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Optimization of 2,3-dihydroxybiphenyl 1,2-dioxygenase expression and its application for biosensor

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ABSTRACT

In this study, two statistical experimental designs, Plackett–Burman design (PBD) and response surface methodology (RSM), were employed to enhance the expression of 2,3-dihydroxybiphenyl 1,2-dioxygenase (BphC_LA-4), which was subsequently used for the construction of catechol biosensor. Ten important factors were evaluated by PBD, and four significant parameters were then optimized by RSM. Under the favorable fermentation conditions, the maximal specific activity of BphC_LA-4 was about 0.58 U/mg with catechol as substrate. Meanwhile, homology modeling and molecular docking were utilized to help understand the interaction between BphC_LA-4 and catecholic substrates, which illustrated that BphC_LA-4 presented lower binding affinity towards 4-methylcatechol in comparison with 3-methylcatechol and catechol. Interestingly, the BphC_LA-4 enzyme electrode prepared by SiO_2 sol–gel showed good response to all these three catecholic compounds. The differences of selectivity to 4-methylcatechol between free and immobilized enzyme implied that the introduction of electro-catalysis might have an effect on the enzyme-catalysis process.

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1. Introduction

The detection of catecholic compounds has drawn great interest in recent years because they are important environmental pollutants that often coexist in environmental samples persistently with potential toxicity and carcinogenicity (Sun et al., 2000; Subramanyam and Mishra, 2008; Lin et al., 2009). Up to now, several methods for determination of these compounds have been developed, such as high performance liquid chromatography (Mizukami et al., 2007), gas chromatography/mass spectrometry (Moldoveanu and Kiser, 2007) and fluorescence (Yuan et al., 2008), etc. These detection techniques can offer simultaneous determination of samples with high sensitivity and wide linear range. However, these precision instruments commonly require complicated pretreatment, costly operation and maintenance. Many efforts have been made to develop more simple and rapid detection methods of catecholic compounds. Recently, enzyme biosensors have attracted a great deal of attention in measuring these compounds due to their good selectivity, high sensitivity and relatively low cost.

So far, the catechol biosensors based on enzymes mainly utilize tyrosinase, laccase, peroxidase as the sensing elements (Liu et al., 2008; López et al., 2009; Ozoner et al., 2009; Xu et al., 2009). The biosensors built up with these enzymes present excellent analytical performances for phenol, catechol and other phenolic compounds. Nevertheless, they generally cannot distinguish the signal of catechol from that of other phenolic compounds in the mixtures, which is due to the broad substrate range of these enzymes. Therefore, it is necessary to exploit novel enzymes with good specificity of catecholic compounds used for biosensors. The extradiol and intradiol catechol dioxygenases can catalyze the ring cleavage of catecholic substrates, which is a key metabolic step in the biodegradation of most aromatic compounds by bacteria (Vaillancourt et al., 2006; Khajamohiddin et al., 2008). Several typical extradiol catechol dioxygenases (such as catechol 2,3-dioxygenase, 2,3-dihydroxybiphenyl 1,2-dioxygenase (BphC), protocatechuate 4,5-dioxygenase) and intradiol catechol dioxygenases (such as catechol 1,2-dioxygenase, protocatechuate 3,4-dioxygenase) have been widely studied for their structures, reaction mechanism, as well as their application in the degradation of aromatic compounds (Bugg and Lin, 2001; Siegbahn and Haeffner, 2004; Brivio et al., 2009). However, there have been very few researches of these catechol ring-cleavage enzymes for biosensors (Lv et al., 2005; Zucolotto et al., 2006).

In our previous study, a novel 2,3-dihydroxybiphenyl 1, 2-dioxygenase named BphC_LA-4 was successfully expressed by an *Escherichia coli* recombinant strain carrying a *bphC* gene (915 bp) from *Dyella ginsengisoli* LA-4 (Li et al., 2009). It was proved that BphC_LA-4 was a novel type I Fe(II)-dependent extradiol

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dioxygenase that could catalyze the meta-cleavage reaction of 2,3-dihydroxybiphenyl, catechol and other catecholic compounds (Li et al., 2009). However, the expression of recombinant protein can be affected by many process parameters during the cultivation of gene-engineering strains, such as pH of medium, the culture medium, induction time and temperature. Moreover, not only each of these variables, but also the interactions of them play an important role in the high expression of recombinant enzyme. Therefore, it was essential to optimize the expression conditions of BphC_LA-4 for further study. Statistical experimental designs are very powerful tools for screening and optimizing the key variables from a multivariable process. Plackett–Burman design (PBD) is one of the most frequently used screening methods, which can help to do the fewest experiments to screen key factors among a large number of various and unsure parameters (Tari et al., 2006). Response surface methodology (RSM) is another typical statistical technique used for optimization design. Since it was first introduced by Roquemore in 1976 (Roquemore, 1976), RSM has been widely applied to the optimization of process parameters for many bacteria culture (Gao et al., 2009; He et al., 2009) and some enzyme catalytic processes (Chen et al., 2010; Liu et al., 2010).

In order to obtain an extradiol dioxygenase with relatively high activity used for a catechol biosensor, we optimized the factors in the expression process of BphC_LA-4 by PBD and RSM. The homology modeling and molecular docking of BphC_LA-4 were also used to illustrate the interaction between enzyme and substrates. Furthermore, the validity of BphC_LA-4 enzyme electrode was investigated by its electrochemical behavior of three catecholic compounds.

2. Methods

2.1. Bacterial strain and growth conditions

The bacterial strain used in this study was a recombinant *E. coli* BL21 (DE3) carrying a *bphC* gene (915 bp) encoding 2,3-dihydroxybiphenyl 1,2-dioxygenase (BphC) from *D. ginsengisoli* LA-4. Seed medium (25 mL) and fermentation medium (100 mL) were both Luria–Bertani (LB) media. *E. coli* BL21 was cultured according to the methods described by Li et al. (2009). Before use, the cells were collected by centrifugation (9820*g*, 5 min), suspended in 0.2 M phosphate buffer solution (PBS, pH 8.0, prepared by $NaH_2PO_4{\cdot}2H_2O$ and $Na_2HPO_4{\cdot}12H_2O$), and treated with sonication (4 °C, 30 min). Subsequently, the suspension was centrifugated at 58545*g*, 4 °C, 20 min. Then the supernatant was taken as the crude extracts.

2.2. Chemicals

Isopropyl-D-thiogalactopyranoside (IPTG) was purchased from TaKaRa, kanamycin was obtained from Solarbio, sodium dodecyl sulfate (SDS) and ammonium persulfate were from Sigma, and acrylamide was from Amresco. All the chemicals above were analytically pure, and other chemicals used in this study, such as tetraethoxysilane (TEOS), poly(vinyl alcohol) (PVA), catechol, 3-methylcatechol, 4-methylcatechol, were of the highest analytical grade available. All the solutions used were prepared by ultrapure water (18.2 MΩ).

2.3. Enzyme activity assays

After mixing the crude extracts with 0.2 M PBS (pH 8.0) containing 0.1 mM catechol at 20 °C, BphC_LA-4 activity was measured by monitoring the reaction product 2-hydroxymuconic semialdehyde at 375 nm with a scanning spectrophotometer (V-560, JASCO, Japan). The total volume of reaction was 2 mL. The activity of BphC_LA-4 was evaluated by the climbing slope of time course curve of the UV absorption. Taking catechol as an example, one unit of enzyme activity was defined as the amount of enzyme that catalyzed 1 μM catechol per minute at 20 °C. Representation formula was as follows:

$$\text{Enzyme activity}\ \ (\text{U}) = \text{EW} \times V \times 10^3/(\varepsilon \times L)$$

$$\text{Enzyme specific activity}\ (\text{U/mg}) = \text{Enzyme activity/protein contents}$$

where EW represented changes of absorbance value at 375 nm per minute, V was the total volume of system, ε was molar extinction coefficient, and L was optical path. The molar extinction coefficient of 2-hydroxymuconic semialdehyde was $\varepsilon_{375} = 67.8\ \text{cm}^{-1}\ \text{mM}^{-1}$ at pH 8.0 (Eltis et al., 1993).

2.4. Homology modeling and docking studies

Due to the absence of three-dimensional structure of BphC_LA-4, homology modeling based on sequence similarity was constructed using SWISS-MODEL server (Arnold et al., 2006). Crystal structure of BphC from *Burkholderia xenovorans* sp. LB400 was identified as template structure for BphC_LA-4 according to the sequence-based blast in protein data bank (PDB). Docking was carried out by Autodock 4.2 (Morris et al., 1998). Ligand structure was generated by PRODRG server (Schuettelkopf and van Aalten, 2004). The GA_r number of individuals in the population was set up to 100. Molegro Molecular Viewer was used for visualizing the docking result and displaying the interaction of protein and ligand.

2.5. Optimization of BphC_LA-4

PBD is a two-level design for investigating at most "$N-1$" parameters in "N" runs. In this study, the number of parameters was selected as 11 with a dummy variable and 10 actual variables. The PBD matrix of $N = 12$ and the levels of all the parameters ("+" for the higher value, "−" for the lower value) were shown in Table 1. The actual values of each level were listed in Table 2. The most significant factors were selected based on their contributions and assigned optimal values in the optimization experiments.

Subsequently, a four-factor, five-level central composite design (CCD) was adopted for determining the optimal levels of those variables screened. Thirty experiments designed were conducted according to Table 3. The analysis of variance (ANOVA) was adopted to analyze the significance of each factor and their interaction effects, and the adequacy of response surface model. Catechol was taken as substrate for the screening and optimization experiments, while the specific activity of enzyme was taken as the target.

2.6. SDS–polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was used to evaluate the quality of the 30 enzymes obtained by RSM. The running buffer consisted of 5× Tris–clycine buffer, 0.125 M Tris, 1.25 M Glycine, and 0.5% (WN) SDS. The acrylamide concentrations were 5% and 15% for the concentrating and separating gels, respectively. Protein staining of the gel was performed with Coomassie brilliant blue R250. A protein marker from 200 to 6.5 kDa was used as standard to estimate the molecular mass of the enzyme sample.

2.7. Preparation of PVA–SiO_2 sol–gel and enzyme electrode

The mixture of 1.45 mL TEOS, 0.5 mL double distilled water and 50 μL HCl (0.1 M) was treated with ultrasonic bath for 90 min to

Table 1
Plackett–Burman design for BphC_LA-4 expression.

No.	A	B	C	D	E	F	G	H	I	Dummy	J	Enzyme specific activity (U/mg)
1	+	+	–	+	+	+	–	–	–	+	–	0.27
2	–	+	+	–	+	+	+	–	–	–	+	0.44
3	+	–	+	+	–	+	+	+	–	–	–	0.50
4	–	+	–	+	+	–	+	+	+	–	–	0.35
5	–	–	+	–	+	+	–	+	+	+	–	0.45
6	–	–	–	+	–	+	+	–	+	+	+	0.41
7	+	–	–	–	+	–	+	+	–	+	+	0.36
8	+	+	–	–	–	+	–	+	+	–	+	0.39
9	+	+	+	–	–	–	+	–	+	+	–	0.41
10	–	+	+	+	–	–	–		–	+	+	0.34
11	+	–	+	+	+	–	–	–	+	–	+	0.39
12	–	–	–	–	–	–	–	–	–	–	–	0.41

Table 2
The levels and effects in Plackett–Burman design.

Variables		Levels		Effects	Contribution (%)
Code	Variable	Low (−)	High (+)	(−) (+)	
A	pH of fermentation medium	7	8	−0.0017	0.0064
B	Seed age (h)	10	12	−0.038	3.11
C	Inoculation amount of seeds (%)	0.8	1.0	−0.024	1.25
D	Inoculation amount of broth (%)	1	2	−0.033	2.39
E	OD_{600} for inducement	0.4	0.6	0.0092	0.19
F	Concentration of IPTG (mg/mL)	0.2	0.24	−0.011	0.26
G	Temperature for inducement (°C)	30	37	0.20	86.41
H	Induction time (h)	2	3	0.023	1.13
I	Concentration of kanamycin (mg/L)	30	40	0.0056	0.069
Dummy	Dummy	−1	1	−0.0046	0.045
J	pH of seed medium	7	8	−0.049	5.15

Table 3
Design and analysis of response surface methodology.

No.	Temperature for inducement (°C)	pH of seed medium	Seed age (h)	Inoculation amount of broth (%)	Observed value[a] (U/mg)	Predicted value[b] (U/mg)
1	35	6.5	9	0.6	0.56	0.54
2	40	6.5	9	0.6	0.45	0.42
3	35	7.5	9	0.6	0.56	0.57
4	40	7.5	9	0.6	0.48	0.44
5	35	6.5	11	0.6	0.51	0.51
6	40	6.5	11	0.6	0.47	0.48
7	35	7.5	11	0.6	0.51	0.48
8	40	7.5	11	0.6	0.44	0.45
9	35	6.5	9	1.2	0.58	0.56
10	40	6.5	9	1.2	0.52	0.54
11	35	7.5	9	1.2	0.53	0.51
12	40	7.5	9	1.2	0.50	0.50
13	35	6.5	11	1.2	0.41	0.44
14	40	6.5	11	1.2	0.54	0.53
15	35	7.5	11	1.2	0.33	0.35
16	40	7.5	11	1.2	0.42	0.43
17	32.5	7.0	10	0.9	0.50	0.51
18	42.5	7.0	10	0.9	0.45	0.46
19	37.5	6.0	10	0.9	0.58	0.58
20	37.5	8.0	10	0.9	0.49	0.51
21	37.5	7.0	8	0.9	0.50	0.54
22	37.5	7.0	12	0.9	0.46	0.44
23	37.5	7.0	10	0.3	0.38	0.42
24	37.5	7.0	10	1.5	0.44	0.42
25	37.5	7.0	10	0.9	0.55	0.55
26	37.5	7.0	10	0.9	0.56	0.55
27	37.5	7.0	10	0.9	0.56	0.55
28	37.5	7.0	10	0.9	0.56	0.55
29	37.5	7.0	10	0.9	0.52	0.55
30	37.5	7.0	10	0.9	0.56	0.55

[a] Observed value: enzyme specific activity observed by experiments.
[b] Predicted value: enzyme specific activity predicted by model.

obtain the SiO_2 stock solution. Then 5% PVA solution was added (PVA:SiO_2 = 8:1, *V*:*V*). BphC_LA-4 crude extracts were mixed thoroughly with PVA modified SiO_2 sol solution (*V*:*V* = 1:1) to form homogeneous sol–gel. Ten microliters of this mixture was dropped

on the surface of clean glassy carbon electrode (GCE) and dried at 4 °C. Finally, the modified GCE was stored at 4 °C and rinsed with PBS before use.

2.8. Electrochemical detection of catecholic compounds

Electrochemical experiments were performed on a CHI660D electrochemical workstation. A standard three-electrode system was used in this study with saturated calomel electrode as reference electrode, platinum plate electrode as auxiliary electrode and modified GCE as working electrode. The cyclic voltammetry (CV) experiment was carried out in 10 mL quiescent PBS (0.2 M, pH 8.0) with the sweep rate of 100 mV/s.

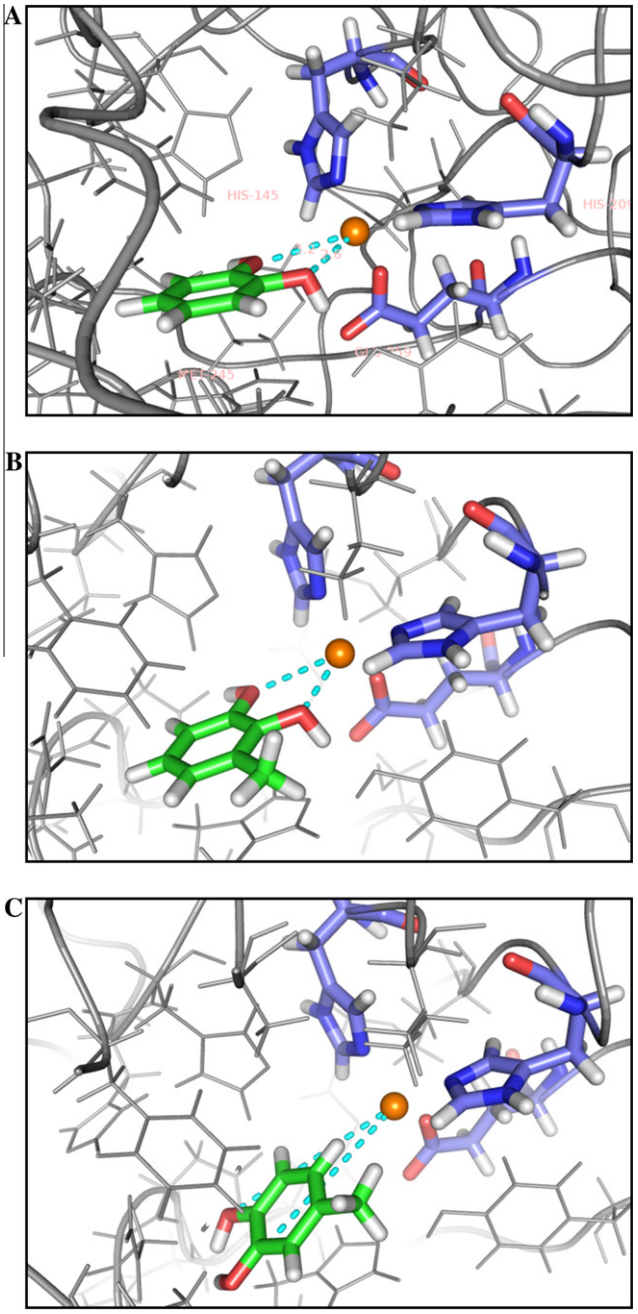

Fig. 1. Model of catechol–BphC_LA-4 complex (A), 3-methylcatechol–BphC_LA-4 complex (B), 4-methylcatechol–BphC_LA-4 complex (C).

3. Results and discussion

3.1. Model of the BphC_LA-4-catechol complex

Three-dimensional structure of BphC_LA-4 gave reasonable insights into the active site geometry, and homology modeling-based docking studies presented the enzyme-substrate interaction in detail. According to the docking studies of the interactions between BphC_LA-4 and catechol (Fig. 1A), 3-methylcatechol (Fig. 1B), 4-methylcatechol (Fig. 1C), it was found that the substrates bound the active site of BphC_LA-4 at the similar position. Since the biological oxygen activation often occurs in extradiol aromatic ring-cleaving dioxygenases after binding to a reduced metal, the distance between Fe(II) and hydroxyl-oxygen atoms seems to be correlated with the substrate selectivity (Emerson et al., 2008). It can be seen that both the hydroxyl-oxygen atoms of 4-methylcatechol were located in the opposite orientation (Fig. 1C) compared with those in 3-methylcatechol and catechol, which illustrated that it was more difficult for BphC_LA-4 to bind 4-methycatechol than 3-methylcatechol and catechol. On the other hand, the results were consistent with the weak BphC_LA-4 activity of 4-methylcatechol in our previous work, which further demonstrated the importance of the Fe–O distance in controlling the enzyme activity (Li et al., 2009).

3.2. Plackett–Burman screening design

There were many variables during the process of enzyme producing in the *E. coli*. Compared with single factor experiments, PBD can select main factors through the fewest experiments. In this study, PBD was adopted to evaluate the effects of 10 factors on the expression of BphC_LA-4. The design matrix and the corresponding responses were shown in Table 1. The specific activity of BphC_LA-4 had a wide variation from 0.27 to 0.50 U/mg, which suggested that it was necessary to optimize the expression conditions for BphC_LA-4.

The levels and effects of experimental variables were analyzed and presented in Table 2. According to the contribution, four significant factors were screened for further optimization studies, which were temperature for inducement, pH of seed medium, seed age, and inoculation amount of broth (Table 2). As is well known, PBD has been applied to screen process parameters for expression of kinds of enzymes, such as P450 monoxygenase, pectinase, alkaline protease, amylase, and so on (Di Gennaro et al., 2006; Reddy et al., 2008; Abdel-Fattah et al., 2009). In the expression of other proteins, inoculum size was also reported to be one of the most significant variables when the different variables excluded (Reddy et al.,

Table 4
ANOVA of response surface model.

Parameter	Coefficient estimate	Standard error	*F*-value	*P*-value
Model			7.97	0.0001
A	−0.011	0.0061	3.40	0.0851
B	−0.019	0.0061	9.44	0.0077
C	−0.026	0.0061	18.50	0.0006
D	−0.0012	0.0061	0.042	0.8405
AB	−0.0006	0.0075	0.007	0.9345
AC	0.024	0.0075	10.63	0.0053
AD	0.027	0.0075	12.93	0.0026
BC	−0.012	0.0075	2.52	0.1330
BD	−0.017	0.0075	5.1	0.0393
CD	−0.019	0.0075	6.72	0.0204
A^2	−0.017	0.0057	8.85	0.0095
B^2	−0.002	0.0057	0.12	0.7336
C^2	−0.016	0.0057	7.59	0.0147
D^2	−0.033	0.0057	33.88	<0.0001

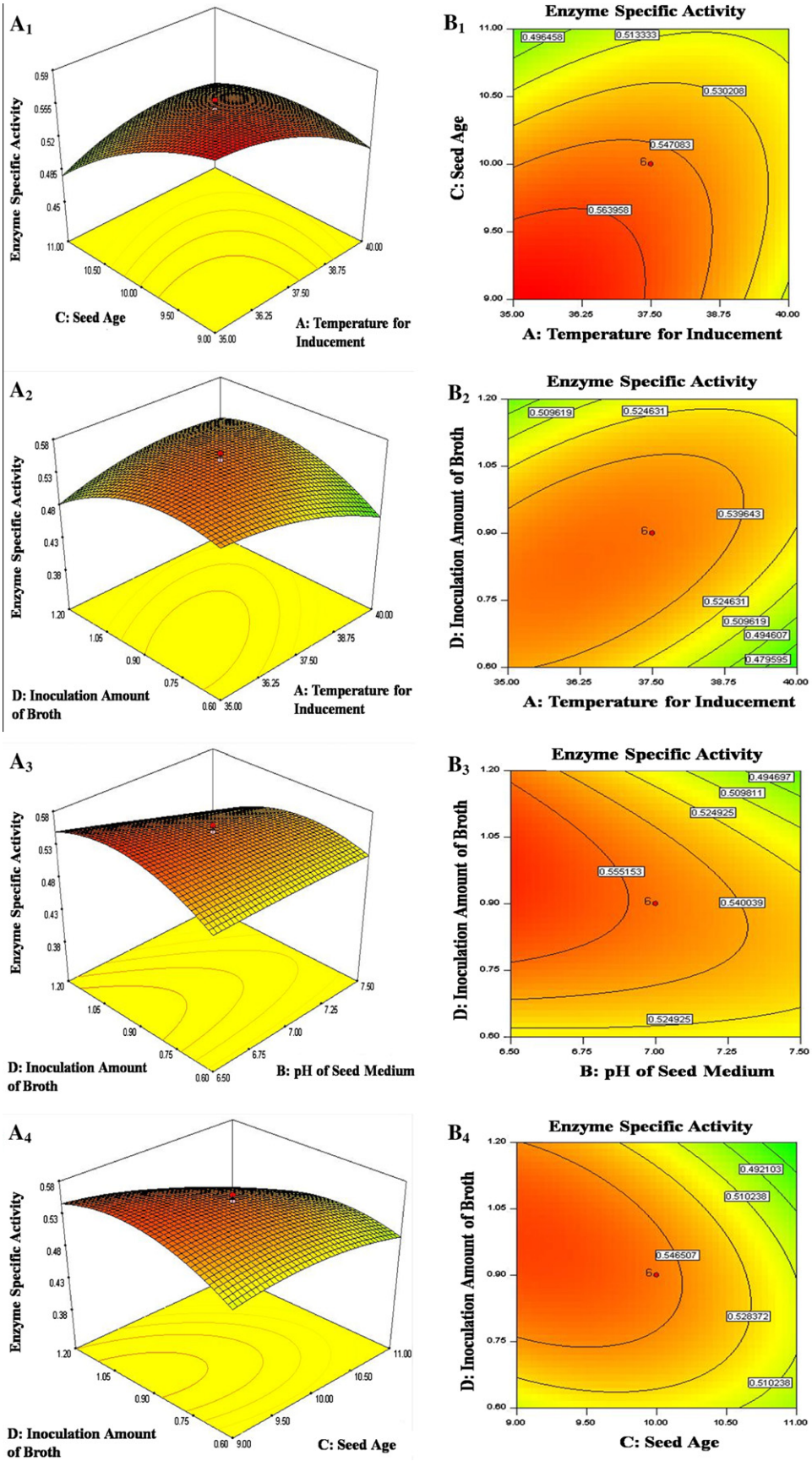

Fig. 2. Three-dimensional response surface plots (A) and 2D contour plots (B) for BphC activity showing the interactive effects of AC (A_1, B_1), AD (A_2, B_2), BD (A_3, B_3), CD (A_4, B_4), respectively. A: temperature for inducement; B: pH of seed medium; C: seed age; D: inoculation amount of broth.

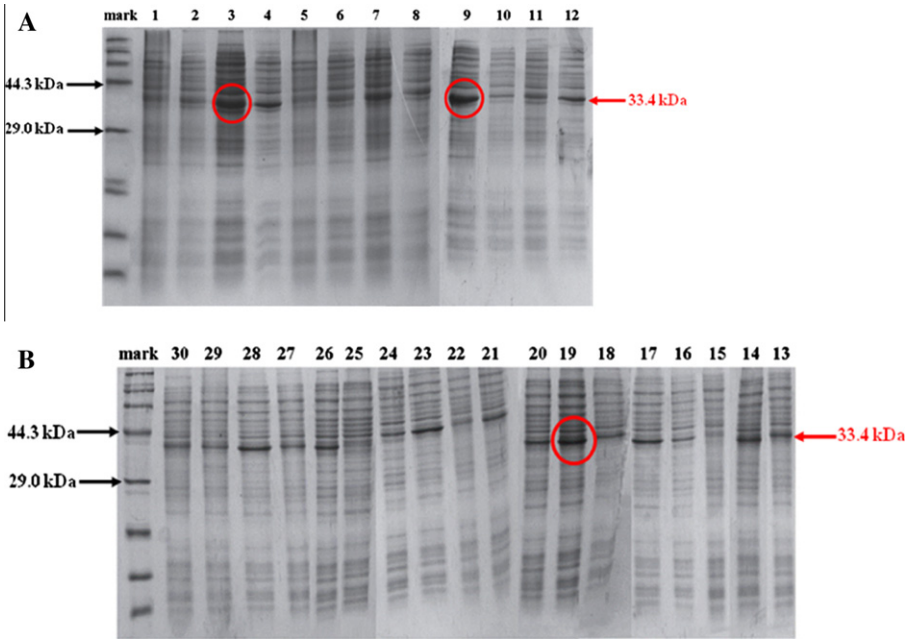

Fig. 3. SDS–PAGE of 30 BphC samples from the optimization process. Mark: protein marker, from top to bottom (200, 116, 97.2, 66.4, 44.3, 29.0, 20.1, 14.3, 6.5 kDa); 1–30 (A for 1–12; B for 13–30): crude cell extracts of 30 BphC_LA-4 samples.

2008). In the same way, incubation temperature was also identified to markedly affect the production of alkaline protease (Abdel-Fattah et al., 2009). As shown in Table 2, temperature for inducement exerted a positive effect, while other three important variables showed negative effects. Therefore, the temperatures around 37 °C were considered for inducement in the optimization section. The values of all other insignificant variables were determined by the effects of them. When the effect was positive, the factor adopted the higher value; otherwise, it adopted the lower level.

3.3. Response surface methodology

The design matrix for RSM and the corresponding responses were exhibited in Table 3. The experimental data were modeled with a second-order polynomial equation to explain the relationship between response (Y) and the factors (A, B, C, D). The following model equation using the coded factors was proposed for BphC_LA-4 activity:

$$Y = 0.55 - 0.011A - 0.019A - 0.026C - 1.250 \times 10^{-3}D - 0.625 \times 10^{-3}AB + 0.024AC + 0.027AD - 0.012BC - 0.017BD - 0.019CD - 0.017A^2 - 1.979 \times 10^{-3}B^2 - 0.016C^2 - 0.033D^2$$

where Y was the specific activity of BphC_LA-4. The four variables (A, B, C, D) were assigned as following: A for temperature for inducement, B for pH of seed medium, C for seed age, and D for inoculation amount of broth, respectively.

The predicted values of enzyme specific activity were also depicted in Table 3. The observed values of Y ranging from about 0.33 to 0.58 U/mg was determined by 30 experiments of CCD (Table 3). According to the ANOVA, the F-values and P-values of each variable in terms of linear, quadratic and interaction were obtained and shown in Table 4. Since P-value (probability > F) less than 0.05 indicated that the model terms were significant, the model was highly significant ($P = 0.0001$), and B, C, AC, AD, BD, CD, A^2, C^2, D^2 were all significant. R^2 is the determination coefficient and its value ($R^2 = 0.8815$) explained the fitness of the model, which indicated that the second order polynomial model equation could explain 88.15% of the total variation.

Design Expert 7.0 was adopted to visualize three-dimensional response surface (Fig. 2A) and two dimensional contour plots (Fig. 2B) of BphC_LA-4 activity relating with independent variables. The effect of both factors (AC, AD, BD, and CD) on the response was presented while the other factor was held at zero level (Fig. 2). It can be seen that each pair of variables interacted with each other. According to the analytical results, the optimal conditions were obtained as follows: inducing temperature 35 °C (A), pH 6.5 for seed medium (B), seed age 9 h (C), and inoculation amount of 0.95% (D). And under the favorable fermentation conditions, the maximal enzyme specific activity of BphC_LA-4 (Y) was up to 0.58 U/mg, which was nearly three times higher than that of previous study. In general, BphC can catalyze 2,3-dihydroxybiphenyl better than catechol (Senda et al., 1996; Yang et al., 2008). However, compared with BphC from *Bacillus* sp. JF8, crude extracts of BphC_LA-4 even displayed higher catalytic activity with catechol as substrate than that of BphC_JF8 with 2,3-dihydroxybiphenyl as substrate (Hatta et al., 2003). Thus, the optimized BphC_LA-4 could be more suitable for the construction of catechol biosensor.

The levels of the crude extracts obtained from those 30 experiments were estimated by SDS–PAGE analysis (Fig. 3). The bands of BphC_LA-4 at about 33.4 kDa were obvious and much brighter than other impurities. As shown in Fig. 3, the bands of BphC_LA-4 marked with red circles displayed more distinctly compared with that of other samples, which was consistent with the results of CCD: the enzymes expressed better under the conditions of team 3, 9 and 19 as a result of their higher activity and protein quality.

3.4. Electrochemical detection of catecholic compounds using BphC_LA-4

To further investigate the possibility of applying BphC_LA-4 to the biosensor, the electrochemical behaviors of three kinds of catecholic compounds on the PVA–SiO_2/BphC_LA-4 enzyme electrode were recorded by cyclic voltammograms (CVs). The reversible redox peaks could be observed in all the three CVs of bare GCE

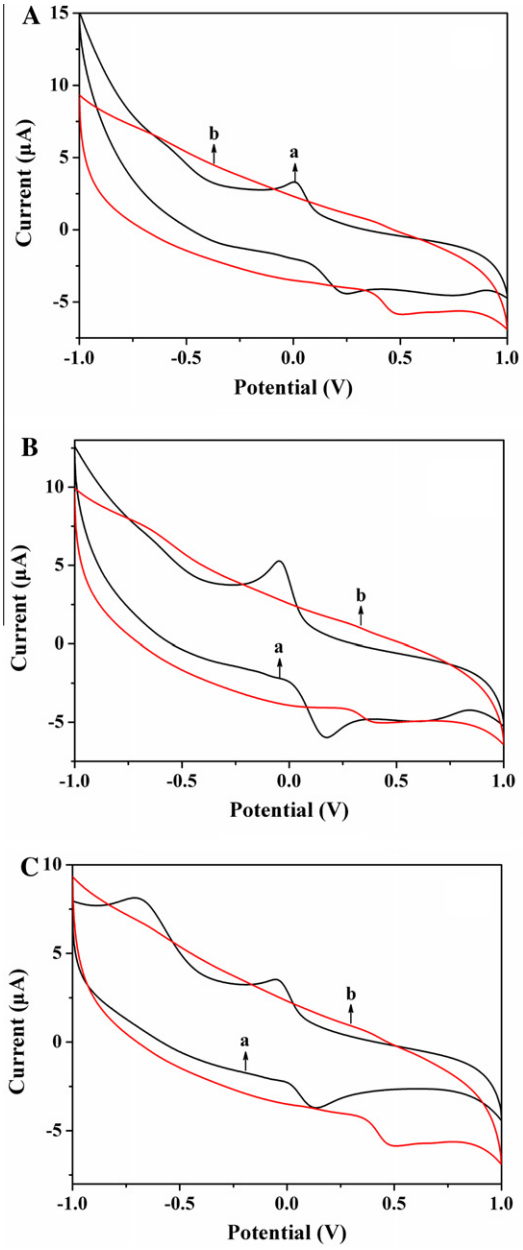

Fig. 4. The cyclic voltammetry curves of two different modified electrodes: line a for PVA–SiO_2 and line b for PVA–SiO_2/BphC_LA-4 modified GCE with 0.4 mM catechol (A), 3-methylcatechol (B), 4-methylcatechol (C).

(Fig. 4 (A)a, (B)a, (C)a), which should be due to the reversible quinine/diphenol redox (Apetrei et al., 2011). However, it was clear that the electrochemical behaviors of these three substrates on the BphC_LA-4 electrode were very different (Fig. 4 (A)b, (B)b, (C)b). All these reduction peaks in curves "a" disappeared with the positive shift of the oxidation peaks. It was evident that BphC_LA-4 played a key role in the electrochemical oxidation of these three catecholic compounds.

Moreover, it was very interesting that the BphC_LA-4 enzyme electrode could give good response to 4-methylcatechol, while only weak catalytic activity could be obtained for free BphC_LA-4. It can be explained that the combination of electro- and enzyme catalysis could change the binding capacity of BphC_LA-4 with its substrate to some extent. Therefore, the results provide potential for the application of BphC_LA-4 in biosensors, and the relevant mechanism requires further study.

4. Conclusions

In this work, the main factors during the expression of BphC_LA-4 were designed and optimized by PBD and RSM. The highest enzyme specific activity of BphC-LA-4 (0.58 U/mg) was obtained under the conditions as following: pH 6.5 for seed medium, seed age of 9 h, inoculation amount of 0.95% and inducing temperature 35 °C. Though the lower binding affinity of BphC_LA-4 towards 4-methylcatechol was illustrated by homology modeling and molecular docking, the BphC_LA-4 electrode showed good response for not only catechol and 3-methylcatechol but also 4-methylcatechol.

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