Optimization of ultrasound-assisted enzymatic extraction of betalains from red beetroot (*Beta vulgaris* L.)

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ABSTRACT

Betalains in red beetroot (*Beta vulgaris* L.) offer health benefits and are commonly used as a food colorant. This study aimed to investigate betalains extraction using ultrasound-assisted enzymatic extraction (UAEE). The most significant factors involved in UAEE such as enzyme concentration, extraction temperature, and extraction time were studied and optimized using the response surface method (RSM) to achieve the highest betalains yield. The results showed that the optimal extraction conditions were as follows: enzyme concentration (32.1 U/mL), extraction temperature (40°C), and extraction time (117 min) gave the highest yield of betalains at the level of 550.51 ± 25.76 mg/L. The findings are promising for the industrial scale of extraction betalains for food applications.

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1. Introduction

Betalains are a class of nitrogen-containing compounds that are highly soluble in water and derived from betalamic acid. They are typically categorized into two subgroups: betacyanins, which produce red to purple pigments, and betaxanthins, which produce yellow to orange pigments. Currently, scientists have identified and isolated approximately 75 betalain compounds, including 42 betacyanins and 33 betaxanthins (Carreón-Hidalgo et al., 2022). One of the most well-known betalains is betanin, which is a red pigment compound classified as a food coloring additive with the code E162. In the food industry, betalains are often used as natural colorants, antioxidants, and functional food ingredients. However, they also have a wide range of other potential applications, such as in biological packaging (Yao et al., 2020), fabric dyes (Guesmi et al., 2012), and metal coatings (Fares & Bani-Domi, 2021).

Unlike anthocyanins, which are widely distributed in different plant orders, betalains can only be found in plants of the Caryophyllales order, such as Amaranthaceae, Basellaceae, Cactaceae, Portulacaceae, Nyctaginaceae, and a few other families (Carreón-Hidalgo et al., 2022). Some notable examples of betalains-containing plants include red beetroot (*Beta vulgaris* L.), the fruit of Malabar spinach (*Basella alba* L.), peels of red-fleshed dragon fruit (*H. polyrhizus*), rose moss flower (*Portulaca grandiflora*), and flowers of the bougainvillea plant (*Bougainvillea* spp.) (Carreón-Hidalgo et al., 2022). Among these plants, red beetroot is the most extensively studied and cultivated source of betalains. It is highly valued for its significant content of betalains and its wide cultivation area, making it the main raw material used for betalains research and commercial applications (Carreón-Hidalgo et al., 2022).

Betalains extracted from red beetroot are gaining significant attention from the scientific community due to their potential applications. To optimize the extraction process, researchers have focused on using the response surface method (RSM) or principal component analysis (PCA) to increase extraction efficiency and ensure betalains stability during extraction (Calva-Estrada et al., 2022). Traditional extraction methods for betalains from red beetroot include pickling extraction using water:ethanol (85:15) (Neagu & Barbu, 2014), water:ethanol (50:50) (Pandey et al., 2023), and continuous diffusion extraction (Wiley & Lee, 1978). However, these methods have drawbacks such as long extraction time, use of organic solvents, high temperatures causing betalains loss, and low extraction efficiency (Tiwari & Cullen, 2012). Currently, modern extraction methods are being increasingly employed for extracting betalains from beetroot, including enzymatic-assisted extraction (Lombardelli et al., 2021), ultrasound-assisted extraction (Silva et al., 2018), and microwave-assisted extraction (Cardoso-Ugarte et al., 2014). These methods offer several advantages such as short extraction time, precise temperature control, reduced use of organic solvents, and improved extraction efficiency, making them more environmentally friendly.

In recent years, there has been an increasing interest in developing new extraction methods for natural compounds. Among these methods, ultrasound-assisted enzymatic extraction (UAEE) stands out due to its numerous advantages. This method combines enzyme-assisted extraction (EAE) and ultrasound-assisted extraction (UAE), where enzymes act as biological catalysts to rapidly hydrolyze the plant cell wall components, while ultrasound waves create high-frequency vibrations and tiny

air bubbles that further disrupt the material's matrix system, allowing for better solvent penetration and component dissolution. The UAEE method has many advantages over using each method individually, as shown by studies on polysaccharide extraction from freshwater mussels (*Corbicula fluminea*) (Liao et al., 2015) and protein extraction from sesame seed husks (Görgüç et al., 2019), which demonstrated significantly higher extraction efficiencies compared to using EAE or UAE alone.

Given that betalains compounds share similarities with highly polar and hydrophilic polysaccharides and proteins, due to their numerous hydroxyl and carboxyl groups, the UAEE method has great potential for the extraction of betalains from beetroot. By optimizing extraction processes through the UAEE method, extraction efficiency, and betalains stability could be improved, making this method an eco-friendly and efficient alternative to traditional extraction methods. Therefore, in this study, the UAEE was applied to the extraction of betalains from red beetroot. Various synergistic modes of UAEE were investigated and multiple UAEE parameters, such as enzyme concentration, enzyme composition, extraction temperature, and extraction time were studied and optimized using the response surface method (RSM).

2. Materials and Methods

2.1. Beetroot powder

Ripe red beetroot (*Beta vulgaris* L.) was provided by a local producer in Ho Chi Minh City, Vietnam. After being thoroughly washed, the peels were removed. The red beetroot fleshes were sliced into pieces of 5 mm thickness, before being dried by hot air at 60°C, until moisture content reached under 5%. The dried red beetroot pieces were then ground and stored in an airtight bag at in the freezer (-20°C) in the dark until used.

2.2. Enzymes and chemicals

Cellulase from Aspergillus niger (2000 IU/g) was provided by Antozyme Biotech Pvt. Ltd. (Vadodara, Gujara, India), pectinase from *Aspergillus niger* (60,000 IU/g) was provided by ICFOOD Co., Ltd. (Yuseong-gu, Daejeon, Korea). Ethanol, acetic acid, sodium hydroxide (NaOH), and sodium acetate were obtained from Sigma Aldrich (St. Louis, MO, USA).

2.3. Selection of synergistic modes in UAEE procedure

Ultrasound-assisted enzymatic extraction (UAEE) can significantly increase in extraction yield compared to techniques using single treatment such as enzyme or ultrasound (Liao et al., 2015; Görgüç et al., 2019). In addition, different synergistic modes of UAEE have different effects on extraction yield (Wu et al., 2014). Thus, various synergistic modes of ultrasound and enzymatic extraction, and conventional extraction methods were studied and an optimal mode was selected. These modes included: (I) C (conventional extraction): 1 g of red beetroot powder was extracted with water:ethanol (75:25), at 25°C for 90 min; (II) EAE (enzymatic-assisted extraction): 1 g of red beetroot was mixed with a buffer solution having cellulase:pectinase (6:4) mixture at 25 U/g concentration, pH 5.5, extraction temperature of 25°C for 120 min before enzyme inactivation; (III) UAE (ultrasound-assisted extraction): 1 g of red beetroot powder was extracted in water:ethanol (75:25), extraction temperature 45°C, ultrasound powder 250 W, frequency of 40 kHz for 90 min; (IV) Eu (enzymatic hydrolyzation first,

followed by ultrasound extraction): 1 g of red beetroot powder was mix buffer solution having cellulase:pectinase (3:2) mixture at 25 U/g concentration, pH 5.5, the mixture was incubated at 25°C with constant stirring for 120 min followed by enzyme inactivation and sonication for 90 min (V) Ue (ultrasound treatment first, followed by EAE) 1 g of red beetroot powder was sonicated for 90 min, followed by EAE (cellulase:pectinase (3:2) mixture at 25 U/g, pH 5.5, extraction temperature of 25°C for 120 min); (VI) UE (ultrasound treatment and enzymatic hydrolyzation happened simultaneously): 1 g of red beetroot powder was mixed with enzyme mixture (cellulase:pectinase (3:2), the concentration of 25 U/g, pH 5.5) when immediately treated with ultrasound (250W, 40 kHz) for 90 min, the enzyme was then inactivated.

2.4. UAEE procedure

After selecting the synergistic UAEE mode, the UAEE was performed as described by Liu et al. (2014)an efficient ultrasound-assisted enzymatic extraction procedure for the water-soluble polysaccharides from the fruit of Lycium barbarum was investigated and optimized. Response surface methodology (RSM with minor modifications. The enzyme mixture solution was prepared by mixing cellulase and pectinase with pH 5.5 acetate buffer at given enzyme concentrations and enzyme compositions. Red beetroot powder (1 g) was mixed with 15 mL of enzyme mixture solution in a 50 mL falcon tube. This mixture was ultrasound treated at the designed temperature and time in an ultrasound cleaning bath (Elma P60H, Singen, Germany) with fixed ultrasound frequency and power at 40 kHz and 250 W, respectively. The extracting solution was centrifuged at 2,500 g for 15 min and the supernatant was collected.

2.5. Determination of total betalains (TB) content

The total betalains (TB) content of red beetroot extract was determined using a spectrometric method as described by Wruss et al. (2015) with slight modifications. The absorbance was measured at two different wavelengths, 485 nm for betaxanthins and 536 for betacyanins, respectively.

The total betalains (TB) content was expressed in mg/L and was the sum of betacyanins (BC) and betaxanthins (BX) content according to formula (1).

$$BC\ or\ BX\left(\frac{mg}{L}\right) = \frac{A \times DF \times M_w \times 1000}{\varepsilon \times L} \quad (1)$$

Whereas, A is the maximum absorbance value at 536 nm for BC and 485 nm for BX; DF is the dilution factor; Mw is the molecular weight of BC (Mw = 550 g/mol) and BX (Mw = 339 g/mol), ε is the extinction coefficient of BC (ε = 60,000 L/mol.cm) and BX (ε = 48,000 L/mol.cm), L is the path length (1 cm).

2.6. Preliminary experiments design

A series of single-factor, completely randomized experiments was conducted before the optimization experiment to identify significant factors and narrow the condition ranges. The independent variables studied were: enzyme concentration (20 U/mL, 25 U/mL, 30 U/mL, 35 U/mL), enzyme ratio (cellulase/pectinase 1/0, 3/2, 2/3 & 0/1), extraction temperature (30°C, 40°C, 50°C & 60°C), and extraction time (60 min, 90 min, 120 min, & 150 min). Total betalains (TB) was the response variable. After each experiment, the condition level resulting in the highest TB was chosen as the control variable for the next experiments.

2.7. Central Composite Design and statistical analysis

Based on single-factor preliminary experiments, central composite design (CCD) was used to optimize the UAEE procedure of betalains from red beetroot. An experimental factorial model with three independent variables (X_1, enzyme concentration; X_2, extraction temperature; X_3, extraction time) at three levels was performed. To ensure the data reproducibility and determine the experimental error, 4 replicated central points were used. The levels and ranges of independent variables were presented in Table 1. The dependent variable studied was total betalains (TB) content (mg/L) and was related to the coded variables by a second-degree polynomial using Equation 2:

$$Y = b_o + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 \quad (2)$$

Where is the dependent response while the coefficients of the polynomial are represented by b_o (constant term); b_1, b_2, b_3 (linear effects); b_{11}, b_{22}, b_{33} (quadratic effects); and b_{12}, b_{13}, b_{23} (interaction effect).

Table 1. Levels of the independent variables used in the central composite design

Independent variables	Levels		
	-1	0	1
Enzyme concentration (U/mL)	25	30	35
Extraction temperature (°C)	40	50	60
Extraction time (min)	90	120	150

2.8. Statistical analysis

All the experiments and analyses were done in triplicates except for the optimization experiment, which had 4 replicated center points. The data analysis was conducted using Excel and JMP Pro statistical software (version 17.0, SAS Institute, Cary, NC, USA). All data are presented as mean values ± SD except for the optimization experiment. In preliminary experiments, one-way analysis of variance (ANOVA) and Tukey's HSD were used to analyze the mean values at $P < 0.05$ (Wilkinson, 1989) to detect significant differences among levels of treatment. In the RSM-CCD experiment, ANOVA tables were developed to determine the effect and regression coefficients of individual linear, quadratic, and interaction terms.

3. Results and Discussion

3.1. Selection of synergistic modes

The betalains extraction yields of conventional (C), enzyme-assisted extraction (EAE), ultrasound-assisted extraction (UAE) methods, and different synergistic modes of UAEE (Eu, Ue, UE) are shown in Figure 1. The EAE and UAE enhanced the betalains extraction yield in comparison with the conventional (C) method ($P < 0.05$) because EAE using cellulase and pectinase to hydrolyze and break down plant cell wall constituents which led to the release of intracellular contents (Karki et al., 2011) and UAE employs ultrasound cavitation to break

down plant tissues, agitate the mixture and enhance extraction yield (Chen et al., 2012).

Moreover, the effects of synergistic modes on betalains extraction yield are shown by the comparison among Eu mode (enzymatic hydrolyzation first, followed by ultrasound extraction), Ue (ultrasound treatment first, followed by enzymatic-assisted extraction), UE (ultrasound treatment and enzymatic hydrolyzation happened simultaneously). In which, UE mode showed the highest betalains extraction yield ($P < 0.05$). This was in line with other reports on UAEE application on polysaccharides and polyphenols (Wu et al., 2014; Li et al., 2017), where the extraction yield was significantly improved by applying both treatments simultaneously. The presence of ultrasound cavitation was found to cause an increase in the contact area between phases and subsequently decreased mass transfer limitations within the enzyme-substrate system. High-intensity focused ultrasound led to a decrease in size, thereby increasing the amount of substrate in contact with the enzyme. Furthermore, the boost in reaction rates observed after ultrasound treatment was believed to be due to a greater number of collisions between the enzyme and substrate (Capelo et al., 2005). Therefore, the synergistic mode UE (ultrasound treatment and enzymatic hydrolyzation happened simultaneously) was selected and used in subsequent UAEE experiments.

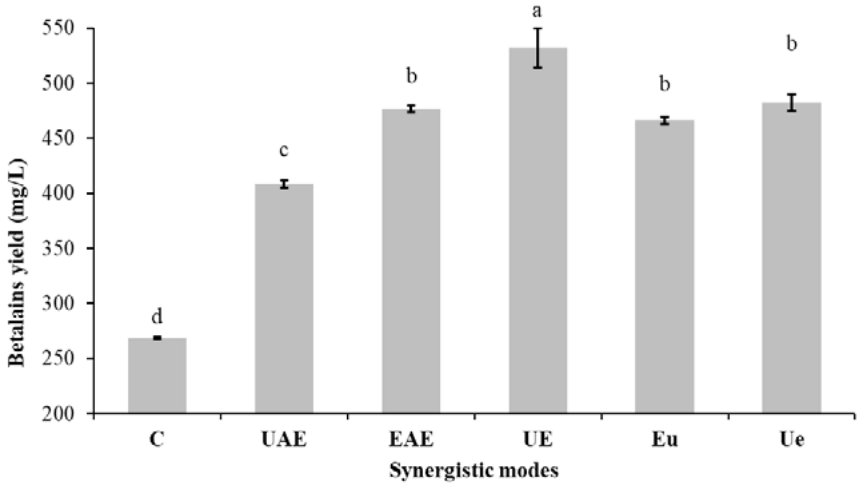

Figure 1. Effect of different synergistic modes on extraction yield of betalains from red beetroot. The models are: C, conventional solvent extraction; UAE, ultrasound-assisted extraction; EAE, enzyme-assisted extraction; Eu, enzymatic hydrolyzation first, followed by ultrasound extraction); Ue, ultrasound treatment first, followed by enzymatic-assisted extraction; UE, ultrasound treatment and enzymatic hydrolyzation happened simultaneously. Values marked by the same letter are not significantly different ($P < 0.05$).

3.2. Effect of enzyme concentration on betalains extraction yield

The effect of enzyme concentration on betalains extraction yield is shown in Figure 2a. The enzyme concentration was studied at different levels while other extraction conditions were fixed as follows: enzyme mixture composition, C:P (3:2); extraction temperature, 40°C; extraction time, 90 min. A significant increase in betalains yield was observed by increasing the enzyme concentration from 20 U/mL to 30 U/mL because higher enzyme concentration can enhance the hydrolyzation of the plant cell wall. The peak yield (516.83 ± 23.44 mg/L) was achieved at an enzyme concentration of 30 U/mL. When higher dosage of the enzyme mix (35 U/mL) were employed, the extraction yields decreased compared to the 30 U/mL dosage. This reduction in yield can be attributed by an end-production inhibition causing by a too fast hydrolysis as proposed in literature (Çinar, 2005; Ranveer et al., 2013). The enzyme concentration was selected as 30 U/mL for subsequent experiments.

3.3. Effect of enzyme mixture composition on betalains extraction yield

To study the effect of enzyme mixture composition on betalains extraction yield, the UAEE process was conducted using different enzyme mixture compositions (C:P). Other extraction conditions were fixed as follows: enzyme concentration, 30 U/mL; extraction temperature, 40°C; extraction time, 90 min. As shown in Figure 2b, when the compositions of C:P were set at 3:2, the betalains yield reached its peak value (513.43 ± 24.28 mg/L). The result indicated that this enzyme composition matches well with the cell wall composition of red beetroot, thus maximizing the hydrolyzation effect. This is in agreement with the cell wall composition of red beetroot determine in another study (Wu et al., 2014), where cellulose was 37% and pectin was 28%. The composition of C:P (3:2) was selected for subsequent experiments.

3.4. Effect of extraction temperature on betalains yield

The impact of extraction temperature on betalains yield is depicted in Figure 2c. Keeping other extraction condition constant (enzyme concentration 30 U/mL; enzyme composition, C:P (3:2); extraction time, 90 min), while various extraction temperatures were employed. An increase in extraction temperature from 30°C to 50°C led to a significant rise in betalains yield due to the enzyme-catalyzed activities that favor the release of betalains. The maximum yield (533.64 ± 14.46 mg/L) was achieved at 50°C, after which the yield decreased significantly ($P < 0.05$) upon further temperature increase. Higher temperatures resulted in a decrease in the number of cavitation bubbles and reduced the impact of cavity collapse on homogenized samples, leading to a decrease in enzyme activity (Palma & Barroso, 2002). As a result, the temperature was chosen to be 50°C for subsequent experiments.

3.5. Effect of extraction time on betalains yield

The UAEE method was conducted at different extraction times while fixing other extraction parameters as follows: enzyme concentration 30 U/mL; enzyme composition, C:P (3:2); extraction temperature, 50°C. As shown in Figure 2d, betalains yield was increased with the increase of extraction time up to 120 min ($P < 0.05$). This was since in the early stages of extraction, the ultrasound-induced damage to cell structures resulted in low diffusion resistance for intracellular matters, which facilitated their extraction (Linares & Rojas 2022). However, a notable decline in the yield of betalains in red

beetroot was observed when the extraction time exceeded 120 min ($P < 0.05$). This suggests that excessive extraction time (beyond 120 min) under ultrasound conditions caused oxidative degradation of betalains, thus resulting in reduced yield (Maran et al., 2015).

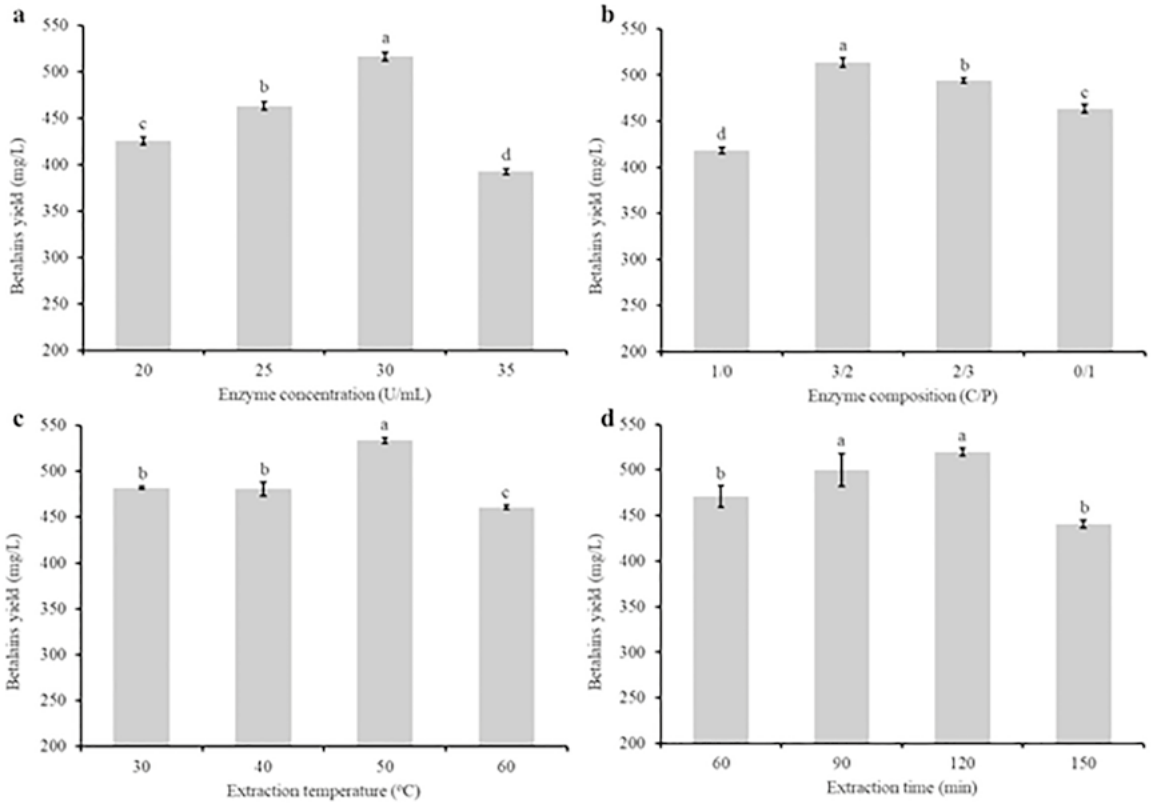

Figure 2. Effect of dependent variables on the extraction yield of betalains from red beetroot: (a) enzyme concentration; (b) enzyme composition; (c) extraction temperature; (d) extraction time. Values marked by the same letter are not significantly different ($P < 0.05$).

3.6. Optimization experiment

All 18 random sequential experiments under different extraction conditions were performed to study the reciprocal influence of independent variables (enzyme concentration, extraction temperature, and extraction time) on betalains extraction yield and to optimize the operating parameters. The actual and predicted values according to the factorial design are presented in Table 2. The experimental results showed that the linear coefficient of extraction temperature (X_2) and extraction time (X_3) were considerable ($P < 0.05$). In addition, enzyme concentration (X_1) and extraction temperature (X_3) had a significant quadratic effect on the yield of betalains ($P < 0.05$). The interaction terms of X_1X_2 was an extremely prominent effect for the betalains yield ($P < 0.01$). The unaffected factors ($P > 0.05$) are excluded from the quadratic equation. By applying multiple regression analysis to the experimental data, the final quadratic equation obtained in terms of actual actors is given below (3):

$$Y=517.75- 20.23X_2-12.51X_3-20.75X_1X_2-27.26X_1^2-41.91X_3^2$$

where, X_1, X_2, and X_3 are enzyme concentration, extraction temperature and extraction time, respectively.

Table 2. Response surface central composite design and results for extraction yield of betalains

Run	X_1 (U/mL)	X_2 (°C)	X_3 (min)	Betalains extraction yield (mg/L)	
				Predicted	Experimental
1	25	40	90	464.64	470.98
2	25	40	150	427.81	432.20
3	25	60	90	468.11	463.90
4	25	60	150	426.42	427.00
5	35	40	90	497.59	500.27
6	35	40	150	489.25	496.72
7	35	60	90	418.06	416.94
8	35	60	150	404.86	401.79
9	25	50	120	487.64	480.53
10	35	50	120	493.34	487.37
11	30	40	120	539.00	518.11
12	30	60	120	498.54	506.36
13	30	50	90	488.34	484.64
14	30	50	150	463.33	453.96
15	30	50	120	517.75	527.88
16	30	50	120	517.75	550.51
17	30	50	120	517.75	509.27
18	30	50	120	517.75	509.50

Enzyme concentration (U/mL).
Extraction temperature (°C).
Extraction time (min).

The prediction models for the yield of betalains was significant ($P < 0.01$) with a coefficient of determination (R2) of 0.923, indicating that there are 92.3% of the corresponding response variations could be explained as a function of three UAEE parameters (Myers et al., 2016) (Figure 3). An R^2 value of at least 0.75 recommended that the model fitted the experimental values well (Wang et al., 2019). In addition, the adjusted determination coefficient R^2_{Adj} value was used to check the overestimation of the R^2. The R^2_{Adj} value was calculated to be 0.837, also demonstrating a good correlation between experimental and predicted values. The results presented in Figure 3 also supported the adequacy of the model, where the points were very close to the fitted line.

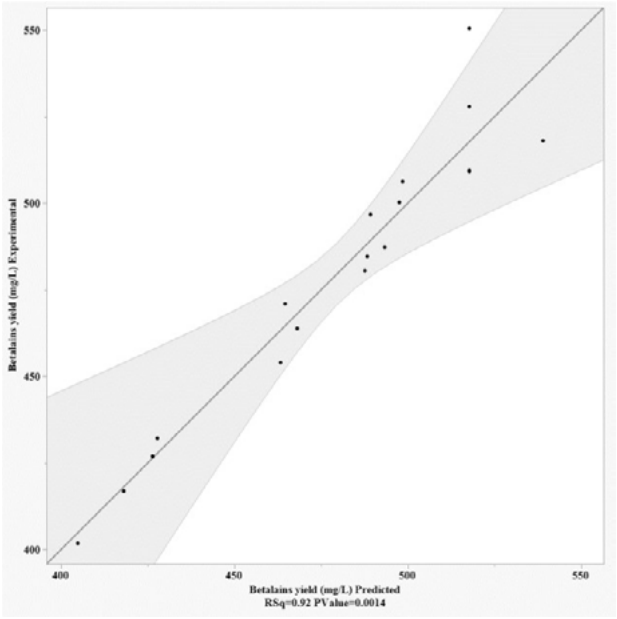

Figure 3. Predicted versus Experimental betalains yield.

To observe the effects of two UAEE parameters on betalains yield, the response surface plots were generated (Figure 4). One variable was kept constant at its respective zero level while two variables varied within their experimental ranges to understand their main and interactive effects on the dependent variable. The results show that increasing enzyme concentration and extraction time up to a threshold limit led to an increase in the betalains yield. However, when those parameters were increased above the threshold, the yield decreased. On the other hand, when increasing the temperature from 40°C to 60°C, the betalains yield decreased. These findings showed that better betalains yield could be obtained when the appropriate enzyme concentration and extraction time were applied. In addition, when the temperature was increased above the optimum level, the surface tension and viscosity of the solvent might be decreased, which altered the ultrasound cavitation and mass transfer intensity, thus reducing the extraction yield (Maran et al., 2015).

The response surface method was employed to predict the optimal conditions of betalains extraction and calculate the maximum betalains content. In this study, the desirability function was used as a tool to find the parameter settings that maximize overall desirability. According to the obtained model, the optimal conditions were determined as enzyme concentration of 32.1 U/mL, extraction temperature of 40°C and extraction time of 117 min with a desirability value of 0.91. Under these conditions, the predicted betalains yield was 544.5 mg/L. The validation experiment was performed, and experimental results showed an optimum betalains extraction yield of 550.51 ± 25.76 mg/L that was not statistically different compared to the predicted model ($P > 0.05$) suggesting that the model was adequate to reflect the expected optimization.

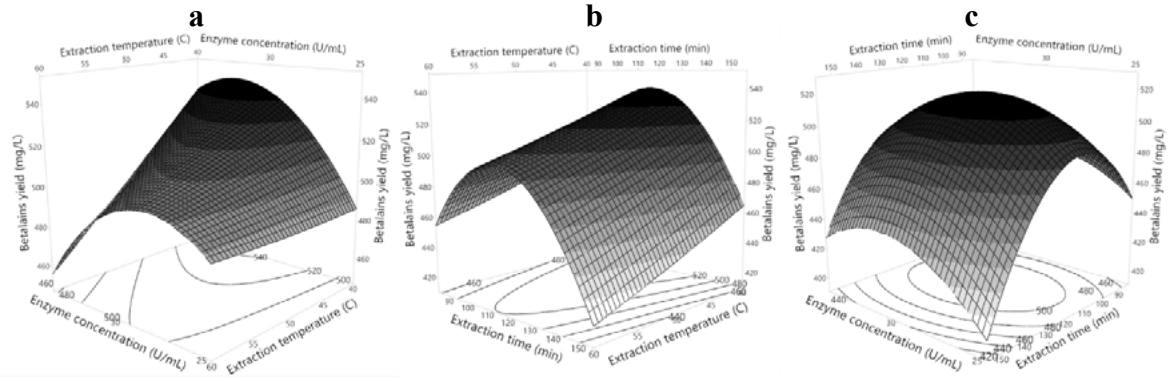

Figure 4. Response surface showing the effects of four variables (enzyme concentration, extraction temperature, extraction time) on extraction yield of betalains from red beetroot.

Conclusions

Ultrasound-assisted enzymatic extraction were employed for betalains extraction from red beetroot. The synergistic mode of simultaneous enzymatic hydrolyzation and ultrasound treatment was chosen for the UAEE process. The response surface methodology optimization of enzyme concentration, extraction temperature and temperature were performed. At the optimum conditions of enzyme concentration (32.1 U/mL), temperature (117°C) and time (117 min) yielded the highest betalains at the level of 550.51 ± 25.76 mg/L. Ultrasound-assisted enzymatic extraction is promissing for extracting betalains from red beetroot. The physicochemical and biological properties of obtained betalains should be further studied to elucidate the potentials of food applications.

Conflict of interest

The authors declare that they have no conflict of interest.

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