overnight, milled to produce material retained through a 120-mesh screen, and stored at room temperature. The chemical composition of citric wastes was determined according to the Laboratory Analytical Procedure (LAP) and biomass analysis of the National Renewable Energy Laboratory (NREL, Technical Report TP-510-42618, U.S. Department of Energy) (data not shown).

Sugarcane bagasse (SCB) was used as the substrate of the fungus *A. niger* LBM 134 for growing and producing the enzymatic cocktail. SCB was sampled from a sugarcane mill at San Javier locality, Misiones, Argentina. The bagasse was dried at a constant temperature without exceeding 60 °C and milled to produce material retained through a 40-mesh screen. SCB was characterized according to the LAP and biomass analysis of the NREL by Díaz et al. (2022).

Production of an enzymatic cocktail by A. Niger LBM 134

The fungus *A. niger* LBM 134 was reactivated on 39 g L^{-1} PDA at 28 ± 2 °C under static conditions for 5 days until mycelial development. Then, the spores were aseptically scraped from the surface of the plates and suspended in sterile Tween 80 (Sigma-Aldrich, St. Louis, Missouri) aqueous solution (0.1% v/v) to obtain a spore suspension at 10^7 spores mL^{-1}. One mL of this spore suspension was inoculated in 100 mL Erlenmeyer flasks containing the culture medium optimized by Díaz et al. (2019) to obtain an enzymatic cocktail to carry out the EAE of citrus waste. The culture medium consisted of Czapek minimal medium supplemented with peptone 2.5 g L^{-1}, yeast extract 2 g L^{-1}, and SCB 15 g L^{-1}. The flasks were incubated at 28 ± 2 °C and 100 rpm for 8 days. Then, the culture broths were centrifuged at 10,000 g and 4 °C for 20 min, clarified, and sterilized by Chromafil Xtra PET-20/25 (0.20 µm) filters (Macherey Nagel; Düren, Germany) to obtain the cell-free enzymatic cocktails. Cellulases, xylanases, amylase, and pectinase activities were determined to confirm these activities in the enzymatic cocktail.

Determination of enzyme activities

Cellulases, EG, BGL, CBH; xylanases, EX, BXL; amylase; and pectinase activities were determined according to Table 1.

Effect of temperature on BGL activity in the cocktail of A. Niger LBM 134

Since BGL enzymes have been reported as the main ones responsible for releasing phenolic compounds from vegetable cell walls (Kaushal et al., 2021), BGL activity and stability were analysed in the enzymatic cocktail from the fungus *A. niger* LBM 134. The effect of the temperature on BGL activity was determined as described previously varying the temperature (20, 30, 40, 50, 60, 70, and 100 °C) to obtain optimal activity. The enzymatic cocktail was also incubated at 50 °C, at different intervals (6, 12, 24, 48, and 72 h) to study the BGL stability. The residual BGL activity was determined as described previously and expressed as a percentage, taking the initial enzymatic activity as 100% corresponding to 11.97 ± 0.10 U mL^{-1}.

Table 1 Protocols for determination of enzyme activities and enzymatic values

Enzyme activity	Substrate	Determination mode	Reference	Enzymatic levels (mL^{-1})
Endoglucanase	CMC 2%	Reducing sugars	Ghose (1987); Zhu et al. (2021)	14.33 ± 0.23 IU
β-Glucosidase	*p*NPG 0.5 mM	*p*-nitrophenol	Herr et al. (1978); Huang et al. (2021)	11.97 ± 0.10 IU
Cellobiohydrolase	*p*NPC 0.5 mM	*p*-nitrophenol	Ghose (1987); Kondaveeti et al. (2020)	11.84 ± 0.08 IU
FPase	Whatman no. 1 filter paper	Reducing sugars	Ghose (1987); Zhu et al. (2021)	0.37 ± 0.00 FPU
Endoxylanase	Beechwood xylan 1%	Reducing sugars	Bailey et al. (1992); Zhu et al. (2021)	46.45 ± 2.93 IU
β-Xylosidase	*p*NPX 1 mM	*p*-nitrophenol	Ghose and Bisaria (1987); Mercado- Flores et al. (2022)	0.08 ± 0.00 IU
Amylase	Soluble starch 2%	Reducing sugars	Abo-Kamer et al. (2023)	2.90 ± 0.90 IU
Pectinase	polygalacturonic acid	Reducing sugars	Mahto et al. (2023)	2.95 ± 0.60 IU

FPase filter paper enzyme, *CMC* carboxymethylcellulose, *pNPG* nitrophenyl glucopyranoside, *pNPC p*-nitrophenyl cellobioside, *IU* international units, *FPU* filter paper units

The fungal cocktail was incubated at 100 °C for 5 min to denaturalize the BGL activity. Then, the cocktail was cooled and the residual BGL activity was determined as described previously.

Effect of pH on BGL activity in the cocktail of A. Niger LBM 134

To study the effect of pH on BGL activity, this activity was determined as described previously, varying the pH (3, 4, 5, and 6). For that, 0.05 M citrate buffer was used for pH 3; 0.05 M sodium acetate buffer, for pH 4 and 5; and 0.05 M sodium phosphate buffer, for pH 6.

The enzymatic cocktail was also incubated at pH 5 during different periods (6, 12, 24, 48, and 72 h) to study the BGL stability. The residual BGL activity was determined as described previously and expressed as a percentage, taking the initial enzymatic activity as 100%.

Detection of BGL activity in polyacrylamide gels

To identify the BGL electro morpho profile in the cocktail of *A. niger* LBM 134 on SDS-PAGE was carried out according to Laemmli (1970) with 4% stacking gel and 12% resolving gel. About 20 µg of proteins from each sample was applied to the gel by adding 10% SDS (w/v); Lane 1 corresponded to the cocktail enzymatic without any incubation (time 0); Lane 2, to the enzymatic cocktail after 72 h incubated at 50 °C; Lane 3, to the enzymatic cocktail treated at 100 °C for 5 min; lane 4, negative control. For that, proteins were determined following the Bradford method (Bradford, 1976) employing the Bradford Protein Assay (Bio-Rad, Hercules, California). Electrophoresis was performed at 100 V for 2 h, by adding 1 g L^{-1} SDS to the running buffer. After protein separation, SDS was cleaned from the gels by soaking them for 30 min in 2.5% Triton X-100 at 25 °C. The gels were then briefly washed for 1 min in 50 mM sodium acetate buffer, pH 5.0, and covered with a solution of 0.1 mM 4-methyl umbelliferyl glucoside (Mu-g; Sigma, MO, USA). After 40 min, gels were exposed to UV light. Electro morpho with positive BGL activity was visualized as bright bands.

Optimization of conditions for extracting phenolics from citrus waste

To optimize the conditions (temperature, pH, and time of incubation) of the EAE of phenolic compounds from citrus waste, a central composite design (CCD) was employed. The CCD had five levels (-α, −1, 0, +1, +α) and four replicates at their central points with 18 experimental runs (Supplementary Table 1). For each experimental run, a sample of 0.3 g (dry weight) of citrus waste was incubated in 15 mL of a mixed reaction to get a 2% (w/v) final concentration. This mixed reaction contained 10 IU of BGL g^{-1} of biomass from the cocktail of *A. niger* LBM 134, 0.05 M sodium acetate buffer at pH according to the experimental run of the CCD. All reactions were incubated at 100 rpm, and different temperatures and times according to the CCD matrix. After the incubation, the reactions were stopped by adding NaOH 0.1 M to reach a pH of 8 and incubating at 50 °C and 100 rpm for 30 min. The reactions were centrifugated at 12,096 g (DCS-16RV Presvac, San Martín, Buenos Aires, ARG, Argentina) for 20 min and the extracts were used to measure total phenolic compounds (TPC) and identify biological compounds, particularly phenolics.

The behavior of CCD is explained by the following equation:

$$y = a_0 + \sum a_i x_i + \sum a_{ii} x^2{}_i + \sum a_{ij} x_i x_j \quad (1)$$

where y is the response, TPC, a_0 is the intercept coefficient, a_i is the coefficient of the linear effect, a_{ii} is the coefficient of quadratic effect, and a_{ij} is the coefficient of an interaction effect, x_i and x_j denote the encoded levels of the variables X_i and X_j in the experiments.

To validate the optimization, six experimental runs were carried out under the incubation conditions predicted by the model. After that, reactions were stopped as described previously.

In addition, four control groups were carried out to evaluate the performance of the enzymatic cocktail of *A. niger* LBM 134 on the extraction of phenolic compounds. The first group was a conventional alkaline extraction (AE) using 1 N NaOH pH 9.5; the second group was a buffer-assisted extraction using only sodium acetate buffer 0.05 M, pH 4.8; the third group was an EAE using the enzymatic cocktail of *A. niger* LBM 134 pretreated at 100 °C for 5 min; and the fourth group was an EAE using 10 IU of the commercial enzyme Viscozyme L (Novozymes, Denmark). In all cases, 0.3 g (dry weight) of citrus waste was incubated in 15 mL of a mixed reaction to get a 2% (w/v) final concentration and all the reactions were incubated under the optimal conditions predicted by the model. After the incubation, the reactions were treated as described by the reactions of the CCD. TPC and biological compounds were also determined in these control groups.

Determination of TPC

TPC was determined by the colorimetric method proposed by Singleton et al. (1999) using 1 N Folin/Ciocalteu reagent (Biopack, Argentina) at alkaline pH via 7.5% Na_2CO_3. After 30 min of incubation, the mixture reaction was read at 765 nm and TPC was expressed in mg of gallic acid equivalents per mL (mg GAE mL^{-1}) against a standard curve.

Identification of biological compounds released from citrus waste

Bioactive compounds released from citrus waste by the buffer-assisted extraction, AE, and EAE with fungal and commercial enzymes were identified by LC-MS/MS analysis on an Acquity UPLC H-Class (Waters) with a quadrupole Xevo TQ-S Micro (Waters) using a Waters BEH C18 analytical column (Waters) at 15 °C. The isocratic mobile phase consisted of A: H_2O MQ/0.5% (v/v) formic acid; B: methanol/0.5% (v/v) formic acid at a flow rate of 0.25 mL min^{-1}. Also, phenolic compounds released from citrus waste by the extractions were identified by HPLC-MS/MS using an Ultimate 3000 RSLC (Dionex, Thermo Scientific) with a TSQ Quantum Access Max triple quadrupole mass spectrometer (Thermo Scientific). The mobile phase consisted of (A) H_2O MQ/0.1% formic acid and (B) acetonitrile/0.1% formic acid at a flow rate of 0.3 mL min^{-1} using a Hypersil GOLD aQ column (Thermo Scientific). The concentration of the phenolic compounds was quantified against pattern curves of *p*-coumaric acid, gallic acid, quinic acid, and hesperetin (Sigma-Aldrich, USA).

Statistical analyses

Factorial design and experimental results of the CCD were performed and analysed, respectively, with the software Statgraphics Centurion XVI.I.

Analysis of the BGL study and control tests of EAE were conducted in triplicate and the results were expressed as mean ± standard deviation. Statistical differences among samples were estimated using Student's t-test using the software GraphPad Prism 5.0 (Graph Pad Software Inc., San Diego, CA).

Cost-effectiveness study

The economic analysis of releasing biological compounds from citrus waste by the EAE using the *A. niger* LBM 134 cocktail was compared with the cost of employing the commercial enzyme Viscozyme.

Results

Production of the low-cost enzymatic cocktail from A. Niger LBM 134

In this work, cellulases xylanases, pectinases, and amylases were determined in the cocktail produced by LBM 134 strain (Table 1). Cellulases were the most abundant enzymes, followed by xylanases, whereas pectinases and amylases were the least abundant.

A. niger LBM 134 enzymatic extract exhibited a high titer of BGL; hence we focused on the evaluation of BGL activity in the enzymatic cocktail from the fungus *A. niger* LBM 134.

Evaluation of the BGL activity and stability in the cocktail of A. Niger LBM 134

The effect of temperature and pH on the activity and stability of the crude *A. niger* LBM 134 BGL is shown in Fig. 1. The maximal enzyme activity occurred at 50 °C and pH 5 (Fig. 1a, b), which was the standard assay temperature and pH.

The enzyme did not lose activity under incubation at 50 °C for 24 h and was stable for 72 h, keeping a residual activity above 75% (Fig. 1c). BGL stability was above 70% after incubation at pH 5 after 72 h (Fig. 1d). These results on enzyme stability are desirable for any bioprocess.

BGL activity of the cocktail of *A. niger* LBM 134 treated at different temperatures was detected in polyacrylamide gels, where bands of different brightness were observed in the Mu-g SDS-PAGE gel under UV light. Bands corresponded to the fungal enzymatic cocktail exposed to different temperatures (Fig. 2). The cocktail without exposition (time 0) showed the most intense band (Lane 1). A relevant finding was that BGL in the cocktail exposed at 50 °C after 72 h of incubation kept its enzymatic activity (Lane 2) and showed activity after being incubated at 100 °C for 5 min (Lane 3), denoted by the presence of bright bands. After the heat treatment, the BGL enzyme was probably renatured by the addition of Triton X-100.

Optimization of the EAE conditions of phenolic compounds from citrus waste using the A. niger LBM 134 enzymatic cocktail

Data from the experimental runs of the CCD showed a large variation in TPC values. The quadratic expressions for all conditions of incubation were significant ($P \leq 0.05$) on levels of TPC. This statistical significance indicated that optimal conditions of incubation were around the central points. The R^2 of the model was 0.87, which means that 87% of the total variability of TPC could be explained by the model. Response surface plots showing interactive effects were drawn from experimental data to show the optimal levels of incubation conditions for releasing the TPC (Fig. 3). The maximal TPC extraction occurred when pH and time were at their center levels, pH 5 and 8 h, respectively, being the temperature fixed at its middle level (Fig. 3a). b) The maximal TPC level was observed when the temperature was about 40 °C (Fig. 3b) and pH around 5 (Fig. 3c).

The equation of the adjusted model for TPC (*y*) without the nonsignificant factors is the following:

$$\mathrm{TPC} = 69.04 - 4.93 * \mathrm{Temperature}^2 - 3.71 * \mathrm{pH}^2 - 6.71 * \mathrm{Time}^2 \quad (2)$$

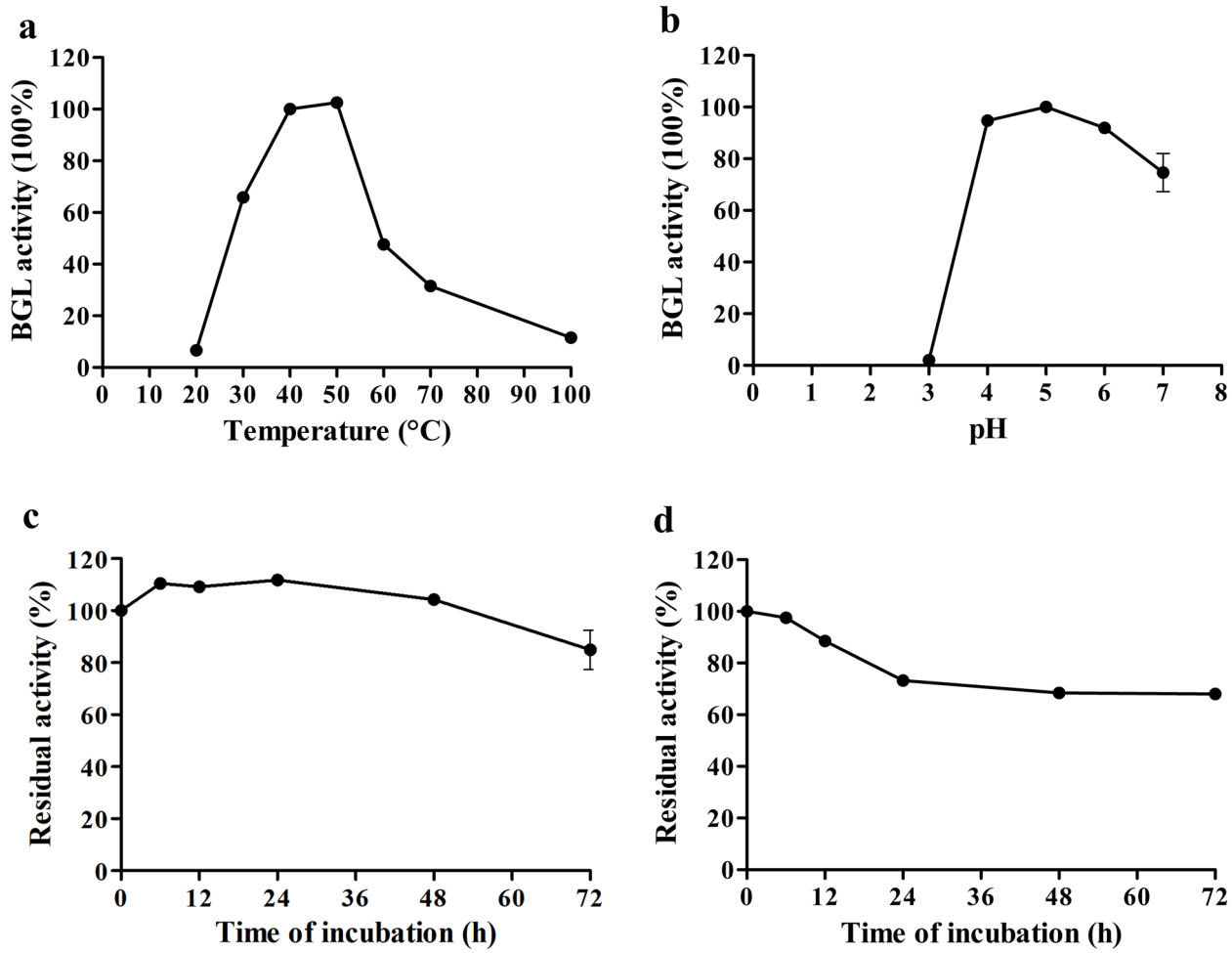


Fig. 1 Effect of temperature on BGL activity and stability from the enzymatic cocktail of *A. niger* LBM 134. **a** Optimal temperature. **b** Optimal pH at 50 °C. **c** Thermostability at 50 °C during 72 h. **d** Stability of pH at 50 °C

This model predicted an optimal TPC of 69.04 mg GAE mL^{-1} under the conditions at level 0: 40 °C, pH 5, and 8 h. Six experimental runs were carried out under these predicted conditions; their average was 71.97 ± 1.71 mg GAE mL^{-1} of TPC, showing concordance with the predicted value.

There was no significant ($P > 0.05$) difference between the TPC values obtained by the extraction with buffer and the AE showing both the lowest TPC yields. The highest release of phenolic compounds from citrus waste was obtained with EAE using the enzymatic cocktail of *A. niger* LBM 134 ($P < 0.05$), releasing about 112% phenolic compounds more than the AE and 30% more than the EAE using the commercial enzyme Viscozyme (Fig. 4). Moreover, the was no statistical difference between TPC released by EAEs using commercial enzyme or the enzymatic cocktail of *A. niger* LBM 134 treated at 100 °C for 5 min.

Identification of phenolic compounds with multifaceted biological properties released from citrus waste

We identified five bioactive naturally occurring compounds with multiple biological properties from citrus waste. Four of them, the *p*-coumaric, tryptophan, quinic acid, and hesperetin were released by EAE using both the commercial enzyme and the cocktail from *A. niger* LBM 134, and the gallic acid was released by AE (Table 2). We identified three phenolic acids in this study, gallic acid in extracts from the AE; quinic, and *p*-coumaric acid in extracts from the EAE carried out by both the fungal and the commercial enzymes. We only detected gallic acid (3,4,5-trihydroxybenzoic acid) in extracts obtained from the treatment with the alkali NaOH. We also identified tryptophan in both enzymatic extractions; but not in the alkaline extraction.

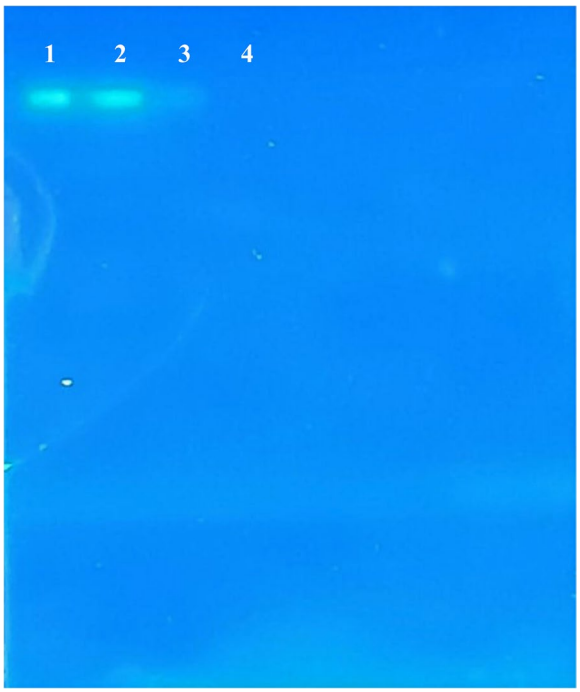


Fig. 2 Determination of BGL of the cocktail of *A. niger* LBM 134 treated at different temperatures by an ND-PAGE using Mu-g as enzyme substrate

Cost-effectiveness study

The cost of using enzymes for EAE of phenolic compounds from citrus waste was analysed by comparing the costs of using the enzymatic cocktail from *A. niger* LBM 134 or the commercial enzyme Viscozyme (Table 3).

In this work, we have shown that the extraction of phenolic compounds from citrus waste using the low-cost enzymatic cocktail of *A. niger* LBM 134 is both efficient and economical.

Although the identified phenolic compounds extracted by both the enzymatic cocktail of *A. niger* LBM 134 and the commercial enzyme were similar, the cost of the global process of fungal enzymes was almost 150 times less expensive than the commercial one. Moreover, as it was mentioned previously, the fungal cocktail can extract about 30% higher phenolic compounds than the commercial cocktail (Fig. 4).

Discussion

Production of the low-cost enzymatic cocktail from A. niger LBM 134 with high titers of BGL

The hydrolytic enzymes degrading the carbon components of the vegetal cell walls were determined in the enzymatic cocktail of *A. niger* LBM 134 grown on SCB. This by-product was reported to be useful in producing a wide variety of hydrolytic enzymes by fungi and bacteria, mostly carbohydrate-active enzymes (Díaz et al., 2019; Pisa et al., 2022; Scarcella et al., 2021). We determined more levels of cellulases in the enzymatic cocktail followed by xylanases; pectinases and amylases were the least abundant. Moreover, the cocktail exhibited a high titer of BGL, which showed to be sufficient as the main activity to release bioactive compounds, particularly phenolics, from citrus waste. Otherwise, several commercial enzymatic cocktails need to be supplemented with exogenous BGL including those of *Trichoderma reesei* (Díaz et al., 2020; Singhania et al., 2013). In addition, BGL has been reported as the main responsible for releasing phenolic compounds from the biomass cell walls by hydrolyzing β-1,4-glycosidic bonds in glycosides and oligosaccharides (Kaushal et al., 2021; Wang et al., 2017). For these reasons, we focused on the evaluation of BGL activity in the enzymatic cocktail from the fungus *A. niger* LBM 134.

The optimal temperature and pH of the BGL activity of this enzymatic cocktail were 50 °C and 5, respectively, similar to those of most reported fungal BGLs (Prieto et al., 2021; Zheng & Shetty, 2000). The enzyme did not lose activity under incubation at 50 °C for 24 h and was stable for 72 h, keeping a residual activity above 75%, which is desirable for any bioprocess.

The good performance of BGL activity and stability showed by the cocktail of *A. niger* LBM 134 increases the potential use for different biotechnological applications, such as the EAE. In this context, BGL-assisted extraction could significantly improve the release of insoluble-bound phenolics content of vegetal matrices by degrading the bounds between these compounds and the cell wall-carbon components (Wang et al., 2017).

Optimization of the EAE conditions of phenolic compounds from citrus waste

The large variation in TPC values from CCD data indicates the importance of optimizing the extraction conditions to obtain the maximum phenolic compounds. The optimal conditions of incubation were 40 °C, pH 5 and 8 h.

The observed increase in TPC by the EAE carried out with the fungal cocktail demonstrates the importance of enzyme activities for improving the extraction yields of these compounds due to the disruption of the bonds between them and the cell wall components of the biomass matrix (Nadar et al., 2018). Therefore, the enzymatic cocktail reported in this work is mandatory for extracting phenolic compounds from citrus waste. The extra goal of the enzymatic cocktail produced by *A. niger* LBM 134 is that it used SCB as substrate instead of using commercial enzymes or pure chemical substrates, becoming the process more cost-effective and environmentally sustainable.

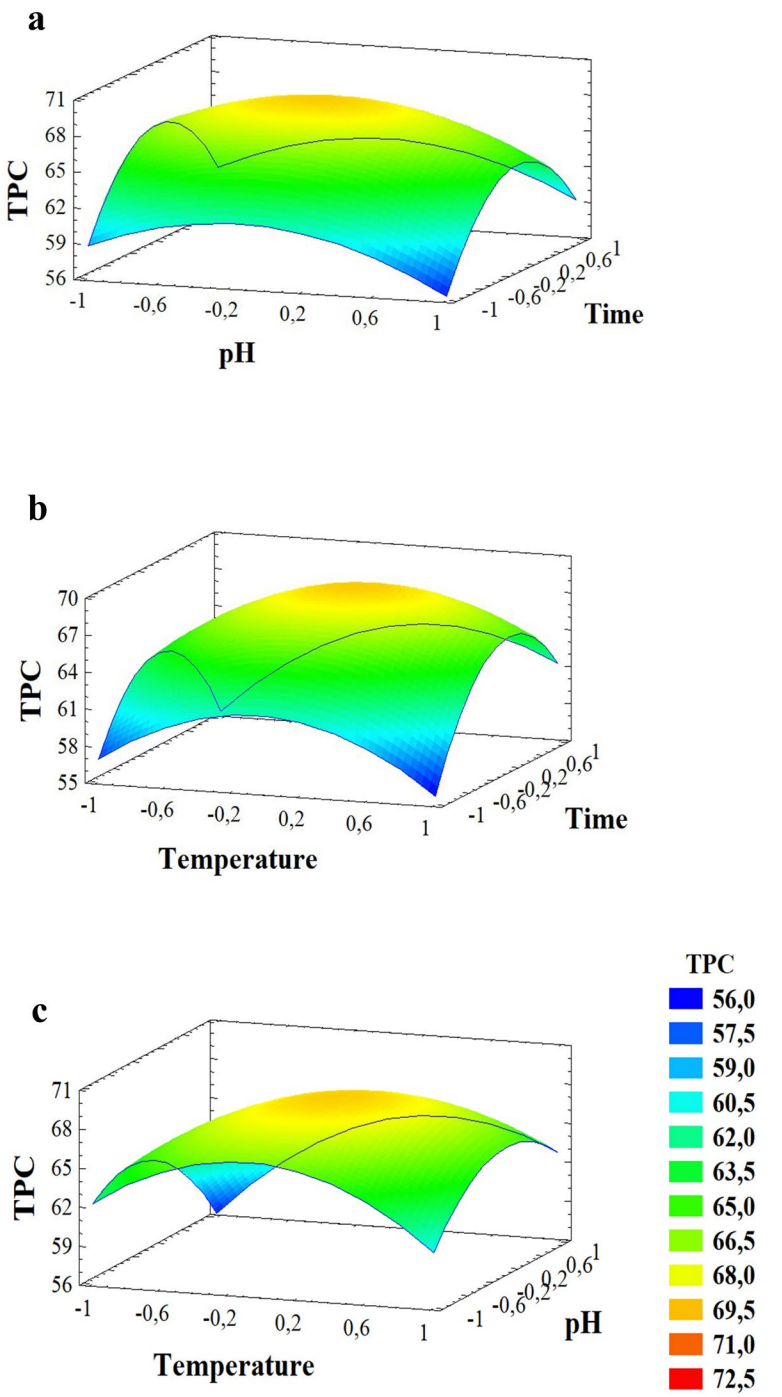


Fig. 3 Response surface plots for interactive effects of the extraction conditions of phenolic compounds from citrus waste released by the enzymatic cocktail of *A. niger* LBM 134. Levels of TPC improved when pH, time, and temperature of incubation were around their central points: pH 5, 40 °C, and 8 h, respectively. TPC: total phenolic compounds

Extraction of phenolic compounds with bioactive properties released from citrus waste

We identified five bioactive naturally occurring compounds with multiple biological properties from the citrus waste, hesperetin, tryptophan, *p*-coumaric, quinic, and gallic acid. As conventional chemical routes to obtain these compounds are expensive, time-consuming, and could produce potential health dangers, the use of natural sources and mild enzymatic conditions is highly advantageous (Bai et al., 2021; Sharma et al., 2017).

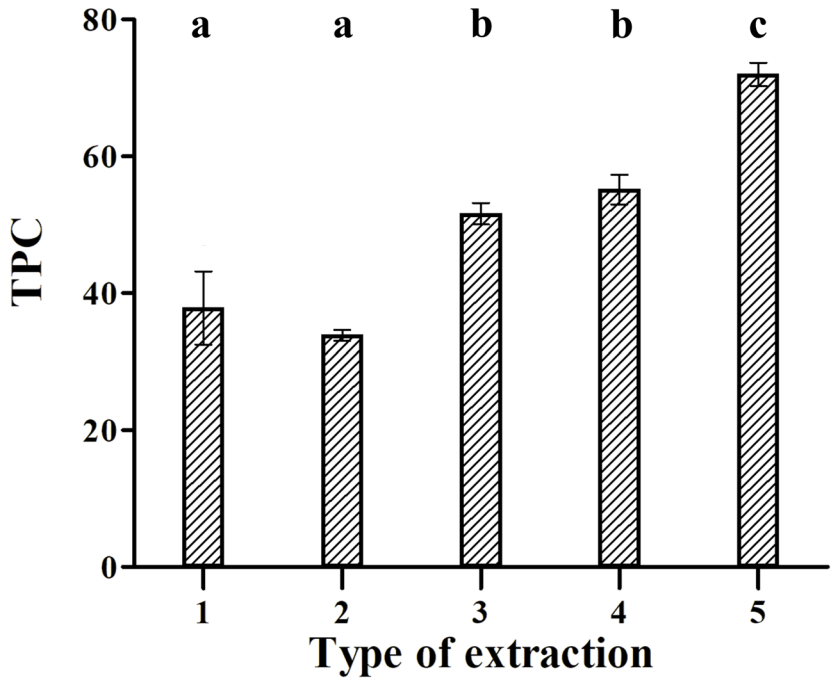


Fig. 4 Comparison of different extractions of phenolic compounds from citrus waste. 1: with buffer; 2: with alkaline extraction; 3: with the fungal enzymatic cocktail pretreated at 100 °C for 5 min; 4: with commercial enzyme Viscozyme; 5: with the fungal cocktail

Table 2 Identification of phenolic compounds released from citrus waste through AE and EAE

Extraction type	Identified phenolic compounds with multiple biological activities				
	p-coumaric acid (µg/L)	Tryptophan	Gallic acid (mg/L)	Quinic acid (mg/L)	Hesperetin (µg/L)
EAE with *A. niger* LBM 134 cocktail	3387.7 ± 12.37	> LOQ	< LOD	11.1 ± 0.06	9.82 ± 0.06
EAE with the commercial cocktail	3275.5 ± 11.04	> LOQ	< LOD	6.14 ± 0.02	9.74 ± 0.07
Alkaline	< LOD	< LOD	5.39 ± 0.03	< LOD	< LOD
With buffer	399.8 ± 5.81	> LOQ	< LOD	< LOD	< LOD

EAE enzymatic assisted extraction, *<LOQ* limit of quantification, *<LOD* limit of detection

Hesperetin, a flavanone known for its diverse pharmacological properties (Agrawal et al., 2021; Eberle et al., 2021a), was successfully identified in extracts obtained from enzymatic cocktails of *A. niger* LBM 134 and a commercial enzyme. Its current industrial production relies on hesperidin as a starting material, using methanol and sulfuric acid as catalysts and solvents (Liu et al., 2022) which carries environmental concerns. The alternative obtention of hesperetin by enzymatic extraction would help to reduce the environmental concerns.

Gallic acid was detected in extracts from the AE while quinic and *p*-coumaric acid were identified in extracts from the EAE carried out by both the fungal and the commercial enzymes. Gallic acid (3,4,5-trihydroxybenzoic acid) is a naturally occurring compound considered a main promising for new drug development due to its preventive role against oxidative damage (Bai et al., 2021; Zahrani et al., 2020). Zahrani et al. (2020) reported the easy release of gallic acid by alkaline hydrolysis of tannins. In this sense, we only detected this compound in extracts carried out by the alkali NaOH.

Quinic acid is a cyclohexane carboxylic acid contained in the extracts of several plants and has exhibited numerous biological activities. Several researchers have focused on its purification from plants by different methods to be used as a chiral compound for producing pharmaceutical products (Benali et al., 2022).

p-Coumaric acid, a phenolic acid belonging to the hydroxycinnamic acid family, is commonly found in plants and mushrooms either in a free or bound form (Pei et al., 2016). The compound shows potential as an antioxidant in

Table 3 Cost-effectiveness study for the extraction of phenolic compounds from citrus waste

	Cost of enzyme production			
Medium components	**Homemade cocktail**			**Commercial enzyme**
	Unit cost (USD kg^{-1})	**(g L^{-1})**	**Total cost (USD L^{-1})**	**(USD L^{-1})**
$NaNO_3$	115.35	2	0.23	
KH_2PO_4	363.65	1	0.37	
KCl	171.71	0.50	0.09	
$MgSO_4{\cdot}7H_2O$	336.49	0.50	0.17	
$FeSO_4{\cdot}7H_2O$	218.84	0.01	0.00	
Meat peptone	185.13	2.50	0.42	
Yeast Extract	331.85	2	0.68	
Sugarcane bagasse	0.01	15	0.000	
Water	0.07	975.99	0.07	
Subtotal		1000	2.03	
Operational parameters	**Unit cost (USD $year^{-1}$)**	**(USD $year^{-1}$)**	**Total cost (USD L^{-1})**	
Fixed asset investment	25.80	8	0.56	
Utilities (electricity, water, natural gas)	5.88	8	0.13	
Labour/fixed cost	5.38	8	0.12	
Consumables	2.78	8	0.06	
Subtotal			0.87	
Total			**2.90**	**6171**
	Cost of bioactive compounds extraction with:			
	homemade cocktail			**commercial enzyme**
BGL activity (IU L^{-1})	393			6000
IU $reaction^{-1}$	10			10
The volume of enzymatic cocktail used (mL $reaction^{-1}$)	15			1.67
Cost of the enzyme (USD $reaction^{-1}$)	0.07			10.28
Extraction of TPC (mg equivalents of GA per mL) from 0.3 g of citrus waste	71.96 ± 2.07			55.13 ± 2.16
Cost of production (USD) of 1 mg equivalents of GA per mL	**0.009**			**0.186**
Production of mg equivalents of GA per mL from 100 g of citrus waste	23,987			18,377
The volume of enzymatic cocktail used (mL $reaction^{-1}$)	5000			557
Cost of extraction (USD) of TPC from 100 g of citrus waste	**23.33**			**3428.72**

SCB sugarcane bagasse, *TPC* total phenolic compounds, expressed in mg of gallic acid equivalents per mL, *USD* United States Dollar, *IU* International Units, *GA* gallic acid

[a] Commercial enzyme Viscozyme from Sigma-Aldrich

the food and nutrition industries, and it has also demonstrated antiplatelet activity, making it a promising candidate for the development of new drugs targeting oxidative stress-induced diseases (Roychoudhury et al., 2021). However, additional research is required to determine whether the *p*-coumaric acid observed in this study originates from citric waste or the fungus *A. niger* LBM 134.

Tryptophan was also identified in both enzymatic extractions; but not in the alkaline extraction. It was reported that strong alkaline conditions, such as those used in this work, partial or complete racemization of tryptophan were observed (Bellmaine et al., 2020) which could explain why tryptophan was not identified in our alkaline extraction.

The presence of these phenolic compounds and the bioactive amino acid in extracts obtained by EAE reinforces the employment of this bioprocess to recover high-added-value compounds from agricultural wastes.

In this work, we demonstrated that the extraction of phenolic compounds from citrus waste using the low-cost enzymatic cocktail of *A. niger* LBM 134 is efficient and economical, about 30% better, and 150 times less expensive than the commercial one. The high levels of these compounds extracted by the fungal cocktail were

feasible for the action and stability of the carbohydrate-degrading enzymes, particularly BGL, during the process (Díaz et al., 2020). Hence, besides formulating an efficient and low-cost media for hydrolytic enzyme production, we propose a potential solution to the problem that the citrus industries have with their waste disposal. We also added value to citrus waste by extracting functional compounds with multifaceted biological activities that can be used in natural therapies or pharmaceutical industries. Although we have focused our work on identifying bioactive compounds in this study, we must continue purifying them and evaluating their cytotoxicity and biological activity, mainly in cellular cultures. The purification of these compounds is the actual challenge. The achievement of pure extracts would provide us with a clearer understanding of the biological activities of these compounds and thus enable us to delve deeper into their mechanisms of action which is essential for conducting rigorous scientific studies and clinical trials.

Conclusions

In this work, we valorized sugarcane bagasse and citrus wastes to obtain high-added-value products: enzymes and compounds with multiple biological properties. The sugarcane bagasse was used as a substrate for *A. niger* LBM 134 cultivation allowed us to produce a low-cost enzymatic cocktail whose composition showed a broad enzyme hydrolytic range, where the BGL role stood out due to its dominance and high thermostability. In addition, we also report here a specific and simple protocol to visualize BGL enzyme activity using polyacrylamide gels.

The enzymatic cocktail was demonstrated to be useful in releasing bioactive compounds from citrus waste by an enzymatic-assisted extraction strategy, which was optimized by applying a CCD. This procedure revealed high concentration values of phenolic compounds and was less expensive than the extractions performed with commercial enzymes. As a result, the bioprocess developed here could be used by the citric industry to improve waste management, generating a sustainable scenario where the environmental stress is reduced, and the waste is converted into functional bioactive compounds.

Abbreviations

AE	alkaline extraction
BGL	β-glucosidases
BXL	β-xylosidases
CBH	cellobiohydrolases
CCD	central composite design
CMC	carboxymethylcellulose
EAE	enzymatic-assisted extraction
EG	endo-β-1,4-glucanases
EX	endo-β-1,4-xylanases
GAE	gallic acid equivalents
LAP	Laboratory Analytical Procedure
NREL	National Renewable Energy Laboratory
PDA	potato dextrose agar
SCB	sugarcane bagasse
TPC	total phenolic compounds

Supplementary Information

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Supplementary Material 1: Supplementary Table 1. Levels of factors evaluated by the CCD for optimizing the extraction conditions of phenolic compounds.

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Not applicable.

Authors' contributions

GVD designed the work, performed the experiments, analysed and interpreted the data, drafted and wrote the manuscript, and approved the submitted version and the authors' contribution. ROC designed the work, interpreted the data, drafted the manuscript, and approved the submitted version and the authors' contribution. LEO performed the experiments and approved the submitted version and the authors' contribution. PDZ drafted and revised the manuscript, and approved the submitted version and the authors' contribution. MAM substantially revised the manuscript and approved the submitted version and the authors' contribution. MIF designed the work, drafted and substantially revised the manuscript, and approved the submitted version and the authors' contribution.

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Data availability

All data generated or analysed during this study are included in this published article [and its supplementary information files].

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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