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Thermostabilization of laccase by polysaccharide additives: Enhancement using central composite design of RSM

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ABSTRACT

Stability of laccase from *Pleurotus florida* NCIM 1243 at high temperature was assessed by combination of polysaccharide additives (Guar Gum and starch). After conducting a series of initial enzyme stabilization experiments, combinatorial additives were selected to construct an appropriate Response Surface Model for optimizing the concentration. Further, sodium acetate buffer was taken as a variable to optimize the polysaccharide additives. The optimum values of these variables for maximum laccase stabilization at 60 °C are Guar Gum (0.098%), starch (6.235%) and sodium acetate buffer (76.84 mM) with a predicted percentage residual activity of 69.91%. The experimental validation for laccase thermostabilization was carried out using optimized variables and was found to be 75%. The significance of the study is that the laccase stabilization was achieved at 60 °C in liquid state rather than in the freeze dried state. The enhancement for stabilization of laccase was 75% and it effectively decolorizes RB198 at 60 °C.

1. Introduction

Laccase (*p*-diphenol oxidase, EC 1.10.3.2) catalyzes the oxidation of various aromatic, particularly phenolic substrates (e.g. hydroquinone, guaiacol or phenylenediamine). The enzymatic reaction is coupled to the reduction of molecular oxygen to water (Messerschmidt, 1997). Laccases are widely distributed in nature, originating from plants (Harvey & Walker, 1999), insects (Sidjanski, Mathews, & Vanderberg, 1997), bacteria (Alexandre & Zhulin, 2000) and especially fungi (Thurston, 1994). Applications of laccase in biotechnology include: textile dye or stain bleaching (Fu & Viaraghavan, 2001; Kirby, Marchant, & McMullen, 2000; Pointing & Vrijmoed, 2000), paper-pulp bleaching (Annunziatini, Baiocco, Gerini, Lanzalunga, & Sjögren, 2005; Grönqvist, Buchert, Rantanen, Viikari, & Suurnäkki, 2003), synthetic dye decolorization (Baldrian, 2004; Nagai et al., 2002), bioremediation (Jaouani, Guillén, Penninckx, Martínez, & Martínez, 2005), biosensors (Timur, Pazarlýoglu, Pilloton, & Telefoncu, 2004), chemical synthesis (Karamyshev, Shleev, Koroleva, Yaropolov, & Sakharov, 2003), immunoassays (Jordaan & Leukes, 2003) and the detoxification of contaminated soil and water (Filazzola, Sannino, Rao, & Giangreda, 1999).

Protein stabilization is becoming a critical technology and greatest challenge in the fields of biomaterials and pharmaceuticals. Temperature raise leads to an increase in molecules collision. Beyond a critical temperature, in consequence, disruption of enzymatic activity and enzyme denaturation occurs (Coughlan, 1992). To avoid destabilization rate, most proteins need a stabilizer in the liquid state for long term storage; these stabilizers are formulation excipients, which are also referred to as chemical additives (Allison et al., 1998). Stabilization of enzymes in aqueous and non aqueous media by polyols and other substances has been widely reported. Many authors have employed these additives in different enzymatic systems (Combes, Graber, & Ye, 1983). In enzymatic stabilization, optimization of the concentration of polysaccharide additives, sodium acetate buffer and enzyme is an important criterion for successful stabilization work.

The purpose of the present work was to optimize the additives concentration used for maximum stabilization of laccase enzyme from *Pleurotus florida*. To achieve this objective, laccase was stabilized and optimized by RSM.

2. Materials and methods

2.1. Microorganism and culture conditions

The wood-rotting fungus *P. florida* (NCIM 1243) was obtained from the National Collection of Industrial Microorganism, National Chemical Laboratory, Pune, India. Microorganism cultivation and laccase production were performed on potato dextrose agar (Poonkuzhali, Sathishkumar, Boopathy, & Palvannan, 2011). The

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laccase containing culture fluid was separated from the mycelium after 8 days by filtration through filter paper (Whatman no. 1).

2.2. Laccase purification

The laccase purification was adapted from a protocol described by Poonkuzhali et al. (2011) with slight modification.

2.3. Laccase assay

Laccase (EC 1.10.3.2) activity was measured at 30 °C using 20 mM ABTS as the substrate (Wolfenden & Wilson, 1982). The absorbance increase of assay mixture was monitored at 420 nm (ε_{420} = 36.0 mM^{-1} cm^{-1}). The enzyme activities were expressed as international units (U), defined as the amount of enzyme needed to produce 1 μmol product min^{-1} at 30 °C and presented as U g^{-1} dry substrate. Protein was determined by the method of Lowry, Rosebrough, Farr, and Randall (1951) with bovine serum albumin as the standard.

2.4. Stability assays

Stability assays were carried out in sealed tubes containing 0.5 U of laccase in a total volume of 2 ml incubated at 60 °C. The combination of two additives (Guar Gum, 0.25%; Starch, 8%) with individual control was tested after 24 h (Poonkuzhali et al., 2011). The pH 5.6 was maintained in the stability assays using 100 mM sodium acetate buffer. All chemicals used are commercially available and were used without further purification.

2.5. Optimization of laccase stabilization by RSM

2.5.1. Central composite design

To find out the optimum level of most effective variables [X_1, X_2, X_3] that have been identified for stabilization of laccase at 60 °C, RSM using central composite design (CCD) was applied. The independent variables selected for this study were (i) X_1, Guar Gum (%); (ii) X_2, starch (%); and (iii) X_3, sodium acetate buffer (mM). For each categorical variable, a 2^3 full factorial CCD for the three variables, consisting of 8 factorial points, 6 axial points and 6 replicates at the center points were employed, indicating that altogether 20 experiments were required, as calculated from the following equation (Azargohar & Dalai, 2005):

$$N = 2^n + 2n + n_c = 2^3 + 2 \times 3 + 6 = 20$$

where N is the total number of experiments required and n is the number of factors.

A 2^3 fractional factorial central composite design for three independent variables each at five levels, with ten star points and six replicates at the center points leading 20 experiments was used in this study.

2.5.2. Statistical analysis

The DESIGN–EXPERT package [Design Expert (Software) ver 6.0, Stat-Ease, Inc., Hennepin Square, Suite 191, 2021 East Hennepin Avenue, Minneapolis, MN 554113] was used for regression and graphical analysis of the data obtained. The center points are used to determine the experimental error and the reproducibility of the data. The independent variables are coded to the (−2, 2) interval where the low and high levels are coded as −2 and +2, respectively. The axial points are located at (±α, 0, 0), (0, ±α, 0) and (0, 0, ±α) where α is the distance of the axial point from the center and makes the design rotatable. In this study, the α value was fixed at 2 (rotatable).

Table 1
Effect of laccase stability on combination of Guar Gum and Starch at 60 °C.

Temperature (°C)	Residual activity of Guar Gum (%)	Residual activity of starch (%)	Residual activity of Guar Gum (0.25%) + Starch (8%)
60	32.72 ± 0.29	16.17 ± 0.18	36 ± 0.31

The standard errors depicted are the averages of three independent experiments.

The experimental sequence was randomized in order to minimize the effects of uncontrolled factors. The three variables were Guar Gum % (X_1), starch % (X_2) and sodium acetate buffer, mM (X_3). Each variable was used to develop an empirical model which correlated the response to three variables using a second degree polynomial equation as given by the following equation:

$$Y = b_0 + \sum_{i=1}^{n} b_i x_i + \left(\sum_{i=1}^{n} b_{ii} x_i \right)^2 + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} b_{ij} x_i x_j$$

where Y is the predicted response, b_0 the constant coefficient, b_i the linear coefficients, b_{ij} the interaction coefficients, b_{ii} the quadratic coefficients and x_i, x_j are the coded values of the three additive variables. Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA).

2.6. Kinetic analysis

The kinetic analysis (K_m, V_{max} and K_{cat}) was performed using stabilized laccase under optimized condition.

2.7. Effect of temperature on dye decolorization

To find out the effect of stabilized laccase using optimized additives on reactive blue 198 decolorization, the reaction mixture was incubated at 60 °C with laccase. Appropriate controls were incorporated in the assay under standard conditions. Reaction mixture contained, 50 mg l^{-1} dye concentrations, 10 U ml^{-1} purified laccase in 100 mM sodium acetate buffer (pH 5.6) in a total volume of 1 ml.

3. Results and discussion

3.1. Thermostability of laccase using additives

Laccase stabilization using various additives to elicit the stabilization of laccase against detrimental temperature conditions is useful for industrial applications. The purified laccase obtained from *P. florida* NCIM 1243 was assessed by recovery yield of enzyme (47%) and purification fold (13). Table 1 shows the effect of combination of additives on laccase activity at 60 °C. The enzyme activity was increased when compared to the control (Table 1). The enhanced stability of laccase was in response to the combination of Guar Gum and starch (36%) than with respect to individual performance of Guar Gum and starch, respectively. Polyol, carbohydrates, organic salts, polymers and weakly hydrophobic surfactant are proven to be effective to stabilize protein and peptide drugs in solution (Baptista, Cabral, & Melo, 2000; Costa et al., 2002; Papinutti, Dimitriu, & Forchiassin, 2008; Fernandez, Villalonga, Cao, Alex, & Villalonga, 2004; Papinutti et al., 2008; Zheng, 2006). An increase of additives ($CuSO_4$ and mannitol) concentration was associated with slight increase in laccase activity at 40 °C (Papinutti et al., 2008).

Table 2
Experimental range and levels of independent additive variables (e.g.: 3 factors).

Z variables	Coded variables	Variable levels					Step change value, ΔZ_i
		−2	−1	0	+1	+2	
Guar Gum (%)	X_1	0.05	0.1	0.15	0.2	0.25	0.05
Starch (%)	X_2	4	5	6	7	8	1
Sodium acetate buffer pH 5.6 (mM)	X_3	20	40	60	80	100	20

Table 3
ANOVA for selected model.

Source	Sum of squares	Degree of freedom	Mean square	*f* value	*t* value
Model	9189.5	9	1021.1	119.4	0.0001
Error	85.5	10	8.5		
Cor total	9275.0	9			

Predicted residual sum of squares (PRESS) = 562.1, R^2 = 0.9908 and ADJ R^2 = 0.9825.

3.2. Response surface methodology

The results (Table 1) were used to evaluate the relationship between experimental factors (additives) and observed results, predicting the response and checking the adequacy of model. The range of variables investigated is given in Table 2. The variables were selected based on our preliminary experiments.

Table 3 shows the ANOVA of the regression model and the variables with higher effect were squared terms of Guar Gum (X_1^2) and sodium acetate buffer (X_3^2). The goodness of the fit model was checked by statistical criteria. The determination of coefficient (R^2 = 0.9908) indicates that only one percent of the total variation was not explained by the model. The Fisher *F* test (119.4) also demonstrates a high significance for the regression model. Each of the observed values (Y_j) was compared with the predicted value (Y) calculated from the model (Table 4). Table 5 shows that higher significance of linear, interaction term and squared terms were (X_1, X_3), ($X_1 \times X_3$) and (X_1^2, X_2^2, X_3^2) over the corresponding interaction terms ($X_1 \times X_2$), which indicate that optimum values for laccase stabilization lies within the experimental values chosen whereas, the linear term (X_2) and interaction term ($X_2 \times X_3$) are insignificant. Tables 6a and 6b explain the optimum predicted levels of Guar Gum (0.098%), starch (6.235%) and sodium acetate buffer (76.84 mM) for laccase stabilization at 60 °C using RSM.

The model can be shown as follows:

$$Y = 69.90693 - 12.91322X_1 - 2.17656X_2 + 13.11296X_3 - 9.64008X_1^2 - 8.93315X_2^2 - 12.11435X_3^2 - 5.62500X_1X_2 - 6.87500X_1X_3 + 0.62500X_2X_3 \quad (1)$$

In developing the regression equation the independent variables were coded according to the equation:

$$X_i = \frac{Z_i - Z_i^*}{\Delta Z_i} \quad (2)$$

where Z_i stands for the uncoded value of *i*th independent variable, Z_i^* denotes uncoded value of *i*th independent variable at center point and ΔZ_i is a step change value. The above equation can be converted into the uncoded unit where

$$X_1 = \frac{Z_1 - 0.15}{0.05} \quad (3)$$

$$X_2 = \frac{Z_2 - 6}{1} \quad (4)$$

$$X_3 = \frac{Z_3 - 60}{20} \quad (5)$$

Furthermore, an analysis of variance (ANOVA) for the response surface quadratic model is presented in Table 3, which also proves that this regression was statistically significant ($P < 0.0001$) at 99% of confidence level. The interaction between variables means that change in level of one variable affects the level of other variable for fixed level of enzyme stabilization.

Maximum and minimum principle of differential calculus was used to maximize Eq. (1) with respect to individual tested variables. The partial differential equations obtained are:

$$\frac{\partial Y}{\partial X_1} = -12.91322 - 19.28016X_1 - 5.62500X_2 - 6.87500X_3 \quad (6)$$

$$\frac{\partial Y}{\partial X_2} = -2.17656 - 5.62500X_1 - 17.8663X_2 + 0.62500X_3 \quad (7)$$

Table 4
Comparison of values obtained and predicted values for laccase stabilization.

Experiment	Coded variables			Laccase stabilization		
	X_1	X_2	X_3	Actual data, Y_j	Predicted data, Y	Residual values
T1	−1	−1	−1	32.0	29.3	3.0
T2	+1	−1	−1	32.0	28.5	3.5
T3	−1	+1	−1	36.0	35.0	1.0
T4	+1	+1	−1	14.0	12.0	2.3
T5	−1	−1	+1	68.0	68.0	−0.0
T6	+1	−1	+1	41.0	40.0	1.2
T7	−1	+1	+1	75.0	76.1	−1.2
T8	+1	+1	+1	25.0	25.3	−0.4
T9	−2	0	0	64.0	64.3	−0.4
T10	+2	0	0	18.0	21.0	−3.0
T11	0	−2	0	45.0	48.2	−3.2
T12	0	+2	0	41.0	41.0	0.0
T13	0	0	−2	9.0	13.5	−4.5
T14	0	0	0	59.0	69.9	1.3
T15	0	0	0	70.0	69.9	0.1
T16	0	0	0	69.0	69.9	−1.0
T17	0	0	0	68.0	69.9	−2.0
T18	0	0	0	72.0	69.9	2.1
T19	0	0	0	72.0	69.9	2.1
T20	0	0	0	69.0	69.9	−2.0

The data are the values obtained in triplicate assays with variations of <5%.

Table 5
Significance of regression coefficients.

Variable	Parameter estimate	Degree of freedom	Standard error	*f* value	*t* value
Intercept	69.9	1	1.1	58.6	
X_1	−13.0	1	0.8	−16.3	0.0001*
X_2	−2.1	1	0.8	−2.7	0.0204
X_3	13.1	1	0.8	16.5	0.0001*
$X_1 \times X_1$	−10.0	1	0.8	−12.5	0.0001*
$X_2 \times X_2$	−9.0	1	0.8	−11.6	0.0001*
$X_3 \times X_3$	−12.1	1	0.8	−15.7	0.0001*
$X_1 \times X_2$	−5.6	1	1.0	−5.4	0.0003*
$X_1 \times X_3$	−6.8	1	1.0	−6.6	0.0001*
$X_2 \times X_3$	0.6	1	1.0	0.6	0.5589

X_1, X_2 and X_3 represents Guar Gum, starch and sodium acetate buffer. Standard error of mean = 0.65.

* Significant level at $P < 0.001$.

Table 6a
Levels of variables on laccase stabilization at 60 °C.

Factor	Name	Level	Low level	High level	Uncoded values	Std. dev.	Coding
X_1	Guar Gum (%)	−0.88	−1.00	1.00	0.098	0.00	Actual
X_2	Starch (%)	0.23	−1.00	1.00	6.235	0.00	Actual
X_3	Sodium acetate buffer (mM)	0.62	−1.00	1.00	76.84	0.00	Actual

Table 6b
Prediction of laccase stabilization based on the variables shown in Table 6a.

Response	Prediction	Actual	SE mean	95% CI low	95% CI high	SE pred	95% PI low	95% PI high
R1	69.91	75.0	1.37	78.24	84.35	3.23	74.09	88.50

SE, standard error; CI, confidence interval; PI, probability index.

Table 7
Enzyme kinetics at stabilized condition.

Enzyme without additives at 60 °C			Enzyme with additives[a] at 60 °C			Enzyme with additives[b] at 60 °C		
K_m (mM)	V_{max} (μmol min^{-1} mg^{-1})	K_{cat} (s^{-1})	K_m (mM)	V_{max} (μmol min^{-1} mg^{-1})	K_{cat} (s^{-1})	K_m (mM)	V_{max} (μmol min^{-1} mg^{-1})	K_{cat} (s^{-1})
0.1	0.23	1.7	0.1	3.3	5.2	0.1	10.4	9.7

The data are the values obtained in triplicate assays with variations of <5%.
[a] Additives (Guar Gum, 0.25%; starch, 8%) without optimized condition.
[b] Additives (Guar Gum, 0.098%; starch, 6.235%; sodium acetate buffer, 76.84 mM) at optimized condition.

$$\frac{\partial Y}{\partial X_3} = 13.11296 - 6.87500X_1 + 0.62500X_2 - 24.2287X_3 \quad (8)$$

The second order differential equations are:

$$\frac{\partial^2 Y}{\partial X_1} = 19.28016 \quad (9)$$

$$\frac{\partial^2 Y}{\partial X_2} = 17.8663 \quad (10)$$

$$\frac{\partial^2 Y}{\partial X_3} = 24.2287 \quad (11)$$

The positive values of second order partial differential equations (Eqs. (6)–(8)) indicate the presence of local maximum and applicability of maximization. Eqs. (3)–(5) are equated to zero and solved for X_1, X_2 and X_3, which give the maximum value of Y.

$$-12.91322 - 19.28016X_1 - 5.62500X_2 - 6.87500X_3 = 0 \quad (12)$$

$$-2.17656 - 5.62500X_1 - 17.8663X_2 + 0.62500X_3 = 0 \quad (13)$$

$$13.11296 - 6.87500X_1 + 0.62500X_2 - 24.2287X_3 = 0 \quad (14)$$

Algebraic solution to the above equations (Eqs. (9)–(11)) were Guar Gum = −1.0383, starch = 0.2345 and sodium acetate buffer pH 5.6 = 0.8418 mM. These values correspond to the uncoded values of Z_1 = 0.098 (%), Z_2 = 6.235 (%) and Z_3 = 76.84 (mM). At these optimum values, Guar Gum and sodium acetate buffer were selected as significant interactive variables and the maximum predicted residual enzyme activity for stabilization of laccase at 60 °C was 69.91%. The Guar Gum and starch in combination stabilized the laccase up to 36%, whereas after optimization using RSM, it imparted 75% of thermostability to laccase which is 39% increase when compared to the unoptimized thermostabilization. After optimization, the enhancement of laccase thermostabilization was 2.16 fold. This may be due to gelling nature of Guar Gum and stabilization effect of sugar (starch) because of preferential exclusion of the protein surface from contact of solvent (normally aqueous medium) (Lee & Timasheff, 1981). Therefore statistical approach plays an important role in optimizing the components for increasing the stability of laccase at high temperature. The good correlation between these two results verifies the validity of response model and the existence of an optimal point.

Table 8
Decolorization of reactive blue 198 using optimized laccase.

Contents	Decolorization of RB198 (%)
Free enzyme	2
Laccase with Guar Gum (0.25%)	36
Laccase with starch (8%)	14
Laccase with combination of Guar Gum (0.25%) and starch (%)	49
Laccase with stabilized laccase under optimized additives	91

3.3. Kinetics of laccase at stabilized condition

The determination of kinetic activity in the stabilized laccase against ABTS was performed at 60 °C with optimized condition. As shown in Table 7, the V_{max} (10.4 μmol min^{-1} mg^{-1}) and catalytic efficiency (9.7 s^{-1}) for optimized laccase was increased at K_m = 0.1 mM when compared to the control. This result indicates the confirmation of laccase thermostabilization using additives at optimized condition.

3.4. Effect of optimized additives on dye decolorization

Table 8 shows the combination of Guar Gum, starch and sodium acetate buffer which increases the decolorization rate up to 91% when compared to the control (2%) at 60 °C. In this study, higher decolorization rate was observed at 60 °C for reactive blue 198 using optimized laccase supplemented with additives.

4. Conclusion

The combination of Guar Gum, starch and sodium acetate buffer may prevent the enzyme denaturation and retain the laccase activity for long period to effectively work in solution state itself. Studies in the area of stabilization of industrially important enzymes using RSM as a tool are limited. This approach from our study enlightens the use of RSM towards optimizing the components for stabiliza-

tion of industrially important enzymes. Thus RSM, which includes factorial designs and regression analysis, can better deal with multifactor influence on experiments towards optimizing the conditions for laccase stabilization.

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