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Effect of pectinases from *Aspergillus niger* on phenolic extraction during grape maceration

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Abstract

The sensory quality of red wines is characterized by, among other parameters, color and flavor, which depend on phenolic compounds such as anthocyanins and tannins. Since these compounds are responsible for the sensory characteristics of wine, it is necessary to use techniques that allow their efficient and optimal extraction. In this study, the extraction efficiency of phenolic compounds using fungal pectinases is analyzed. Pectinases were produced with *Aspergillus niger* NRRL 332 in submerged fermentation, analyzing different temperatures (22, 25, 30, 35, and 38 °C), initial pH (3.1, 3.5, 4.5, 5.5 and 6.1), and growth kinetics were performed for 120 hours. The optimal pectinase production points were found to be at a temperature of 30 °C, pH of 5.5, and an incubation time of 72 h, with a maximum of 9.6 $U \cdot mL^{-1}$. Subsequently, the enzymes produced were applied in the wine maceration process. The use of pectinases significantly increased the concentration of total soluble phenols, favoring color attributes such as hue angle and chromaticity.

Keywords: Enzymes; submerged fermentation; process optimization; microbial biotechnology.

Introduction

Wine contains phenolic compounds such as anthocyanins and tannins that influence its color, aroma and flavor, contributing to the complexity and stability of the final product. In addition, they have a significant impact on its commercial value, as they are determinants of the sensory quality of wine and are used as markers of authenticity in the wine industry (Gutiérrez-Escobar et al., 2021). These secondary metabolites are mainly located in the vacuole, and their release depends on the rupture of the cell wall during the maceration stage (Gao et al., 2016). Their concentration and profile can be modulated during the production process to obtain desired characteristics that bring uniqueness to the wine (Gutiérrez-Escobar et al., 2021; Merkyte et al., 2020). Extraction of phenolic compounds from grapes has been tested using sonication (Osete-Alcaraz et al., 2019), microwave application (Caldas et al., 2018; Wang et al., 2019) and enzyme incorporation (Gao et al., 2019).

In the last-mentioned option, it has been shown that the use of pectinases can help break the polymeric chains that make up the cell wall and foster the release of secondary metabolites (Satapathy et al., 2020). During the maceration stage, these enzymes optimize the extraction of anthocyanins, tannins and other phenolic compounds (Fernández-González et al., 2024). Pectinases occur naturally in plants; however, industrial production is mainly carried out with microbial systems, including a large number of bacterial strains such as *Bacillus* spp. (Mercimek Takcı & Turkmen, 2016), some yeasts such as *Kluyveromyces marxianus* and *Saccharomyces cererevisiae* (Bilal et al., 2022; Poondla et al., 2016) and many

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filamentous fungi, mainly of the genera *Aspergillus* and *Penicillium* (Li et al., 2015). *Aspergillus niger* is the most widely used microorganism in this biotechnological field, since it has been generally recognized as safe (GRAS) by the Food and Drug Administration (FDA) of the United States government (Reginatto et al., 2017). The production of pectinases has been carried out by solid-state fermentation (SsF) (Pitol et al., 2016; Poletto et al., 2017) and submerged fermentation (SmF) (Fratebianchi et al., 2017; Silva et al., 2019). In the latter, the microorganism grows in a liquid medium and is characterized as a process with controlled and aseptic physicochemical conditions, with homogeneously dissolved nutrients always available in the culture medium (Ravindran & Jaiswal, 2016). The control of this bioprocess depends on several factors, especially pH and temperature (Amin et al., 2019), which must be evaluated to define the best conditions in the enzyme production process.

In this context, this work aimed to characterize the conditions for pectinase production through submerged cultures with *A. niger* and apply them during the wine maceration process to evaluate their effect on the extraction of phenolic compounds.

Materials and methods

Preparation of inoculum and submerged cultures

The strain *Aspergillus niger* NRRL 332, provided by the United States Department of Agriculture, was used. It was propagated in Petri dishes with potato dextrose agar (PDA, BD Bioxon brand) culture medium and incubated at 30 °C for 5 days. Spores were then scraped and resuspended in a 0.05 % v/v solution of Tween 20 for quantification with a Neubauer chamber. Dilutions were made until reaching a concentration of 1×10^5 spores$\cdot$mL^{-1} (Reginatto et al., 2017).

Pectinase production

Erlenmeyer flasks were used with 50 mL of culture broth composed of 1.0 % yeast extract (analytical grade, Meyer), 0.48 % $MgSO_4$ (analytical grade, Meyer), 0.018 % $FeSO_4$ (analytical grade, Meyer), 0.0075 % $CaCl_2$ (analytical grade, Meyer), 0.02 % $KH_{24}PO_4$ (analytical grade, Meyer), 0.0125% $(NH_4)_2$ SO_4 (analytical grade, Fermont) and 1.0% reagent grade pure citrus pectin (analytical grade, Fermont), and then they were inoculated with the previously prepared spore solution. Treatments were incubated with constant stirring at 200 rpm for 72 h (Reginatto et al., 2017). Pectinase production was evaluated using a central composite response surface experimental design (Table 1) with two factors, initial pH (3.1, 3.5, 4.5, 5.5 and 6.1) and temperature (22, 25, 30, 35 and 38 °C), each with two levels (+1 and -1), two axial points (+1.5 and -1.5) and a central point (0), resulting in a total of nine treatments with their respective replicates (five replicates for the central point and three for the axial points and levels), with Matlab 2013 software. The response variable was the enzyme activity of the culture broths expressed in U$\cdot$mL^{-1}. One unit of enzyme activity (U) was defined as the amount of enzyme required to produce one micromole of galacturonic acid per minute under the assay conditions. The central composite design was based on the following second-order polynomial equation (1):

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i<j} \beta_{ij} X_i X_j \quad (1)$$

Where: Y is the predicted pectinase activity (U$\cdot$mL^{-1}), X_i and X_j are the parameters temperature (°C) and pH), β_0 is the intercept term, and β_i, β_{ii} and β_{ij} are the linear, quadratic and interaction coefficients, respectively. The predicted responses obtained from the central composite design were compared with the actual responses to estimate the accuracy of this methodology.

Based on the conditions that generated the highest pectinase activity, kinetics of biomass production in triplicate, enzyme activity, substrate consumption and pH variation were performed, measuring each parameter at 12-h intervals for five days.

Biomass production was determined using the dry weight method described by Reginatto et al. (2017) by separating the pellets formed from the liquid phase with Whatman No. 1 filter paper and drying them at 80 °C for 24 h; the results were expressed in g$\cdot$L^{-1}. Substrate consumption was quantified using the anthrone method described by Sim et al. (1997); the absorbance of the sample was measured in a spectrophotometer (GENESYS™ 10, Thermo Scientific™, USA) at 650 nm and the results were expressed in g$\cdot$L^{-1}. To determine enzyme activity, the culture broth was subjected to microfiltration to concentrate the enzyme using a 50 mL Amicon® cell and a 10-kDa cellulose membrane at 40 psi pressure and 180 rpm. The retained fraction was recovered by resuspension in 10 mL of 0.1 M acetate buffer at pH 4.5, adding 1 % (w/v) citrus pectin as substrate; subsequently, the concentration of galacturonic acid released was measured using the 3,5-dinitrosalicylic acid (DNS) method proposed by Miller (1959). Samples were analyzed in a spectrophotometer (GENESYS™ 10, Thermo Scientific™, USA) at 540 nm and the results were expressed in U$\cdot$mL^{-1}. With the same enzyme concentrate, protein content was measured by the Bradford (1976) technique, using a calibration curve with bovine serum albumin (BSA) as a standard. The results were expressed in mg$\cdot$L^{-1}.

SDS-PAGE electrophoresis

The molecular weight of the pectinases produced was estimated by SDS-PAGE following the method of (Laemmli, 1970) with some modifications, using a separating gel and a concentrating gel. The separating gel was prepared with 2.5 mL of 1.5 M Tris-HCl solution (pH 8.8, Sigma Aldrich brand), 4.82 mL of distilled water, 5 mL of acrylamide

Table 1. Central composite experimental design for pectinase production by A. niger NRRL 332.

Treatment	Combination of coded points	Temperature	pH
1	0, 0	30	4.5
2	-1, -1	25	3.5
3	+1, -1	35	3.5
4	-1, +1	25	5.5
5	+1, +1	35	5.5
6	-1.5, 0	22	4.5
7	+1.5, 0	38	4.5
8	0, -1.5	30	3.1
9	0, +1.5	30	6.1

(Sigma Aldrich brand), 0.125 mL of 10 % SDS (BioRad brand), 0.06 mL of PSA (BioRad) and 17.5 µL of TEMED (N, N, N', N'-tetramethylethylenediamine, analytical grade, BioRad). The concentraing gel was made with 0.15 mL of 0.5 M Tris-HCl solution, pH 6.8 (analytical grade, Sigma Aldrich), 3.78 mL of distilled water, 0.75 mL of acrylamide (analytical grade, Sigma Aldrich), 0.1 mL of 10 % SDS (analytical grade, BioRad), 0.025 mL of 10 % PSA (analytical grade, BioRad) and 7 µL of TEMED (analytical grade, BioRad). Electrophoresis was carried out in a Mini Protean® Tetra Cell system (BioRad, USA) at 180 V for 45 minutes. Protein staining was performed with Coomassie Brilliant Blue R-250 (analytical grade, BioRad). The obtained protein resolutions were analyzed with a dual-wavelength chromatogram scanner (Shimadzu, CS-910, Japan) and data acquisition was performed using Chromatography Station CSW software from DataApex Ltd.

Application of the enzymatic extract in the wine maceration process

First, 1200 g of common grape vine (*Vitis vinifera*) fruits Carignan variety, harvested in Ensenada, Baja California, Mexico, with a total soluble solids content of 29 °Brix, were used. The fruits were divided into three groups of 100 g each, which were ground in a mortar and placed in 250-mL Erlenmeyer flasks. The material from the first group was placed at 25 °C, was taken as the positive control (PC) and represented the initial concentration of phenolic compounds. The second group constituted the negative control (NC) and was formed with grape macerate at 40 °C. The third group (E) consisted of grape macerate added with 3 mL of the enzyme concentrate with a hydrolytic activity of 4.66 $U \cdot mg^{-1}$ and subjected to a temperature of 40 °C. All treatments were under constant stirring at 200 rpm for 30 min. The phytochemical profile of the grape macerates was quantified using calibration curves with standards. The concentration of total phenols (TP) was determined according to the Folin-Ciocalteu colorimetric method (Singleton & Rossi, 1965). Absorbance was measured at 760 nm. Gallic acid was used as a standard (analytical grade, Sigma Aldrich) and results were expressed as mg of gallic acid equivalent ($GAE \cdot mL^{-1}$). Total flavonoids (Fl) were quantified according to the quercetin aluminum chloride method ($meq\text{-}Q \cdot mL^{-1}$, analytical grade, Sigma Aldrich) at 510 nm (Zhishen et al., 1999). Condensed tannins (CT) were measured by the vanillin method using catechin as a standard ($meq\text{-}C \cdot mL^{-1}$, analytical grade, Sigma Aldrich) at 550nm (Price et al., 1978). The anthocyanin concentration was quantified using the pH differential method to determine the total monomeric anthocyanin (Ant) content of the samples (Lee et al., 2005) with cyanidin-3-glucoside $\cdot mL^{-1}$ ($C3G \cdot mL^{-1}$, analytical grade, Sigma Aldrich). The absorbance of the samples diluted with potassium chloride (pH 1.0) and sodium acetate (pH 4.5) buffer solutions was measured using a spectrophotometer at wavelengths of 520 and 700 nm. The absorbance values were calculated according to equation (2):

$$A = (A_{520} - A_{700})pH_{1.0} - (A_{520} - A_{700})\,pH_{4.5} \quad (2)$$

Finally, color was evaluated with a Hunter Lab colorimeter (Mini Scan XE Plus 45/0-L, USA) and expressed as lightness, hue angle and chromaticity (Carvajal-Herrera et al., 2011).

This phase was carried out with a completely randomized design, where the PC, NC and E treatments constituted the source of variation. An analysis of variance was applied, complemented with comparison tests of treatment means performed with the Tukey statistic, with a significance level of 0.05.

Results and discussion

Aspergillus niger NRRL 332 showed the highest pectinase production at a temperature (T°) of 25 and 30 °C and a pH that varied from 5.0 to 5.8 with a range of variation between 8.91 and 9.09 $U \cdot mL^{-1}$ (Figure 1), with no significant differences between these levels. The model used (Equation 1) on the response surface fitted the experimental data by 84.8 % (Table 2), expressed in Equation 3.

$$Y = -49.3309 + 3.3713*A + 2.8385*B + 0.0678*A*B - 0.0623*A2 - 0.4439*B22 \quad (3)$$

Table 2. Model fit to experimental data on pectinase production by *Aspergillus niger* NRRL 332

Temperature (°C)	pH	Experimental Y(U·mL^{-1})	Predicted Y (U·mL^{-1})	Error (%)
22	4.5	4.1093	5.1810	26.0792
25	3.5	8.3822	6.4436	23.1282
25	5.5	9.5884	7.5204	21.5678
30	4.5	8.0292	8.6754	8.0475
30	2.9	5.6354	6.1352	8.8690
30	6.1	8.2866	8.9428	7.9188
35	3.5	4.5175	5.1496	13.9924
35	5.5	7.0805	7.5824	7.0881
38	4.5	5.2125	4.1954	19.5127
			Average:	15.1337

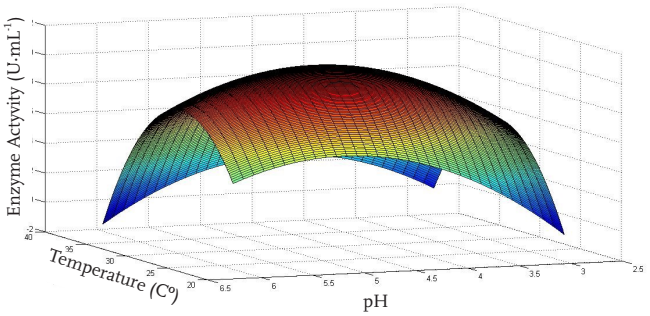

Figure 1. Response surface of the interaction of temperature and pH on pectinase production by *A. niger* NRRL 332.

An analysis of variance ($P \leq 0.05$) confirmed that both temperature and pH significantly influenced pectinase production (Table 3). Several studies have investigated the optimal conditions for pectinase production by *Aspergillus niger*. For example, El Enshasy et al. (2018) evaluated the effect of initial pH on pectinase production by the NRC1ami strain, finding that a pH of 5.5 was optimal for pectinase production in submerged culture. On the other hand, (Sandri & da Silveira, 2018) studied pectinase production by *Aspergillus niger* LB-02-SF in solid-state culture, using a pH of 4.0 and a temperature of 30 °C, obtaining a maximum enzyme activity of 68 U·g^{-1}. These studies indicate that, although optimal conditions may vary depending on the strain and culture method, a pH in the range of 4.0 to 5.5 and a temperature of 30 °C are common for efficient pectinase production by *Aspergillus niger*.

Growth kinetics and pectinase production

Figure 2 shows that the strain produced a maximum of 9.1 U·mL^{-1} at 72 h of incubation. Likewise, it is observed that the highest biomass production occurred at 65 h. Therefore, it is inferred that the production of pectinases was directly related to the growth of the microorganism. In addition, it is shown that enzyme production increased as the substrate (pure citrus pectin) was consumed; it was also found that for each gram of pectin consumed, 1.12 units of enzyme activity were produced (Table 4). Some studies have indicated that pectinase production in *Aspergillus niger* is regulated by the expression of the pg1 gene, which is induced in the presence of pectin in the culture medium, resulting in greater pectinase production (Lin et al., 2021).

Likewise, an initial decrease in pH is observed in the first 24 hours, followed by an increase after 60 hours of incubation. This behavior has been attributed to the production of acidic compounds such as citric acid and gluconic acid, which are known products of *A. niger* metabolism (Shrestha et al., 2021). In addition, this species is capable

Table 3. Analysis of variance of the effect of temperature and pH on pectinase production of *A. niger* NRRL 332.

Source	SC	GL	CM	F statistic	P-value
Regression	57.78	5	11.55	7.14	0.001
A	15.48	1	15.48	9.57	0.005
B	0.85	1	0.85	0.53	0.476
A*A	23.48	1	23.48	14.52	0.001
B*B	2.53	1	2.53	1.57	0.224
A*B	1.38	1	1.38	0.85	0.366
Error	33.97	22	0.27		

R^2=0.95; Coefficient of Variation= 8.4249; A= Temperature; B= pH.

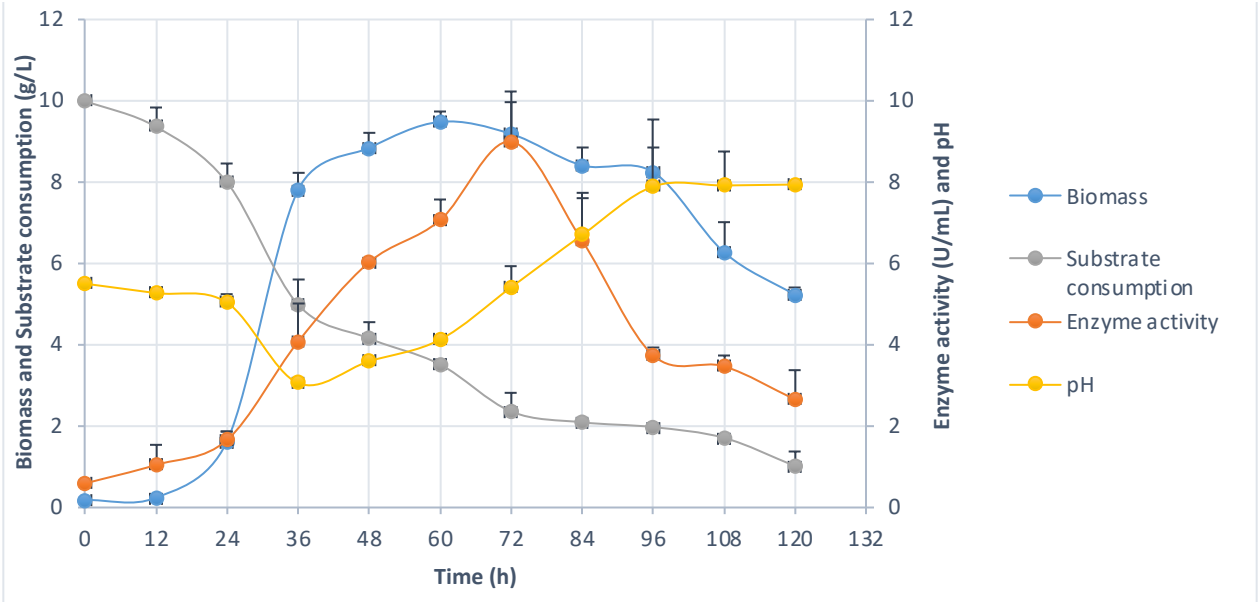

Figure 2. Growth kinetics and pectinase production by *A. niger* NRRL 332.

Table 4. Kinetic parameters of A. niger NRRL 332.

μmax (h^{-1})	Y x/s (g biomass·g substrate^{-1})	Y p/s (U·g substrate^{-1})
0.0769	1.52	1.12

of synthesizing other important hydrolytic enzymes, such as phytases and amylases, which reinforces its relevance in biotechnological processes (Sahu & Sevda, 2022).

Characterization by SDS-PAGE

The results of the zymogram presented by the SDS-Page electrophoresis method showed two bands with molecular weights of 45 and 70 kDa (Figure 3), which indicated that they were probably the weights of the pectinases produced by the microorganism under study. According to (Okonji et al., 2019), the molecular weight of pectinases ranges from 30 to 70 kDa. The results obtained are consistent with studies that have characterized polygalacturonases with similar molecular weights. For example, (Nawaz et al., 2021) purified a polygalacturonase from *Penicillium notatum* with a molecular weight of 38 kDa. The evaluation of the enzyme activity was supported by the DNS method, based on the determination of the free carbonyl groups (C=O) of the reducing sugars, which are produced when the pectin chain is hydrolyzed by pectinase. These occur when the pectin chain is hydrolyzed by pectinase, so it was accepted that a polygalacturonase was among the pectic enzymes produced.

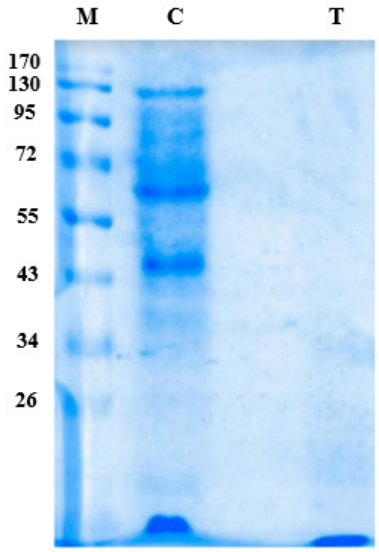

Figure 3. SDS-PAGE electrophoresis of pectinases produced by *A. niger* NRRL 332. M: Molecular weight marker (kDa). C: Bands corresponding to pectinases. T: Control treatment (culture broth without pectinases).

Application of the enzymatic extract in the wine maceration process

With the use of pectinases in grape fruit maceration, the concentrations of total soluble phenols, flavonoids, anthocyanins and condensable tannins increased by 90, 44, 104 and 43 %, respectively, with respect to the positive control (Table 5). The use of pectinases during the maceration of grapes and other fruits has been widely studied due to their ability to break down the cell wall and facilitate the release of phenolic compounds and other beneficial metabolites. According to (Guler, 2023), maceration techniques that include the combination of enzymatic and heat treatments not only increase the extraction of

Table 5. Comparison of means for the extraction of phenolic compounds.

Treat	Response variables						
	TP GAE·mL^{-1})	Fl (meq-Q·mL^{-1})	Ant (C3G·mL^{-1})	CT (meq-C·mL^{-1})	L*	H* (°H)	C*
PC	234.2 ± 17.9^{b}	95.1 ± 3.5^{b}	49.9 ± 11.1^{b}	310.5 ± 36.3^{b}	6.1 ± 1.3^{a}	305.9 ± 9.9^{b}	5.2 ± 1.2^{b}
NC	248.2 ± 8.9^{b}	97.7 ± 8.4^{b}	67.1 ± 3.3^{b}	315.1 ± 46.3^{b}	6.3 ± 0.9^{a}	310.5 ± 12.4^{b}	5.2 ± 0.5^{b}
E	447.2 ± 31.3^{a}	137.7 ± 15.0^{a}	102 ± 11.2^{a}	445.9 ± 42.9^{a}	6.4 ± 1.41^{a}	347.8 ± 3.8^{a}	6.4 ± 0.6^{a}

Treat: treatments PC: positive control; NC: negative control; E: enzyme treatment. Response variables. TP: total soluble phenols; Fl: total flavonoids; Ant: total anthocyanins; CT: condensed tannins; L*: lightness; H*: hue angle; C*: chromaticity. Different letters indicate significant differences ($P < 0.05$); GAE·mL^{-1}: mg gallic acid equivalents·mL^{-1}; meq-Q·mL^{-1}: mg quercetin equivalents; C3G·mL^{-1}: mg cyanidin-3-glucoside equivalents·mL^{-1}; meq-C·mL^{-1}: mg catechin equivalents.

anthocyanins and flavonoids, but also improve the antioxidant capacity of the final product. This study showed that microwave treatment combined with pectinases increased the content of catechin and trans-resveratrol, key compounds for the antioxidant activity of grape juices. This is evidence that the synergy between thermal and enzymatic treatments can enhance the release of phenolic compounds from grape skins and seeds. Similarly, (Osete-Alcaraz et al., 2022) discussed the importance of using pectinases not only to increase the extraction of phenolic compounds during maceration, but also to improve wine clarity and stability. Their research demonstrated that the use of pectinases can reduce suspended material in the must, facilitating sedimentation and promoting a clearer final product with better chromatic characteristics. These findings underline how optimizing enzymatic processes can influence the phenolic composition and visual quality of wine. On the other hand, (Ngadze et al., 2018) analyzed the impact of the combination of enzymatic treatment and heat on the extraction of phenolic compounds in Strychnos cocculoides, finding an increase in juice yield and in the concentration of compounds such as quercetin and caffeic acid. The combination of pectinases and heat favored the decomposition of pectin, which not only improved the extraction of phenolic compounds but also the physicochemical quality and clarity of the juice. This supports the idea that heat treatment conditions and the selection of specific enzymes are decisive factors in maximizing the utilization of the bioactive components of fruits.

On the other hand, the type of treatment used in the maceration of the grape fruit did not have a significant effect on lightness, but it did have a significant effect on hue angle and chromaticity (Table 5), indicating that the appearance of the extract obtained was different in color and, therefore, the wine obtained is expected to have a different appearance. Among the characteristics that define the quality of a wine, color is a determining factor and, in this regard, sensory attributes are the first to be observed in the tasting. Through color, in its aspects of intensity and tonality, information is received about possible defects and virtues, structure, age and evolution in the wine (Acosta et al., 2013). Therefore, the color of the maceration extract was characterized using the classic parameters of lightness, chromaticity and hue angle. /Lightness represents whether a color is dark, gray or light, ranging from zero for black to 100 for white (Carvajal-Herrera et al., 2011). The lightness (L*) values obtained in the extracts processed with the PC, NC, and E treatments were 6.1, 6.3, and 6.4, respectively, with no significant difference ($P > 0.05$) among them.

The hue angle (H*) represented the color of the product obtained from the maceration and it has been assumed that it would represent the hue that the wine produced from it would have. The positive control (PC) presented a hue of 305.9°, that is, the characteristic purple color of the grape fruit. With the NC treatment the angle increased to 310.5°, equivalent to a more intense purple tone than the previous one, while with the application of pectinases it was possible to increase to 347.8°, referring to a reddish tone characteristic of the pigments of phenolic compounds such as anthocyanins. Chromaticity represents the purity of the color and indicates how pale or intense the hue is. The E treatment produced the highest chromaticity value with 6.4, while the NC and PC treatments both had 5.2, with no differences among them ($P > 0.05$). Although lightness was not significantly affected by the treatments, enzyme application showed an increase in the hue angle of the maceration extract, as this attribute was more similar to an absolute value of red and higher chromaticity values indicated that this hue was displayed with greater intensity.

The hue and color of wines are fundamental aspects in the perception of their quality and attractiveness. In young red wines, purple and violet-red tones are valued, indicative of freshness and richness in anthocyanins, the main pigments present in grapes (He et al., 2010). With ripening, a more intense and brighter red is sought, associated with greater complexity and structure. The transition to more orange or brownish tones is typical of aged wines and, although it can indicate maturity, it is also associated with loss of freshness and vibrancy (Waterhouse et al., 2016).

Phenolic compounds play a crucial role in wine copigmentation. This phenomenon occurs when anthocyanins interact with other phenols, forming stable complexes that intensify and stabilize the color (Boulton, 2001). Copigmentation contributes to deep red-violet hues, especially desirable in young red wines. Furthermore, the presence of anthocyanins and flavonoid compounds in the wine matrix influences visual perception, enhancing the chromaticity and purity of the color (He et al., 2010).

Over time, the color of wines changes due to polymerization and oxidation reactions. Free anthocyanins decrease and combine with tannins and other compounds, forming larger and less soluble complexes that lead to more orange and brown hues. This transition is associated with the oxidation of phenolic compounds and the loss of copigmentation properties (Ribéreau-Gayon P. et al., 2006). The application of enzymes, such as pectinases, has been shown to favor the release of anthocyanins and other phenols, initially contributing to increased color intensity, but may also influence long-term color stability due to changes in pigment structure (Morata et al., 2003).

In this context, the discussion of the results suggests that the application of pectinases, by increasing the hue angle and chromaticity, enhances the reddish hue, probably by the release and modulation of anthocyanins and other phenolic compounds. This behavior is consistent with that reported in studies highlighting how enzymes can intensify color perception through the breakdown of grape cell walls and the release of pigments (Morata et al., 2003). Therefore, the use of enzymes not only improves the extraction of phenolic compounds, but also has significant implications for the stability and evolution of color during storage.

Conclusions

The use of pectinases produced by *Aspergillus niger* in the maceration process of grape fruits favored the release of phenolic compounds. As a result, color attributes were increased, specifically hue angle and chromaticity, which could favor the production of wines with superior sensory characteristics.

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