

Optimization of ethanol and bioactive compounds fermentation of *Elaeocarpus hygrophilus* Kurz by using response surface methodology

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Abstract

Elaeocarpus hygrophilus Kurz is used for food such as jam, lactic fermentation, pickling with rice wine, etc. To enhance the value and contribute to product diversification of *Elaeocarpus hygrophilus*, the optimizing wine fermentation by response surface methodology (RSM) was conducted with 2 factors including initial total soluble solids (20-30°Brix) and pH (3.5-4.5). The 3-level fractional design used for optimizing the effect of initial total soluble and pH were in agreement with the quadratic regression models well with $R^2 > 0.85$. Based on the response surface plots, the optimal parameters of fermentation were 23°Brix and pH 4.4. In the optimized condition, the ethanol content of *E. hygrophilus* wine was 7.33 % v.v⁻¹. In 100 g of dry matter, the content of phenolic, flavonoid and tannin in wine was 0.47 g TAE, 0.20 g QE and 1.67 g TAE, respectively.

1. Introduction

Elaeocarpus hygrophilus Kurz is found in Southeast Asia. In Vietnam, *E. hygrophilus* grows wild or along rivers and canals in some provinces of the Southwest such as An Giang, Dong Thap, Vinh Long, Can Tho, Hau Giang, Soc Trang and more (Niem *et al.*, 2017). *Elaeocarpus hygrophilus* is a green fruit, oval-shaped, acidic and sour. Each fruit has one seed which is rhombic in shape and hard coat (Ha *et al.*, 2021). Currently, the situation of growing *E. hygrophilus* is also of interest and is developed in An Giang province, especially in some districts such as Phu Tan, Thoai Son, Chau Phu, Tan Chau and more. *Elaeocarpus hygrophilus* is processed into attractive dishes that people in rural and urban love. However, *E. hygrophilus* is only used for processing products on a household scale such as lactic fermentation, jam and pickling with rice wine.

Wine production is indeed a promising processing technology. Wine is a type of alcohol that is fermented from fruit juices by yeast and not through distillation. The wine has a delicious fruit flavour, with light alcohol content (10-15%) brings many health benefits and is suitable for women and the elderly (Ngoc *et al.*, 2018). The potential for the production of typical fruit wine of Vietnam in general and of the Mekong Delta, in particular, has been studied such as rags of jackfruit (Ngoc *et al.*, 2018), palm (Thuy, Tuyen, Cuong *et al.*,

2011), pineapple (Thanh *et al.*, 2013), dragon fruit (Tam, 2018). Wine fermentation is affected by many factors, of which the initial total soluble solids and pH of juice are important factors that determine the ethanol content and quality of the wine (Phong *et al.*, 2021). To enhance the value and contribute to product diversification of *E. hygrophilus*, the study used a 3-level fractional design and response surface method (RSM) to optimize and determine the influence of initial total soluble solids (°Brix) and pH on the content of ethanol and bioactive substances in wine.

2. Materials and methods

2.1 Materials

Elaeocarpus hygrophilus fruits were harvested at Thoai Son district, An Giang province, Vietnam at 3 cm in size, slightly green in colour and without damage, then transported to The Experimental-practical Area – An Giang University (Vietnam) within 1 hr. *Elaeocarpus hygrophilus* were washed and flesh was collected for further analysis. *Saccharomyces cerevisiae* (3×10^{10} CFU.g⁻¹) was provided by Angel Yeast Co. Ltd (China). Saccharose was produced by Bien Hoa Company (Vietnam). Food-grade Na₂CO₃ was a commercial product of Merck (Germany).

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2.2 Experimental design

The optimization of fermentation parameters was designed according to Response Surface Methodology (RSM) using 3-level factorial design (3^2) (STAGRAPHICS centurion, version 16.1), including 5 central points and 13 treatments. The experiment was conducted with 2 factors including initial total soluble solid content ($^{\circ}\text{Bx}$) (X_1) ($20\div 30^{\circ}\text{Brix}$) and initial pH (X_2) ($3.5\div 4.5$). The level of encrypted variables and experimental layout were shown in Table 1.

2.3 Experimental methods

Each 500 g flesh was crushed with water at a ratio of 1:2 (w.v^{-1}) for 5 mins and adjusted to $^{\circ}\text{Bx}$ and pH (with saccharose and Na_2CO_3 , respectively) as designed in Table 1. It was heated at 80°C for 10 mins, cooled to 40°C , and then 0.1% (w.v^{-1}) *Saccharomyces* was added and fermented at room temperature for 10 days (Thuy, Cuong, Tuyen *et al.*, 2011).

Table 1. Variable coding and survey levels of initial total soluble solids content and pH

Variables	Codes	Levels		
		-1	0	1
Initial total soluble solid ($^{\circ}\text{Bx}$)	X_1	20	25	30
Initial pH	X_2	3.5	4	4.5

2.4 Analysis methods

2.4.1 Determination of total soluble solid content and pH

The total soluble solid content ($^{\circ}\text{Bx}$) and pH of samples were measured by using Atago hand-held refractometer (Japan) with $0\text{-}53^{\circ}\text{Bx}$ of detection level range and pH meter (Hanna, USA), respectively.

2.4.2 Determination of total acid content

The total acid content was measured by titration (Erinc *et al.*, 2009). Acid-base titration was a quantitative analytical method to determine the concentration of acid by precisely neutralizing it with a standard solution of a known base concentration (0.1 N NaOH). The concentration of total acid was calculated using Equation 1:

$$A_x = \frac{n \times V_1}{V_2 \times V_3} \quad (1)$$

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

2.4.3 Determination of total phenolic content

Total phenolic content (mg TAE.kg^{-1} of dry matter) was determined using the Folin-Ciocalteu reagent

(Singleton *et al.*, 1999). Approximately 150 μL of the sample was mixed with 1200 μL of distilled water and 450 μL of 5% (w.v^{-1}) Na_2CO_3 in the test tube. The mixture was added with 0.1 mL of Folin-Ciocalteu reagent and let at room temperature for 90 mins. Phenolics in extract react with Folin-Ciocalteu to form a blue complex in an alkaline medium that is phosphomolybdenum complex. The concentration of total phenolics was calculated as equal 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.

2.3.4 Determination of total flavonoid content

This assay was performed using the aluminium chloride colourimetric method described by Barros *et al.* (2008) with some modifications. The principle related to AlCl_3 creating a stable acid complex with the C-4 keto groups and the hydroxyl C-3 or C-5 group of the flavon and flavonol. Approximately 100 μL of the sample was added with 1200 μL of distilled water and 30 μL of 5% (w.v^{-1}) NaNO_2 . After 5 mins, the mixture was mixed with 10% (w.v^{-1}) $\text{AlCl}_3 \cdot \text{H}_2\text{O}$ (60 μL). A volume of 200 μL of 1 M NaOH and 110 μL of water was mixed into the solution. The solution was analyzed at 510 nm. The concentration of total flavonoid was calculated as equal to 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 in the tube.

2.4.5 Determination of tannin content

Tannin content was determined by the Folin-Denis method (Laitonjam *et al.*, 2013). Approximately 0.5 mL of sample was added to 0.5 mL of distilled water, 0.5 mL of Folin-Denis and 2 mL of 20% Na_2CO_3 . The mixture was shaken well, warmed in boiling water for 1 min and cooled to room temperature. The solution was measured at 700 nm. The concentration of tannin was calculated as equal to the 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 the solution in the tube.

2.4.6 Determination of ethanol content

Ethanol content ($\% \text{v.v}^{-1}$) was measured by the distillation method (Food Safety and Standard of Authority of India, 2015). A volume of 200 mL of sample was put into a 500 mL distillation flask containing 20-30 mL of saturated NaCl, distilled and collected into a 200 mL volumetric flask and cooled to 20°C . Ethanol content ($\% \text{v.v}^{-1}$) was determined by an ethanol meter (PET 109, Atago, Japan).

2.4.7 Determination of saccharose content

Saccharose content (g.100 g⁻¹ of dry matter) was measured by the DNS method (Miller, 1959) with some modifications. A volume of 1 mL of sample was put in the test tube and then 2 mL of reagent DNS was added. This method is based on the oxidation of the C = O group by 3,5-Dinitrosalicylic acid from a yellow colour to orange-red in an alkaline medium. The tubes of the solution of standard glucose and samples were placed in boiling water for 10 mins. After that, 7 mL of distilled water was added to the tubes. The solution was analyzed with an absorption of 575 nm. The concentration of saccharose was based on a standard curve of glucose, $y = 23885x + 0.126$ ($R^2 = 0.9999$), where y is the absorbance and x is the concentration of the solution in the tube.

2.5 Data analysis

Data were collected and processed by STAGRAPHS 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 . Equations optimize the response surface of general form experiments according to equation 2.

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

Where Y is the object function, β_0 is constant, β_i is the linear coefficient, β_{ii} is the square coefficient, β_{ij} is

the interaction coefficient and X_i , X_j are survey variables.

3. Results and discussion

3.1 Effect of initial total soluble solid and pH of *Elaeocarpus hygrophilus* juice on total soluble solid content, pH, saccharose and total acid of the wine

The response surface models showed the effect of initial °Bx and pH on °Bx, pH, saccharose and total acid contents of wine (Figure 1).

The result showed that the experiment factors had both independent and interacting effects on the Y values when $p < 0.05$ were quite complicated. Specifically, in the equation to predict the total soluble solid of wine, the initial pH of *E. hygrophilus* (X_2) had a positive coefficient of influence, it was the factor that increased the total soluble solid (°Bx) of wine. In contrast, the negative coefficients of the initial total soluble solids and interaction ($X_1 X_2$ and X_2^2) were the factors that reduced the °Bx of wine. The pH and contents of saccharose and total acid were explained similarly.

The results showed that the more the initial values of total soluble solids and pH were, the higher the value of total soluble solids, pH and saccharose content in the product. In addition to that, the pH values of the wine decreased slightly compared to the initial pH after fermentation. The contents of CO₂ and some organic acids (such as acetic acid, lactic acid, pyruvic acid, etc.) were produced and dissolved in wine (Thanh *et al.*, 2013; Ngoc *et al.*, 2018). Therefore, the total acid content in the pH values of the wine decreased slightly compared to the initial pH after fermentation and total

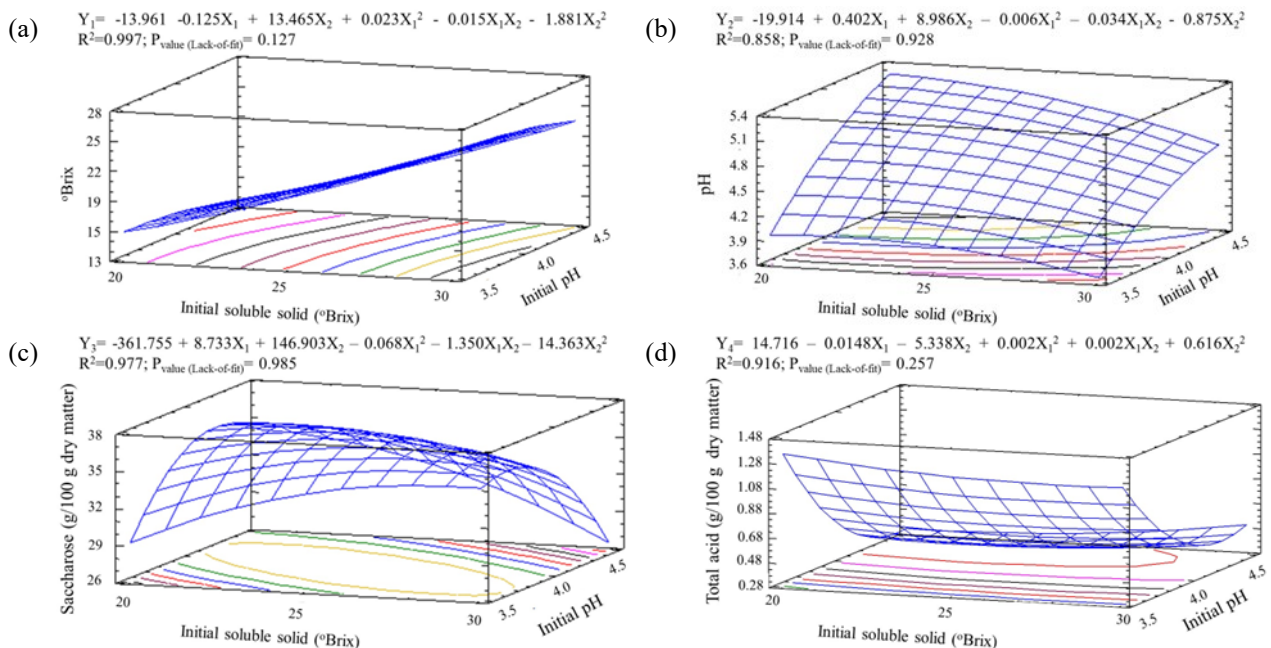


Figure 1. The response surface and contour plots show the effect of initial total soluble (°Brix) and pH on (a) °Brix, (b) pH, (c) saccharose and (d) total acid of wine.

acid content was inversely proportional to the pH of the wine.

3.2 Effect of initial total soluble solids and pH of *Elaeocarpus hygrophilus* juice on ethanol production

A response surface model and the level of influence illustrated the correlation between initial °Bx and pH of juice to the ethanol content were built (Figure 2).

The results showed that the initial °Bx and pH of *E. hygrophilus* juice had a quadratic influence on the ethanol content of wine (Figure 2a). In which, the initial total soluble solid showed more effectiveness on the ethanol production than the initial pH (Figure 2b). The content of ethanol in wine increased to an optimal value and then gradually decreased with increasing °Bx. Similar results were obtained for increasing the initial pH of juice. The optimal value of ethanol was obtained at 24.96°Bx and pH at 4.21.

Yeast needs carbohydrates to grow and metabolize. Sugar is an essential substrate for fermentation and it greatly affects the achievement of fermentation. *Saccharomyces cerevisiae* can convert the sugars in juice into ethanol (Fleet, 2003) and the ethanol production is

high or low depending on the sugar content of the juice (Attri, 2009; Ton *et al.*, 2010). Therefore, the initial °Bx must be suitable for yeast to grow and develop. When the content of the initial sugars of juice is low, the yeast lacks a carbon source for biomass growth and competes for nutrients with each other, resulting in low ethanol content. However, if the sugar content is too high, it increases the osmosis pressure and causes imbalances in the physiological state and metabolism of yeast (Attri, 2009). In addition, the initial pH of juice also greatly affects the activities of yeast, capable of changing the charge of the cell wall and increasing or decreasing the osmosis level of nutrients and fermentation direction (Pamulha and Louriero-Dias, 1989). The ability of yeast to produce ethanol was reduced in a low pH medium (Ton *et al.*, 2008).

3.3 Effect of initial total soluble solids and pH of juice on the bioactive description of wine

The response surface models showing the effect of initial °Bx and pH on phenolic, flavonoid and tannin are presented in Figure 3.

The results showed that initial °Bx and pH affected the content of bioactive compounds in the wine,

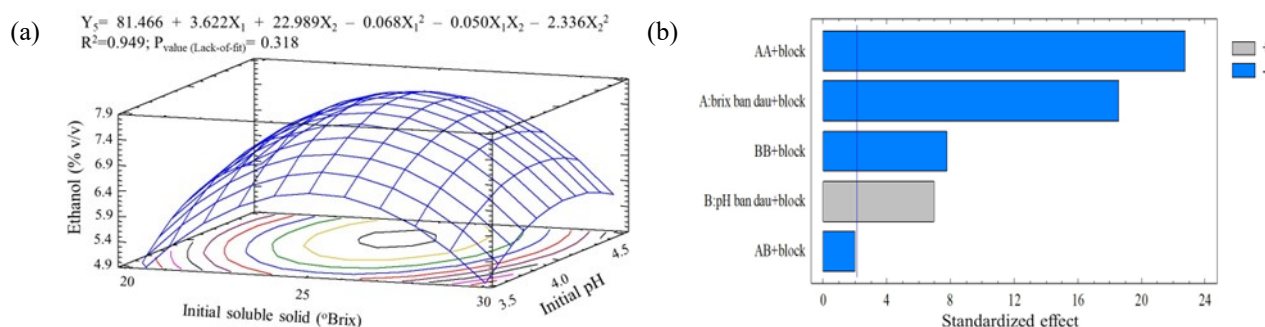


Figure 2. The response surface plot (a) and the Pareto chart (b) show the effect of total soluble (°Brix) on ethanol content.

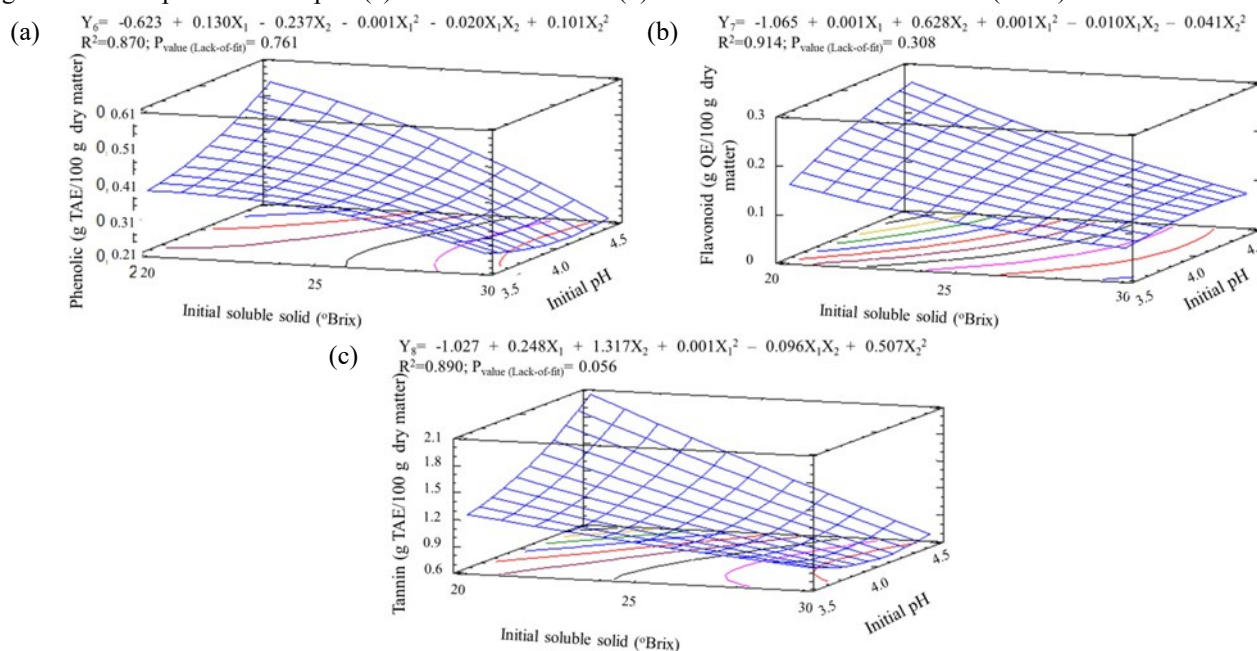


Figure 3. The response surface plots (a) phenolic, (b) flavonoid and (c) tannin of wine influenced by fermentation of initial total soluble (°Brix) and pH.

exception of flavonoids that had no statistically significant difference when increasing the initial pH ($p>0.05$). The contents of phenolic, flavonoid and tannin increased with increasing the initial °Bx and decreased with increasing the initial pH of juice. The optimal results of phenolic, flavonoid and tannin (in 100 g of dry matter) (respectively) were 0.55 g TAE, 0.27 g QE and 2.06 g TAE when fermenting at 20°Bx and pH 4.5.

Phenolic, flavonoid and tannin are the main factors determining the quality of the wine. These compounds play a crucial role in the sensory characteristics of wine such as colour, flavour and preference levels (Morata *et al.*, 2016; Perez-Jiménez *et al.*, 2019). Phenolic compounds in wine mainly came from raw materials or were produced during the fermentation with microorganisms' activities (Lingxi and Baoshan, 2019; Morata *et al.*, 2020). Therefore, the content of bioactive compounds can be changed during fermentation depending on the type of yeast. Wine yeast is one of the causes of reducing the bioactive compounds by absorption (Caridi *et al.*, 2004). Tannin can interact with the mannoprotein of the cell wall of yeast to form complex structures through polymerization and galloylation (Karas *et al.*, 2017). In addition, bioactive compounds may diffuse through the periplasmic and

plasma membrane of dead or inactivated yeasts to interact with cytoplasmic components, mainly the protein of yeast (Mekoue Nguela *et al.*, 2016). Besides, hydrolytic enzymes derived from raw materials or microorganisms can promote the hydrolysis of bioactive compounds in wine (Villena *et al.*, 2007). Furthermore, the decrease in phenolic compounds during fermentation was due to esterification with methanol and ethanol, as a result of yeast metabolism (Monagas *et al.*, 2005).

The regression equations expressing the relation of the independent variables to the ethanol, phenolic, flavonoid, and tannin was described in Figure 2 and 3. A good correlation model requires a correlation coefficient (R^2) to be higher than 0.8 (Guan and Yao, 2008). A good correlation model needs to be consistent between experimental and theoretical data, the model with Lack-of-fit without statistical ($p>0.05$) is essential. Moreover, the degree of compatibility between ethanol and phenolic of experimental and predicted data was expressed in Figure 4 with a high correlation coefficient ($R^2>0.87$). Similar results for flavonoid, tannin, total acid content, °Bx, pH and saccharose ($R^2>0.85$).

In addition, the results of optimization of multiple response surfaces found that the ethanol, phenolic,

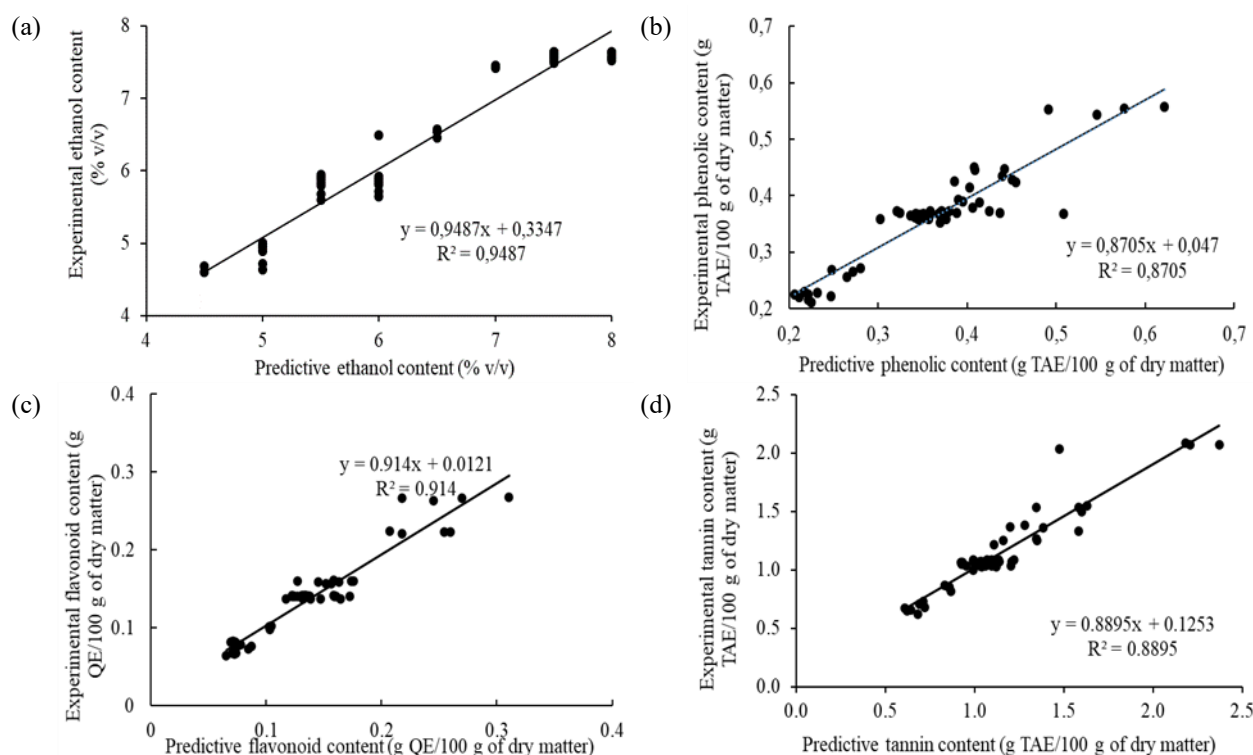


Figure 4. Correlation between experimental and predictive data for (a) ethanol, (b) phenolic (c) flavonoid and (d) tannin.

Table 2. The results of optimization model and actual data

Target functions	Unit	Values of experiment	Values of optimal model
Ethanol	% v.v ⁻¹	7.33±0.29	7.20
Phenolic	g TAE.100 g ⁻¹ of dry matter	0.47±0.02	0.48
Flavonoid	g QE.100 g ⁻¹ of dry matter	0.20±0.01	0.20
Tannin	g TAE.100 g ⁻¹ of dry matter	1.67±0.04	1.66

Values are presented as mean±SD of three repetitions.

flavonoid and tannin content reached the highest values at 22.86°Bx and pH 4.42. With these optimal parameters, the contents of phenolic, flavonoid and tannin (in 100 g of dry matter) were 0.48 g TAE, 0.20 g QE and 1.66 g, respectively, and ethanol content was 7.20% v.v⁻¹. The experimental results of fermentation at 23°Brix and pH 4.4 were equivalent to the results predicted from the model (Table 2). The difference is about 0.31-6.67%. The initial °Bx and pH were also consistent with the studies of Thu (2019) and Ngoc *et al.* (2018).

4. Conclusion

3-level fractional design and response surface methodology were effectively used to calculate and predict the content of ethanol and bioactive compounds in *E. hygrophilus* wine. The optimum parameters in fermentation were found at 23°Bx and pH 4.4. By application of these results can lead to maximum levels of ethanol and bioactive compounds in wine.

Conflict of interest

The authors declare no conflict of interest.

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