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Decolorization of some synthetic dyes using optimized culture broth of laccase producing ascomycete *Paraconiothyrium variabile*

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ABSTRACT

Optimization of the medium components for production of laccase by *Paraconiothyrium variabile* using response surface methodology was investigated. Initial screening by Plackett–Burman design was performed to select major variables out of eleven medium components, among which peptone, $CuSO_4$, and xylidine were found to have significant effects on laccase production. After application of the steepest ascent to approach the proximity of the optimum point, a central composite design was employed to optimize the level of the selected variables. In optimum concentrations of the most effective parameters, including peptone, 2.2 g/L, $CuSO_4$, 0.03 g/L, and xylidine 1.29 mM, extracellular laccase activity was enhanced from 970 U/L (in basal medium) to 16,678 U/L, which means a 17-fold increase in laccase production in the optimized medium. Supernatant of the optimized medium was used for decolorization of five synthetic dyes, among which 93% of Remazol brilliant blue R (with initial concentration of 600 mg/L) disappeared after 3 h treatment in the presence of 5 mM hydroxybenzotriazole.

1. Introduction

Laccase (benzenediol: oxygen oxidoreductase; EC 1.10.3.2), a blue multi-copper oxidase, catalyzing oxidation of phenolic compounds, in addition to non-phenolic ones, in the presence of specific substrates is termed as a mediator by reducing molecular oxygen to water [1]. Laccases have been found in higher plants and fungi [2]; however, there are also some reports of the existence of bacterial laccases [3]. In addition to the basidomycete phylum of white-rot fungi, which are the main producers of the enzyme, laccase activity of other fungal families such as deuteromycete and ascomycetes has also been reported [4,5]. Due to the broad spectrum of laccase substrates, this copper-containing enzyme has been used in many biotechnological processes such as pulp delignification, textile dye bleaching, effluent detoxification, and organic synthesis and production of complex medical compounds [6], as well as development of biosensors in the field of nanobiotechnology [7]. All of the above-mentioned applications of laccase, especially on an industrial scale, have increased the demand for production of high amounts of this enzyme. This purpose may be achieved by heterologous expression of laccase genes or through fermentation optimization [8,9]. Medium optimization has traditionally been performed one factor at a time, by evaluating the effect of one variable while the others are maintained at a certain level. The disadvantages of such a classical method are that it is time consuming and high cost, in addition to ignoring total interactions between medium components. Researchers are encouraged to apply statistical experimental approaches, e.g., the Plackett–Burman (PB) design and response surface methodology (RSM), which provide a great amount of information based on only a small number of experiments [10].

In a recent study [5], a newly laccase producing ascomycete, identified as *Paraconiothyrium variabile*, was isolated from soil and its purified laccase was applied for decolorization of the synthetic dye malachite green. The present work aims at the application of statistical experimental designs (Plackett–Burman design followed by central composite design) to optimize medium components for laccase production in a submerged culture of *P. variabile*. A supernatant of the optimized medium was applied in the decolorization of five synthetic dyes.

2. Materials and methods

2.1. Chemicals

2,2′-Azinobis-(3-ethylbenzthiazoline-6-sulphonate) (ABTS), asparagine, and veratryl alcohol were purchased from

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Sigma–Aldrich (St. Louis, MO, USA). 2,5-Xylidine, Sabouraud-2%-dextrose broth (SDB), glucose, peptone, yeast extract, and hydroxybenzotriazole (HBT) were obtained from Merck (Darmstadt, Germany). All other chemicals were of analytical grade.

2.2. Microorganism

The ascomycete used in the present study was isolated from soil and identified as *P. variabile*, based on rDNA sequence and morphological analysis [5]. The fungal strain was grown on Sabouraud-2%-dextrose agar (SDA) containing 0.1% guaiacol at 30 °C and maintained at 4 °C for brief storage. Subculturing was performed every two weeks.

2.3. Culture media and cultivation

The production basal medium was composed of (g/L): glucose, 10; peptone, 5; yeast extract, 1; $MgSO_4{\cdot}7H_2O$, 0.5; KH_2PO_4, 1.3; K_2HPO_4, 0.4; NH_4NO_3, 1; $CaCO_3$, 1, and $FeSO_4{\cdot}7H_2O$, 0.01 [11]. 250 mL-Erlenmeyer flasks containing 60 mL of basal medium were inoculated by two five-day-old culture plugs (3 mm in diameter) of SDA plates containing 0.1% guaiacol, and incubated at 30 °C and 150 rpm. To prevent negative effect of xylidine on fungal growth [12] xylidine and $CuSO_4$, two potent laccase inducers, were aseptically added to culture medium to reach the final concentration of 250 μM and 100 μM, respectively, after 4 days of cultivation [5]. Cultivation was continued for further 8 days and during this 12-day period, daily sample volumes of 1 mL were taken from culture media followed by measurement of laccase activity of culture broth after centrifugation (12,000 × *g* for 15 min). The acquired peak day of laccase production was used for statistical experiments.

2.4. Laccase assay

Laccase activity was determined using ABTS as the substrate [13]. The reaction mixture consisted of 0.5 mL ABTS (5 mM) dissolved in 100 mM acetate buffer (pH 4.5) and 0.5 mL of culture supernatant followed by incubation at 37 °C and 120 rpm. Oxidation of ABTS was monitored by an increase in absorbance at 420 nm (ε_{420} = 36,000 M^{-1} cm^{-1}) [14]. One unit of laccase activity was defined as the amount of enzyme required to oxidize 1 μmol of ABTS/min.

Table 1
Media components and test levels for Plackett–Burman design.

Variables	Symbol	Low level (−1)	High level (+1)
Glucose (g/L)	X_1	20	50
Peptone (g/L)	X_2	1	5
$CuSO_4$ (g/L)	X_3	0.01	0.1
$MgSO_4$ (g/L)	X_4	0.25	0.75
$FeSO_4$ (g/L)	X_5	0.01	0.03
$CaCO_3$ (g/L)	X_6	0.5	2
Yeast extract (g/L)	X_7	0.5	1.5
NH_4NO_3 (g/L)	X_8	0.5	2
K_2HPO_4 (g/L)	X_9	0.2	0.6
KH_2PO4 (g/L)	X_{10}	1	2
Xylidine (mM)	X_{11}	0.05	1

2.5. Experimental design and statistical analysis

2.5.1. Screening design

Plackett–Burman design was employed to select the most effective parameters for laccase production by the fungus *P. varibile* out of 11 medium components including glucose (X_1), peptone (X_2), $CuSO_4$ (X_3), $MgSO_4$ (X_4), $FeSO_4$ (X_5), $CaCO_3$ (X_6), yeast extract (X_7), NH_4NO_3 (X_8), K_2HPO_4 (X_9), KH_2PO_4 (X_{10}) and xylidine (X_{11}) at two levels: high (+1) and low (−1) (Table 1). As there is no interaction between variables, the main effect of each variable could be estimated directly by the size of the coefficients. These variables were attained from the result of the primary literature review and from previous experience to determine the most important factor influencing laccase production. This design characterizes a model that identifies the significant variables when no interaction among the factors is expected. Therefore, a first-order multiple regression can model the data properly:

$$Y = \beta + \Sigma\beta_i x_i (i = 1, \ldots, k) \quad (1)$$

where Y is the predicted response (laccase activity), β_0 is the intercept, and β_i is the linear coefficients.

Table 2 represents the design matrix created for a study of 11 variables in 12 experiments using the statistical software package Design-Expert 7.0.0 (Stat-Ease, Inc., Minneapolis, MN, USA). Three replicates in the center point were also performed to find the curvature that may exist in the model and pure experimental error that shows lack of fit (trials 13–15). The statistical significance of the first-order model was identified using Fisher's test for analysis of variance (ANOVA). Moreover, the multiple correlation coefficient (R^2) was used to express the fit of this first model. The influence of each variable on laccase production was calculated by subtracting the mean responses of variables at their lower levels

Table 2
Plackett–Burman design matrix (trial nos. 1–12) and center point experiments (trail nos. 13–15) with corresponding results.

Run no.	Variable											Laccase activity (U/L)
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	
1	+1	−1	+1	−1	−1	−1	+1	+1	+1	−1	+1	3507
2	+1	+1	−1	+1	−1	−1	−1	+1	+1	+1	−1	1536
3	−1	+1	+1	−1	+1	−1	−1	−1	+1	+1	+1	2918
4	+1	−1	+1	+1	−1	+1	−1	−1	−1	+1	+1	7184
5	+1	+1	−1	+1	+1	−1	+1	−1	−1	−1	+1	6126
6	+1	+1	+1	−1	+1	+1	−1	+1	−1	−1	−1	15 071
7	−1	+1	+1	+1	−1	+1	+1	−1	+1	−1	−1	4082
8	−1	−1	+1	+1	+1	−1	+1	+1	−1	+1	−1	7200
9	−1	−1	−1	+1	+1	+1	−1	+1	+1	−1	+1	5598
10	+1	−1	−1	−1	+1	+1	+1	−1	+1	+1	−1	1106
11	−1	+1	−1	−1	−1	+1	+1	+1	−1	+1	+1	5182
12	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	4462
13	0	0	0	0	0	0	0	0	0	0	0	9138
14	0	0	0	0	0	0	0	0	0	0	0	9363
15	0	0	0	0	0	0	0	0	0	0	0	8944