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High-efficiency novel extraction process of target polyphenols using enzymes in hydroalcoholic media

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Author contribution

Conceptualization: MRM, DMP, DR**; Methodology:** MRM, DMP; **Formal analysis and investigation:** DMP**; Writing - original draft preparation:** DMP; **Writing - review and editing:** MRM**; Funding acquisition:** MRM, DR; **Resources:** MRM, DR; **Supervision:** MRM, DR.

Abstract

Agro-industrial by-products are a sustainable source of natural additives that can replace the synthetic ones in the food industry. Grape pomace is an abundant by-product that contains about 70 % of the grape's polyphenols. Polyphenols are natural antioxidants with multiple health-promoting properties. They are secondary plant metabolites with a wide range of solubilities. Here, a novel extraction process of these compounds was developed using enzymes that specifically liberates target polyphenols in the appropriate hydroalcoholic mixture. Tannase, cellulase, and pectinase retained 22, 60, and 52 % of their activity, respectively, in ethanol 30 % v/v. Therefore, extractions were tested in ethanol concentrations between 0 and 30 % v/v. Some of these enzymes presented synergistic effects in the extraction of specific polyphenols. Maximum yield of gallic acid was obtained using tannase and pectinase enzymes in ethanol 10 % v/v (49.56 ± 0.01 mg L^{-1} h^{-1}); in the case of *p*-coumaric acid, by cellulase and pectinase treatment in ethanol 30 % v/v (7.72 ± 0.26 mg L^{-1} h^{-1}), and in the case of *trans*-resveratrol, by pectinase treatment in ethanol 30 % v/v (0.98 ± 0.04 mg L^{-1} h^{-1}). Also, the effect of enzymes and solvent polarity was analyzed for the extraction of malvidin-3-*O*-glucoside, syringic acid, and quercetin. Previous studies were mainly focused on the maximization of total polyphenols extraction yields, being the polyphenolic profile the consequence but not the driving force of the optimization. In the present study, the basis of a platform for a precise extraction of the desire polyphenols is provided.

Key points

- *Enzymes can be used up to ethanol 30 % v/v.*
- *The specific enzymes´ action determines the polyphenolic profile of the extracts.*
- *The yields obtained of target polyphenols are competitive.*

Keywords: Polyphenols; Grape pomace; Gallic acid; *p*-Coumaric acid; *trans*-Resveratrol; Enzymes

Introduction

Every year over 60 million tons of grapes are produced, being these one of the largest fruit crops in the world (Antoniolli et al. 2015). Around the 80 % of those grapes are used for winemaking, generating a great deal of grape pomace waste per year worldwide (Kammerer et al. 2005). Grape pomace (comprised mainly of skins, seeds, and residual pulp and stems) represents around the 30 % of the initial grape weight (Beres et al. 2017) and constitutes a significant disposal problem for wine industry due to its high pollution load (Devesa-Rey et al. 2011). In view of achieving a more sustainable winemaking process, grape pomace is generally reused for alternative purposes (Beres et al. 2017). However, a great proportion of bioactive compounds present in this residue are discarded. Approximately 70 % of polyphenols present in grapes remain within the pomace after winemaking process (Dwyer et al. 2014). Simple flavonoids (flavonols, anthocyanins and catechins), phenolic acids (benzoic and hydroxycinnamic acids) and tannins, and proanthocyanidins are among the main constituents of grapes and their derivatives (Fontana et al. 2013; Unterkofler et al. 2020).

Polyphenols' extraction from grape pomace has drawn great attention in recent years, mainly because this residue constitutes an economical source of these compounds. There is a growing demand from consumers for safer and functional foods, which implies the replacement of synthetic additives for natural additives (Ueda et al. 2022). Therefore, efforts are being directed towards the development of technologies that allow the usage of natural derived additives, such as plant extracts, to replace the synthetic ones (Ueda et al. 2022). Phenolic compounds are extensively used in the pharmaceutical, cosmetic, and food industries due to their multiple health-promoting properties (Fontana et al. 2013; Antoniolli et al. 2015), among which stand out its antioxidant, anti-microbial, anti-inflammatory, antiaging, anticancer, antidiabetic, and antihypertensive properties (Xu et al. 2005; Pandey and Rizvi 2009; Sri Harsha et al. 2013; Milke et al. 2018; Fernández-Fernández et al. 2019). Therefore, the recovery of polyphenols from grape pomace allows to upcycle this residue by obtaining high-value bioactive compounds that can be used as natural additives.

The extraction procedure applied plays a key role in the isolation, identification and use of polyphenols and there is no unique and standard method (Ignat et al. 2011). Among the different methods available, solid-liquid extraction is the most used technique for the extraction of polyphenols from grape pomace. This method uses organic solvents and, thus, it has several disadvantages such as low quality of the extracts due to remaining solvent traces, environmental risks, and safety hazards (Puri et al. 2012;

Fontana et al. 2013; Moro et al. 2021). In recent years, this extraction technique has been gradually replaced by greener alternatives which are mainly focused on reducing the usage of organic solvents and increasing the sustainability of the process (Fontana et al. 2013). One of these alternatives is enzyme-assisted extraction in aqueous media. This approach is based on the ability of enzymes to degrade or alter the complex materials of the plant cell wall, which facilitates the recovery of the compounds present there (Puri et al. 2012; Sirohi et al. 2020; Xavier Machado et al. 2021).

The complex network of grape skins' cell walls is comprised of different macromolecules which form a barrier that prevents the release of polyphenols since, in grapes, these can be either in soluble form, inside vacuoles of plant cells, or in insoluble form (Pinelo et al. 2006). The later are so called since they are linked to cell-wall polysaccharides through hydrophobic interactions, hydrogen bonds or covalent bonds and, in general, they are not recovered with traditional extraction methods (Pinelo et al. 2006; Xavier Machado et al. 2021). Therefore, the enzymatic treatment of cell-wall structures might be an efficient way to recover these polyphenols. Among the most used enzymes for this purpose, cellulase, pectinase, and tannase stand out (Xavier Machado et al. 2021). Cellulase enzymes hydrolyse the major component of plant cells, cellulose, into fermentable sugars, pectinase enzymes hydrolyse pectic polymers in plant cell walls, and tannase enzymes break down hydrolysable tannins or gallic acid esters, releasing gallic acid and glucose (Chamorro et al. 2012; Xavier Machado et al. 2021).

In recent years, the use of enzymes for the recovery of polyphenols from grape pomace has been seen as a promising alternative for replacing solvents partially or totally (Chamorro et al. 2012; Meini et al. 2019). However, comparison of several extraction techniques showed that water mixtures with organic solvents (mainly ethanol and methanol) are still the most efficient for polyphenols' extraction due to their low solubility in water (Fontana et al. 2013; Brazinha et al. 2014). To date, only a few studies have been reported to use enzymes and solvents sequentially for extracting polyphenols from grape pomace. In those cases, grape pomace is first subjected to a pre-treatment with enzymes, and then a solid-liquid extraction is carried out using organic solvents (Martins et al. 2016; Ferri et al. 2017). However, there are no studies in which polyphenols are extracted using enzymes and solvents simultaneously. Enzymes are commonly used in aqueous media since, in these, they generally have optimum activity. Nonetheless, reactions' productivities might be negatively affected by the poor solubility of substrates and/or products in such media (van Schie et al. 2021). Some studies have tested the activity of the enzymes presented here in non-aqueous reaction systems, showing that they can tolerate considerable percentages of organic solvents

(Marco et al. 2009; Nguyen and Quyen 2012; Pham et al. 2012; Reynolds et al. 2018). In the present study, the aim was to test whether the recovery of polyphenols from grape pomace could be improved in yield and composition through an enzymatic treatment in hydroalcoholic media. Moreover, previous studies have dealt with the optimization of extraction conditions using total phenolic yields as the response (Zhang et al. 2013; Brazinha et al. 2014). Yet, information regarding how individual polyphenols' composition is affected by the extraction procedure is lacking. The present work seeks to evaluate how the variables of the extraction process under study, i.e, solvent composition and enzymes used, influence the polyphenolic profile. In this way, the most accurate extraction conditions can be found for the extraction of target polyphenols.

Materials and methods

Materials

Grape pomace of red wine grapes (*Vitis vinifera* L.) of Cabernet Sauvignon variety, harvested in the year 2021, was supplied by the Argentinian winery BordeRío (Victoria, Entre Ríos Province).

Pectinase (EC 3.2.1.15) from *Aspergillus niger* (17389, > 1 U/mg) and cellulase (EC 3.2.1.4) from *A. niger* (C1184, ≥ 0.3 U/mg) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Tannase KT-FH (EC 3.1.1.20) from *Aspergillus oryzae* (≥ 500 U/g) was provided by Kikkoman (Minato-ku, Tokyo, Japan). Folin-Ciocalteu's phenols reagent, ABTS (2,2'-azino-bis (3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), methyl gallate, Rhodanine (2-thioxo-4-thiazolidinone), 3,5-dinitrosalicylic acid (DNS), carboxymethylcellulose sodium salt (CMC), pectin from citrus peel, 4-nitrophenyl β-D-glucopyranoside (*p*NPG), and 4-nitrophenol (*p*NP), were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). High-performance liquid chromatography (HPLC) grade standards of gallic acid, *p*-coumaric acid, syringic acid, malvidin-3-*O*-glucoside, quercetin, and *trans*-resveratrol were from Sigma-Aldrich (St. Louis, Missouri, USA). Absolute ethanol 99.5 % was obtained from Cicarelli (San Lorenzo, Santa Fe Province, Argentina).

Preparation of grape pomace

After red winemaking process, grape pomace (GP), composed of skins, seeds and residual pulp and stems, was collected, and stored frozen at - 20 °C. Subsequently, grape pomace was dried in a drying oven with forced air flow (San-Jor, SL60SDB, San Andrés, Buenos Aires, Argentina) at 60 °C for 24 h,

and milled in a blade grinder at an approximate speed of 15,000 rpm (Tecnodalvo TMDC, Santa Fe, Argentina) to a particle size smaller than 0.841 mm (using a mesh No. 20) before the extraction process. The expression "g of GP" refers to the dried and milled grape pomace.

Determination of enzymatic activities

For the determination of enzymatic activities in media containing different ethanol concentrations, the protocols were modified as describe in each case.

Tannase

Tannase activity was determined by the spectrophotometric methanolic rhodanine method, which uses methyl gallate as substrate (Sharma et al. 2000). The reaction was performed following the procedure previously reported (Meini et al. 2021), with slight modifications. In this case the protocol was carried out in a media containing 50 mM sodium acetate buffer at pH 5.00 and ethanol concentrations ranging between 0 % and 40 % v/v. The absorbance was measured at 520 nm using an UV-visible spectrophotometer (UV-3100 spectrophotometer, Paralwall, Santa Fe, Argentina). One enzyme unit (U) is defined as the µmol of gallic acid formed per min at 30 °C and pH 5.00.

Cellulase and pectinase

Endoglucanase and polygalacturonase activities were determined by the DNS method (Miller 1959) which measures the production of reducing sugars. The substrates used were pectin from citrus peel and CMC for pectinase and cellulase, respectively. In the case of cellulase, one U is defined as the amount of enzyme which liberates 1 µmol of glucose from CMC in 10 min at pH 5.30 and 50 °C; and for pectinase, one U corresponds to the amount of enzyme which liberates 1 µmol galacturonic acid per min at pH 4.20 and 40 °C. Both activities were assayed following the procedure previously reported (Meini et al. 2021), with slight modifications. In these cases, enzymatic activity assays were performed in media with ethanol concentrations varying from 0 % to 40 % v/v. The absorbance was measured at 560 nm using an UV-visible spectrophotometer (UV-3100 spectrophotometer, Paralwall, Santa Fe, Argentina). The initial concentrations of reducing sugars in the enzyme solutions were considered by carrying out controls without the addition of the substrates.

β-Glucosidase

The residual β-glucosidase activity of the commercial enzymes' preparations was assayed using *p*NPG as substrate, following the method previously reported (Pino-García et al. 2020) with slight

modifications. The reactions were performed by adding 150 µL of the enzyme solution to a mixture containing 50 µL of 10 mM *p*NPG prepared in 50 mM sodium phosphate buffer, pH 7.00, in a final volume of 500 µL. After incubating at 40 °C for 5 min, the reaction was stopped by adding 500 µL of 1 M Na_2CO_3. The absorbance was measured at 400 nm using a microplate spectrophotometer (Multiskan GO, Thermo Fisher Scientific, Waltham, MA, USA). One unit (U) is defined as the amount of enzyme that catalyses the formation of 1 µmol *p*NP per min under the conditions of the assay. Only in the case of pectinase preparation, in which a considerable amount of β-glucosidase activity was detected, the activity was measured in media containing ethanol concentrations varying from 0 % to 40 % v/v.

Enzymatic extraction of polyphenols in hydroalcoholic media

Polyphenols' extractions from grape pomace were carried out using tannase, cellulase and pectinase individually or combined, at a concentration of 200 U/g GP based on previous results (Meini et al. 2019). They were added after being manually weighted and solubilized in 50 mM acetate buffer at pH 5.00, taking into consideration the U/g of preparation specified by the manufacturers. According to β-glucosidase activity determinations, this also led to the addition of approximately 400 U/g GP of β-glucosidase in the case of pectinase preparation, and approximately 150 U/g GP in the case of cellulase preparation. For each extraction, grape pomace was mixed with buffer, enzymes, and ethanol in different proportions, at a solid:solvent ratio of 1:10 w/v. Control extractions were carried out without enzymes, replacing them with buffer solution in the adequate proportion. Samples were incubated in continuous agitation (36 rpm) in a rotary shaker (Bioelec, Santa Fe, Argentina), at 40 °C (San-Jor SL60C precision oven, San Andrés, Buenos Aires, Argentina) for 2 h. Afterwards, they were centrifuged at 10397 × *g* for 10 min (Sigma 3-18KH laboratory centrifuge, Rotor No. 19776-H, St. Louis, Missouri, USA). Finally, the supernatant from each tube was transferred to Eppendorf tubes that were stored at - 20 °C until analysed.

Determination of total phenolic content

The Folin-Ciocalteu method was used to estimate the total concentration of phenolic compounds in the extracts (Ainsworth and Gillespie 2007). The results were obtained by comparing the absorbance at 765 nm (UV-3100 spectrophotometer, Paralwall, Santa Fe, Argentina) of the samples (or a convenient dilution of them) with a standard curve of gallic acid. Each determination was made in triplicates and results were expressed in g of gallic acid equivalents (GAE)/100 g of GP.

Determination of antioxidant activity

The antioxidant activity of the extracts was evaluated using the improved $ABTS^{\bullet+}$ radical cation decolorization assay (Re et al. 1999). The samples were diluted to fall in the linear range of a standard curve performed with known Trolox solutions. The absorbance was measured at 734 nm using an UV-visible spectrophotometer (UV-3100 spectrophotometer, Paralwall, Santa Fe, Argentina). Determinations were made in triplicates and results were expressed in mmoles of Trolox equivalents (TE) per 100 g of GP (mmol TE/100 g GP).

Analysis of the phenolic content of the extracts by HPLC-DAD

Phenolic compounds were resolved, identified, and quantified using a Dionex Ultimate 3000 SD HPLC system with a diode array detector (DAD) (Thermo Fisher Scientific, Waltham, MA, USA). After filtering the samples or a convenient dilution, they were injected in an Hypersil Gold C18 3 µm; 2.1 mm × 100 mm column (Thermo Fisher Scientific, Waltham, MA, USA). Elution was carried out at a flow rate of 0.25 mL/min with a mobile phase consisting of a mixture of deionized water acidified by acetic acid 0.5 % v/v, and acetonitrile. The separation method used has been previously reported (Meini et al. 2019). Chromatograms were recorded at 280, 320, 370 and 520 nm. The identity of the peaks was confirmed by their retention time and absorbance spectrum, in comparison with available standards. Polyphenols were quantified using calibration curves of the following standard compounds at each maximum absorbance wavelength: gallic acid (280 nm), *p*-coumaric acid (320 nm), syringic acid (280 nm), malvidin-3-*O*-glucoside (520 nm), *trans*-resveratrol (320 nm), and quercetin (370 nm). Results were expressed as mg/100 g GP.

Statistical data analysis

Each experiment was performed in triplicate and the results were presented as means ± standard deviation. Statistical analysis was performed using GraphPad Prism software for Windows (Version 8.0, GraphPad Software, San Diego, CA, USA). Data were subjected to a two-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests. Significant differences were established in respect to the control experiments of each study: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$.

Results

Enzymatic activity assessment in hydroalcoholic media

With the aim of developing a novel enzymatic extraction process of polyphenols in hydroalcoholic media, the activity of the enzymes (tannase, cellulase and pectinase) was first evaluated in different concentrations of ethanol (between 0 % and 40 % v/v). It has been previously suggested that the presence of secondary activities in the commercial enzyme's preparations, such as β-glucosidase, can also affect polyphenols' extraction (Kammerer et al. 2005). Therefore, β-glucosidase activity was measured in the three enzymes' preparations. Since a considerable activity was detected in pectinase preparation (2.00 ± 0.03 U/mg), this activity was also assayed in different concentrations of ethanol. The results obtained for each enzyme are presented in Fig. 1. In this way, the maximum concentration of ethanol that can be used during the extraction process was established, seeking to maintain a considerable percentage of the enzymes' activities.

In the case of tannase (Fig. 1a), its activity showed a sharp decrease with the increasing ethanol concentrations. Nonetheless, a conservation of about 81 % of activity in 10 %, 57 % in 20 %, and 22 % in 30 % v/v of ethanol was observed, which might be enough to get an effect in the enzyme-assisted extractions.

Fig. 1 here

Cellulase enzyme (Fig. 1b) was more resistant than tannase to the increasing concentrations of ethanol, showing a plateaued behaviour in the range of 10 - 20 % v/v of ethanol, in which around 80 % of the activity is preserved. Moreover, cellulase preserves more than 50 % of its activity at 30 % v/v of ethanol, while tannase retains only 22 % of the activity at the same solvent concentration.

Regarding pectinase activity (Fig. 1c), the enzyme preserved more than 70 % of its activity at a solvent concentration of 10 % v/v. Between 20 and 40 % v/v of ethanol, the activity was reduced gradually, retaining about 41 % of its activity at 40 % v/v of ethanol. Pectinase enzyme was the most tolerant to the highest ethanol concentration tested. β-Glucosidase activity of the pectinase enzyme preparation (Fig. 1d) showed a sharp decrease with the ethanol concentrations tested. However, a considerable amount of activity was preserved in 10, 20 and 30 % v/v of ethanol.

Analysis of different polyphenols' extraction processes using enzymes and ethanol simultaneously

Extractions of polyphenols from red grape pomace were carried out using enzymes in ethanol solutions with concentrations ranging between 0 % and 30 % v/v. The pomace was firstly processed, and then the extractions were carried out as previously described in materials and methods section. After obtaining the extracts, these were first analysed in terms of total phenolic content and antioxidant activity (Fig. 2). The best results in both assays were obtained using enzymes in media containing 20 % and 30 % v/v of ethanol. The total phenolic content of these extracts ranged from 1.3 to 2.3 g gallic acid equivalents/100 g GP, and their antioxidant activity ranged between 8.3 and 14.5 mmol of Trolox equivalents/100 g GP.

When comparing the extracts obtained using the enzymes individually to their respective controls, significant increases in the total phenolic content (Fig. 2a) and in antioxidant activity (Fig. 2b) were observed for cellulase in ethanol 10 % and 20 % v/v, and for pectinase in ethanol 20 % v/v. In the case of the extracts obtained with tannase, no increase in the total concentration of polyphenols was noted, while this enzyme significantly improved the extracts' antioxidant activity at all ethanol concentrations.

As regards the extracts obtained with different combinations of enzymes, a significant increase in the total phenolic content was observed at every ethanol concentration assayed in comparison to their respective controls (Fig. 2a). The sole exception to this was the extraction carried out with cellulase and pectinase in aqueous media (ethanol 0 % v/v). Regarding the antioxidant activity of these extracts (Fig. 2b), only in the case of ethanol 20 % v/v, all the enzymes' combinations showed significant increments when contrasting with their controls. In addition, a negative effect on the antioxidant activity of the extracts was noted when using pectinase in the extraction process, since the values obtained with the three enzymes were lower than the combination "cellulase + tannase".

Fig. 2 here

Identification and quantification of polyphenols present in the extracts by HPLC-DAD

In general, studies on this topic are focused on maximizing the extraction yields of total phenolics and antioxidant activity of the extracts. However, the analysis and quantification of individual phenolic compounds is important to understand the effect of the extraction process on the composition of the extracts, as well as the relationship between the presence of certain polyphenols and the bioactivities

281 of the extracts. Therefore, the extracts obtained using enzymes in hydroalcoholic media were analysed by
282 HPLC-DAD to identify and quantify some of the phenolic compounds present in them.

283 One of the phenolic compounds found in every extract was gallic acid (Fig. 3a). The increasing
284 concentrations of ethanol in the control extractions did not significantly modify its extraction yield.
285 However, when tannase was added, individually or simultaneously with other enzymes, it had a positive
286 effect on gallic acid's concentration.

287 The concentration of syringic acid in the extracts was positively affected when enzymes were
288 used (Fig. 3b). In general, the content of this polyphenol was higher than the controls when using
289 enzymes individually or combined, in media with 0 %, 10 % and 20 % v/v of ethanol. However, when 30
290 % v/v of ethanol was used, only the combination of tannase, cellulase, and pectinase had a significant
291 effect on the content of syringic acid. Also, a positive effect on the extraction yield of this compound was
292 observed with the increasing concentrations of the solvent in the control extractions. The highest yield of
293 syringic acid was obtained when using the three enzymes combined in ethanol 20 % v/v.

294 Fig. 3 here

295 As regards to *p*-coumaric acid (Fig. 4a), no positive effect on its extraction yield was achieved
296 when using tannase enzyme; while the other enzymes, cellulase and pectinase, benefited its recovery in
297 media containing ethanol. Besides, when contrasting the enzymatic extractions with their controls, in
298 general, a greater concentration of *p*-coumaric acid was obtained when using the enzymes simultaneously.
299 Lastly, it is worth mentioning that *p*-coumaric acid's extraction yield considerably increases in the
300 extractions carried out with cellulase and/or pectinase, with the growing concentrations of ethanol, while
301 this effect is only slightly appreciated in the controls without enzymes.

302 In the case of the anthocyanin malvidin-3-*O*-glucoside (Fig. 4b), its extraction is benefited by the
303 increasing ethanol concentrations. In addition, not only tannase, but also cellulase had a positive effect on
304 this compound's extraction yield. However, when pectinase was used, either alone or combined with the
305 other enzymes, malvidin-3-*O*-glucoside was not detected in the extracts.

306 Fig. 4 here

307 Another two phenolic compounds were identified and quantified in the extracts: quercetin (Fig.
308 5a) and *trans*-resveratrol (Fig. 5b). These polyphenols were only extracted in media containing ethanol 20

% v/v and 30 % v/v. Quercetin was only recovered when the extractions were carried out with enzymes. In ethanol 20 % v/v low concentrations of this polyphenol were obtained, while higher concentrations were achieved in media with 30 % v/v of ethanol, being "cellulase + tannase" the highest among them. As regards *trans*-resveratrol, it was not recovered in the controls, while its extraction was notably favoured by pectinase. With 20 % v/v of ethanol this compound was present only in the extracts obtained with pectinase individually and combined with cellulase; and in ethanol 30 % v/v, higher concentrations of *trans*-resveratrol were recovered but only in the conditions where pectinase was used.

Fig. 5 here

Discussion

With the aim of developing a polyphenols' one-step extraction process using enzymes and ethanol simultaneously, in the first place, the activities of three commercial enzymes (tannase, cellulase and pectinase) were determined in media with varying concentrations of the solvent. Currently, the information available regarding the effect of organic solvents on these enzymes' activity is narrow since, in general, the assays evaluate the residual activity after a preincubation in organic solvent mixtures but not the activity within the organic solvent mixture itself. As regards to tannase enzyme, the effects of various organic solvents were reported for a tannase enzyme from for *A. niger* GH1, giving clues that these enzymes could at least resist 20 % v/v of multiple organic solvents (Marco et al. 2009). In the case of cellulase, endoglucanase enzymes from *A. niger* VTCC-F021 and *A. oryzae* VTCC-F045 preserved over 80 % of their activity after being preincubated in ethanol 10 % and 20 % v/v for 2 h, and ethanol 20 % v/v for 4 h, respectively (Nguyen and Quyen 2012; Pham et al. 2012). The tolerance to solvent seen in the case of pectinase enzyme was not reported before, since a progressive reduction on the polygalacturonase activity of the commercial pectinase enzyme preparation Scottzyme® Color X (Scott Laboratories, Pickering, ON, Canada) was observed, but it was only evaluated up to an ethanol concentration of 10 % v/v (Reynolds et al. 2018). Based on the results discussed above, ethanol concentrations ranging from 0 % to 30 % v/v were chosen for testing the enzymes' effect on the extraction of polyphenols from grape pomace. The highest ethanol concentration (40 % v/v) was not considered since most of the enzymes showed very low activity in that condition.

Polyphenols' extractions were carried out using the enzymes' preparations individually or in combinations of two or three in ethanol concentrations ranging from 0 to 30 % v/v of ethanol. The

extracts were analysed in terms of total polyphenols' concentration and antioxidant activity. In addition, the individual polyphenols present in them were quantified by HPLC-DAD. As regards to the extracts obtained with the enzymes individually, although tannase did not have a significant effect in the total phenolic content, an increase in the antioxidant activity was observed when using this enzyme. Tannase enzyme hydrolyses tannins present in the plant cell wall, releasing gallic acid, which is a phenolic compound with a high antioxidant capacity (Badhani et al. 2015). Results obtained by HPLC showed a significant increase in gallic acid's concentration in the presence of tannase (Fig. 3a). Therefore, the positive correlation found between the enhancement in the extracts´ antioxidant activity and the increase in gallic acid concentration, indicates that gallic acid plays a key role in the antioxidant activity of the extracts.

In addition, it was noted that by using the enzymes simultaneously, higher recovery yields of polyphenols and greater antioxidant activities were obtained (Fig. 2). This might occur because the hydrolytic enzymes act synergistically favouring the release of polyphenols that are bound to the cell wall (Xavier Machado et al. 2021). On the other hand, a negative effect in the antioxidant activity of the extracts was noted when comparing the ones obtained with three enzymes with the ones obtained using cellulase and tannase combined. This effect seen in presence of pectinase enzyme correlates with the disappearance of malvidin-3-*O*-glucoside as determined by HPLC (Fig. 4b) when using this enzyme preparation. As stated before, the pectinase preparation used have a considerable amount of β-glucosidase activity. Anthocyanins are more prone to degradation when the hydrolysis of the glycosylic bond occur (Kammerer et al. 2005; Monteiro et al. 2019). Therefore, the loss of malvidin-3-*O*-glucoside and the concomitant decrease in antioxidant activity, can be attributed to the β-glucosidase activity of the pectinase enzyme preparation.

When using pectinase and cellulase individually and/or combined, higher concentrations of *p*-coumaric acid were obtained in the extracts (Fig. 4a). This polyphenol is a *p*-hydroxycinnamic acid mostly found in plants covalently linked to lignocellulose (insoluble form) or on its soluble form. The latter is stored in vacuoles, where it can be found forming ester bonds with small molecules or in its free form (Robbins 2003; Xu et al. 2005). Therefore, the hydrolysis of cell wall components by cellulase and pectinase enzymes might be enhancing the recovery of both fractions of *p*-coumaric acid. These results are consistent with what was previously reported when using cellulase in aqueous extractions (Meini et al.

2019). But the point that stands out in the present study is that a synergistic effect is observed when using these enzymes and hydroalcoholic media, up to ethanol 30 % v/v.

Also, when using pectinase and ethanol a significant increase in the concentration of *trans*-resveratrol was noted (Fig. 5b). In general, this polyphenol is largely found in grape skins in its glycoside form called polydatin or piceid (resveratrol-3-*O*-β-D-glucoside) (Averilla et al. 2019). However, polydatin presents a lower bioavailability than *trans*-resveratrol (Kuo et al. 2016), and therefore, the latter is the desired form that is mostly sought to be recovered (Wang et al. 2019). It has been previously reported that the action of β-glucosidase enzymes can efficiently convert polydatin to *trans*-resveratrol, and the advantage of performing the reaction with an enzyme tolerant to organic solvents has been highlighted (Zhou et al. 2022). Pectinase might be enhancing the recovery of this compound not only because it helps to reduce the cell wall integrity, but also the β-glucosidase activity present in the pectinase preparation is probably hydrolysing the glycosidic bond of piceid favouring the release of *trans*-resveratrol. As stated in the case of *p*-coumaric acid, there is a synergist effect between the application of pectinase preparation and the % of ethanol used in the extraction.

In recent years, the production of certain polyphenols has gained great importance due to its increasing demand on the market (Xavier Machado et al. 2021). Typically, the best vegetable source of each polyphenol is used, and a conventional solvent extraction is performed. But these processes require great quantities of biomass, and they present inherent drawbacks regarding environmental impact. Within this context, recent advances have been reported in obtaining genetically modified microorganisms that produce specific polyphenols with high yield (Borja et al. 2019; Kanpiengjai et al. 2020; Yuan et al. 2020). Alternatively, valuable polyphenols can be recovered from agro-industrial wastes through greener methods as stated in the present study. The volumetric productivity obtained for *p*-coumaric acid using cellulase and pectinase in ethanol 30 % v/v was almost two times the productivity reported in one of these studies (Borja et al. 2019), where they used a genetically modified *Saccharomyces cerevisiae* strain, and xylose as carbon source (Table 1). In the case of *trans*-resveratrol, the volumetric productivity obtained using pectinase in ethanol 30 % v/v was very similar to the one reported in a study where they used an *Escherichia coli – S. cerevisiae* consortium, and glucose as carbon source (Table 1) (Yuan et al. 2020). On the other hand, for gallic acid's production, tannic acid is generally used as the carbon source in fermentation processes (Kanpiengjai et al. 2020). This compound is a greater source of gallic acid per unit of weight than grape pomace so, as expected, higher yields were obtained by Kanpiengjai et al. (2020) in

contrast with the present study. Even so, a considerable volumetric productivity of gallic acid was obtained (Table 1), being this polyphenol one of the compounds found in higher concentrations in the extracts.

Table 1. Comparison of the volumetric productivity of gallic acid, *p*-coumaric acid, and *trans*-resveratrol obtained in this study and other published studies.

Study	Volumetric productivity (mg L^{-1} h^{-1})		
	***p*-Coumaric acid**	***Trans*-resveratrol**	**Gallic acid**
Present study[a]	7.72 ± 0.26	0.98 ± 0.04	49.56 ± 0.01
(Borja et al. 2019)[b]	4.03 ± 1.00	-	233.33 ± 0.01
(Yuan et al. 2020)[c]	-	0.96 ± 0.14	-
(Kanpiengjai et al. 2020)[d]	-	-	-

[a] The conditions selected were: *p*-coumaric acid ("cellulase + pectinase" ethanol 30 % v/v); *trans*-resveratrol ("pectinase" ethanol 30 % v/v); gallic acid ("tannase + pectinase" ethanol 10 % v/v).

[b] Production of *p*-coumaric acid from xylose after 60 h of culture, using a genetically modified *S. cerevisiae*.

[c] De novo resveratrol production from glucose through a synthetic *E. coli – S. cerevisiae* consortium.

[d] Gallic acid production from tannic acid and glucose after 48 h of culture, using the yeast *Sporidiobolus ruineniae* A45.2.

In conclusion, a novel polyphenol's extraction process was developed. This method allows the extracts' enrichment on specific polyphenols by making slight adjustments of the extraction's conditions. This selectivity is achieved through the combination of the specificity of hydrolytic enzymes, their ability to act in a range of concentrations of organic solvent, and the solubility of the desired polyphenols. This method represents a further improvement of the classical optimized extraction methods, which generally seek to maximize the extraction yields of total polyphenols without targeting specific polyphenols. Hydroxycinnamic acids, *trans*-resveratrol, and gallic acid's market are growing, boosted by the preference for safer alternatives in the food and cosmetic industries. Therefore, the process presented here represents a greener alternative for extracting these high-value polyphenols. Also, according to the results obtained, the addition of an organic solvent in the reaction system might represent a key factor when seeking to achieve competitive yields. Although the scalability of the process should be evaluated for

industrial application, it has been demonstrated that the usage of enzymes and ethanol in a one-step process has a considerable perspective on the production of valuable phenolic compounds. Moreover, this method showed volumetric productivities in the range of recently developed methods for producing individual polyphenols by microorganisms' fermentations, thus being a competitive process. Additionally, the principles of the extraction method under study could provide the basis for recovering other bioactive molecules trapped in biomasses that have moderate solubility in water.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Statements and Declarations

Ethical approval. This article does not contain any studies with human or animals performed by any of the authors.

Conflict of interest. The authors declare that they have no conflict of interest.

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Figure captions

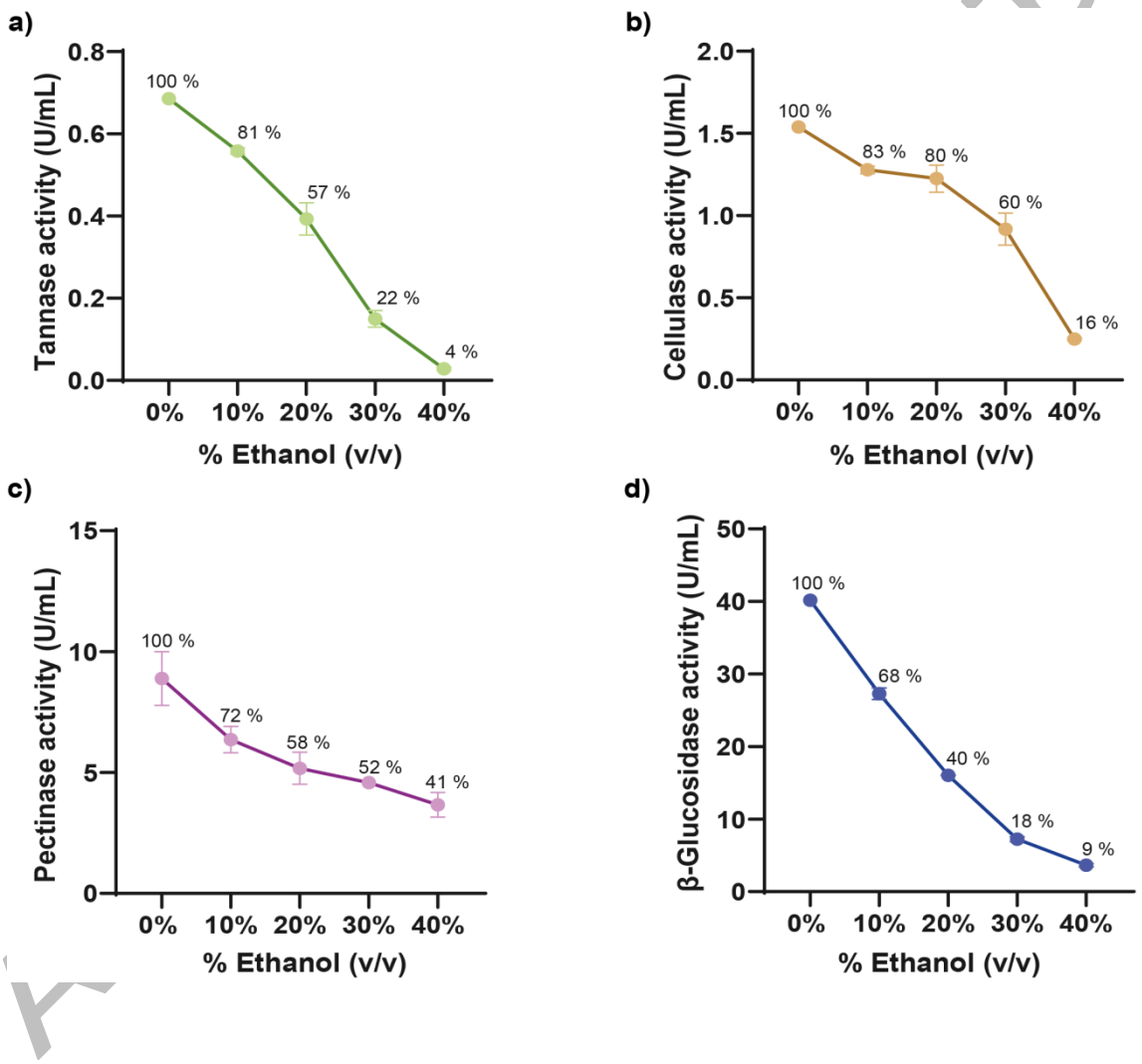
Fig. 1. Evaluation of (a) tannase, (b) cellulase, (c) pectinase and (d) β-glucosidase activities at different ethanol concentrations. Enzyme activity assays were carried out in media with 0 %, 10 %, 20 %, 30 % and 40 % v/v of ethanol. Assay conditions such as temperature, pH, and substrate were the ones established in the standard protocols for each enzyme. The percentages shown on top of each activity value correspond to the remaining activity of the enzymes. The activity obtained in the absence of ethanol was considered as 100 % of activity.

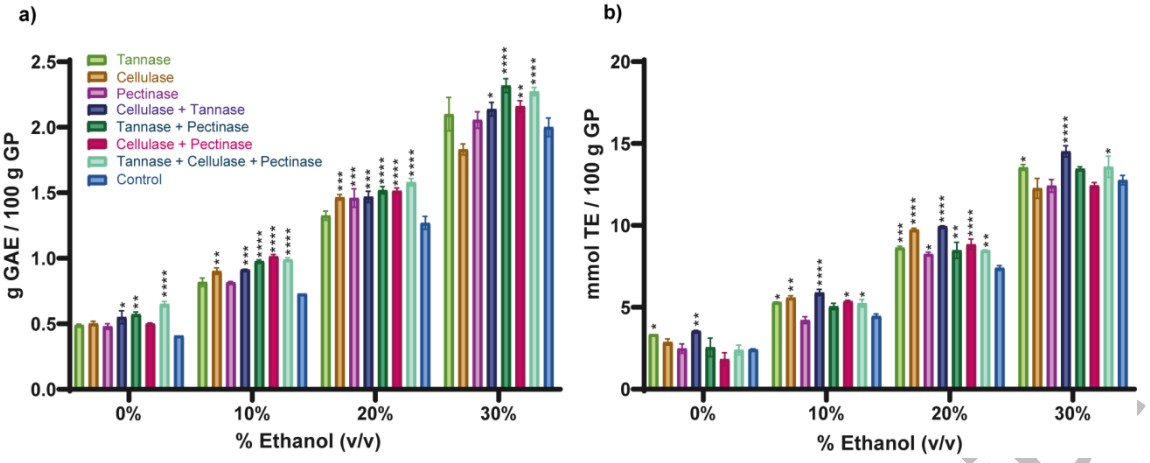
Fig. 2. Characterization of grape pomace extracts in terms of (a) total phenolic content and (b) antioxidant activity. The extractions were carried out using 200 U/g GP of tannase, cellulase, and pectinase enzymes (individually or combined). They were performed at 40 °C for 2 h, under continuous agitation, in media containing 50 mM acetate buffer at pH 5.00, and ethanol concentrations ranging from 0 % to 30 % v/v. Significant differences are indicated in respect to the control extractions performed without enzymes: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. GP: grape pomace; GAE: gallic acid equivalents; TE: Trolox equivalents.

Fig. 3. Identification and quantification of individual phenolic compounds present in the extracts by HPLC-DAD: (a) gallic acid and (b) syringic acid. The extractions were carried out using 200 U/g GP of tannase, cellulase, and pectinase enzymes (individually or combined). They were performed at 40 °C for 2 h, under continuous agitation, in media containing 50 mM acetate buffer at pH 5.00, and ethanol concentrations ranging from 0 % to 30 % v/v. Significant differences are indicated in respect to the control extraction performed without enzymes: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. GP: grape pomace.

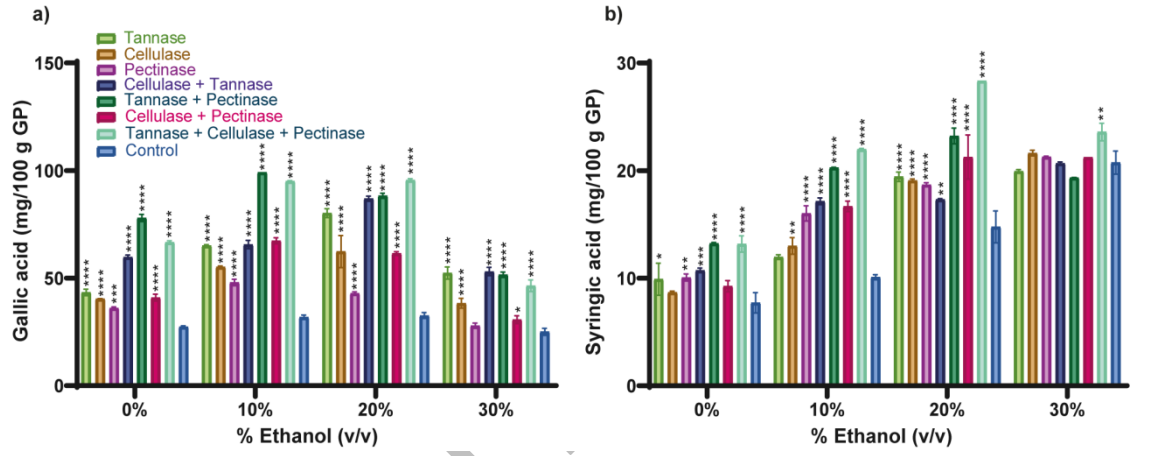
Fig. 4. Identification and quantification of individual phenolic compounds present in the extracts by HPLC-DAD: (a) *p*-coumaric acid and (b) malvidin-3-*O*-glucoside. The extractions were carried out using 200 U/g GP of tannase, cellulase, and pectinase enzymes (individually or combined). They were performed at 40 °C for 2 h, under continuous agitation, in media containing 50 mM acetate buffer at pH 5.00, and ethanol concentrations ranging from 0 % to 30 % v/v. Significant differences are indicated in respect to the control extraction performed without enzymes: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. GP: grape pomace.

Fig. 5. Identification and quantification of individual phenolic compounds present in the extracts by HPLC-DAD: (a) quercetin and (b) *trans*-resveratrol. The extractions were carried out using 200 U/g GP of tannase, cellulase, and pectinase enzymes (individually or combined). They were performed at 40 °C for 2 h, under continuous agitation, in media containing 50 mM acetate buffer at pH 5.00, and ethanol concentrations ranging from 0 % to 30 % v/v. Significant differences are indicated in respect to the control extraction performed without enzymes: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. GP: grape pomace.

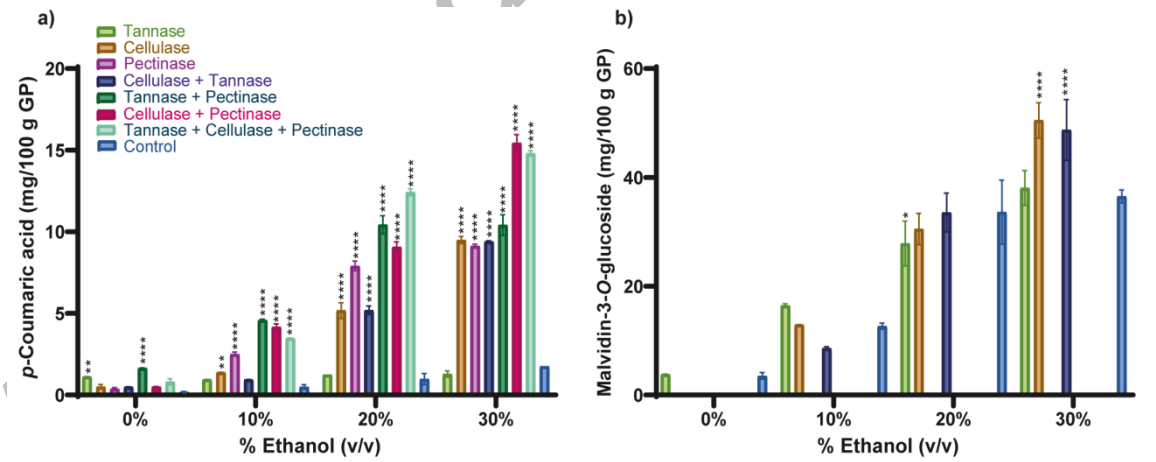



a)
b)
Tannase
Cellulase
Pectinase
Cellulase + Tannase
Tannase + Pectinase
Cellulase + Pectinase
Tannase + Cellulase + Pectinase
Control
g GAE / 100 g GP
mmol TE / 100 g GP
0%
10%
20%
30%
% Ethanol (v/v)

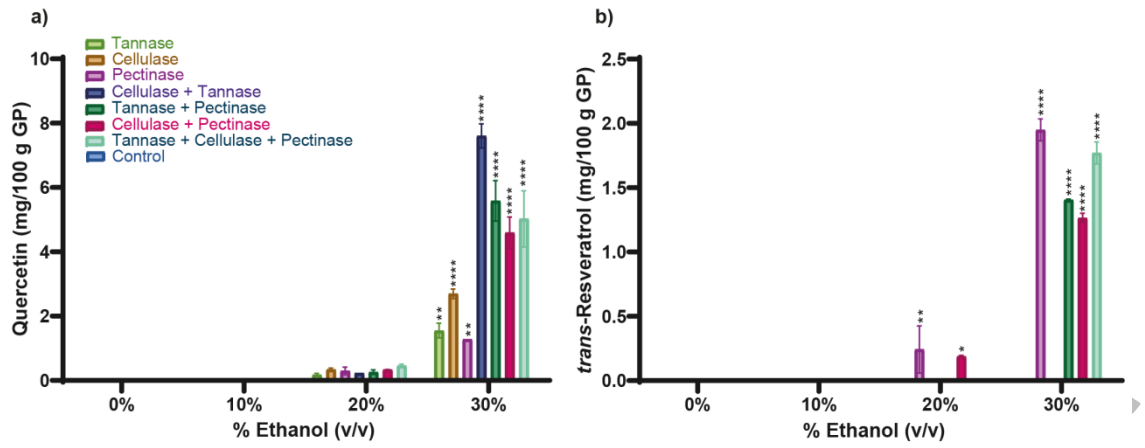
607


a)
b)
Tannase
Cellulase
Pectinase
Cellulase + Tannase
Tannase + Pectinase
Cellulase + Pectinase
Tannase + Cellulase + Pectinase
Control
Gallic acid (mg/100 g GP)
Syringic acid (mg/100 g GP)
0%
10%
20%
30%
% Ethanol (v/v)

608


a)
b)
Tannase
Cellulase
Pectinase
Cellulase + Tannase
Tannase + Pectinase
Cellulase + Pectinase
Tannase + Cellulase + Pectinase
Control
p-Coumaric acid (mg/100 g GP)
Malvidin-3-O-glucoside (mg/100 g GP)
0%
10%
20%
30%
% Ethanol (v/v)

609



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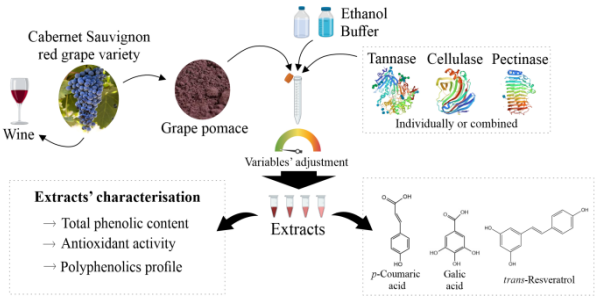

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