OPTIMIZATION OF PEROXIDASE ACTIVITY OF TURNIP (*BRASSICA RAPA*) USING RESPONSE SURFACE METHODOLOGY

– Research paper –

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Abstract: The present study is focused on the determination of optimized activity conditions of peroxidase (POD) of turnip (*Brassica rapa*). The enzyme is extracted from turnip with 0.5M Na₂HPO₄ (pH7.0). Peroxidase activity is evaluated by monitoring the oxidation of guaiacol to tetraguaiacol in the presence of hydrogen peroxide (H₂O₂). Before the optimization of peroxidase activity from a turnip by response surface methodology (RSM), a preliminary step is conducted for each studied parameter (pH, temperature, and NaCl concentration). Further, RSM following Box-Behnken design is used in order to refine the optimization of the conditions. Response surface analysis showed that parameter values with the highest peroxidase activity are 5.9, 29.8°C, and 1.07M for pH, temperature, and NaCl, respectively. Under these experimental conditions, peroxidase activity is found to be 4027.25U/mg. The obtained experimental value agrees with that of the calculated one, indicating the pertinence of the developed model for the optimization of peroxidase activity.

Keywords: Peroxidase activity, turnip, optimization, response surface methodology

INTRODUCTION

Peroxidases (POD) is belonging to oxidoreductase enzymes that responsible for catalyzing reactions in which H₂O₂ is the acceptor of hydrogen atoms and another compound is a donor (Ikehata et al., 2005; Passardi et al., 2007). According to their amino acid sequences, non-animal peroxidases are divided into three groups: ascorbate type (class I), fungal secretory (class II), and guaiacol type that is synthesized by plants (class III) (Das et al., 2011). The specificity of POD to its substrate is high, but it has low specificity to the hydrogen donor (Passardi et al., 2007). Peroxidases are implicated in several plant functions such as hormone regulation, plant development, defense mechanisms, lignin biosynthesis, and indoleacetic acid degradation (Chisari et al., 2007; Manu and Rao, 2009; Pandey et al., 2017). However, the release of POD from plant tissues is responsible for the oxidation of a wide range of phenolic compounds, such as guaiacol, catechol, pyrogallol, catechin, and chlorogenic acid (Onsa et al., 2004). It is well known that many factors affect enzymatic activity in fruits and vegetables and which can be

divided into physical and chemical parameters such as temperature, pH, substrate, and composition of the medium (Shu et al., 2016; Pandey et al., 2018). The enzyme activity is also influenced by the season, the stage of development, and the use of pesticides and fertilizers (Horotan and Oancea, 2013; Serrano-Martínez et al., 2014). The studies on peroxidases provided from different plants such as turnip shown that physicochemical parameters, as well as the used substrate, produce significant variations on kinetic properties of enzymes (Serrano-Martínez et al., 2008; Zeyadi and Almulaiky, 2020).

Traditional optimization methods are complex processes for multi-variable systems; they permit to examine one factor at a time in which all others are fixed at the same levels. It takes a lot of time and can lead to bad conclusions. While Response Surface Methodology (RSM) is a helpful statistical and mathematical technique for studying and modeling the influence of numerous variables on responses, and widely useful to optimize processes (Tsapatsaris and Kotzekidou, 2004; Benkerrou et al., 2018; Saci et al., 2018). This method permits to improve the statistical interpretation and reveal the interaction between the studied variables while

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reducing the number of required essays (Yim et al., 2012). The RSM is widely used to optimize synthesis of bioactive molecules, extraction of desired compounds, and activity of enzymes (Yamaguchi et al., 2006; Zhang et al., 2017; Pramana et al., 2018).

To the best of our knowledge, this work is the first study concerning the enzymatic activity

optimization of the peroxidase extracted from the turnip. The sequential methodology is used in the first time to study the influence of three independent physicochemical parameters (pH, temperature, and NaCl concentration) on POD activity in the turnip. Thereafter, the results are used to optimize POD activity with Surface Response Methodology.

MATERIALS AND METHODS

Extraction of crude enzyme

The turnip samples used in this study are from Bejaia department (North of Algeria). The turnip roots were cleaned and crushed, then homogenized with 1/2 (w/v) citrate-phosphate buffer solution (0.5M, pH6.0) by ultra-turrax for 2min. The homogenate was centrifuged (Sigma 2-16K, Germany) under 4°C at 5000 rpm for 10min and the supernatant containing peroxidases was transferred to another tube.

Determination of protein concentration

Total soluble proteins were measured by a colorimetric assay based on measuring the absorbance of dye Coomassie Brilliant Blue G-250 bonded to basic amino acid residues (arginine, histidine, and lysine) Bradford (1976). The absorbance was measured at 595 nm using a UV-VIS spectrophotometer (UviLine 9400, France) and results are expressed referring to Bovine serum albumin (BSA).

Determination of peroxidase activity

POD activity was determined based on the absorbance of tetraguaiacol formed from guaiacol in the presence of hydrogen peroxide within 1min. The reaction mixture consisted of 100 μ L of guaiacol (45mM), 50 μ L of H₂O₂ (22.5mM), and 50 mL of citrate phosphate buffer solution (0.5M) in addition to 20 μ L of the crude enzyme extract. The absorbance was measured by a spectrophotometer at 470 nm. One unit of enzymatic activity (U/mg) was defined as the amount of enzyme that catalysis the production of 1 μ mol of tetraguaiacol/min under test conditions (Şahin Ercan and Soysal, 2018).

Optimization of enzymatic activity with the sequential methodology

Effect of pH on peroxidase activity

POD activity was measured at variable pH (3, 4, 5, 7, 8, and 9) using 0.5M citrate phosphate buffer solution. It consists of the preparation of a mixture

containing 50 μ L of guaiacol, 25 μ L of H₂O₂, 25mL of citrate phosphate buffer (0.5M) with different pH 3-9, and 20 μ L of the crude enzyme extract.

Effect of temperature on peroxidase activity

The solution of the reaction mixture contains 50 μ L of guaiacol, 25 μ L of H₂O₂, and 25mL of citrate phosphate buffer solution (0.5M, pH 6.0). After incubation for 10 min under different temperatures (5-70°C), the crude enzyme extract was added and absorbance measurements are carried out every 1min.

Effect of NaCl concentration on peroxidase activity

The ionic strength influence on POD activity was measured at variable NaCl concentrations (0.5-2 M) using citrate phosphate buffer solution pH 6.0 (0.5M) at fixed concentrations of guaiacol and H₂O₂.

Optimization of enzymatic activity with Response surface methodology

Experimental design

In order to refine the optimal conditions for the peroxidase activity of turnip, an experimental design following the response surface methodology was carried out. For this, the Box-Behnken (BBD) design was used with three variables and factorial levels. The independent variables used were pH (x_1), temperature (x_2 ; °C), and NaCl concentration (x_3 ; mol/L), while the dependent variable response was peroxidase activity. The results of optimization of POD activity by sequence methodology were used to show the levels of the three parameters: pH (0.5-1.5), temperature (25-35°C), and NaCl concentration (5-7M) for optimization with RSM. The levels of the three factors were coded as + (low), 0 (middle), and - (high). The experimental design was conducted in duplicate (Table 1).

Construction and validation of the model

The construction of the experimental plan and the analysis of data were achieved using JMP 10 software. The significance of the elaborated mathematic model was tested by analysis of variance (ANOVA) and the obtained data were fitted to a second-order polynomial model; regression coefficients of the response surface analysis were obtained according to the general equation (Eq. 1).

$$y = \alpha_0 + \sum_{i=1}^3 \alpha_i x_i + \sum_{i=1}^3 \alpha_{ii} x_i^2 + \sum_{i=1}^3 \sum_{j=1}^3 \alpha_{ij} x_i x_j \quad (i \neq j) \quad (\text{Eq. 1})$$

RESULTS AND DISCUSSION

Effect of pH on POD activity

POD activity of turnip (*Brassica rapa*) was tested at variable pH. As illustrated in Figure 1a, the best pH value is found around pH 6. Generally, most plants are characterized by a maximum enzymatic activity around neutral pH (Arnnok et al., 2010). For pH values above 9 and below 3, the POD loses almost completely its activity. This reduction of activity at extreme pH is due to the denaturation of enzymes (Amiour and Hambaba, 2016).

The optimum pH for enzymatic activity depends on plant material, substrate, and reaction medium (Arnnok et al., 2010; Rezaei et al., 2017). The optimum pH of Kiwifruit peroxidase, using *p*-phenylenediamine as substrate, is 6.0-8.5 (Fang et al., 2008), it is equal to about 5.0 for POD extracted from loquat and sapota fruits with guaiacol (Zhang and Shao, 2015), potato POD is most active at pH 6.0, and for garlic bulb, it is around pH 5.5 to 6.5 with guaiacol as substrate (Cai et al., 2012), using the same substrate, the optimum pH of Ghars and Deglet Nour dates peroxidases are 6.4 and 5.6, respectively (Amiour and Hambaba, 2016). The optimum pH for chestnut peroxidase can reach up to pH 7.0 with 2-methoxy phenol as substrate (Gong et al., 2015).

Effect of temperature on POD activity

The use of different mediums with variable temperatures (5-70°C) indicated a gradual increase of peroxidase activity with temperature increasing from 5 to 30°C and then quickly declined (Figure 1b). Beyond 50°C, the POD completely inactivated. High temperatures will break the bonds responsible for maintaining the 3D structure of the enzyme that induces the modification of enzyme shape and consequently disrupts the active site. In this case, the enzymatic activity will be

α_0 , α_i , α_{ii} , and α_{ij} are regression coefficients for intercept, linear, quadratic, and interaction terms, respectively; x_i and x_j are independent variables.

Optimal conditions of peroxidase activity of turnip depending on pH, temperature, and NaCl concentration were predicted with the predictive equation of RSM. The optimal values obtained experimentally and calculated were compared in order to verify the validity of the model.

reduced or completely will be suppressed (Agostini et al., 2002; Sáez et al., 2019). The enzymatic activity usually depends on the temperature of the medium. Several studies have been devoted to study the influence of temperature on POD activity. The optimal temperature for peroxidases activity of sweet potato and lettuce is around 30°C and is 40°C for radish, garlic, sorgho, and dates (Saraiva et al., 2007; Diao et al., 2011; Amiour and Hambaba, 2016).

Effect of NaCl concentration on POD activity

The POD activity increased with NaCl concentration rising from 0 to 1M and then decreased for higher concentrations due to the modification of enzyme structure (Figure 1c). The most commonly used reagents for enzyme extraction are NaCl and buffer solutions. The NaCl increases the ionic strength of the buffer, which enhanced extraction of the plant enzymes. Plants possess hydrogen peroxide enzymes as POD and CAT, which lead to detoxification of reactive oxygen, thus protected cells against the harmful of hydroperoxides. Some authors reported that POD activity depends significantly on the salinity of the reaction medium (Dicko et al., 2006; Zeyadi and Almulaiky 2020).

Analysis of the model

The combination of the three factors (pH, temperature, and NaCl concentration) as well as the observed (experimental) and predicted (predicted) of response (enzymatic activity) are summarized in Table 1. From the results, it can be observed that experimental values of enzymatic activity are close to that of predicted by the model. The results of the model analysis of the peroxidase activity optimization are listed in Tables 2 and 3. The regression coefficients (intercept, quadratic, linear, and interaction terms) of the mathematic

model are resolved with the least square technique (Table 2). According to these results, the linear terms x_2 and x_3 and the three quadratic and the interaction terms x_1 - x_2 are significant ($p < 0.05$), whereas the linear term of x_1 and the interactions x_1 - x_3 and x_2 - x_3 are not significant. It is indicated that the linear term of temperature, the three

quadratic parameters, and the interaction of pH-NaCl concentration terms influenced negatively the enzymatic activity. However, only NaCl concentration positively affected this activity. The fitted quadratic model for peroxidase activity considering significant terms is given in equation 2 (Eq. 2):

$$\text{Peroxidase activity (U/mg)} = 3726.23 - 20.55x_2 + 498.94x_3 - 46.60x_1x_2 - 1076.32x_1^2 - 48.62x_2^2 - 2552.40x_3^2 \text{ (Eq. 2)}$$

x_1 : pH, x_2 : temperature ($^{\circ}\text{C}$), x_3 : NaCl concentration (M), *: statistically significant ($p < 0.05$)

Figure 1. Influence of pH (a), temperature (b), and NaCl concentration (c) on turnip POD activity

Table 1. Box-Behnken design matrix of experiments runs and observed and predicted results of POD activity

Run	Variable levels			Enzymatic activity	
	x_1	x_2	x_3	Observed	Predicted
1	5 (-)	25 (-)	1 (0)	1725.854	1601.069
2	7 (+)	25 (-)	1 (0)	2154.853	2049.225
3	5 (-)	25 (-)	1 (0)	1725.854	1601.069
4	7 (+)	25 (-)	1 (0)	2154.853	2049.225
5	5 (-)	30 (0)	1.5 (+)	2751.505	2634.364
6	7 (+)	35 (+)	1 (0)	1291.925	1367.333
7	6 (0)	30 (0)	1 (0)	4063.155	4009.188
8	6 (0)	25 (-)	1.5 (+)	2100.611	2388.734
9	6 (0)	30 (0)	1 (0)	4003.983	4009.188
10	6 (0)	25 (-)	0.5 (-)	2085.818	2137.997
11	7 (+)	35 (+)	1 (0)	1324.799	1367.333
12	6 (0)	30 (0)	1 (0)	3872.489	4009.188
13	6 (0)	30 (0)	1 (0)	4018.776	4009.188

14	6 (0)	30 (0)	1 (0)	4092.741	4009.188
15	5 (-)	35 (+)	1 (0)	1727.498	1851.206
16	5 (-)	30 (0)	0.5 (-)	2118.692	1973.034
17	6 (-)	25 (-)	1.5 (0)	1469.442	1601.069
18	5 (-)	35 (+)	1 (0)	1763.659	1851.206
19	5 (-)	30 (0)	0.5 (-)	1750.509	1973.034
20	6 (0)	35 (+)	1.5 (+)	2087.462	2327.446
21	7 (+)	30 (0)	0.5 (-)	1840.911	2211.178
22	6 (0)	35 (+)	1.5 (+)	2608.506	2327.446
23	7 (+)	30 (+)	0.5 (-)	2411.265	2211.178
24	6 (0)	25 (-)	0.5 (-)	2149.100	2137.997
25	5 (-)	30 (0)	1.5 (+)	2687.402	2634.364
26	6 (0)	35 (+)	0.5 (-)	1880.359	1767.528
27	6 (0)	35 (+)	0.5 (-)	1942.819	1767.528
28	7 (+)	30 (0)	1.5 (+)	2342.231	2360.504
29	7 (+)	30 (0)	1.5 (+)	2455.644	2360.504
30	6 (0)	30 (0)	1 (0)	4003.983	4009.188

x_1 : pH, x_2 : temperature ($^{\circ}\text{C}$), x_3 : NaCl concentration (M), peroxidase activity expressed as U/mg. *: statistically significant ($p < 0.05$)

Table 2. Calculation and statistical significance check of regression coefficients of the peroxidase activity model

Parameters	Estimated	Standard error	t-ratio	Prob> t
Intercept	3726.23	386.59	9.64	<0.0001*
x_1	67.09	44.85	1.50	0.1503
x_2	-20.55	9.28	-2.21	0.0387*
x_3	498.94	95.86	5.20	<0.0001*
x_1 - x_2	-46.60	12.34	-3.78	0.0012*
x_1 - x_3	-256	12.39	-1.98	0.0618
x_2 - x_3	30.92	28.33	1.09	0.2882
x_1 * x_1	-1076.32	68.31	-15.76	<0.0001*
x_2 * x_2	-48.62	2.72	-17.87	<0.0001*
x_3 * x_3	-2552.40	273.25	-9.34	<0.0001*

x_1 : pH, x_2 : temperature ($^{\circ}\text{C}$), x_3 : NaCl concentration (M), *: statistically significant ($p < 0.05$)

The fitted data following the quadratic model are summarized in Table 3. The analysis of the variance of the developed peroxidase activity model denoted a high statistical significance with a probability lower than 0.0001. This is supported by the high value of the correlation coefficient (0.9704) indicating that 97.03% experimental response is explained by the model and only less than three of 3% of the information is lost, attesting the goodness of the quadratic model for the POD activity. In addition, the checking of model lack of fit is found not significant ($p=2.7221$). This result implied that the mathematical model has a high performance for the prediction of the peroxidase activity of turnip.

Response surface analysis

In order to illustrate the interaction between the three studied variables and peroxidase activity, Figure 2 showed the graphical plots of the three-

dimensional surface of the model. The effect of pH and temperature of the reaction medium on the response is given in Figure 2.a which indicates that both factors influenced together peroxidase activity. These latter increased until the optimum pH (5.9) and temperature (29.8 $^{\circ}\text{C}$) and thereafter decreased. Plants peroxidase display an optimal enzymatic activity at temperatures ranging generally from 30 to 50 $^{\circ}\text{C}$ and pH between 3 and 7 (Pandey et al., 2017). The effects of pH-NaCl concentration and temperature-NaCl concentration are shown in Figure 2b and c. The quadratic effect is well observed for the studied parameters. This indicated that at high or low levels of these three factors, the peroxidase activity is reduced; the increase of these parameters induced the increase of the response, but beyond certain levels, an inverse effect is observed and this is due to the ionization or denaturation of the enzyme.

Table 3. Analysis of variance for the peroxidase activity model.

Source	Degree of freedom	Sum of squares	F-ratio	Prob> F
x_1	1	74909	2.2372	0.1503
x_2	1	163890	4.8947	0.0387*
x_3	1	907055	27.0898	<0.0001*
x_1 - x_2	1	477556	14.2626	0.0012*
x_1 - x_3	1	131074	3.9146	0.0618
x_2 - x_3	1	39860	1.1905	0.2882
x_1 * x_1	1	8311763	248.2368	<0.0001*
x_2 * x_2	1	10689979	319.2640	<0.0001*
x_3 * x_3	1	2921390	87.2494	<0.0001*
Model	9	21917617	2435291	<0.0001*
Lack of fit	3	217298.98	72433.0	0.0767
Error	20	669664.02		
Total model	29	22587281		
Pure error	17	452365.04		
$R^2 = 0.97$				
Adj $R^2 = 0.96$				

x_1 : pH, x_2 : temperature ($^{\circ}\text{C}$), x_3 : NaCl concentration (M), *: statistically significant ($p < 0.05$)

Figure 2. Three-dimensional response surfaces representing the influence of pH-temperature (a), pH-NaCl concentration (b), and temperature-NaCl concentration (c) on POD activity.

Model Verification

Optimal values of three factors on peroxidase activity are obtained by the desirability profiler of JMP. The adequate conditions that manifested the highest peroxidase activity from the turnip are pH of 5.9, the temperature of 29.8°C, and 1.07 M for NaCl concentration. Under these optimal

conditions, the predicted enzymatic activity is 4027.25U/mg. The experimental test of these maximized conditions (4031.10 ± 23.77 U/mg) indicated the agreement with the predicted value of peroxidase activity and attesting thus the validity of the developed quadratic model.

CONCLUSION

In this study, the sequential and response surface methodologies allowed to determine the optimal conditions for the activity of the peroxidases extracted from the turnip. The three studied parameters (pH, temperature, and NaCl

concentration) significantly influenced POD activity. The conditions that maximized POD activity are 5.9, 29.8°C, and 1.07M, respectively. The observed POD activity is in agreement with the calculated value indicating the relevance of the established quadratic model for peroxidase activity.

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