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The effect of pectinase addition on clarity and quality of Ca na (*Elaeocarpus hygrophilus*) wine

The hydrolysis roles a crucial play in the winemaking process, and Ca na wine is a novel alcoholic beverage. In order to produce good quality products, the hydrolysis temperature at 30, 45 and 60 °C and time for 3, 5 and 7 hours, which were studied with the investigated addition of pectinase concentration at 1.0, 2.5 and 4.0 % (v.v^{-1}). The evaluation of the product was based on the clarity, the content of ethanol, saccharose and the bioactive compounds. The optimal condition of the pectinase hydrolysis was figured out using the Response Surface Method (RSM). The optimum parameters were 1 % (v.v^{-1}) pectinase addition and the hydrolysis conducted at 39.96 °C during 5 hours 45 minutes. With these optimal parameters, the clarity (expressed under the transparency T %), the content of saccharose, ethanol, phenolic, flavonoid and tannin (per 100 g of dry matter) were 25.85g, 9.28 % v.v^{-1}, 38.24 g, 10.34 g tannic acid equivalent (TAE), 4.06 g quercetin equivalent (QE) and 3.50 g TAE, respectively. The compatibility between the predictive model and the research's objectives was highly matched and could be used as a reference in calculating and optimizing the processing design for the food industry, especially in the wine or alcoholic beverage industry.

Descriptors: Bioactive compounds, Elaeocarpus hygrophilus, hydrolysis process, pectinase, winemaking process.

1 Introduction

Pectinases are a large group of enzymes that break down pectic polysaccharides of plant tissue into simpler molecules such as galacturonic acid. It has been used widely to increase the yield and clarity of fruit juices. Since pectic substances are a very complex group of macromolecules, various pectinolytic enzymes are required to degrade it completely [1]. Pectinase is one of the most widely distributed enzymes from microorganisms (yeasts, fungi, bacteria) and grapes but also performed by commercial enzymatic preparations added exogenously [2]. The mechanism of pectinase enzyme in clarifying fruit juice is breaking down pectin and causing suspended particles to settle, without unwanted changes in color, taste and stability [3]. The pectinase was first used commercially in 1930 in wine and fruit juices [4]. It is better to use pectinase enzymes in wine, bringing high yield when adding enzymes before fermentation. As the performance of pectinase enzyme in making banana wine enhances clarity without affecting any other characteristics of the wine [5]. Pectinase pretreatment does not affect yeast growth, nor ethanol and glycerol production, but significantly increases juice/alcohol yield [6].

Ca na (*Elaeocarpus hygrophilus*) fruit is olive-like, about 3 cm long with a pointed tip, its color is green and turns to pale yellow when it ripes [7]. This fruit is rich in phenolic compounds and triterpenoids, including tannin, gallic acid, ferulic acid and rutin, the main macronutrient is carbohydrates (12 %), other micronutrients include calcium, iron, vitamin C [8–10]. Ca na is mainly processed to produce lactic fermented product, jam, pickle with rice wine, but they are still under a household scale and discrete, so the yield and selling-value are not efficient. In order to enhance its commercial value, this fruit can be used as a raw material for creating a variety of beverage products that will help increase not only the value and diversify products from Ca na, but also raise income for growers. Meanwhile, the process of making fruit beverages from Ca na, especially wine processing, is facing some unqualified criteria due to the turbidity caused by juice solid matters, cloudifiers from polysaccharides or combinations of these. Clarity is one of the important features that affect the evaluation and decision making of almost all customers in the market. Hydrolysis process with the addition of pectinase enzyme can enhance the speed of process and improve clarification and filtration [2]. Therefore, this paper aimed to determine the optimal condition for the hydrolysis process and proper amount of pectinase enzyme addition in making good quality Ca na wine.

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2 Materials and methods

2.1 Materials

Ca na (*Elaeocarpus hygrophilus*) fruits was collected at Thoai Son district, An Giang province, Vietnam with the required maturity, weight and without damage. They were transported to The Experi-

mental Practical Area – An Giang University (Vietnam) within 1 hour. Ca na was washed and its flesh was separated for the research.

Saccharomyces cerevisiae ($3x10^{10}$ cfu.g^{-1}) was provided by Angel Yeast Co. Ltd (China). Before entering to the main fermentation *S. cerevisiae* passed the activation twice. In the first activation, 2 g of yeast was added into 10 mL of sterilized Hansen liquid medium (is a type of culture medium used to grow microorganisms for faster growth as their energy source) in the test tube, incubated in the room temperature for 24 hours. After that, 10 mL of yeast medium that had been activated in the first time was put into the 250 mL erlen, which contained 45 mL Hansen medium and 45 mL of Ca na juice. Saccharose was added to the mixture for the fermentation to get 18 °Brix and the pH was modified to get 4.5 by using Na_2CO_3. The mixture was ready for the incubation and it was carried out at room temperature (28 – 30 °C) on the shaking machine in 24 hours to perform the 2nd activation of *S. cerevisiae*. In this state, the density of yeast cell was 10^8 cfu.mL^{-1} [7, 11].

Pectinase enzyme was synthesized by *Aspergillus niger* with activity of 300 U. mL^{-1}. Pectinase was provided by Nanjing Duly Biotech Co., Ltd (China).

2.2 Experimental Design

The research was designed with 3 factors including pectinase concentration (1 – 4 %), hydrolysis temperature (30 – 60 °C) and time (3 – 7 hours) using Response Surface Methodology (RSM) with Box-Behnken Design (BBD), a mid-level between the original low- and high-level of the factors. In order to collect and analyze data, the STAGRAPHICS centurion software, version 16.1 was used, including 5 replications of central points and 17 numbers of treatments. Each factor was surveyed with 3 levels, coded from -1 to +1. The level of encrypted was shown in table 1.

2.3 Experimental Methods

Each 500 g of flesh was crushed with water at ratio of 1:2 (w.v^{-1}) for 5 minutes and adjusted to 23 °Bx and pH 4.2 (with saccharose and Na_2CO_3, respectively); heated at 85 °C for 15 minutes and cooled to 40 °C. Saccharomyces cerevisiae after 2nd activation was added with the optimum condition with the yeast content at 0.24 % (w/w, compared to raw materials) to the solution and fermented at room temperature (28 – 30 °C) for 11.5 days [7]. After the fermentation, the mixture was filtered through PE filter bag (10 µm filter hole diameter), and the fruit fluid (supernatant) was used for the hydrolysis process. Each sample was 400 mL of fruit fluid, the amount of pectinase addition and hydrolysis temperature and time were as designed in table 2.

2.4 Analytical Method

2.4.1 Determination of the clarity

The clarity of the solution after pectinase enzyme treated was presented through the transmittance (T (%)) of the solution based on the theory of Lambert-Beer. Briefly, this assay was measured using a UV-visible spectrophotometer (V730, Jasco, Japan) with absorption of 435 nm.

Table 1 Variable coding and survey levels of pectinase content, hydrolysis temperature and time

Variables	Codes	Levels		
		– 1	0	+ 1
Pectinase concentration (% v.v^{-1})	A	1	2.5	4
Hydrolysis temperature (°C)	B	30	45	60
Hydrolysis time (hours)	C	3	5	7

Table 2 Experiment layout according to Box-Behnken Design

Runs	Pectinase concentration (% v.v^{-1})	Hydrolysis temperature (°C)	Hydrolysis time (hours)
1	2,5 (0)	45 (0)	5 (0)
2	4,0 (+ 1)	60 (+ 1)	5 (0)
3	2,5 (0)	30 (– 1)	7 (+ 1)
4	1,0 (– 1)	45 (0)	3 (– 1)
5	2,5 (0)	45 (0)	5 (0)
6	1,0 (– 1)	60 (+ 1)	5 (0)
7	4,0 (+ 1)	45 (0)	3 (– 1)
8	2,5 (0)	45 (0)	5 (0)
9	4,0 (+ 1)	30 (– 1)	5 (0)
10	2,5 (0)	45 (0)	5 (0)
11	2,5 (0)	60 (+ 1)	7 (+ 1)
12	2,5 (0)	45 (0)	5 (0)
13	2,5 (0)	30 (– 1)	3 (– 1)
14	1,0 (– 1)	45 (0)	7 (+ 1)
15	2,5 (0)	60 (+ 1)	3 (– 1)
16	1,0 (– 1)	30 (– 1)	5 (0)
17	4,0 (+ 1)	45 (0)	7 (+ 1)

2.4.2 Determination of colour

Color analysis was measured through L, a, b values using a colorimeter (Konica Minolta CR400) of the solution after the hydrolysis process.

2.4.3 Determination of phenolic content

Phenolic content (g TAE.100 g^{-1} of dry matter) was indicated by the Folin-Ciocalteu reagent [12] with some modifications. Briefly, 0.15 mL of the sample was mixed with 1.2 mL of distilled water and 0.45 mL of 5 % (w/v) Na_2CO_3 in a test tube. The mixture was added to 0.1 mL of Folin-Ciocalteu reagent and left at room temperature for 90 minutes for the reaction. Phenolic in the extract reacts with Folin-Ciocalteu to form a phosphomolybdenum complex with a blue color in the alkaline medium. The concentration of total phenolics was calculated equally to the standard tannic acid graph (TAE), $y = 0.0021x + 0.0064$ ($R^2 = 0.9999$), where y is the absorbance and x is the concentration of the solution in the tube.

2.4.4 Determination of flavonoid content

Flavonoid content can be observed through the colorimetric

reaction using the aluminum chloride, a stable acid complex is formed by $AlCl_3$ with the C-4 keto groups and the hydroxyl C-3 or C-5 group of the flavone and flavonol. According to the assay of [12] with some modifications, 0.1 mL of the sample mixed with 1.2 mL of distilled water and 30 µL of 5 % (w/v) $NaNO_2$. After 5 mins, the mixture was added with 10 % (w/v) $AlCl_3.H_20$ (60 µL), 0.2 mL of 1 M NaOH and 0.11 mL of distilled water. The absorbance of the samples was measured at 510 nm. The total flavonoid concentration was calculated based on the standard quercetin graph (QE), y= 8.2634x + 0.0182 (R^2 = 0.9999), where y is the absorbance, and x is the concentration of the solution.

2.4.5 Determination of tannin content

This assay was carried out based on the Folin-Denis method [13]. Briefly, a sample (0.5 mL) with the addition of distilled water (0.5 mL) and Folin-Denis (0.5 mL) and 2 mL of 20 % Na_2CO_3. The mixture was vortexed well and kept in boiling water for 1 min and cooled at room temperature. The absorbance of the samples was measured at 700 nm. The concentration of tannin was calculated using standard tannic acid graph (TAE), y= 0.0098x + 0.0478 (R^2 = 0.9999), where y is the absorbance and x is the concentration of solution.

2.4.6 Determination of saccharose content

Determination of saccharose content (g/100 g dry matter) was conducted using the DNS method [14] with certain adaptations. This technique relies on the oxidation of the C=O group facilitated by 3,5-Dinitrosalicylic acid, causing a color change from yellow to orange-red in an alkaline environment. In the procedure, an aliquot (1 mL) of the sample was placed in a test tube, followed by the addition of 2 mL of DNS reagent. Subsequently, the tubes containing the blank, standard glucose solution, and samples were subjected to boiling water for 10 minutes. Afterward, 7 mL of distilled water was introduced, and the resulting solution was analyzed at an absorbance of 575 nm. The concentration of sucrose was determined based on a standard curve of glucose, expressed by the equation y = 23885x + 0.126 (R^2 = 0.9999), where y represents the absorbance, and x corresponds to the concentration of the solution in the tube.

2.4.7 Determination of total acid content

This method is based on a quantitative analytical method using an acid-base titration [15]. A standard base solution (0.1 N NaOH) was prepared to precisely neutralize the total acid of the samples. The total acids content is calculated using equation (1):

$$A_x = \frac{n\ x\ V_1}{V_2\ x\ V_3} \quad \text{(Eq. 1)}$$

Where, n is the volume of 0.1 N NaOH to titrate (mL); V_1 is the capacity of used volumetric flask (mL); V_2 is the volume of raw sample (mL), and V_3 is the volume of diluted sample to titrate (mL).

2.4.8 Ethanol content

Ethanol content (%) was measured using spectrophotometric method [16]. The assay based on the reaction by solvent extraction and dichromate oxidation with some modifications. the liquid ethanol in the sample was extracted using Tri-n-butyl phosphate (TBP, Sigma Aldrich, USA). A mixture of 1 mL of wine and 1 mL of distilled water was vigorously vortexed for 1 minute. The mixture was centrifuged to obtain 0.5 mL of the clear and transparent upper layer. This was then combined with 0.5 mL of dichromate reagent (containing 10 % w/v of $K_2Cr_2O_7$ in 5 M H_2SO_4) and vortexed vigorously for 1 minute. The mixture was left undisturbed for 10 minutes at room temperature to allow oxidation in the lower phase, resulting in a blue-green color. The oxidation product (1 mL) was diluted with 9 mL of deionized water. The optical density at 595 nm of the tested sample was measured using a spectrophotometer (T80+ UV/Vis Spectrometer, PG Instrument Ltd., USA). The ethanol concentration in the sample was determined from the ethanol standard curve, expressed by the equation y = 0.0164x + 0.013 (R^2 = 0.9999), where y represents the absorbance, and x corresponds to the concentration of the solution in the tube.

2.4.9 Sensory evaluation

The sensory attributes of Ca na wine were assessed based on parameters such as color, flavor, taste, clarity, and preferred preference. The evaluation employed the Quantitative Descriptive Analysis method (QDA). In this approach, sensory panelists were provided with instructions to assess the Ca na wine's sensory attributes concerning color, taste, flavor and clarity. Each attribute was represented on a descriptive scale spanning from 1 to 5, indicating sensory values from poor to excellent [17]. The level of preference was determined using the Hedonic scale from 1 to 9.

2.5 Data analysis methods

Data were collected and processed by STAGRAPHICS Centurion 16.1 software for analysis variance (ANOVA), LSD test to conclude the difference between the average of experiments at 5 % confidence (P = 0.05) and Microsoft Excel software for calculating and graphing.

The appropriateness of the predicted model was assessed through the correlation coefficient R^2. Equation optimize the response surface of general form experiments according to equation 2.

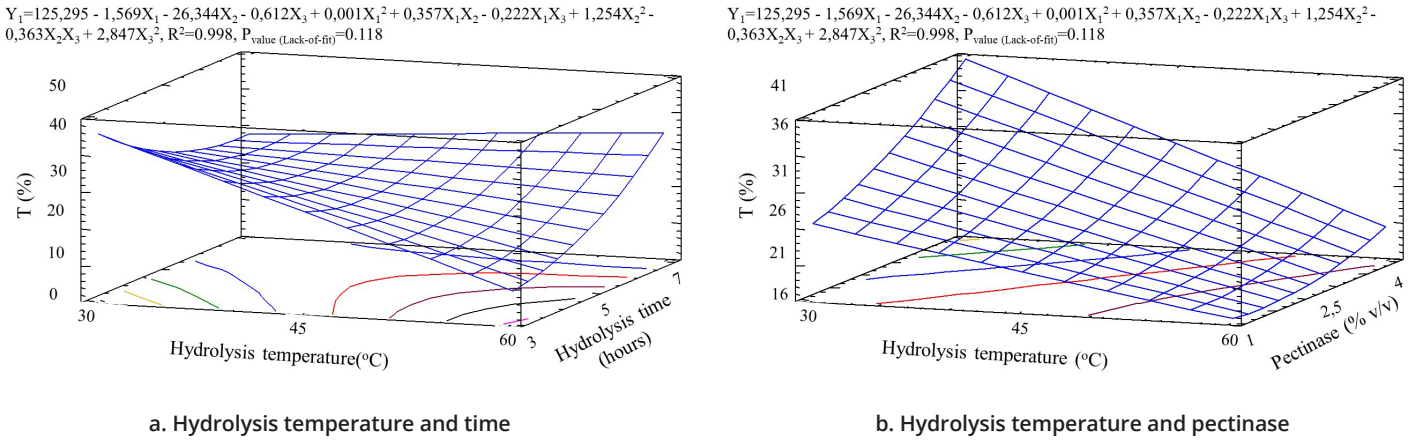
$$Y = \beta_0 + \sum_{i=1}^{k} \beta iXi + \sum_{i=1}^{k} \beta_{ii} {X_i}^2 + \sum_{i=1}^{k} \sum_{j=1}^{K} \beta_{ij} X_i X_j \quad \text{(Eq. 2)}$$

Where, Y is object function, β_o is constant, β_i is linear coefficient, β_{ii} is square coefficient, β_{ij} is interaction coefficient and X_i, X_j are survey variables.

3 Results and discussions

3.1 Effect of added pectinase enzyme concentration, hydrolysis temperature and time on clarity and color of the wine product

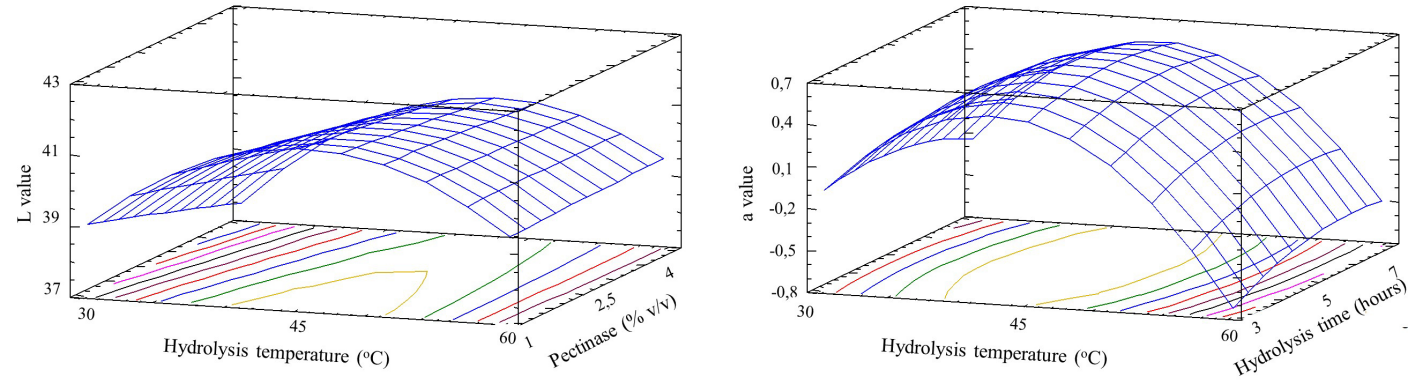
Pectin, polyphenols, tannins, and yeast residues coexisting in fruit wines for a long time, which produce turbid colloids and precipitates, making wines cloudy and possibly damaging during the storage [18]. The application of pectinase enzyme in making fruit wine is an effective treatment in terms of cost and quality aspects [19]. The



a. Hydrolysis temperature and time

b. Hydrolysis temperature and pectinase

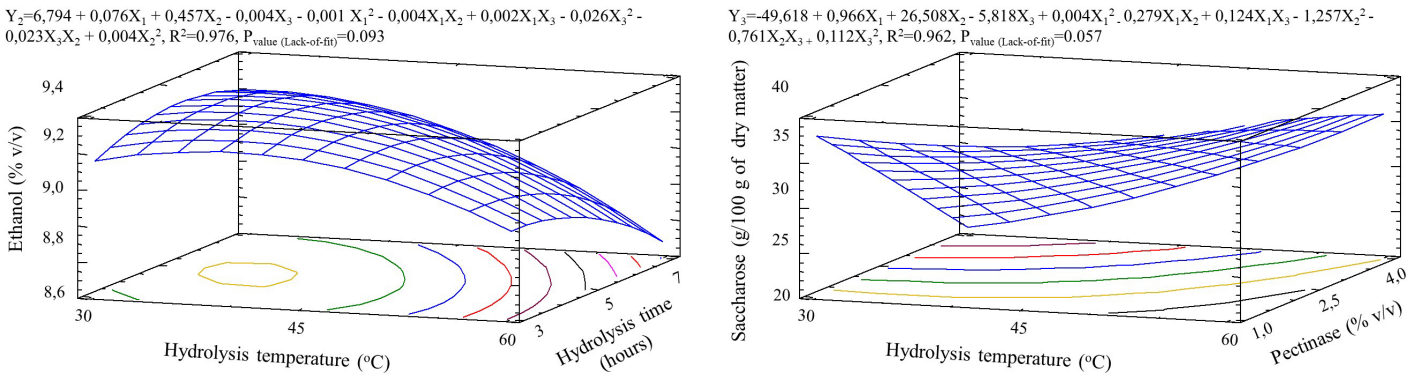
Fig. 1 Correlation of added pectinase enzyme concentration, temperature and hydrolysis time to the clarity (expressed in transparency, T (%)) of the wine (a factor fixed in a central position)



a. L value

b. a value

Fig. 2 Correlation of added pectinase enzyme concentration, temperature and hydrolysis time to (a) L-value and (b) a-value of the wine (a factor fixed in a central position)



a. Ethanol

b. Saccharose

Fig. 3 Correlation of added pectinase enzyme, hydrolysis temperature and time to the content of (a) ethanol and (b) sucrose of the product (a factor identified in the central position)

effects of additional pectinase enzymes on the temperature and time of the hydrolysis process including the clarity and the color of Ca na wine are shown in figure 1 and figure 2.

The results showed that the clarity of the product (expressed in transparency, T (%)) decreased with the increase in hydrolysis temperature. In contrast, the transparency T increased while increasing pectinase concentration and hydrolysis time (Fig. 1). The results also presented that the color parameters (including L and a values) increased to an optimal value and then gradually decreased with the increase in pectinase concentration, hydrolysis time and temperature (Fig. 2). The wine product color was brighter when hydrolyzing with pectinase enzyme at 45 °C.

Among polysaccharides, pectin is a soluble heteropolysaccharide. Pectinase enzyme reacts with the pectin molecule, the de-esterifying (pectinesterase) or degrading (polygalacturonases, polymethyl galacturonase, pectin and pectate lyases) of specific

Table 3 Experimental and the optimization models results

Target functions	Unit	Values of experiment *	Values of optimization models
T	%	25,84 ± 0,01	25.85
Ethanol	% $v.v^{-1}$	9,3 ± 0,25	9.28
Saccharose	g.100 g^{-1} of dry matter	38,23 ± 0,25	38.24
Phenolic	g TAE.100 g^{-1} of dry matter	10,33 ± 0,25	10.34
Flavonoid	g QE.100 g^{-1} of dry matter	4,06 ± 0,05	4.06
Tannin	g TAE.100 g^{-1} of dry matter	3,50 ± 0,01	3.50

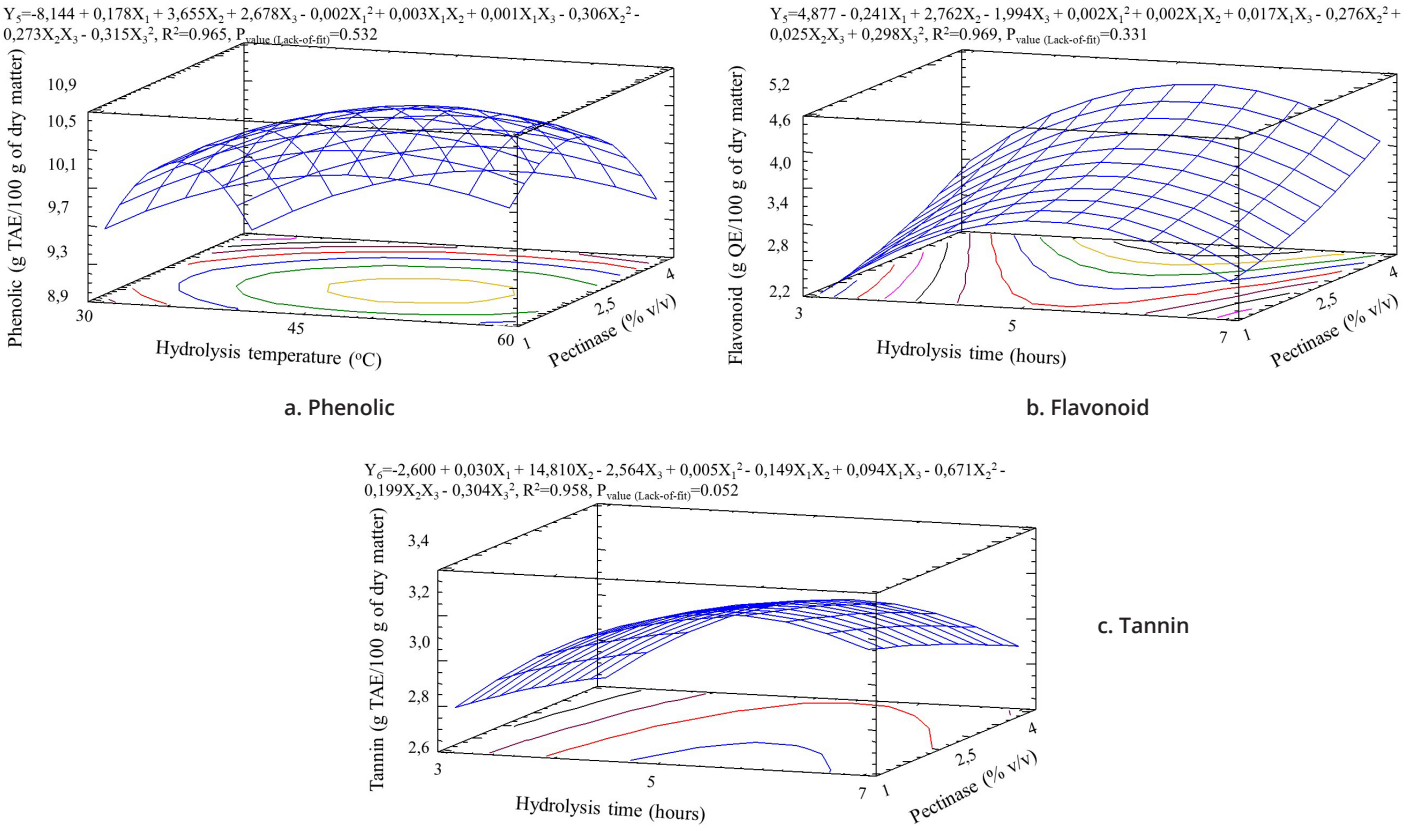
*Values are expressed as means of triplicate testing with the Standard Deviation (SD)

pectin substrates. The benefit of the pectinase treatment is that the particles settle more quickly. This improves the overall color intensity, as well as the color fastness of the wine [3, 20, 21]. In addition, pectinase enzyme activity was optimal at 45 – 50 °C and decreased rapidly at 60 °C [18].

3.2 Effect of added pectinase enzyme concentration, hydrolysis temperature and time on the quality of Ca na wine

A response surface model shows the effects of added pectinase concentration, hydrolysis temperature and time on the ethanol, sucrose, phenolic, flavonoid and tannin content of wine was illustrated in figure 3 and figure 4. The use of pectinase shows the potential of the polysaccharide hydrolysis into soluble sugars [20]. In addition, enzyme-treated wines showed higher phenolic content [22]. An increase in the amount of additional pectinase affects the rate of the hydrolysis, which was up to an optimal point and then gradually decreased due to the presence of several factors that directly affect the hydrolysis. The reaction is illustrated by pectinase enzyme through its characteristics and its hydrolysis including the presence of the substrate and inhibitors, the conditions of the hydrolysis, etc. [23]. However, an increase in the concentration of the added pectinase enzyme was not synonymous with a significant increase in the hydrolysis rate [24]. In addition, the structure of the enzyme is very susceptible to destruct at high temperatures, so the enzyme activity declined with the increase in temperature, which was recorded at over 50 °C. This phenomenon can be explained due to the heat provides enough energy to disrupt the enzyme structure so the enzyme is slow down its activity or inactive during the hydrolysis [25].

The results obtained from multiple response surfaces exhibited the optimum parameters of the winemaking process using pectinase enzyme, which was 1 % pectinase enzyme addition and the hydrolysis conducted at 39.96 °C for 5.77 hours (Fig. 5). With these optimal parameters, the clarity (expressed under the transparency T %), the content of sucrose, ethanol, phenolic, flavonoid and tannin (per 100 g dry matter) were 25.85 %, 9.28 % $v.v^{-1}$, 38.24 g, 10.34 g TAE, 4.06 g QE and 3.50 g TAE, respectively.

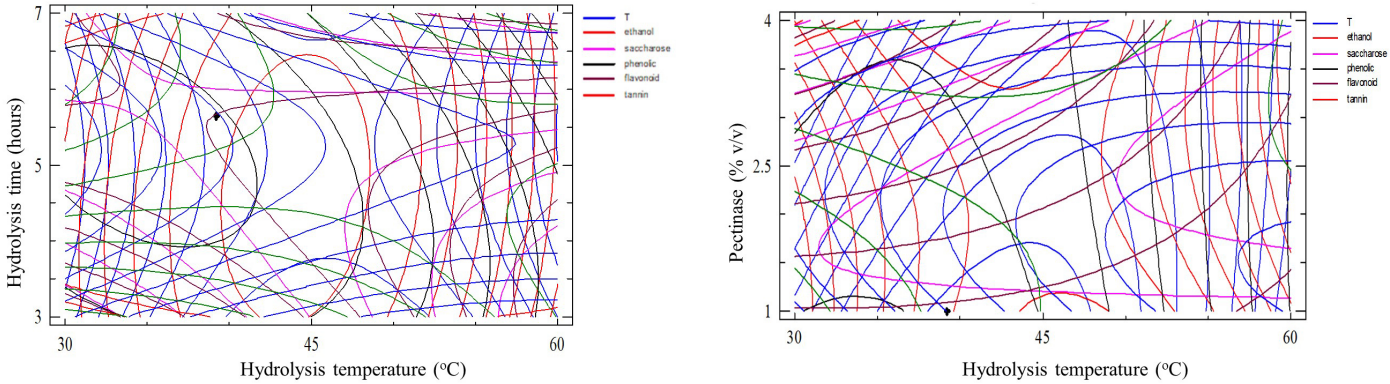


a. Phenolic

b. Flavonoid

c. Tannin

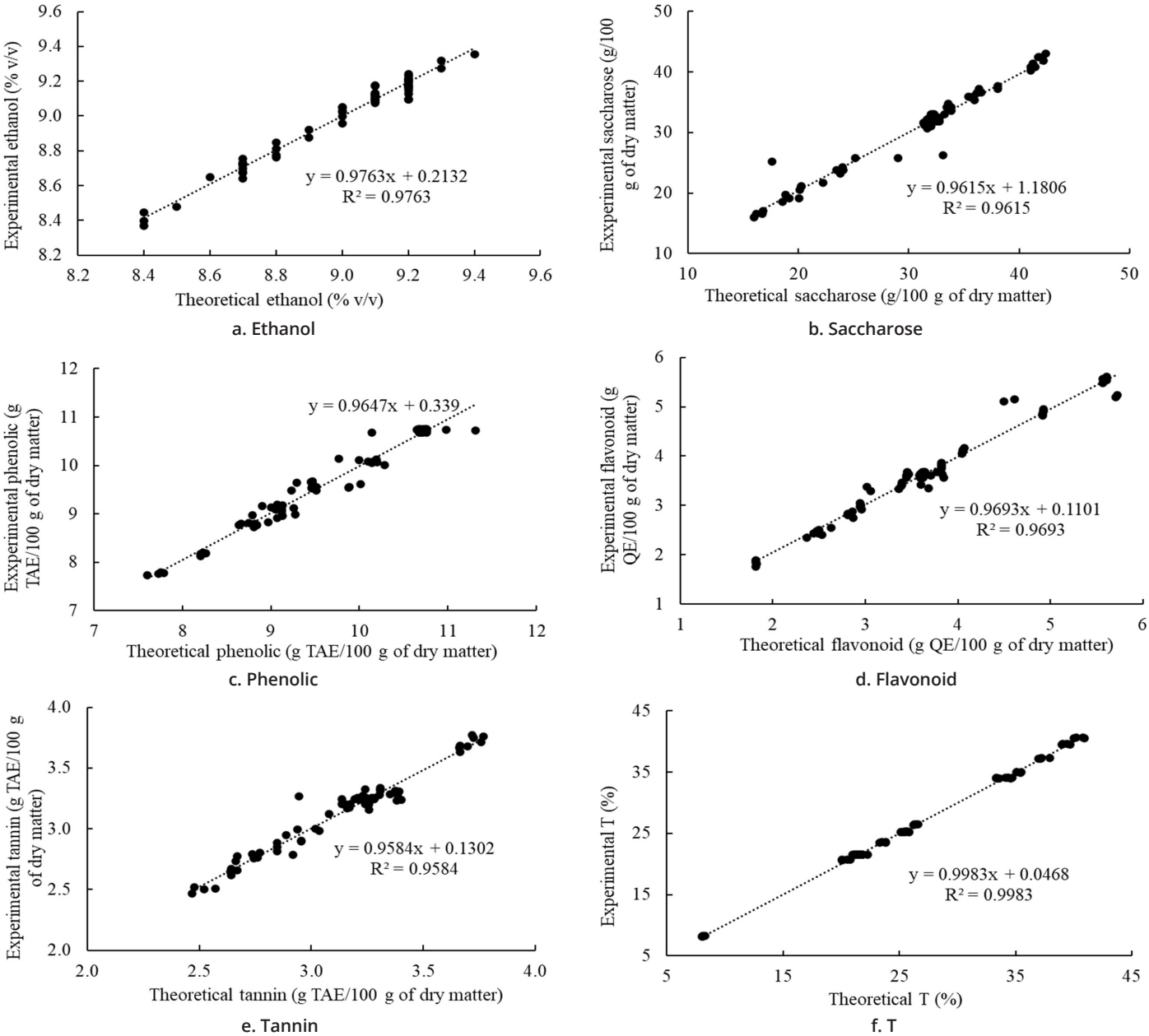
Fig. 4 Correlation of added pectinase enzyme, hydrolysis temperature and time to the content of (a) phenolic, (b) flavonoid and (c) tannin of the product (a factor identified in the central position)



a. Hydrolysis temperature and time

b. Temperature and pectinase content

Fig. 5 **The contour plot shows the simultaneous optimization of multiple response surfaces (T %, ethanol, sucrose, phenolic, flavonoids, tannins) according to (a) hydrolysis temperature and time, (b) temperature and pectinase content (a factor identified in the central position)**



a. Ethanol

b. Saccharose

c. Phenolic

d. Flavonoid

e. Tannin

f. T

Fig 6. Correlation between experimental and predicted data by regression equation for (a) ethanol, (b) sucrose, (c) phenolic, (d) flavonoids, (e) tannins and (f) the clarity (T)

The process of making wine was carried out experimentally with the addition of 1 % pectinase for the hydrolysis at 40 °C for 5.75 hours (equal to 5 hours 45 minutes). Table 3 shows the clarity and the content of ethanol, sucrose and bioactive compounds were equivalent to the values from the model, with a difference of 0.1 – 0.5 %.

Besides, the regression equation shows the relationship of independent variables to the clarity, ethanol, sucrose and bioactive substances contents of Ca na wine. All of the parameters had the $R^2 > 0.96$, value Lack-of-fit has no statistical significance ($p > 0.05$) (Fig. 1, Fig. 3 and Fig. 4) and a high compatibility between experimental data and predicted data from the model ($R^2 > 0.96$) (Fig. 6) shows that the ability of the predictive model to fit the objectives was very high. The results also show that the factors of the winemaking process with pectinase enzyme independently affect the Y value ($p < 0.05$).

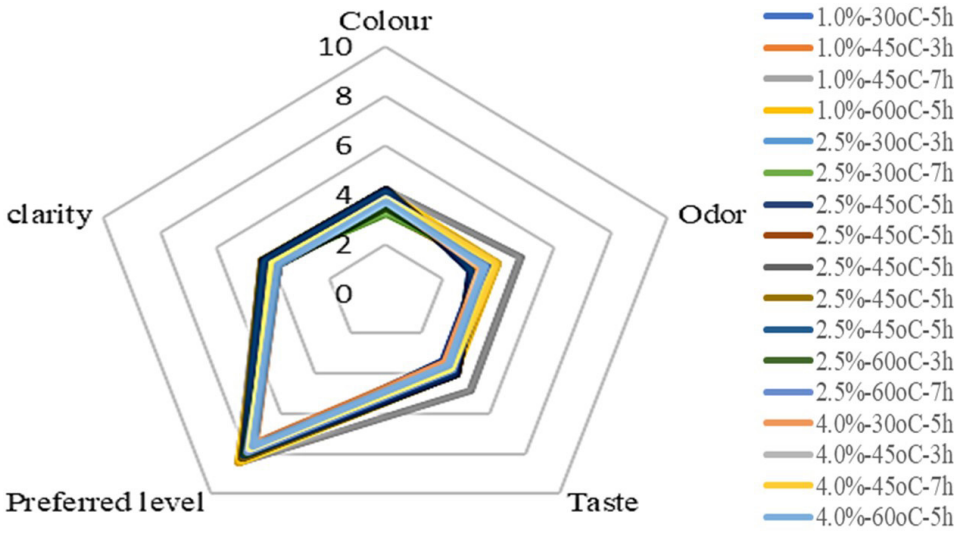


Fig 7. The sensory evaluation for clarity, color, aroma, taste, and preference of Ca na wine at different hydrolysis conditions related to pectinase

Moreover, figure 7 illustrates a sensory assessment of various hydrolysis conditions involving pectinase, focusing on color, flavor, taste, clarity, and overall acceptance. Most panelists remarked that pectinase treated samples had the bright color, due to the application of pectinase improve the lightness of the Ca na wine, which was similar with the results of L-value when increased the concentration of pectinase. This could be explained based on the decomposing of the structural tissues and releasing colored components, which are caused by the effect of pectinase [26]. This enzyme also contributes to enhancing the clarity of the wine, the more the enzyme concentration, the less the turbidity of the wine, this was illustrated through the transmittance (T) above.

However, employing high concentrations of pectinase for treatment resulted in lower sensory scores, particularly in the aspects of flavor and taste. Previous studies have similarly indicated substantial alterations in beverage flavor following pectinase treatment [27, 28]. This change could be attributed to the conversion of volatile compounds into non-volatile forms during pectinase treatment. Additionally, the wine treated with pectinase exhibited a noticeable sour taste, aligning with the observed titratable acidity and pH values. Consistent with this, Ninga et al. [29] reported a significant decrease in the pH of pectinase-treated guava juices. there was no significant difference between samples treated with 1 % and 2.5 % pectinase enzyme addition ($p > 0.05$). Overall, there was a noteworthy disparity in the overall acceptance of various enzyme-treated conditions, and the sample with 1 % pectinase enzyme addition was deemed to possess the highest level of preference.

4 Conclusion

The quality of Ca na wine including the clarity, ethanol, saccharose and bioactive contents among different hydrolysis conditions under the application of pectinase enzyme. A mathematical equation was introduced to design the preferential processing conditions for temperature and time of the hydrolysis during the winemaking process using pectinase enzyme. The optimum parameters were 1 % pectinase enzyme addition and the hydrolysis conducted at 40 °C during 5 hours 45 minutes. Therefore, the approach of this research is useful for optimizing the processing design for the food industry, especially in the wine or alcoholic beverage industry.

Conflict of interest

The authors declare no conflict of interest.

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