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Optimization of an Activity Assay of *Coprinus Cinereus* Peroxidase

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ABSTRACT

To seek a simple, rapid and sensitive Coprinus cinereus Peroxidase (CIP) activity assay, a convenient one-factor-at-a-time (OFAT) method and a response surface methodology (RSM) were used. The recombinant CIP expressed in Pichia pastoris was purified with the Ni-NTA spin column. Based on the results of catalytic efficiency (k_{cat}/Km*) analysis, 2,2'-azinobis (ethylbenzthiazoline -6-sulfonate) (ABTS) was selected as the optimal enzyme substrate. Results of the OFAT method showed that enzymatic reaction performed in 0.1 mol/L sodium acetate (pH 5.0) buffer in a 200-µl reaction mixture containing 0.5 mmol/L ABTS, 10 mmol/L hydrogen peroxide (H_2O_2), 49.7 ng CIP at 25°C gave an average CIP activity of 88 U/mL. The ABTS and H_2O_2 concentrations were then further optimized to improve the sensitivity of the assay. To do that, RSM was conducted through centra1 composite design, and a reduced quadratic model with good fit regression equation was generated. ANOVA analysis of this model indicated that the concentrations of ABTS and H_2O_2 and their interaction had significant impact on the assay sensitivity. The optimal reaction mixture was determined to include an initial ABTS concentration of 0.82 mmol/L 49.7 ng CIP and 16.36 mmol/L H_2O_2, and the activity under this condition was determined to be 138.89 U/mL.*

Key words: enzymatic activity assay, one-factor-at-a-time (OFAT) method, response surface methodology (RSM), *Coprinus cinereus* peroxidase (CIP)

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INTRODUCTION

Peroxidases (EC 1.11.1.7) are heme-containing enzymes that are widely distributed in animals, plants and microorganisms. They oxidize a variety of organic and inorganic compounds with the presence of hydrogen peroxide[1,2,3,4]. Their powerful oxidizing ability makes peroxidases very useful in many fields, including analytical chemistry, immunochemistry, and biosensor construction. In addition, they have great potential and prospect in the textile, paper and pulp bleaching industries[1,5]. Although peroxidases are important in many industries, it is difficult to produce these enzymes in large scale, because most of the peroxidases are isolated from plants. The production and properties of these enzymes are restricted by geographical and climate conditions. A fungal peroxidase secreted from *Coprinus cinereus* was first isolated and characterized by Shinmen et al.[6]. This peroxidase (CIP) has high activity and broad substrate specificity similar to that of horse radish peroxidase (HRP), although there is less than 10%-16% sequence similarity between CIP and HRP, and thus has attained considerable attention since it was isolated[7,8]. CIP has higher thermostability than HRP[9,10]. It has been used successfully to remove phenolic compounds from wastewater[11-16], to degrade benzene homologs and derivates[17,18], to decolourate dyes[19-21], and to produce functional polyaromatics[22-26]. CIP can also be used as cleaners[27] and biosensors[28]. Therefore, from a commercial viewpoint, CIP is a good candidate for replacing HRP in industry applications, and has broad market prospect. Unfortunately, a standard CIP activity assay is not available so far.

Because CIP can catalyze a wide range of substrates, there are several methods to measure CIP activity, such as the ABTS method[23,29,30], the phenol method[17,21,28,31], the guaiacol method[32], the pyrogallol method[12], and the o-phenylenediamine method[33]. When measuring specific activity, researchers use different assay mixtures, including different substrates, different types of buffers, different pH, different temperature, and different concentration of substrates according to their needs, which lead to difficulties in comparing the activity of CIPs from multiple sources, and thus hinder their applications in the industry. Phenol, guaiacol and o-phenylenediamine are slightly soluble in water, and the preparation of substrate solutions and reaction mixtures require much effort. Furthermore, phenolic compounds (i.e. guaiacol, phenol and pyrogallol) and o-phenylenediamine are toxic, insensitive for the CIP activity assay, and the residue reaction mixture needs to be treated before release to the environment. Hence, there is an urgent need to develop a simple, rapid and sensitive CIP activity assay with good reproducibility.

CIP activity is greatly affected by pH, buffer, temperature, type of substrate, and substrate and H_2O_2 concentrations. The widely accepted one-factor-at-a-time (OFAT) method for optimizing enzymatic activity assays involves varying one parameter at a time while keeping the other parameters constant. This technique is time-consuming, and ignores the interactions among different parameters, so it may lead to wrong conclusions[21,34]. An assay with orthogonal design can evaluate the interactions among different parameters accurately, but requires too much experimentation, and is time-consuming and labor intensive.

Response surface methodology (RSM) is a useful mathematical and statistic method to overcome these difficulties. RSM requires minimal experimentation, but can help researchers to optimize conditions from multiple variables rapidly and efficiently, and to provide sufficient information that leads to sound results[34-37]. RSM has been successfully applied in areas of food science research[38-40], enzyme production from microorganisms[41-43], producing target products using enzymatic catalysis[35,39,44-46],

biodiesel production[47], and dye decoloration[21,34,48]. To the best of our knowledge, there has been no report on the optimization of enzymatic activity assay using RSM.
As part of the optimization process of the enzymatic activity assay, the optimal substrate was determined by comparing the CIP catalytic efficiencies (k_{cat}/K_m) of different substrates, and ABTS was selected as the optimal substrate for the assay. Then, to investigate the interaction between ABTS and hydrogen peroxide concentrations and its effect on enzymatic activity assay, both the OFAT method and the RSM were used to improve the sensitivity of the assay using the specificity of the assay as the response value.

MATERIAL AND METHODS

Chemicals

All chemicals and reagents were of analytical grade, [2,2'-azinobis(ethylbenzthiazoline-6-sulfonate)] (ABTS), phenol, 2,6-Dimethoxyphenol (2,6-DMP), guaiacol, and 2,4-dichlorophenol (2,4-DCP) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Peptone and yeast extract were purchased from Oxoid (Basingstoke, England). Ni-spin column was obtained from QIAGEN (Hilden, Germany). Other chemicals were purchased from Shanghai Sangon (Shanghai, China).

Strain, media and growth condition

Engineered strain *P. pastroi* CIP/GS115, harboring the CIP gene with *P. pastroi* codon bias integrated into the *Pichia* genome, was used to produce recombinant CIP efficiently. Solid YPD (1% yeast extract, 2% peptone, 1% glucose, and 1% agar) was used to produce single clone inoculum. BMGY (1% yeast extract, 2% peptone, 1.34% YNB, 0.1 mol/L phosphate buffer pH 6.0 , 0.4 mg/L biotin and 1% glycerol) and BMMY (the same as BMGY, except that 0.5% methanol was used instead of 1% glycerol) were used to induce protein expression. Protein was induced at 28°C, while at other times P. pastroi CIP/GS115 was grown at 30°C.

Production and purification of recombinant CIP

Single clones of CIP/GS115 were prepared on YPD agar plate. Cells from single clone were inoculated into 5 mL of BMGY medium in a 20-mL screw cap test tube. Cultures were grown at 30°C with agitation (220 rpm) overnight, and then transferred to 500-ml shake flask containing 100 ml BMGY medium sealed with a four-layer sterile gauze and continued to culture with agitation (220 rpm) at 30°C.When OD600 of the culture reached 4.0-6.0, cells were centrifuged at 4,000 g for 5 min, and resuspended in BMMY medium to an OD600 of 10. Methanol was added to the culture at a final concentration of 0.5% every 24 h to maintain induction, and cultivation continued at 28°C for 5 days with agitation. After induction, the extracellular CIP with a polyhistidine (6×His) tag was purified using Ni NTA spin column according to the instructions of the manufacturer in a cold room.

Determination of the concentration of the recombinant CIP

The purity of recombinant CIP was estimated by sodium dodecyl sulfate polyacryl amide gel electrophoresis (SDS-PAGE). Protein concentration was determined using Nanodrop 2000 ultraviolet spectrophotometer (ThermoFisher, CA, USA) at 280 nm (ε = 1.597×104 $L \cdot mol^{-1}cm^{-1}$). NPI-500 (50 mmol/L NaH_2PO_4, 300 mmol/L NaCl, 500 mmol/L imidazole) was used as the blank control.

Selection of optimal substrate according to kinetic parameters

To investigate the substrate specificity of the purified recombinant CIP, 5 substrates were selected, and the kinetic parameters (Km and kcat) of the recombinant enzyme were determined by assaying the enzymatic activity in the 0.1 mol/L sodium acetate (NaAC) buffer (pH 5.0) at 25°C with ABTS ranging from 0.0625-1.5 mmol/L, phenol ranging from 0.125-7.5 mmol/L, guaiacol ranging from 0.25-5 mmol/L, 2,4-DCP ranging from 0.125-12.5mmol/L, or 2,6-DMP ranging from 0.0625-2.95 mmol/L as the substrate. A total of 0.63 mmol/L 4-aminoantipyrine (4-AAP) was added to the assay mixture when using phenol as substrate. Values for the maximum velocity and half-saturation coefficient (Km) were determined by fitting the data of the substrate concentration vs. the initial velocity of each reaction to the nonlinear regression of the Michaelis-Menten equation. Kinetic analyses by curve fitting were performed with the Graphpad prime 5 software. The substrate with the highest *Kcat/Km* value was selected as the best substrate.

One factor at a time method

CIP activity was measured in a microplate reader Bio-Rad EXL 800 with ABTS (ε_{420nm} = 36,000 $M^{-1}cm^{-1}$) as the substrate. One unit of CIP activity was defined as the amount of CIP required to produce 1 μmol ABTS cation per min from ABTS under specified conditions.

Enzyme amount of 49.7 ng was selected according to previous experience, since excess enzyme might lead to the absorbance value to exceed the machine reading range. Assay of mixture optimization was carried out by changing an independent variable while fixing the others at certain levels. PH, temperature, buffer, and H_2O_2 and ABTS concentrations play crucial roles in the CIP activity measurement. Therefore, these parameters were optimized using the OFAT method.

The experiments were conducted in microplates containing 200 μL mixture containing ABTS, buffer, CIP, and H_2O_2, which were placed on the microplate reader at different experimental conditions as bellow:49.7 ng of CIP, ABTS concentrations (0.063-1.5 mmol/L), H_2O_2 concentrations (0.05-50 mmol/L), temperature (10-60°C) and pH (2.2-9.0). The buffers used in optimal pH tests were the Mcllvaine's citrate-phosphate (pH 2.2-2.6) and the Britton-Robinson buffer (pH 3.0-9.0). Furthermore, different buffers, including the Mcllvaine's citrate-phosphate buffer, the Britton-Robinson buffer and 0.1 mol/L NaAC were selected and adjusted to pH 5.0 to seek for the most sensitive one. Table 1 lists different experimental conditions for the OFAT method. All experiments were conducted in triplicate and average values were reported.

Table 1 Experimental conditions used in the OFAT method

Parameter	pH	H_2O_2 (mmoll/L)	Temp (ºC)	ABTS (mmol/L)	Buffer
Effect of Ph	2.2-9.0	0.1	25	0.5	A B
Effect of Buffer	5.0	0.1	25	0.5	ABC
Effect of H_2O_2 conc	5.0	0.05-50	25	0.5	C
Effect of temperature	5.0	0.1	10-60	0.5	C
Effect of ABTS conc	5.0	0.1	25	0.063-1.5	C

A: Mcllvaine's citrate-phosphate buffer, B: Britton-Robinson buffer, C: NaAC Buffer

Response surface analysis

A response surface methodology (RSM) with a two-factor (i.e. ABTS concentration and H_2O_2 concentration) and five-level (Table 2) central composite design (CCD) was carried out to improve the sensitivity of the CIP activity assay according to the design of the statistical software package "Design Expert 8.05", leading to 13 different experiments in random with 5 replicates at the center point. Other parameters, including a temperature of 25°C, 0.1 mol/L NaAC buffer (pH 5.0), and 49.7 ng CIP, were kept constant, all the experiments were carried out in triplicate. The level of different variables with code and actual value and average values of activity were reported in Table 2. The program of "Design Expert 8.05" was used to analyze the experimental design and results.

Table 2 central composite design with two independent variables

Run	ABTS (mmol/L)		H_2O_2 (mmol/L)		Activity((U/mL)
	Coded value	Actual value	Coded value	Actual value	
1	0	0.5	0	10	80.926
2	-γ	0.05	0	10	41.667
3	0	0.5	0	10	91.269
4	0	0.5	0	10	82.407
5	1	0.82	-1	3.64	65.185
6	1	0.82	1	16.36	129.63
7	0	0.5	-γ	1	27.593
8	γ	0.95	0	10	135.741
9	0	0.5	Γ	19	102.037
10	0	0.5	0	10	95.371
11	0	0.5	0	10	90
12	-1	0.18	-1	3.64	31.852
13	-1	0.18	1	16.36	47.593

γ = 1.41

RESULTS AND DISCUSSION

Production, purification and concentration measurement of recombinant CIP

The expression of recombinant CIP was induced with methanol, and the protein was then purified using the Ni-NTA spin column. On SDS-PAGE gel, a clear band of estimated size of 43kDa was observed (Fig.1), and this size was in accordance with the predicted molecular mass. The concentration of purified recombinant CIP was 14.916 mg/mL.

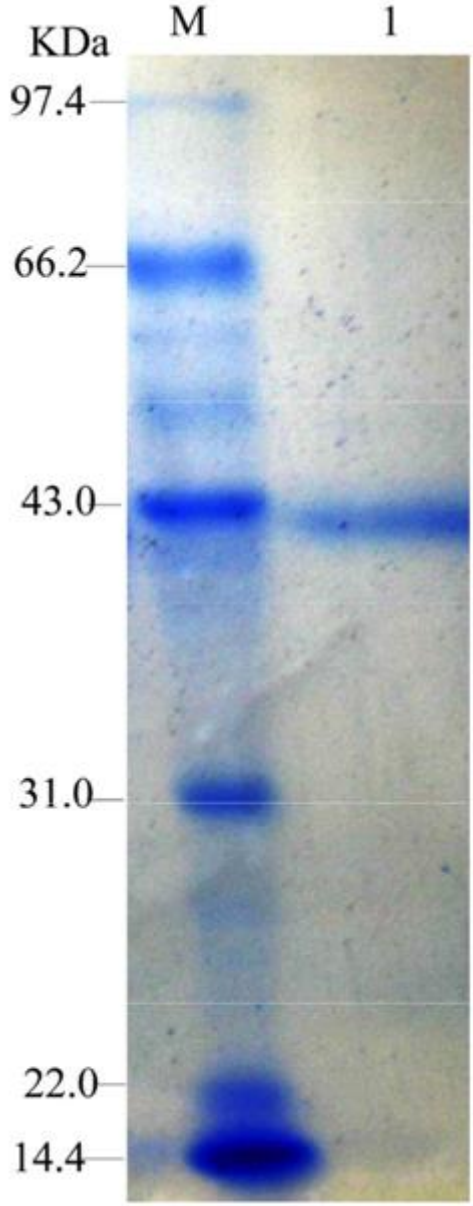

Figure 1. SDS-PAGE analysis of the purified recombinant CIP. M, standard protein molecular mass markers (TaKaRa, sizes in kilodaltons are indicated on the left); Lane 1, recombinant CIP purified by Ni-NTA spin column.

Selection of optimal substrate

In general, the catalytic efficiency (k_{cat}/K_m) is considered as a measurement of the enzymatic specificity.

The initial reaction rates with various substrate concentrations were measured, and the kinetic parameters for the enzymatic activity of the recombinant CIP using different substrates were summarized (Table 3).

ABTS was the best substrate with the highest catalytic efficiency, followed by guaiacol, 2, 6-DMP, 2,4-DCP and phenol. We also found that the minimal time to convert various amount of 2,4-DCP or phenol into steady brownish red products was 30-65 min using 9.94 µg enzyme, while the minimal time to convert various amount of 2,6-DMP into steady brownish red products was 5-10 min using 4.97 µg enzyme. Using ABTS as substrate saved time and effort, and it was more water soluble and less toxic than other substrate. Therefore, ABTS was used as the substrate for further experiments.

Table 3 Kinetic parameters and specific activity of CIP with different substrate

Substrate	Km (M)	Kcat (S-1)	Kcat/Km (S-1mM-1)
ABTS	62.80 ± 7.771	17.190 ± 0.314	0.274
2,6-DMP	711.00± 9.403	0.122± 0.006	1.718×10-4
Guaiacol	150.200± 3.047	0.200 ± 0.005	0.001
Phenol	240.200± 5.090	0.003± 0.001	1.249 ×10-5
2,4-DCP	1494.000 ± 128.400	0.054± 0.0001	3.614×10-5

Effects of pH and buffer on CIP activity

PH plays crucial roles in enzyme activity assays. It influences not only the dissociative state of the substrate and the enzyme, but also the structure of the catalytic active center of the enzyme. Most enzymes are active only within a narrow pH range, and drastic changes in pH often lead to denaturation of the enzyme[44]. To investigate the effect of pH on enzyme activity, experiments were performed at 25°C and pH conditions ranging from 2.2 to 9.0 with 0.5 mmol/L ABTS, 0.1 mmol/L H_2O_2, and 49.7 ng purified recombinant enzyme (Fig. 2A). The optimum assay pH was determined to be 5.0. The enzyme activity increased with the increase in pH between 2.2 and 5.0. However, CIP activity decreased notably at above pH 5.0.

Buffer type also affected CIP activity assay greatly. Three types of buffer was used in this study, including 0.1 mol/L sodium acetate (NaAC) buffer, the Britton-Robinson buffer and the Mcllvaine's citrate-phosphate buffer adjusted to pH 5.0. The sensitivity of the enzymatic activity assay was the highest in 0.1 mol/L NaAC buffer, followed by the Britton-Robinson buffer and the Mcllvaine's citrate-phosphate buffer (Fig. 2B). Therefore, 0.1 mol/L NaAC buffer (pH 5.0) was selected

as the optimal buffer for further tests.

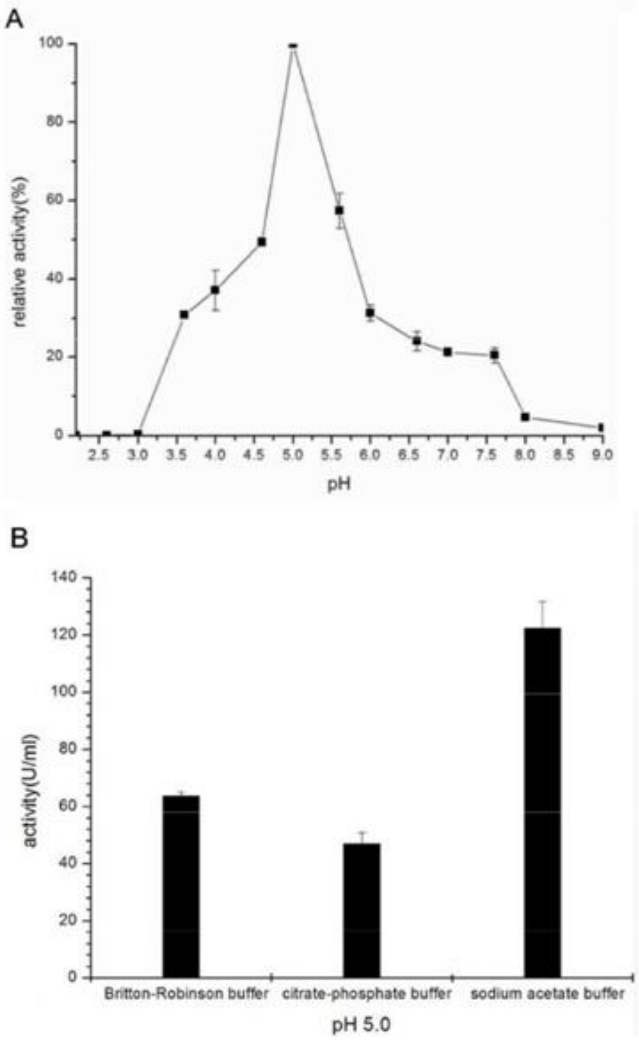

Figure 2. Effects of pH (A) and types of buffer (B)on CIP activity. Data points are the average of triplicate measurements. Error bars represent ± 1SD.

Effect of temperature on CIP activity

The effect of temperature on enzyme activity was measured and summarized (Fig. 3). The CIP activity increased with temperature increase until 25°C, above which the activity decreased significantly, because high reaction temperature led to irreversibly denatured CIP, whereas low temperature slowed down the reaction due to a lack of energy for additional substrates to enter into transition state[44]. According to our result, 25°C was selected as the optimal reaction temperature.

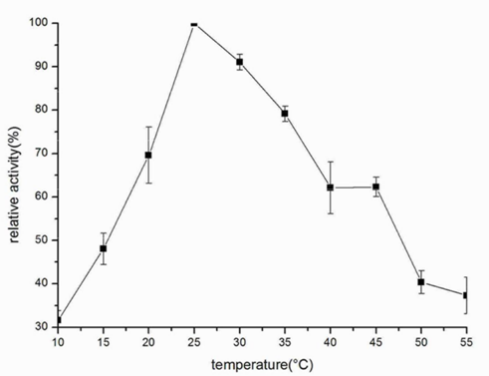

Figure 3. The effect of temperature on CIP activity. Data points are the average of triplicate measurements. Error bars represent ± 1SD.

Effects of ABTS concentration and H_2O_2 concentration on enzymatic activity

Substrate concentration significantly affects the enzyme activity. In general, activity increases with the increase in substrate concentration before the enzyme is saturated, and then keeps constant and even decreases slowly. Experiments were performed to investigate the effects of ABTS and H_2O_2 concentrations on enzyme activity (Fig. 4). The optimal ABTS concentration was 0.5 mmol/L, and the enzymatic activity drastically increased when the ABTS concentration increased from 0 to 0.3125mmol/L. The enzymatic activity increased slowly when the ABTS concentration was between 0.3125 and 0.5 mmol/L, and decreased slowly over 0.5 mmol/L (Fig. 4A).

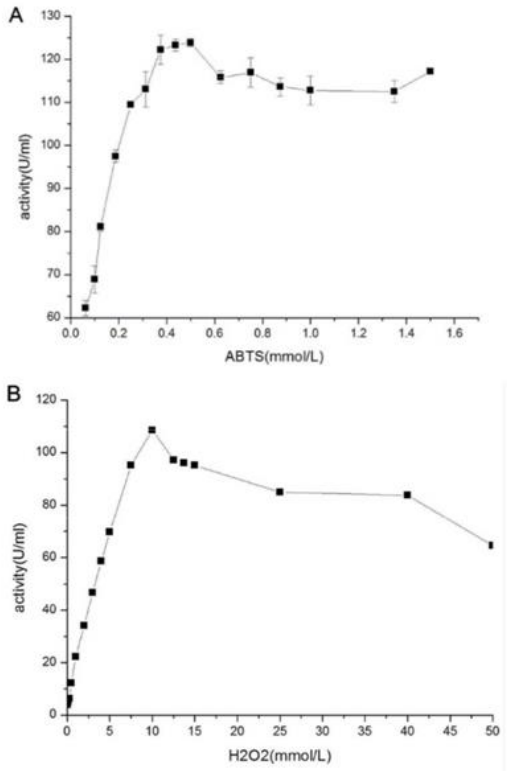

Figure 4. The effects of ABTS (A) concentration and H_2O_2 (B) concentration on CIP activity. Data points are the average of triplicate measurements. Error bars represent ± 1SD.

H_2O_2 is essential for CIP activity. The optimum H_2O_2 concentration was determined to be 10 mmol/L, above which the enzymatic activity decreased gradually (Fig. 4B).

Previous studies have shown that excess hydrogen peroxide is suicidal, resulting in heme destruction, protein oxidation and CIP inactivation[3,49,50]. It has been reported that high concentrations of H_2O_2 inhibit the activity of peroxidase in dye decoloration[21,51].

In conclusion, using the OFAT method, we determined that the CIP enzymatic activity assay was optimal at 25ºC with 0.1 mol/L NaAC (pH 5.0) buffer, 10 mmmol/L H_2O_2, 0.5 mmol/L ABTS and 49.7 ng CIP.

The activity of CIP was 88 U/mL under these conditions.

Response surface analysis A 2-factor-5-level central composite design was adopted to optimize the assay mixture using the statistical software package "Design Expert 8.05". The experimental data were analyzed and fitted to a second order polynomial regression model described as follow: Activity = 87.99 + 31.05A + 23.18B + 12.18AB - 1.69A^2 - 13.64B^2 where A is the concentration of ABTS, and B is the H_2O_2 concentration.

The analysis of variance for the quadratic polynomial model was summarized in Table 4. The model F-value of 48.36 (P value < 0.0001) implied that the model was significant and adequate to represent the actual relationships between the response (CIP activity) and the significant variables (ABTS concentration and H_2O_2 concentration). There was only a 0.01% chance that a model F-value could occur due to noise. The lack-of-fit p-value of 0.2264 indicated that the lack of fit was not significant relative to the pure error, and that the non-significant lack of fit was good. In this case, factor A, factor B, their combinant reaction and B^2 were significant model terms (Table 4). The "Pred R-Squared" of 0.8583 was in reasonable agreement with the "Adj R-Squared" of 0.9518. "Adeq Precision" measured the signal to noise ratio. A ratio greater than 4 was desirable, and the ratio of 21.578 indicated an adequate signal.

Therefore, this model can be used to investigate the design space. The closer the values of adjusted R^2 are to 1, the better is the correlation between the experimental and predicted values[46,52].

Table 4. Analysis of variance (ANOVA) for the quadratic regression model

Source	Sum of squares	Df	Mean square	F value	P value	
Model	13900.72	5	2780.14	48.36	< 0.0001	Significant
A-A	7713.50	1	7713.50	134.18	< 0.0001	
B-B	4300.00	1	4300.00	74.80	< 0.0001	
AB	593.01	1	593.01	10.32	0.0148	
A^2	19.96	1	19.96	0.35	0.5742	
B^2	1294.15	1	1294.15	22.51	0.0021	
Residual	402.39	7	57.48			
Lack of fit	252.05	3	84.02	2.24	0.2264	Not significant
Pure error	150.34	4	37.58			
Cor Total	14303.11	12				

The effects of ABTS concentration, H_2O_2 concentration and their interaction on response values could be graphically presented as three dimensional response

surface curves and contour plots (Fig. 5). CIP activity was enhanced with the increase in ABTS concentration and H_2O_2 concentration from 0 to 10 mmol/L. However, CIP activity decreased gradually when ABTS and H_2O_2 concentrations increased further. Too high or too low concentrations of ABTS and H_2O_2 would reduce the sensitivity of the enzyme activity assay. The highest enzyme activity was predicted as 139.073 U/mL by the "design expert 8.05" software, with 16.36 mmol/L H_2O_2 and 0.82 mmol/L ABTS.

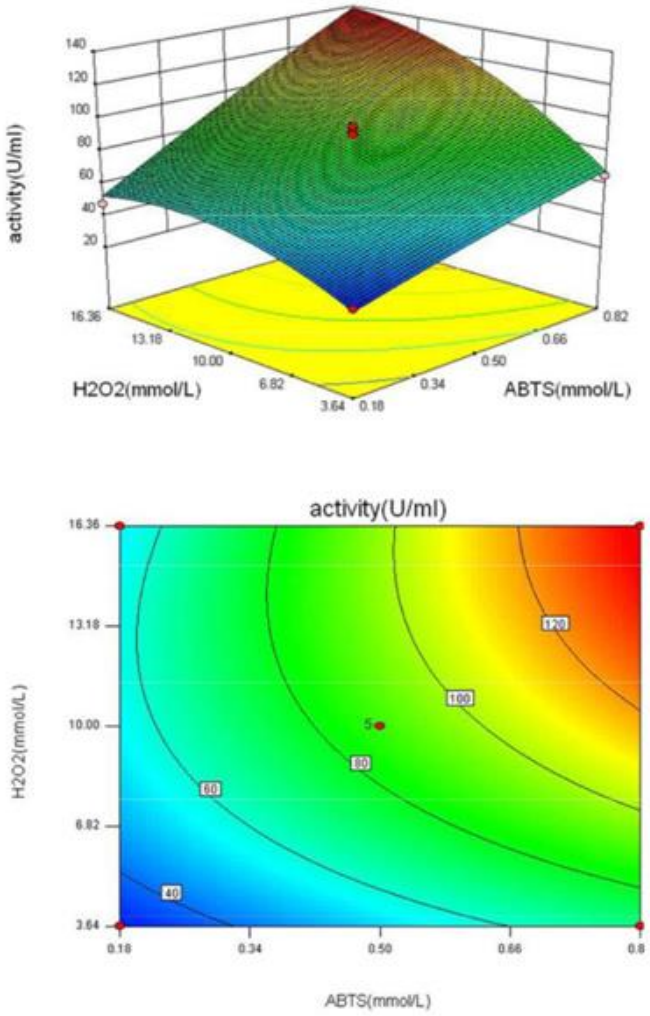

Figure 5. Response surface plot and contour plot of ABTS and H_2O_2 concentrations.

Verification of optimal reaction condition

To test the effectiveness and accuracy of the above model, enzyme activity assay was carried out at the predicted optimal conditions in triplicate, and the average enzyme activity was 138.89 U/mL, which was quite close to the predicted value. The results indicated that the results of the theoretical analysis matched well with the experimental results, and that the optimization method for CIP assay was feasible based on the results of the response surface analysis. We also measured the CIP activity using the traditional ABTS method[23], Chang's Guaiacol method[32] and Sakurai's phenol method[31], and the results showed that the activity of CIP was 46.24 U/mL, 20.9 U/mL and 0.17 U/mL, respectively.

CONCLUSIONS

According to the kinetic parameters, ABTS was the best substrate. By using OFAT, we determined that the optimal reaction condition was 25°C, 0.1 mol/L NaAC (pH 5.0), and 200 μl mixture containing 0.5 mmol/L ABTS, 10 mmol/L H_2O_2 and 49.7 ng CIP, and the average CIP activity was 88 U/mL under these conditions. Furthermore, the optimal reaction mixture obtained from RSM was an initial ABTS concentration of 0.82 mmol/L, 49.7 ng CIP, 16.36 mmol/L H_2O_2, and 25°C. The maximum CIP activity was 138.89 U/ml, which was 1.58 fold, 3 fold, 6.65 fold and 817 fold higher than that obtained using the OFAT method, the traditional ABTS method, the Guaiacol method, and the phenol method, respectively.
The model is adequate to represent the relationships between predicted values and experimental values.

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