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Original article

Partial purification of a polygalacturonase from a new *Aspergillus sojae* mutant and its application in grape mash maceration

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Summary The use of polygalacturonase (PG) preparations in winemaking promotes the release of phenolic compounds. A PG from a new source, *Aspergillus sojae* mutant, was semi-purified and tested for grape mash maceration. Crude extract (CE), a commercial pectinase, and two high PG activity semi-purified preparations, F_I and F_{II}, were applied for maceration at PG activity of 3.5 U g^{-1} of grape for 46 h. Enzyme-assisted maceration significantly ($P < 0.05$) increased the total phenolic content from 255.8 to 916.3 ± 5.2, 5732.9 ± 9.9, 563.4 ± 6.7 and 620.6 ± 18.4 mg L^{-1} for CE, commercial pectinase, F_I and F_{II}, respectively. The content of individual phenolics such as gallic, protocatechuic, chlorogenic and *p*-coumaric acids was improved. Principal component and hierarchical clustering analyses suggested that CE has a better performance upon the release of phenols. Semi-purified preparations acted similar to commercial pectinase. These findings open an opportunity for the potential use of PG from the mutant strain as an alternative macerating enzyme.

Keywords *Aspergillus sojae*, *C. Sauvignon*, maceration, phenolic compounds, polygalacturonase, purification.

Introduction

Pectinolytic enzymes are a set of enzymes that degrade pectin as substrate, the major component of primary cell wall of plants (Pedrolli *et al.*, 2009). Pectinases are classified as de-esterifying and depolymerising enzymes. Polygalacturonase (PG) cleaves pectic acid into oligosaccharidic units (Saito *et al.*, 2004; Jayani *et al.*, 2005).

Pectinases are abundant in plants and microorganisms. Their production is mainly through fermentation processes with fungi. Although *Aspergillus niger* is the major producer (Murad & Azzaz, 2011), other fungal species also produce PG (Jayani *et al.*, 2005). *Aspergillus sojae* has recently been shown to produce PG in solid-state (SSF) and submerged (SmF) fermentations (Demir & Tari, 2014; Heerd *et al.*, 2014). Furthermore, a mutant strain from *A. sojae* produced high levels of PG as compared to *A. niger* under optimised SSF (Heerd *et al.*, 2012, 2014). Thus, the mutant strain can be an attractive alternative to produce PG.

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Commercial pectinases are applied in the clarification of fruit juices and in winemaking. For the improvement of wine quality, several techniques such as cold maceration (Cejudo-Bastante *et al.*, 2014), use of heat (Zimman *et al.*, 2002), must freezing (Busse-Valverde *et al.*, 2011), longer contact time (Zimman *et al.*, 2002; Gambacorta *et al.*, 2011) have been used as prefermentation steps to promote the release of phenolic compounds that will further improve wine quality. On the other hand, enzyme-assisted maceration, used with the same purpose, is an area of great interest. Enzymatic degradation of pectic substances during maceration increases the juice yield and extraction of bioactive phenolic compounds from grape tissue into the juice (Pinelo *et al.*, 2008; Tapre & Jain, 2014).

Various types of phenolic compounds in grape skins, pulp and seeds are partially transferred into must during maceration (Ribéreau-Gayon *et al.*, 2006); the presence of stems has also been described to contribute to the improvement of proanthocyanidins in red wine (Suriano *et al.*, 2015). However, it has been also reported that stems can release highly astringent, herbaceous and bitter proanthocyanidins as well. Therefore, the grapes are usually destemmed (Hashizume & Samuta, 1997; Del Llaudy *et al.*, 2008). Hydroxycinnamic and hydroxybenzoic acids, flavonols

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(quercetin and myricetin), flavan-3-ols (catechin and epicatechin), stilbene (resveratrol) and anthocyanins are commonly studied phenolic compounds in wines (Monagas *et al*., 2005). Sensorial characteristics of wines such as colour, astringency and bitterness are affected by phenolics (Ribéreau-Gayon *et al*., 2006). Thus, the increase in the content of such compounds in wine plays a crucial role for consumer acceptance. Besides, phenolic compounds have health-promoting, *that is,* antioxidant, anti-inflammatory and cancer protective effects (Fresco *et al*., 2006). Thus, extraction of phenolic compounds from red grape tissue to must is of great interest for wine industry.

Despite the potential that the new *A. sojae* mutant holds to degrade pectin, demonstration of the effectiveness of PG from this new strain has not been explored yet. Therefore, the aim of this study was to investigate the possibility of enhanced extraction of phenolic compounds from grape skin to the juice considering enzymatic treatment by a new PG source. The performances of the crude extract (CE) and the semi-purified preparations of PG on the release of phenolic compounds from *Cabernet Sauvignon* grapes were evaluated.

Materials and methods

Reagents and materials

Sugar beet molasses Goldsaft® (Grafschafter Krautfabrik, Meckenheim, Germany) was bought from a local market in Bremen, Germany. Wheat bran was obtained from a local market in Monterrey, México. Sugar beet pellets were from Nordzucker AG (Uelzen, Germany). Lallzyme Ex was kindly donated by Lallemand Inc. (Petaluma, CA, USA). Pure endo-PG (Megazyme, No. E-PGALUSP) was bought from Megazyme International, Ireland. Polygalacturonic acid (Mr = 25 000–50 000, No. 81325), D-(+)-galacturonic acid monohydrate (No. 48280-F), gallic acid (No. 27645), protocatechuic acid (No. 03930590), chlorogenic acid (No. 00500590), *p*-coumaric acid (No. C9008), ferulic acid (No. Y0001013) and formic acid (No. 09676) were purchased from Sigma-Aldrich Co. LLC (Toluca, México). HPLC grade methanol was from Merck Millipore (Darmstadt, Germany). *Cabernet Sauvignon* grapes were kindly donated by Casa Madero S.A. de C.V. located in Parras de la Fuente, Coahuila, Mexico.

Microorganism and fermentation

The mutant M3, used here to produce PG, was generated from the parental *A. sojae* ATCC 20235 strain, which was purchased from Procochem Inc. (Teddington, UK), an authorised distributor of the ATCC (American Type of Culture Collection) in Europe. The parental strain was subjected to mutagenesis by ultraviolet irradiation, which resulted in the mutant M3 strain (Heerd *et al*., 2014). Enzyme production was performed by SSF as described before (Heerd *et al*., 2014). Briefly, the solid medium was composed of 7 g wheat bran and 3 g sugar beet, wetted at 160% with 0.2 N HCl. The sterilised medium was inoculated with spores (2×10^6 spores g^{-1} solid medium), which were produced on slants containing molasses medium (Heerd *et al*., 2014). After incubation at 30 °C for 120 h, crude extracts (CEs) were recovered and dialysed using a 10 000 MWCO dialysis tubing (Thermo Scientific, Rockford, IL, USA) against distilled water (pH 5) for 36 h and used for further steps.

Fractionation by IEXC

To obtain a semi-purified PG preparation, CE from mutant was fractionated by ion-exchange chromatography (IEXC) centrifugal units having a DEAE adsorbent membrane (Vivapure Maxi H spin column, Sartorius stedim, Gottingen, Germany. Two experimental sets were constructed. In the first set, column was equilibrated with 10 mM Na-acetate buffer solutions at pH 4.8 or 5.5. Then, CE, diluted in the same buffer (pH 4.8 or 5), was loaded onto the column and a washing step was carried out to remove unbound proteins. The protein bound onto the membrane was eluted by applying a five step-gradient of NaCl prepared in equilibration buffer. Salt concentration was varied from 0.1 to 0.5 mol L^{-1} with increments of 0.1 mol L^{-1}. After this, 1 mol L^{-1} NaCl was applied to finish the process and membrane regeneration was carried out by applying equilibration buffer. Fractions of 5 mL were collected and stored at −20 °C for further steps. The second set was performed at pH 5.5 by varying salt concentration from 0.025 to 0.300 mol L^{-1} with increments of 0.025 mol L^{-1}. Fractions were desalted using PD-10 columns (GE Healthcare, Marlborough, MA, USA). All fractions were analysed for protein content and enzyme activity.

SDS-PAGE

Purification was monitored by SDS-PAGE (Laemmli, 1976). Desalted protein samples were mixed with sample buffer (6X), containing 0.027 mol L^{-1} dithiothreitol, in a 5:1 ratio. Samples were heated at 95 °C in a thermoblock for 5 min. Then, a 15% polyacrylamide gel was loaded with 15 μL of sample. Protein separation was performed at 120 V during 45 min in a Mini-PROTEAN® Tetra Cell (Bio-Rad, Hercules, CA, USA). Protein bands were stained with colloidal Coomassie staining as previously described (Dyballa & Metzger, 2009).

Enzyme activity assay and protein content

Polygalacturonase activity was assayed by mixing 200 μL of polygalacturonic acid (2.4 g L^{-1}) and 43 μL of the enzyme. Reaction was performed at 40 °C during 10 min in acetate buffer pH 4.8. Afterwards, galacturonic acid released from reaction was quantified according to Panda *et al.* (1999). After reaction, 200 μL aliquots were transferred into a microplate. Absorbance was read at 620 nm (Epoch, BioTek Instruments, Inc., Winooski, VT, USA). Galacturonic acid was used as standard. One unit of enzyme activity was defined as the amount of enzyme that releases 1 μmol of product per mL of enzyme preparation under tested conditions. Assays were conducted in triplicates.

Protein content was estimated using a micro-assay based on the method of Bradford (1976). Bovine serum albumin was used as standard.

Maceration and experimental design

The potential use of PG from *A. sojae* as a macerating enzyme was tested on *C. Sauvignon* grapes (*Vitis vinifera* L.) collected during August in 2013. Grapes were firstly separated from stems and then rinsed with sterile deionised water to reduce the background microbial load. Afterwards, grapes were manually cracked to blow up the skin. Ten grams of cracked grapes was placed into 50-mL tubes followed by addition of different enzyme sources, *that is* CE and commercial PG at the desired enzyme activity. Then, the cracked grapes were crushed for a better interaction with enzyme preparations and the pulp was kept under maceration conditions.

Response surface methodology considering face-centred composite design was applied for CE and a commercial pectinase (Lallzyme Ex) using Design-Expert 7.0.0 software (Stat-Ease, Inc. Minneapolis, MN, USA). Enzyme activity (x_1), maceration time (x_2) and enzyme type (x_3: CE and commercial pectinase) were considered as factors, while total free phenolic content (TPC) and colour density (CD) were responses. Three levels were selected for enzyme activity (0.5; 2.0; 3.5 U g^{-1} of grape) and maceration time (2, 24, 46 h) according to dosage and maceration time recommended in the technical data sheet of the commercial pectinases used here. To ensure accuracy, factorial points were duplicated and four central points were added into the design. Nystatin (720 U mL^{-1}) was added to avoid yeast growth and alcoholic fermentation. Enzymatic maceration was conducted in duplicate in a dark room at 20 °C as tested by others (Cejudo-Bastante *et al.*, 2014). Afterwards, the samples were centrifuged at 6000 ***g*** for 10 min and assayed for TPC and CD. The conditions, which yielded maximum TPC and CD, were selected as macerating conditions.

Validation was performed in duplicate considering optimal conditions. Individual phenolic compounds were also assayed. Moreover, semi-purified PG preparations were included at the optimum conditions. Control samples were prepared without any added enzyme.

Total phenolic content and colour

Total phenolic content was determined in triplicates according to Waterhouse (2003). TPC was expressed as milligram gallic acid (GA) L^{-1}. CD of macerated juice samples was determined spectrophotometrically (Cliff *et al.*, 2007) and calculated as follows:

$$CD = [(A_{520\,nm} - A_{700\,nm}) + (A_{420\,nm} - A_{700\,nm})]. \quad (1)$$

Individual phenolic compounds

Separation of phenols was performed as in Cantos *et al.* (2000) using an HPLC unit (Agilent 1200 Infinity Series) equipped with an autosampler, a reverse phase Luna C18 column (250 × 4.6 mm, 5 μm particle size, 100 Å, Phenomenex) at 25 °C and a diode array detector. Formic acid (5%) and methanol were used as mobile phases A and B, respectively. Elution was performed at 0.8 mL min^{-1} with a gradient: 98% A/2% B in 30 min; 68% A/32% B in 10 min; 60% A/40% B in 10 min; 5% A/95% B in 5 min; isocratic for 15 min, and finally (98% A/2% B) in 5 min. Gallic and protocatechuic acids were detected at 280 nm, while chlorogenic, *p*-coumaric and ferulic acids were monitored at 320 nm. Peak identification was performed based on the retention times and UV-visible absorption spectra of standards. Concentrations of phenolic compounds were expressed in mg L^{-1}.

Statistical analysis

Data analyses were performed by Excel 2010 (Microsoft Excel, Redmond, WA, USA) and Minitab 16 (Minitab Inc., State College, PA, USA). ANOVA and least significant difference (LSD) test were applied. Means were compared by Tukey's pairwise comparison test ($P < 0.05$). The observed and predicted values were compared by chi-square (χ^2) goodness-of-fit test. Principal component analysis (PCA) was performed to visualise the data structure. Hierarchical cluster analysis (HCA) was computed to show the similarity/dissimilarity of enzymatic treatments.

Results and discussion

Purification

The extract was fractioned by two sets of IEXC experiments. Figure 1 shows the results from the first

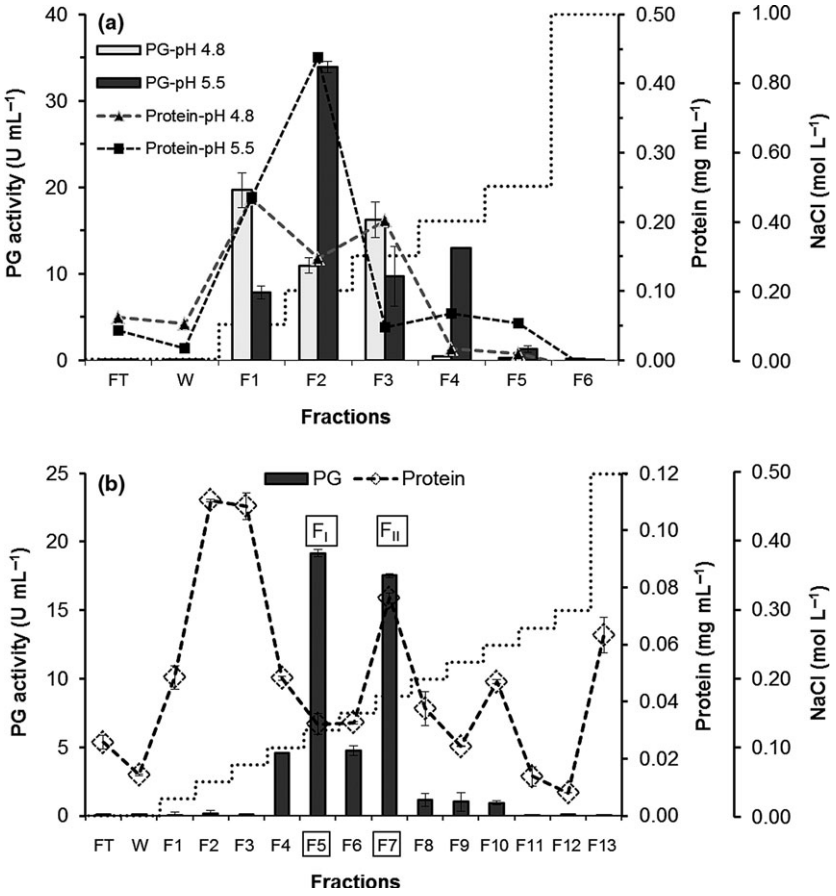


Figure 1 Fractionation of polygalacturonase crude extract from *Aspergillus sojae* mutant by ion-exchange chromatography. (a) First experimental set: NaCl (0.1–1 mol L^{-1}) in 10 mM acetate buffer at pH 4.8 or 5.5; (b) second experimental set: NaCl (0.025–0.5 mol L^{-1}) in 10 mM acetate buffer at pH 5.5. FT, flow through; W, wash; F, fraction eluted with the corresponding NaCl concentration in parenthesis. Dotted line indicates the step-gradient of NaCl. Fractions F5 and F7 in the black rectangles were selected for next experiments and renamed as F_I and F_{II}.

experimental set. Fraction 2 (F2) had the highest PG activity and protein content as 33.9 ± 0.7 U mL^{-1} and 0.4 ± 0.1 mg mL^{-1}, respectively. At pH 5.5, above pI of PG (~4.5), the net charge of PG is more negative than at pH 4.8. Thus, a greater amount of PG was bound onto the adsorbent and eluted at higher concentration with high activity.

In the second experimental set, therefore, 0.025–0.5 mol L^{-1} NaCl solutions at pH 5.5 were used (Fig. 1). Fractions 5 and 7, eluted with 0.125 and 0.175 mol L^{-1} NaCl, showed the highest PG activity of 19.2 ± 0.3 and 17.5 ± 0.2 U mL^{-1}, respectively. SDS-PAGE reveals an intense band with a molecular mass of ~37 kDa in both fractions, $F5_{125}$ and $F7_{175}$ (Fig. 2). These protein bands could be isozymes due to similar molecular mass and desorption at different ionic strength. Semi-purified fractions $F5_{125}$ and $F7_{175}$ were renamed as F_I and F_{II}, respectively.

Ion-exchange chromatography increased specific activity up to 7.7 and 3.0 times, with a recovery of 37.3% and 34.2%, for F_I and F_{II}, respectively. F_I exhibited higher specific activity (596.2 U mg^{-1}) than F_{II} (229.3 U mg^{-1}) (Table 1). This can be due to the fact that contaminant proteins were eliminated under tested conditions obtaining a purer PG preparation. PG was recovered in F_I and F_{II} with a purity of 74% and 35%, respectively. The PG active fractions (F_I and F_{II}) were then used for the validation step of maceration.

Maceration

To determine the maceration conditions of grape mash which yield in maximum TPC and CD, face-centred composite design and a quadratic model were applied for crude and commercial preparation. According to ANOVA, the models were significant enough ($P < 0.0001$) to explain the main, interaction and quadratic effects on TPC and CD with nonsignificant 'lack-of-fit' value ($P > 0.05$).

Enzyme activity (x_1), maceration time (x_2) and enzyme type (x_3) as main effects, interaction of time and enzyme type (x_2x_3) and quadratic term ${x_1}^2$ showed significant effect ($P < 0.05$) on TPC of macerated grape mash. R^2 and adjusted R^2 values of TPC were 0.9821 and 0.9758, respectively. The eqns 2 and 3 used for the estimation of TPC of CE and commercial PG treated samples are given below in coded terms:

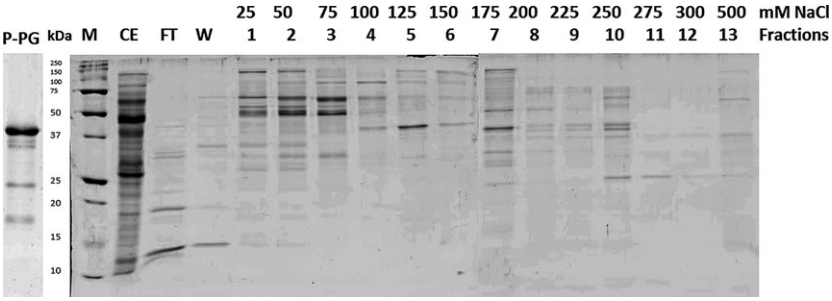


Figure 2 SDS-PAGE gel of ion-exchange chromatography fractions stained with colloidal Coomassie G-250 staining. P-PG, standard pure polygalacturonase, M, marker; CE, crude extract; FT, flow through; W, wash; numbers (1–13) indicate the fractions; F_I and F_{II}, partially purified fractions.

Table 1 Summary of the partial purification of PG from *Aspergillus sojae* mutant

Treatment	Protein (mg)	Activity (U)	Sp. activity (U mg^{-1})	Fold	Recovery (%)	Purity (%)
Crude extract	3.3	256.5	77.1	1.0	100.0	–
F_I	0.2	95.8	596.2	7.7	37.3	74.0
F_{II}	0.4	87.6	229.3	3.0	34.2	35.0

F_I and F_{II} were obtained using 0.125 and 0.175 mol L^{-1} NaCl solution, respectively. Purity was estimated by densitometry analysis of the gel using ImageJ 1.48v software.

$$\mathrm{TPC_{crude}}\ (\mathrm{mg\ L^{-1}}) = 605.41 + 20.06 * x_1 + 232.90 * x_2 + 31.82 * x_2^2, \quad (2)$$

$$\mathrm{TPC_{commercial}}\ (\mathrm{mg\ L^{-1}}) = 392.85 + 19.67 * x_1 + 142.27 * x_2 + 31.82 * x_2^2. \quad (3)$$

Total phenolic content of control samples was 172.5 ± 0.7, 225.5 ± 5.9 and 255.8 ± 0.2 mg L^{-1} for 2, 24 and 46 h, respectively. TPC of mashed grape treated with CE was found in the range of 397.9 to 911.9 mg L^{-1}, whereas that of grape mash samples macerated by the commercial pectinase varied from 257.3 to 579.9 mg L^{-1}. Thus, TPC content of macerated grape mash by crude PG and commercial enzyme showed a remarkable difference considering enzyme type, enzyme activity and maceration time. CE yielded a higher amount of TPC than commercial PG preparation according to both observed and predicted values. This increase can be attributed to the action of other pectin-degrading enzymes present in the CE, which were described by Mata-Gómez *et al.* (2015), that may contribute to degrade pectin besides PG, thereby contributing to the release of phenolic compounds from tissue to grape mash. Besides, some other pectin-degrading activities such as β-glucosidase, xylanase, cellulose, α- and β-galactosidase and arabinofuranosidase have been found in the crude extract from the *A. sojae* mutant (data not shown). A significant increase in the release of total phenolics from muscadine grape skin (Noble) has been found after enzymatic hydrolysis by a commercial pectinase from *A. niger*. Enzyme hydrolysis has been claimed to shorten the extraction time (Xu *et al.*, 2014).

Regarding the CD, enzyme activity (x_1), maceration time (x_2) and enzyme type (x_3) as main effects were significant ($P < 0.05$). In accordance with TPC, similar trend was also observed for CD as the enzyme activity and time increased. CE resulted in colour change from 2.0 to 5.7, whereas colour of mash treated with commercial PG varied between 1.3 and 4.7. Colour densities were also predicted using the equations below in coded factors. Codes x_1 and x_2 stand for enzyme activity and maceration time, respectively.

$$\mathrm{CD_{crude}} = +3.59 + 0.28 * x_1 + 1.51 * x_2, \quad (4)$$

$$\mathrm{CD_{commercial}} = +2.64 + 0.14 * x_1 + 1.42 * x_2. \quad (5)$$

Consequently, the models were significant enough to explain the data. Predicted values for TPC and CD were in a reasonable agreement with the observed data having R^2 of 0.9821 and 0.9611, respectively. Chi-square (χ^2) goodness-of-fit test revealed that chi-square values for TPC were 10.23 and 9.32 for crude and commercial preparations, respectively. Likewise, chi-square values for colour of samples treated with CE and commercial preparations were 0.09 and 0.19, respectively. As the chi-square values of TPC and colour were smaller than the $\chi^2_{\mathrm{critical}}$ (15.51), it can be concluded that the predicted values were in accordance with the experimental data.

Figure 3 presents the effect of enzyme activity and maceration time on TPC and CD for both crude and commercial enzyme treatments. TPCs and CDs were increased with the enzyme activity and time in both applications. However, CE resulted in higher TPC and CD. In this study, maximum 46 h was studied in accordance with the objective of examining the effect of PG from *A. sojae* mutant as macerating enzyme. Longer contact time enhanced phenolic compounds and colour in different grape cultivars (Gambacorta *et al.*, 2011; Cejudo-Bastante *et al.*, 2014).

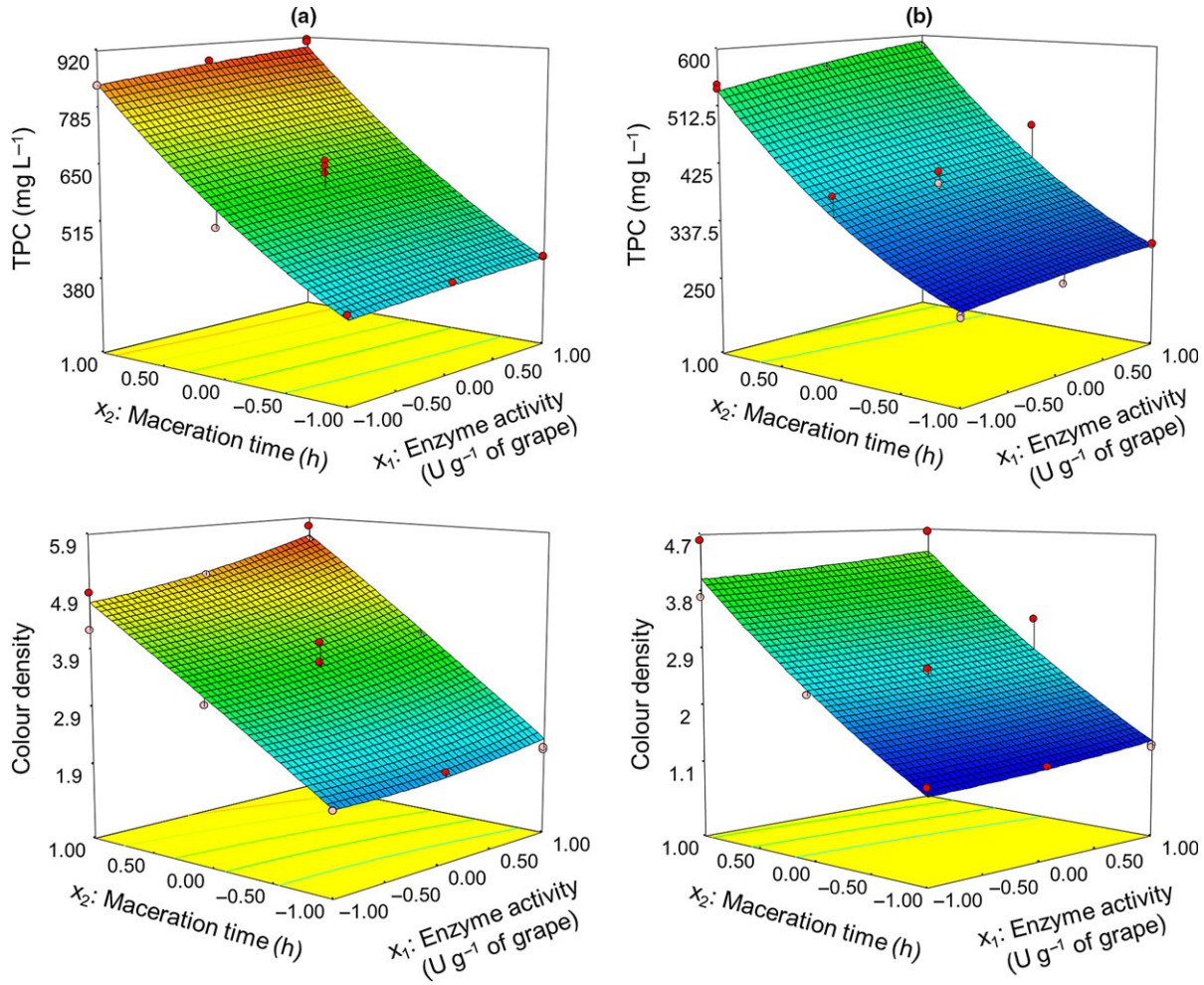


Figure 3 Response surface plots of the total phenolic contents (TPCs) and colour densities of macerated grape mashes for (a) crude extract and (b) commercial pectinase. Levels of variables in the axes were presented in codes. [Colour figure can be viewed at wileyonlinelibrary.com].

In this work, high factor levels for enzyme activity and maceration time (3.5 U g^{-1} of grape and 46 h) were determined as the optimised maceration conditions for both crude and commercial enzyme with the desirability of 0.919 and 0.521, respectively. Desirability was obtained by Design-Expert software (Design-Expert 7.0.0, Stat-Ease, Inc.) as an output of face-centred composite design. Note that the desirability ranges from 0 to 1, being the latest the maximum indicator of desirability in optimisation goals. High levels of activity are needed for a better performance because PG is working out below its optimal pH (~5) (Tari *et al.*, 2008).

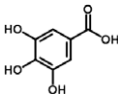
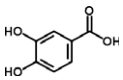
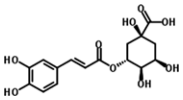
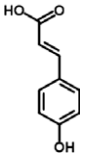
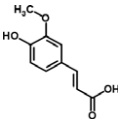
Validation of maceration conditions

For validation, maceration experiments were ran under optimal conditions (3.5 U g^{-1} of grape and 46 h) including F_I and F_{II} as well as commercial enzyme and CE. Tukey's pairwise comparison test showed that CE resulted in a significant difference for TPC (916.3 ± 5.2 mg L^{-1}) as compared to control (255.8 ± 13.0 mg L^{-1}). Fraction F_{II} was the second significant treatment with TPC of 621.0 ± 18.4 mg L^{-1}. Commercial pectinase and F_I exhibited similar behaviour for the extraction of phenolic compounds. The significant increase by CE is probably raised from other possible pectin-degrading enzymes present in CE, and not in the semi-purified preparations, that may also act on pectin as described by Mata-Gómez *et al.* (2015). No significant differences were found for CDs of macerated grape mash among treatments (Table 2).

Individual phenolic compounds

The analysed phenolic compounds in must are presented in Table 2. Gallic acid, protocatechuic acid,

Table 2 Concentrations of individual phenolic compounds and colour density of macerated grape mash before and after enzymatic treatments

			Individual phenolic compounds (mg L^{-1})				
			Gallic acid	Protocatechuic acid	Chlorogenic acid	*p*-coumaric acid	Ferulic acid
Treatment	**TPC (mg L^{-1})**	**CD**					
Control	255.8 ± 13.0^{d}	3.6 ± 0.0^{a}	6.2 ± 0.7^{c}	1.9 ± 0.2^{d}	0.8 ± 0.0^{c}	6.3 ± 0.7^{c}	nd
Crude	916.3 ± 5.2^{a}	3.9 ± 0.5^{a}	17.8 ± 0.0^{a}	6.9 ± 0.0^{a}	2.6 ± 0.0^{a}	11.1 ± 0.1^{a}	0.7 ± 0.0^{a}
Commercial	572.9 ± 9.9^{c}	4.4 ± 0.4^{a}	13.2 ± 0.2^{b}	6.1 ± 0.4^{ab}	1.9 ± 0.1^{b}	9.9 ± 1.2^{ab}	0.4 ± 0.4^{a}
F_I	563.4 ± 6.4^{c}	4.3 ± 0.2^{a}	14.7 ± 1.3^{b}	5.1 ± 0.0^{bc}	2.2 ± 0.2^{ab}	7.9 ± 0.4^{bc}	0.7 ± 0.5^{a}
F_{II}	620.6 ± 18.4^{b}	4.1 ± 0.4^{a}	13.4 ± 0.8^{b}	4.9 ± 0.4^{c}	1.9 ± 0.1^{b}	8.1 ± 0.1^{bc}	1.0 ± 0.0^{a}

nd, not detected; TPC, total phenolic content; CD, colour density.
Grapes were macerated for 46 h by adding 3.5 U PG activity g^{-1} of grape.
F_I and F_{II} are the semi-purified enzyme preparations.
Different letters (a–d) indicate significant differences between treatments.

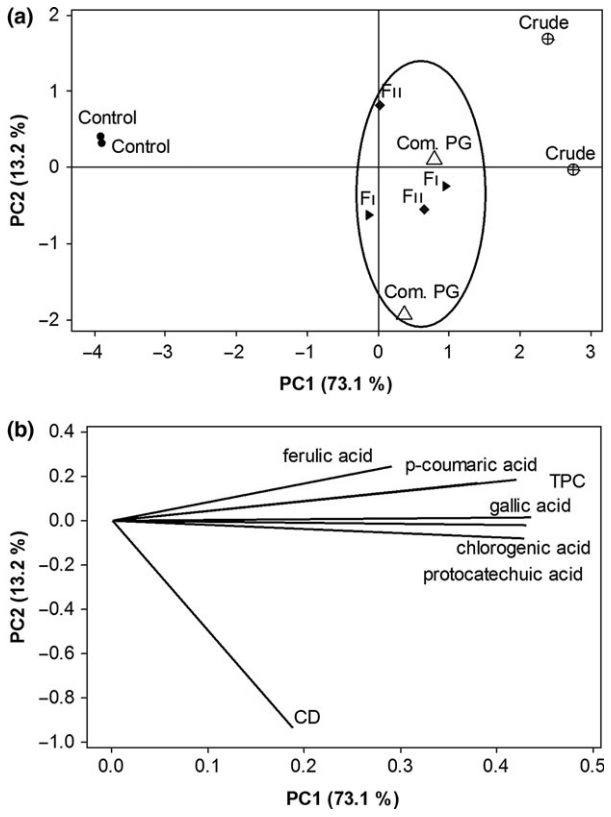


Figure 4 Principal component analysis output showing (a) score and (b) loading plots for discrimination of enzyme treatments considering colour density and phenolic compounds. Com. PG, commercial pectinase; F_I, fraction I; F_{II}, fraction II; TPC, total phenolic content; CD, colour density.

chlorogenic acid and *p*-coumaric acid were found in higher amounts in must treated with CE. Samples treated with commercial PG, F_I and F_{II} had similar amounts of these compounds. No significant change was observed for ferulic acid among treatments. *Cabernet Sauvignon* wine samples contain gallic acid in the range of 22.8–101.6 mg L^{-1}, while *p*-coumaric acid and ferulic acid content varies between 0.7 and 8.3; and 0.6–7.5 mg L^{-1}, respectively (Radovanović *et al.*, 2010). In our study, gallic acid increased from 6.2 ± 0.7 to 17.8 ± 0.0 mg L^{-1} by enzymatic treatment. *p*-Coumaric acid was in the range of 6.3–11.1 mg L^{-1} in accordance with Radovanović *et al.* (2010).

Furthermore, PCA was able to discriminate control samples and CE treatments from others as illustrated in score and loading plots (Fig. 4). The first two components, PC 1 and PC 2, explained 73.1% and 13.2% of the total variance, respectively. According to PC 1, samples treated with any type of enzyme were distinguished from control in the score plot (Fig. 4a). CE treatment showed a different outcome in comparison with semi-purified preparations and commercial PG. Treatments of semi-purified fractions and commercial pectinase exhibited similar results in terms of TPC, colour density and individual phenolic compounds as suggested by PCA. The loading plot showed how phenolic compounds vary in relation to the enzyme treatments by expressing the dominating correlation structure (Fig. 4b).

A similarity/dissimilarity dendrogram of treatments is presented in Fig. 5. The plot illustrates how enzymatic treatments show dissimilarity, distance on *y*-axis,

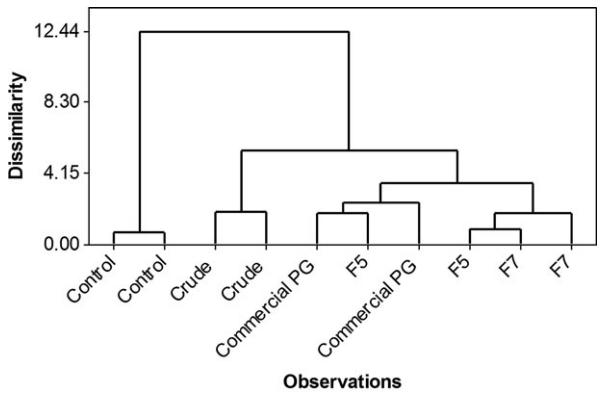


Figure 5 Similarity/dissimilarity of treatments by hierarchical cluster analysis.

comparing to control samples, which were macerated during the same time without addition of any enzyme preparation. It is observed that commercial PG and semi-purified fractions F_I and F_{II} showed higher similarity due to their less dissimilarity distance in *y*-axis. Based on these, semi-purified PG preparations can be recommended as macerating enzymes in wine processing as they showed similar extraction behaviour as the commercial pectinase. However, the use of concentrated fractions may need attention to increase the concentration of phenolic compounds.

Conclusion

Maceration is an important stage before fermentation to release phenolic compounds that will further contribute to wine quality. Here, it was demonstrated that PG from a new source, *that is A. sojae* mutant, improved the release of phenolic compounds from grape considering relatively low maceration time. Thus, it is noticeable to indicate that PG from mutant strain M3 has a potential to increase the TPC and the individual phenolic compounds content as the commercial enzyme does. Most of commercial pectinases are produced from *A. niger*. Thus, these findings open an opportunity for the potential use of PG from the *A. sojae* mutant as an alternative enzyme for maceration of grapes prior to fermentation. As anthocyanins have a great influence on development of colour and enhancement of antioxidant capacity of the final product, further research is warranted to understand the impact of the PG from this new fungal source on release of anthocyanins during maceration. Besides, a maceration scale up study can be suggested.

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