Ann Microbiol (2015) 65:185–196
DOI 10.1007/s13213-014-0849-4

ORIGINAL ARTICLE

Optimization and hyper production of laccase from novel agaricomycete *Pseudolagarobasidium acaciicola* AGST3 and its application in in vitro decolorization of dyes

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Received: 17 August 2013 / Accepted: 12 February 2014 / Published online: 14 March 2014

Abstract A novel white rot fungus *Pseudolagarobasidium acaciicola* AGST3 was investigated for the production of laccase under solid state fermentation. The effects of fourteen medium components were screened by the initial screening method of Plackett-Burman. Each of the components was screened on the basis of a 'p' (probability value) that was above 95 % confidence level. Tween 80, $CuSO_4 \cdot 5H_2O$, glucose and $FeC_6H_5O_7 \cdot NH_4OH$ were identified as significant components for laccase production. Central composite design using response surface methodology with four significant variables was used in this study to optimize significant correlations between the effects of these variables on laccase production. The optimum concentrations of Tween 80, glucose, $FeC_6H_5O_7 \cdot NH_4OH$ and $CuSO_4 \cdot 5H_2O$ were (g/l): 0.6625, 0.437, 0.037 and 0.6625, respectively, for maximum laccase production. Compared to the unoptimized medium, the laccase production (5.35×10^5 U/g of substrate) increased 1.6-fold under optimized condition. Furthermore, the crude laccase obtained was used for the decolorization of structurally different dyes, and 11–96 % of decolorization was obtained after 24 h of incubation.

Keywords *Pseudolagarobasidium acaciicola* · Laccase · Solid state fermentation · Plackett-Burman design · Response surface methodology · Decolorization

Mathematical notation

ε Molar extinction coefficient

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Introduction

Laccase (benzenediol: oxygen oxidoreductase, E.C. 1.10.3.2) is a multinuclear copper-containing enzyme that oxidizes diverse substrates and exists widely in nature (Liu et al. 2009; Thakur et al. 2012). Most of the laccases studied are from fungal origin, especially from white rot fungi (Abou-Mansouri et al. 2009; Fu et al. 2013). Laccases catalyze the oxidation of a variety of phenolic and non-phenolic compounds with a concomitant reduction of molecular oxygen to water (Zhang et al. 2012). The broad substrate specificity of laccase results in a large number of biotechnological applications. These include decolorization and detoxification of textile dyes and effluents, biobleaching, biopulping, and transformation of antibiotics, steroids, and other aromatic compounds (Cerrone et al. 2011; Dhillon et al. 2012; Thakur et al. 2012). Thus, the production of laccase in large amounts at a low cost is required, and hence, the current focus is towards the search for an efficient production system. The use of solid state fermentation (SSF) as a method of laccase production can offer certain advantages, such as high volumetric productivity, concentrated product, lower capital investment and operating cost (Chhaya and Gupte 2010). It is known that the production of laccase is affected by media components. The development of an economically productive medium requires selection of components such as carbon, nitrogen and metal ions (Liu et al. 2009). Large numbers of reports are available on the optimization of the media components by means of the classical methods of changing one independent variable, while fixing other variables at definite levels. Compared to the classical methods, statistical experimental designs are useful tools to screen the main variables rapidly from a multivariable system for medium optimization. Less process variability, overall cost and closer confirmations are a few advantages of the statistical design. Plackett-Burman (PBD) and response surface methodology (RSM) have been successfully applied for the optimization of laccase production

by various researchers (Liu et al. 2009; Chhaya and Gupte 2010; Tinoco et al. 2011). The present work is directed towards the medium optimization for the maximum production of laccase by novel fungal isolate *Pseudolagarobasidium acaciicola* AGST3. Until now, there have been no reports on the medium optimization for enhancing laccase production by the genus *Pseudolagarobasidium* (Agaricomycetes) belonging to the phylum Basidiomycota, Phanerochaetaceae family and order polyporales. Furthermore, the laccase produced was used for the in vitro dye decolorization of chemically different dyes.

Materials and methods

Isolation and screening of new fungal strain

Different samples, such as decayed wood, tree bark and mushrooms, were collected from Vallabh Vidyanagar, Vadtal and nearby regions of Anand District, Gujarat, India. Samples were transferred on 2 % Malt extract agar (MEA) plates with antibiotic streptomycin (25 μg/ml), and incubated at 30°C for 8–10 days. Samples were then screened for the presence of laccase enzyme. Primary screening was carried out on Sabouraud dextrose agar plate containing 0.01 % ortho-dianisidine and guaiacol.

Cultivation conditions for laccase production under SSF

Five grams of wheat bran was added to 250 ml Erlenmeyer flasks and moistened with a medium described by Asther et al. (1988) to give a final substrate to moisture ratio of 1:4 (w/v). The moistening medium containing (g/l) KH_2PO_4 0.2, $CaCl_2{\cdot}2H_2O$ 0.0132, $MgSO_4{\cdot}7H_2O$ 0.05, $FeC_6H_5O_7{\cdot}NH_4OH$ (ammonium ferric citrate) 0.085, $ZnSO_4{\cdot}7H_2O$ 0.0462, $MnSO_4{\cdot}7H_2O$ 0.035, $CoCl_2{\cdot}6H_2O$ 0.007, $CuSO_4{\cdot}5H_2O$ 0.007, L-aspargine 1.0, NH_4NO_3 0.5, thiamine-HCl 0.0025, yeast extract 0.5, Glucose 10, Tween-80 0.1, and pH 5.0 was used. Each flask was inoculated with five agar plugs (9 mm diameter) of actively growing fungal mycelia from MEA plate and incubated at 30°C for 12 days.

The enzyme was extracted using 100 mM sodium acetate buffer (pH 5.0), and the contents were filtered through a muslin cloth. The extract obtained was then centrifuged at 8000×*g* for 20 min at 4 °C, and the clear supernatant obtained was used as a source of laccase.

Experimental design and statistical analysis

Screening of important medium components by Plackett-Burman Design (PBD)

PBD aims to select the most important components that influence overall enzyme production in the system or medium. In the present study, total fourteen medium components (k=14) were selected for study, with each variable being represented at two levels; high (+) and low (−). The numbers of positive and negative signs per trial are (k+1)/2 and (k − 1)/2, respectively. Each row represents a trial and each column represents an independent (assigned) or dummy (unassigned) variable. The effect of each variable was determined by the following Equation:

$$E(x_i) = 2\left(\sum M_i^+ - M_i^-\right)/N \quad (1)$$

where $E(xi)$ is the concentration effect of the tested variable, and M_i^+ and M_i^- are the laccase production from the trial where the variable (xi) measured was estimated by calculating the variance among the dummy variables as follows:

$$V_{eff} = \sum\left(E_d{}^2\right)/n \quad (2)$$

where V_{eff} is the variance of the concentration effect, E_d is the concentration effect for the dummy variables, and n is the number of dummy variables. The standard error (S.E.) of the concentration effect was the square root of the variance of an effect and the significance level (p value) of each concentration effect was determined using student's t test.

$$t(x_i) = Ex_i/\text{S.E.} \quad (3)$$

where, Ex_i is the effect of variable x_{i}.

Response Surface Methodology (RSM)

RSM consists of an empirical modeling system that evaluates the relationship between a group of independent variables and observed responses (Revankar et al. 2007). RSM was used to optimize the screened components by PBD for enhanced laccase production using central composite design (CCD). The effect of each component on laccase production was studied at five different levels, viz., −α, −1, 0, +1, +α. The behavior of the system was explained by the following quadratic Equation:

$$Y = \beta_0 + \sum\beta_i x_i + \sum\beta_{ij} x_i x_j + \sum\beta_{ii} x_i^2 \quad (4)$$

where Y is predicted response, β_0 is the offset term, βi is the linear coefficient, βii is the squared coefficient, βij is the interaction effect, and x_i is the dimensionless coded value of X_i. The above equation was solved using the software Design-Expert (Version 7.0.2, State ease inc., USA). A 2^5 factorial design with five replicates at the center point with a total number of 30 trials was employed. The coded and uncoded values of the variables at various levels are given in Table 4.

Laccase assay

Laccase activity (E.C. 1.10.3.2) was determined by monitoring the A_{420}change related to the rate of oxidation of 2,2-azino-bis-3-ethyl-benzthiozoline-6-sulphonic acid (ABTS, ε=36,000 cm^{-1} M^{-1}) at 30 °C for 3 min (Niku-Paavola et al. 1990). The reaction mixture contained 100 μl of 50 mM ABTS, 800 μl of 100 mM Na-Acetate buffer (pH 5.0), and 100 μl of appropriately diluted enzyme extract. One unit of enzyme activity (U) was defined as the amount of enzyme that leads to the oxidation of 1 μM of substrate/min under the standard assay condition.

In vitro decolorization of dyes by laccase produced in-house

Decolorization activity was determined by measuring the decrease in the dye absorbance at the respective absorption maxima. Twenty-one different dyes (Violet P3P, Blue M4GB, Green ME4BL, Acid Red BN, Blue 3R, Brown 18, Acid green 10H, Reactive red M5B, Direct black 22, Acid black 194, Green HE4G, Reactive red C2G, Red BS, Violet 5B, Reactive red HE8B, Direct orange 5B, Direct red 4BS, Turquoise blue H5G, Direct red 12B, Reactive turquoise blue G and Acid red F2R) were investigated with crude filtrate of enzyme. The reaction mixture contained 100 mM Sodium acetate buffer (pH 5.0), dye (100 mg/l) and crude enzyme filtrate (final concentration 5000 U/ml). The reaction was initiated with enzyme and incubated at 30 °C under shaking condition (150 rpm). Samples were withdrawn at regular time intervals and subsequently analyzed for decolorization. Absorbance was recorded at the respective absorption maxima using Shimadzu UV-1800, Japan. A control in which the crude enzyme filtrate was replaced by denatured enzyme was kept in parallel. Dye decolorization efficiency was expressed in terms of percentage (%) as compared to initial concentration of dye used. All the experiments were carried out in triplicate, and the data represent the mean values of the experiments.

Results and discussion

Isolation and screening of new fungal strain

One of the parameters widely used in the detection of ligninolytic enzymes is the chromogen. In the present study, ortho-dianisidine and guaiacol were used as a chromogen. The dark brown colored zone surrounding the mycelia of the culture on the plate supplemented with ortho-dianisidine and guaiacol was an indication of Bevandamm's reaction. Out of fifteen isolates, five isolates showed positive Bevandamm's reaction for laccase activity and were selected for further quantitative detection of laccase enzyme. Similar results have also been reported by Vishwanath et al. (2008), Patel et al. (2009) and Gao et al. (2011). The culture designated as AGST3 showed highest laccase activity (3.30×10^5 U/g of substrate) among five positive isolates. The identification of isolates was further corroborated by studies on their ITS1 and ITS2 gene sequences, carried out by Xplorigen, Pvt. Ltd. (New Delhi, India). The isolate AGST3 was identified as *Pseudolagarobasidium acaciicola* AGST3 (GenBank accession no. HQ323693).

Screening of important medium components for laccase production by PBD

PBD is a saturated fractional factorial design that allows a balanced estimation of all main components with the smallest possible variance. Moreover, the design is also orthogonal in nature (Plackett and Burman 1946; Joshi et al. 2012). In the present study, PBD was applied for the screening of important medium components for the production of laccase. The independent variables and their respective high and low concentrations are shown in Table 1. Table 2 shows the PBD matrix of 20 trials and the corresponding laccase production in terms of units per gram of substrate. The variables X_1-X_{14} signify the medium components, whereas D_1-D_5 represents the dummy variables. Table 3 represents the effect, standard error, t (x_i), p value and confidence level for each component. The components were screened at a confidence level of 95 % on the basis of their effects. The confidence levels for X_1 (glucose), X_4 ($CuSO_4{\cdot}5H_2O$), X_8 ($FeC_6H_5O_7{\cdot}NH_4OH$), X_{12} (Tween 80) were above 95 %, and were hence considered as significant while the remaining components were considered to be insignificant. The components having 95 % or above confidence level but having a negative effect (glucose and

Table 1 Variables showing medium components used in Plackett-Burman design

Variables	Medium component	+ Values (g/l)	- Values (g/l)
X1	Glucose	10	1
X2	Yeast extract	0.2	0.02
X3	Ammonium nitrate	0.5	0.05
X4	$CuSO_4{\cdot}5H_2O$	0.007	0.0007
X5	$CoCl_2{\cdot}6H_2O$	0.007	0.0007
X6	$MnSO_4{\cdot}7H_2O$	0.035	0.0035
X7	$ZnSO_4{\cdot}7H_2O$	0.0462	0.00462
X8	$FeC_6H_5O_7{\cdot}NH_4OH$	0.085	0.0085
X9	$MgSO_4{\cdot}7H_2O$	0.05	0.005
X10	$CaCl_2{\cdot}2H_2O$	0.0132	0.00132
X11	KH_2PO_4	0.2	0.02
X12	Tween 80	0.1	0.01
X13	Aspargine	1.0	0.1
X14	Thiamine	0.0025	0.00025

Table 2 Plackett-Burman design matrix of fourteen variables (X1–X14) and five dummy variables (D1–D5) along with observed response (laccase production)

Run no.	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	X_{12}	X_{13}	X_{14}	D_1	D_2	D_3	D_4	D_5	Laccase (U/g of substrate)
1	+	+	-	-	+	+	+	+	-	+	-	+	-	-	-	-	+	+	-	5.55×10^4
2	-	+	+	-	-	+	+	+	+	-	+	-	+	-	-	-	-	+	+	3.62×10^4
3	+	-	+	+	-	-	+	+	+	+	-	+	-	+	-	-	-	-	+	1.82×10^5
4	+	+	-	+	+	-	-	+	+	+	+	-	+	-	+	-	-	-	-	2.65×10^5
5	-	+	+	-	+	+	-	-	+	+	+	+	-	+	-	+	-	-	-	8.50×10^4
6	-	-	+	+	-	+	+	-	-	+	+	+	+	-	+	-	+	-	-	8.10×10^4
7	-	-	-	+	+	-	+	+	-	-	+	+	+	+	-	+	-	+	-	4.66×10^5
8	-	-	-	-	+	+	-	+	+	-	-	+	+	+	+	-	+	-	+	6.23×10^4
9	+	-	-	-	-	+	+	-	+	+	-	-	+	+	+	+	-	+	-	1.50×10^5
10	-	+	-	-	-	-	+	+	-	+	+	-	-	+	+	+	+	-	+	6.79×10^4
11	+	-	+	-	-	-	-	+	+	-	+	+	-	-	+	+	+	+	-	6.65×10^4
12	-	+	-	+	-	-	-	-	+	+	-	+	+	-	-	+	+	+	+	5.00×10^5
13	+	-	+	-	+	-	-	-	-	+	+	-	+	+	-	-	+	+	+	3.91×10^4
14	+	+	-	+	-	+	-	-	-	-	+	+	-	+	+	-	-	+	+	7.40×10^4
15	+	+	+	-	+	-	+	-	-	-	-	+	+	-	+	+	-	-	+	2.82×10^4
16	+	+	+	+	-	+	-	+	-	-	-	-	+	+	-	+	+	-	-	1.20×10^5
17	-	+	+	+	+	-	+	-	+	-	-	-	-	+	+	-	+	+	-	6.86×10^5
18	-	-	+	+	+	+	-	+	-	+	-	-	-	-	+	+	-	+	+	2.41×10^5
19	+	-	-	+	+	+	+	-	+	-	+	-	-	-	-	+	+	-	+	1.30×10^5
20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	7.93×10^5

$FeC_6H_5O_7{\cdot}NH_4OH$) have an effect on laccase production, but the amount required is lower than the indicated low (−) concentration in the Plackett-Burman experiment. Moreover, a positive effect ($CuSO_4{\cdot}5H_2O$, Tween 80) indicates a requirement of high concentration for increased laccase production. The results obtained indicate that the PBD is a very useful statistical method for screening important medium components for laccase production.

Optimization of screened medium components by RSM (CCD)

The experimental design performed by the RSM method is based on mathematical techniques that enable us to investigate the interactions between variables of the medium components. Plackett–Burman results indicated $CuSO_4{\cdot}5H_2O$, Tween 80, glucose and $FeC_6H_5O_7{\cdot}NH_4OH$ had a significant influence on

Table 3 Statistical analysis of medium components in relation to laccase production as per Plackett-Burman design

Factors	Medium component	Effect (Exi)	S.E.	t (xi)	P value	Confidence level (%)
X1	Glucose	−67874.8	16132.38	−4.2	0.008	99
X2	Yeast extract	−35859	16132.38	−2.22	0.07	93
X3	Ammonium nitrate	−240061	16132.38	−14.88	2.4×10^{-5}	ND
X4	$CuSO_4{\cdot}5H_2O$	119784.7	16132.38	7.42	0.0007	99.99
X5	$CoCl_2{\cdot}6H_2O$	−197208	16132.38	−12.22	6.4×10^{-5}	ND
X6	$MnSO_4{\cdot}7H_2O$	−66306	16132.38	−4.11	0.009	99
X7	$ZnSO_4{\cdot}7H_2O$	−16799	16132.38	−1.041	0.34	66
X8	$FeC_6H_5O_7{\cdot}NH_4OH$	−93493.6	16132.38	−5.79	0.002	99
X9	$MgSO_4{\cdot}7H_2O$	−276042	16132.38	−17.1	1.2×10^{-5}	ND
X10	$CaCl_2{\cdot}2H_2O$	−73295	16132.38	−4.5	0.006	99
X11	KH_2PO_4	−11465	16132.38	−0.71	0.51	49
X12	Tween 80	46591	16132.38	2.88	0.034	96.6
X13	Aspargine	−57264	16132.38	−3.54	0.016	98.4
X14	Thiamine	−32666	16132.38	−2.02	0.09	91

laccase production, and these were further selected for optimization of their concentration by CCD. A total of 30 experiments with four variables (components of the medium) and five coded levels (five different concentrations) were performed. Based on the results of PBD, components that are significant were set at their higher or lower level, and the other components were set at their middle level in CCD. Table 4 documents the experimental design, concentration of different variables, and their actual and predicted values of laccase production. The quadratic model was used to explain the mathematical relationship between the independent variables and the dependent response. The mathematical expression of the relationship to the predicted response '*Y*' of the laccase production with the variables A ($CuSO_4 \cdot 5H_2O$), B (Tween 80), C (glucose) and D ($FeC_6H_5O_7 \cdot NH_4OH$) is shown below in terms of coded factors:

$$Y = 415730.83 + 11159.58\,A - 17847.16\,B - 7961.25C - 2004.66\,D + 11776.12\,AB - 26108.12\,AC - 20756\,AD + 9308.12\,BC - 28894.5\,BD + 37471.25\,CD + 3469.33\,A^2 + 7268.45\,B^2 - 2976.91\,C^2 + 1241.08\,D^2$$

The statistical significance of the quadratic model for the experimental responses was evaluated by the analysis of variance (ANOVA). The results of ANOVA are shown in Table 5. The ANOVA for the selected quadratic model showed that the model was significant with lack of fit, model *F* and model *P*>*F* values of 0.2699, 34.59 and <0.0001, respectively. The

Table 4 Central composite design matrix with coded values and actual values for laccase production

Run no.	$CuSO_4 \cdot 5H_2O$		Tween 80		Glucose		$FeC_6H_5O_7 \cdot NH_4OH$		Actual response	Predicted response
	Actual value	Coded value	Actual value	Coded value	Actual value	Coded value	Actual value	Coded value		
1	0.55	0	0.55	0	0.775	+2	0.0465	0	3.79×10^5	3.87×10^5
2	0.6625	+1	0.4375	−1	0.6625	+1	0.037	−1	3.63×10^5	3.54×10^5
3	0.55	0	0.325	−2	0.55	0	0.0465	0	4.79×10^5	4.80×10^5
4	0.6625	+1	0.4375	−1	0.6625	+1	0.056	+1	4.46×10^5	4.42×10^5
5	0.4375	−1	0.4375	−1	0.6625	+1	0.056	+1	5.31×10^5	5.37×10^5
6	0.4375	−1	0.4375	−1	0.6625	+1	0.037	−1	3.68×10^5	3.66×10^5
7	0.6625	+1	0.6625	+1	0.6625	+1	0.037	−1	4.11×10^5	4.19×10^5
8	0.6625	+1	0.6625	+1	0.4375	−1	0.056	+1	3.55×10^5	3.65×10^5
9	0.55	0	0.55	0	0.325	−2	0.0465	0	4.21×10^5	4.19×10^5
10	0.55	0	0.55	0	0.55	0	0.0655	+2	4.12×10^5	4.16×10^5
11	0.4375	−1	0.6625	+1	0.6625	+1	0.037	−1	3.92×10^5	3.84×10^5
12	0.55	0	0.775	+2	0.55	0	0.0465	0	4.02×10^5	4.09×10^5
13	0.4375	−1	0.6625	+1	0.6625	+1	0.056	+1	4.47×10^5	5.37×10^5
14	0.4375	−1	0.4375	−1	0.4375	−1	0.056	+1	4.43×10^5	4.42×10^5
15	0.4375	−1	0.6625	+1	0.4375	−1	0.056	+1	3.15×10^5	3.08×10^5
16	0.55	0	0.55	0	0.55	0	0.0465	0	4.10×10^5	4.15×10^5
17	0.4375	−1	0.6625	+1	0.4375	−1	0.037	−1	3.91×10^5	4.04×10^5
18	0.6625	+1	0.4375	−1	0.4375	−1	0.056	+1	4.60×10^5	4.54×10^5
19	0.55	0	0.55	0	0.55	0	0.0465	0	4.28×10^5	4.15×10^5
20	0.775	+2	0.55	0	0.55	0	0.0465	0	4.49×10^5	4.51×10^5
21	0.55	0	0.55	0	0.55	0	0.0465	0	4.07×10^5	4.15×10^5
22	0.325	−2	0.55	0	0.55	0	0.0465	0	4.02×10^5	4.07×10^5
23	0.6625	+1	0.6625	+1	0.6625	+1	0.056	+1	3.96×10^5	3.90×10^5
24	0.55	0	0.55	0	0.55	0	0.0465	0	4.25×10^5	4.15×10^5
25	0.6625	+1	0.6625	+1	0.4375	−1	0.037	−1	5.64×10^5	5.43×10^5
26	0.55	0	0.55	0	0.55	0	0.0465	0	4.16×10^5	4.15×10^5
27	0.55	0	0.55	0	0.55	0	0.0465	0	4.04×10^5	4.15×10^5
28	0.6625	+1	0.4375	−1	0.4375	−1	0.037	−1	4.99×10^5	5.16×10^5
29	0.55	0	0.55	0	0.55	0	0.0275	−2	4.22×10^5	4.24×10^5
30	0.4375	−1	0.4375	−1	0.4375	−1	0.037	−1	4.33×10^5	4.24×10^5

Table 5 Analysis of variance (ANNOVA) for the quadratic model

Source	Sum of squares	Mean df	*F* Square	*p* value	Prob>*F*
Model	71628298055	14	5116307004	34.5922393	< 0.0001
A-$CuSO_4{\cdot}5H_2O$	2988871204	1	2988871204	20.20827676	0.0004
B-Tween 80	7644512593	1	7644512593	51.68587592	< 0.0001
C-Glucose	1521156038	1	1521156038	10.28479988	0.0059
D-$FeC_6H_5O_7{\cdot}NH_4OH$	96448522.67	1	96448522.67	0.652105195	0.4320
AB	2218833920	1	2218833920	15.00192109	0.0015
AC	10906147056	1	10906147056	73.7383524	< 0.0001
AD	6892984576	1	6892984576	46.60466461	< 0.0001
BC	1386259056	1	1386259056	9.372737987	0.0079
BD	13358274084	1	13358274084	90.31760867	< 0.0001
CD	22465513225	1	22465513225	151.8932326	< 0.0001
A^2	330137795	1	330137795	2.232118909	0.1559
B^2	1449064774	1	1449064774	9.79737834	0.0069
C^2	243072900.8	1	243072900.8	1.643458054	0.2193
D^2	42247895.05	1	42247895.05	0.285645348	0.6009
Residual	2218549785	15	147903319		
Lack of Fit	1734457286	10	173445728.6	1.791452346	0.2699

R^2 =0.969, Adj R^2 =0.941

value of the adjusted determination coefficient (AdjR^2= 0.9699) was also very hig,h reconfirming the significance of the model. The lack of fit (0.2699) was found to be not significant. This indicates an excellent correlation between the experimental and predicted values of laccase production. The adequate precision value measures signal-to-noise ratio, and a ratio greater than 4.0 is desirable. In the present study, the value of this ratio was higher for laccase production, and suggested that the polynomial quadratic model can be used to navigate the design space and further optimization. Three-dimensional (3-D) surface plots were obtained when the data of laccase production were fed into the design expert software (version 7.0.2, Stat ease Inc., USA). The 3-D surface plots demonstrate the response over a region of interesting factor levels, the relationship between the response and experimental levels of each variable, and the type of interactions between the test variables in order to assume the optimal composition of the culture medium. Here, each 3-D surface plot represents the effect of two medium components at their studied concentration range and at fixed concentration of the remaining two medium components. The value of the remaining two components was then varied for that situation, using the software to determine the optimum values. The interaction between $CuSO_4{\cdot}5H_2O$ and Tween 80 (Fig. 1a) indicated that laccase production was minimal at lower concentrations of $CuSO_4{\cdot}5H_2O$ and higher concentrations of Tween 80. Moreover, at higher concentrations of $CuSO_4{\cdot}5H_2O$, the laccase production increased with decreasing concentrations of tween 80. 3-D surface plot (Fig. 1b) shows the effect of glucose and $CuSO_4{\cdot}5H_2O$ on laccase production. Lower laccase production was observed at low concentrations of $CuSO_4{\cdot}5H_2O$ and glucose, while at higher concentrations of glucose with gradual increase in $CuSO_4{\cdot}5H_2O$ concentration, significant increase in laccase production was observed. The same trend of results was also observed in the case of $FeC_6H_5O_7{\cdot}NH_4OH$ and $CuSO_4{\cdot}5H_2O$ (Fig. 1c). It is possible to observe in Fig. 2a that the laccase production was maximal at lower concentrations of both Tween 80 and glucose. Although at higher concentrations of glucose, the laccase production starts to increase with decreasing tween 80 concentration. Figure 2b depicts the effect of $FeC_6H_5O_7{\cdot}NH_4OH$ and Tween 80 on laccase production. Decreased laccase production was observed at higher concentrations of both components, while at lower concentrations of $FeC_6H_5O_7{\cdot}NH_4OH$, decreasing concentration of tween 80 results in increased laccase production. The interaction between glucose and $FeC_6H_5O_7{\cdot}NH_4OH$ (Fig. 2c) indicates that the laccase production increased at higher concentrations of ammonium ferric citrate, with decrease in glucose concentrations. Thus, the predicted optimal concentrations for the components were as follows (g/l): $CuSO_4{\cdot}5H_2O$ 0.6625, Tween 80 0.6625, glucose 0.437, $FeC_6H_5O_7{\cdot}NH_4OH$ 0.037. The model predicted a value of 5.31×10^5 U/g of substrate for laccase production using the above optimal concentration of variables.

Validation of the model

Model validation was performed (Fig. 3) using statistically optimized media components, and results showed a higher laccase production of 5.35×10^5 U/g of substrate (9.55×10^4

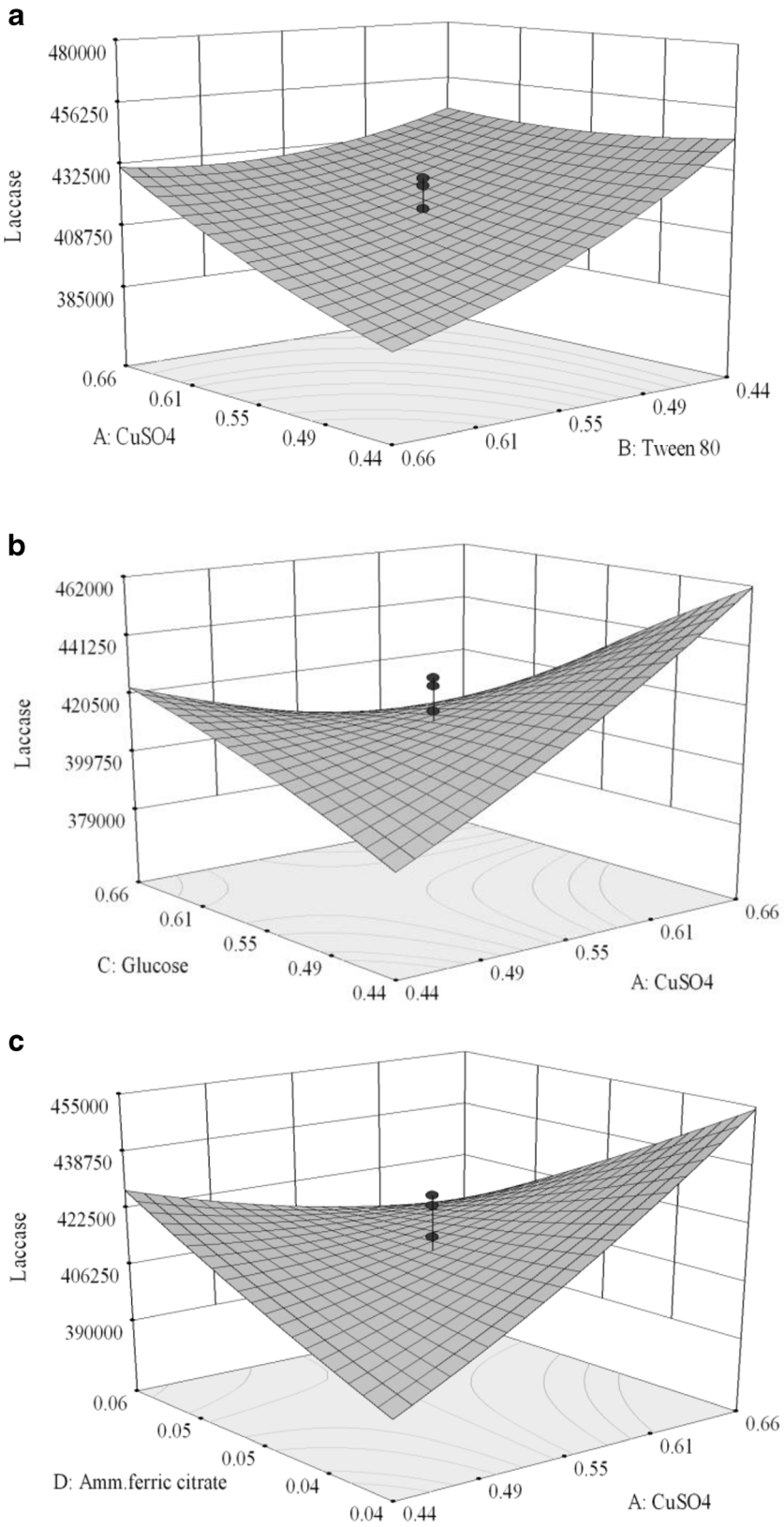

Fig. 1 (**a**) Three-dimensional plot showing the effect of $CuSO_4 \cdot 5H_2O$ and Tween 80. (**b**) Three-dimensional plot showing effect of $CuSO_4 \cdot 5H_2O$ and glucose. (**c**) Three-dimensional plot showing effect of $CuSO_4 \cdot 5H_2O$ and $FeC_6H_5O_7 \cdot NH_4OH$ (ammonium ferric citrate)

U/ml) in optimized media as compared to unoptimized media (3.30×10^5 U/g of substrate & 5.8×10^4 U/ml). Thus, a 1.6-fold increase in laccase production was obtained by statistical optimization. In earlier studies, other researchers have also used statistical methods for medium optimization to improve laccase production using white rot fungi (Revankar et al.

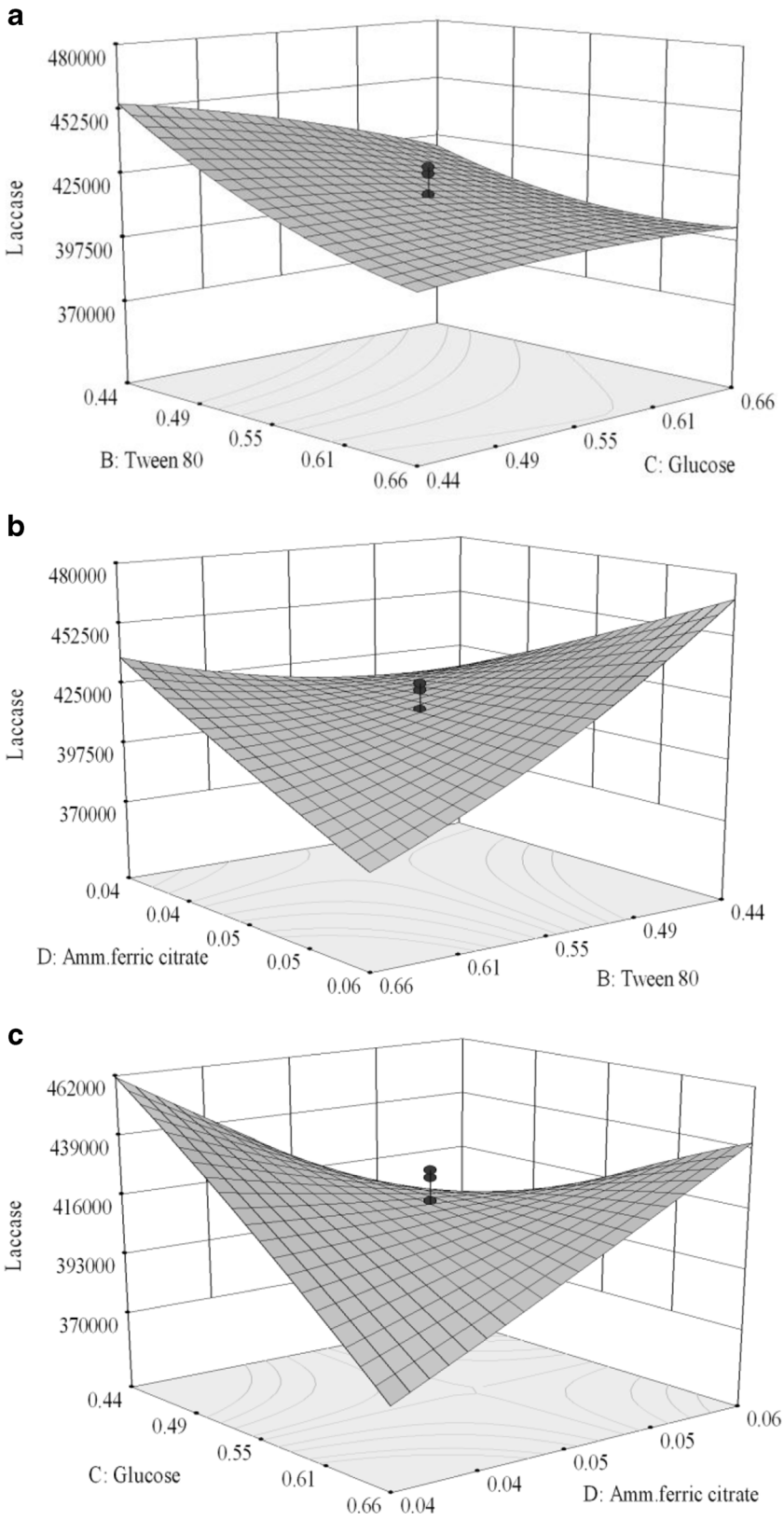

Fig. 2 (**a**) Three-dimensional plot showing effect of Tween 80 and glucose. (**b**) Three-dimensional plot showing effect of Tween 80 and $FeC_6H_5O_7{\cdot}NH_4OH$. (**c**) Three-dimensional plot showing effect of glucose and $FeC_6H_5O_7{\cdot}NH_4OH$

2007; Liu et al. 2009; Tinoco et al. 2011). However, this is the first report of laccase production from *Pseudolagarobasidium acaciicola* AGST3. Revankar et al. (2007) reported a four-fold higher production of laccase with statistically optimized medium CCD using *Ganoderma* sp. Similarly, a three-fold increase in laccase production has also been reported by

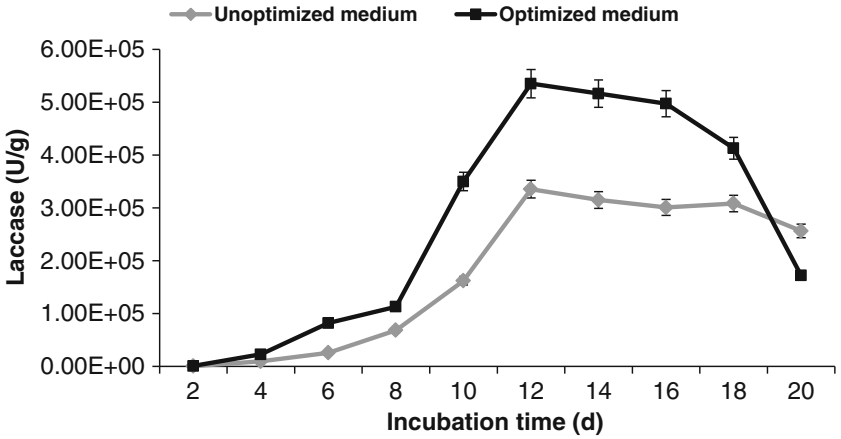

Fig. 3 Time course profile of laccase production with unoptimized medium and optimized medium using *Pseudolagarobasidium acaciicola* AGST3

Niladevi et al. (2007). CCD was performed for the production of laccase from *Fusarium incarnatum* LD-3, and a 16-times higher production of laccase was obtained as compared to unoptimized medium (Chhaya and Gupte 2010). Liu et al. (2009) also reported the application of statistical methods and obtained a 2.5-times higher laccase yield as compared to initial unoptimized medium using *Pleurotus ostreatus* strain 10969. Zhang et al. (2012) reported 1.87-fold increases in laccase production by *Mycena purpureofusca* using PBD followed by the RSM method. The data obtained corroborates the findings of Gao et al. (2013), who reported the optimization of various parameters of fermentation for the production of laccase using PBD and RSM. The laccase yield from *Trichoderma harzianum* ZF-2 was 59.68-times higher in optimized media as compared to unoptimized media. Similarly, Niladevi et al. (2009) reported that statistical optimization by RSM resulted in a three-fold increase in laccase production by *Streptomyces psammoticus* MTCC 7334. Moreover, Mishra et al. (2008) reported maximum laccase production of 163.32 U/g of substrate using *Coriolus versicolor* MTCC138 under statistically optimized media as compared to unoptimized media (65.42 U/g of substrate). Poojary and Mugeraya (2012) also reported that the laccase yield from *Phellinus noxius hp*F17 increased up to 780 U/l, an approximately 1.4-fold improvement in laccase production under optimized media as compared to unoptimized media (545.50 U/l).

In vitro decolorization of dyes by laccase produced in-house

The use of crude enzymes is currently a requirement for application in environmental engineering because of the high cost related to the enzyme purification procedure (Yu et al. 2006). In the present study, we assessed the potential of crude in-house produced laccase from *Pseudolagarobasidium acaciicola* AGST3. Figure 4 shows the time course profile of in vitro dye decolorization of Violet P3P, Green ME4BL and Blue 3R by crude laccase produced in-house. Maximum decolorization of Violet P3P, Green ME4BL and Blue 3R was found to be 96±1.2 %, 76±0.65 % and 85.8±0.91 %, respectively, in 24 h. Table 6 depicts the percent decolorization of different dyes along with their maximum wavelength (λ_{max}). The results indicate that laccase produced in-house decolorized structurally different dyes with variable decolorization efficiencies between 11 and 96 % after 24 h of incubation. The difference in the decolorization rates of different dyes could be due to chemical structural differences, electron distribution, and steric factors between the dyes that affect their decolorization (Knapp et al. 1995; Levin et al. 2010). There are few

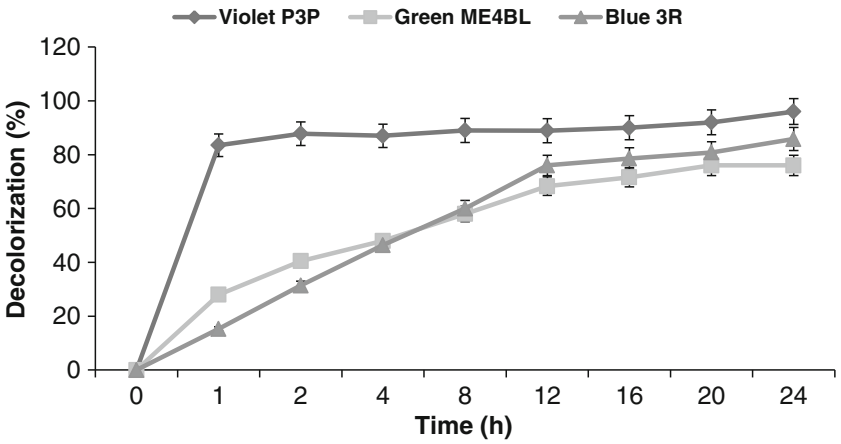

Fig. 4 Time course profile of in vitro dye decolorization by crudelaccase produced in-house

Table 6 Different dyes used for decolorization with laccase produced in-house

Dye	Maximum wavelength (λ_{max}) in nm	Decolorization (%) after 24 h
Violet P3P	560	96.00±1.20
Blue M4GB	616	52.00±0.80
Green ME4BL	639	76.00±0.65
Blue 3R	577	85.80±0.91
Acid red BN	532	54.60±0.54
Green HE4G	646	58.70±0.38
Acid black 194	564	50.60±0.88
Direct black 22	645	72.52±1.50
Acid green 10H	617	51.50±0.55
Reactive red M5B	538	69.37±0.90
Reactive red C2G	502	42.90±0.75
Brown 18	474	37.70±0.60
Red BS	517	11.00±0.50
Violet 5B	560	12.70±0.45
Reactive red HE8B	559	31.50±0.55
Direct orange 5E	493	29.00±0.85
Direct red 4BS	495	23.43±0.77
Turquoise blue H5G	609	11.32±0.30
Direct red 12B	523	14.43±0.55
Reactive turquoise blue G	623	26.00±0.92
Acid red F2R	510	28.20±1.25

reports on in vitro dye decolorization using crude laccase, and the results obtained in the present work are comparable with the previously reported work. Roriz et al. (2009) reported 10 % decolorization of Reactive black 5 by crude laccase from *Trametes pubescens* in 24 h. Extracellular liquid of *Trametes trogii* was able to decolorize 69 %, 14 % and 6 % of Janus green, Azure B and Poly R-478, respectively (Levin et al. 2010). Similarly, Dhillon et al. (2012) reported that crude laccase from *Trametes versicolor* was able to decolorize structurally different dyes with variable decolorization efficiencies between 31.30 and 87.70 % after a 48 h incubation period. The results obtained in the present work show that the crude laccase of *Pseudolagarobasidium acaciicola* AGST3 was better for decolorization of different dyes, without addition of any mediator.

Effect of dye concentration on dye decolorization

The decolorization ability of the crude laccase was further evaluated by varying the concentration of Violet P3P, Green ME4BL and Blue 3R (100 mg/l–500 mg/l) at a fixed concentration of laccase. Maximum decolorization of Violet P3P (97.2 ±2.2 %), Green ME4BL (80.3±1.8 %) and Blue 3R (91.3± 1.5 %) was observed at a concentration of 100 mg/l. The reduction in decolorization ability was observed with an increase in Violet P3P and Blue 3R concentration thereafter (Fig. 5). However, significant reduction in decolorization ability (41.9–28 %) was observed with 400 and 500 mg/l of Green ME4BL. Moreover, the reason for this can be attributed to possible damage of laccase activity at high dye concentrations (Yu et al. 2006). The results obtained indicate that the initial concentration of laccase used was sufficient for decolorization.

Effect of laccase concentration on dye decolorization

Figure 6 (a), (b) and (c) shows the effect of laccase concentration (1,000–40,000 U/ml) on decolorization of Violet P3P, Blue 3R and Green ME4BL (100 mg/l), respectively. Decolorization of dyes gradually increased with increase in concentration of laccase, and remained comparable after a threshold activity (10,000 U/ml for Violet P3P and Blue 3R, and 20,000 U/ml for Green ME4BL). Maximum decolorization of Violet P3P and Blue 3R was 97.20 % and 91.30 %, respectively, at 10,000 U/ml laccase concentrations, whereas 81.59 % decolorization of Green ME4BL was obtained at 20,000 U/ml of laccase. The extent of decolorization may depend upon the reaction rate, which is directly related to enzyme activity and property of the dyes (Yu et al. 2006). The lower decolorization at low enzyme concentration might imply damage to enzyme activity during

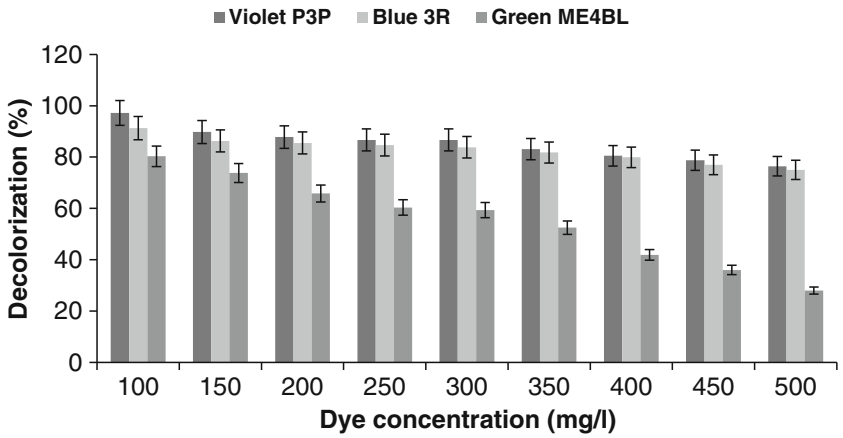

Fig. 5 Effect of dye concentration on in vitro dye decolorization

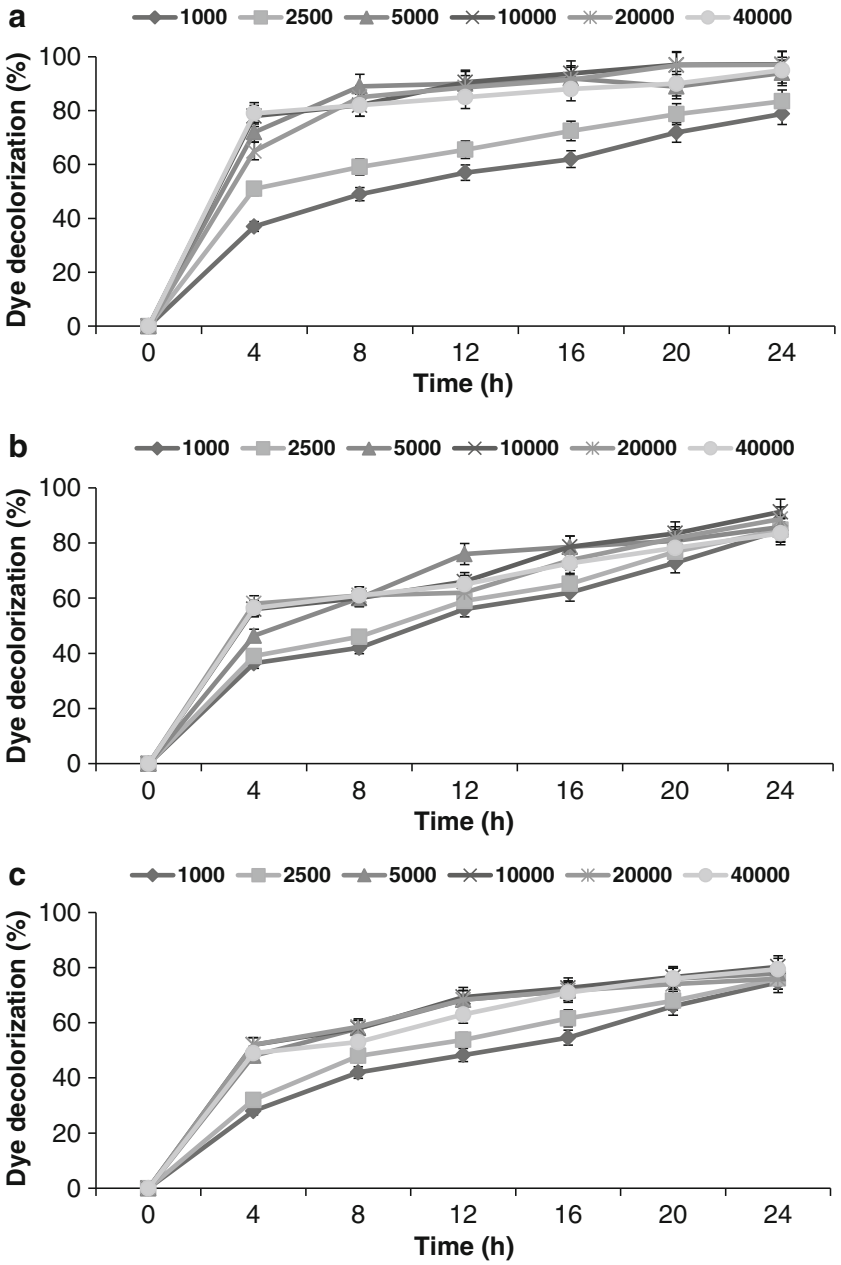

Fig. 6 Effect of laccase concentration on in vitro dye decolorization of (a) Violet P3P, (b) Blue 3R, (c) Green ME4BL

decolorization, while high enzyme concentration could be attributed to the higher reaction rate during decolorization, as well as better protection of the enzyme from unfavorable environmental conditions (Yu et al. 2006).

Conclusion

The novel isolate *Pseudolagarobasidium acaciicola* AGST3 is an excellent producer of laccase. The methodology of PBD was found to be useful to determine important medium variables with a significant effect; these can be further optimized using RSM. RSM was successfully applied to determine the optimal concentration of the relevant variables for maximal laccase production. Furthermore, the crude laccase showed higher potential for dye decolorization. Laccase-based biocatalysts are promising alternatives for dye decolorization for highly efficient, sustainable and ecofriendly industrial processes. Thus, *Pseudolagarobasidium acaciicola* AGST3 can be used for the large scale production of laccase for various industrial applications.

Acknowledgments The authors are very grateful to the Department of Biotechnology (DBT Sanction no. BT/PR9197/PBD/26/120/2007), Ministry of Science and Technology, New Delhi, India for their financial support. Ms. Shilpi Thakur wishes to acknowledge the Council of Scientific and Industrial Research (CSIR), New Delhi, India for Senior Research Fellowship. Shilpi Thakur also wants to acknowledge the Sophisticated Instrumentation Centre for Applied Research and Testing (SICART), Vallabh Vidyanagar, Gujarat, India for providing instrumentation facilities.

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