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Research Article

Application of Asymetrical and Hoke Designs for Optimization of Laccase Production by the White-Rot Fungus *Fomes fomentarius* in Solid-State Fermentation

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Statistical approaches were employed for the optimization of different cultural parameters for the production of laccase by the white rot fungus *Fomes fomentarius* MUCL 35117 in wheat bran-based solid medium. first, screening of production parameters was performed using an asymmetrical design $2^5 3^3//16$, and the variables with statistically significant effects on laccase production were identified. Second, inoculum size, $CaCl_2$ concentration, $CuSO_4$ concentration, and incubation time were selected for further optimization studies using a Hoke design. The application of the response surface methodology allows us to determine a set of optimal conditions ($CaCl_2$, 5.5 mg/gs, $CuSO_4$, 2.5 mg/gs, inoculum size, 3 fungal discs (6 mm Ø), and 13 days of static cultivation). Experiments carried out under these conditions led to a laccase production yield of 150 U/g dry substrate.

1. Introduction

Laccases (benzenediol: oxygen oxidoreductases [EC 1.10.3.2]) are copper-containing enzymes catalyzing the oxidation of a broad number of phenolic compounds and aromatic amines by using molecular oxygen as the electron acceptor, which is reduced to water. Laccase can also oxidize nonphenolic substrates in the presence of appropriate redox mediators [1].

This group of enzymes has attracted increasing scientific attention in the recent years due to their application in several biotechnological processes. Such applications include the detoxification of industrial effluents, the use as a tool for medical diagnostics, and the use as a bioremediation agent to clean up herbicides, pesticides, and certain explosives in soil [2].

The application of these oxidative enzymes to biotechnological processes requires the production of high amounts of enzyme at low cost. In this context, solid state fermentation (SSF) appeared as an interesting alternative for the enzymes production [3]. SSF offers advantages over liquid cultivation, especially for the fungal cultures, as there is higher productivity per unit volume, reduced energy requirements, lower capital investment, low waste water output, higher concentrations of metabolites obtained, and low downstream processing cost [4].

The selection of an adequate support for performing SSF is essential, since the success of the process depends on it. Various agricultural substrates/byproducts such as banana waste, orange peelings, coconut flesh, chestnut shell, barley bran, and wheat bran have been successfully used in solid-state fermentation for laccase production by white-rot fungi [5–8].

Wheat bran, an abundant byproduct formed during wheat flour preparation, has been selected to perform the present study, because it has the physical integrity to serve as a supporting material and it provides the fungus, an environment similar to its natural habitat which is conducive for the high secretion of lignolytic enzymes. In addition,

wheat bran is an abundant source for hydroxycinnamic acids, particularly ferulic and *p*-coumaric acids, which are known to stimulate laccase production [9]. Indeed, these hydroxycinnamic acids, covalently bound to cell wall polymers (pectins, arabinoxylans, and xyloglucans) through ester linkages, could be released after feruloyl esterase action as described in several white-rot fungi [10].

Production of laccase under SSF is affected by diverse typical fermentation factors such as moisture level, carbon and nitrogen source supplementation, and inoculum size [11–13]. Moreover, different compounds have also been widely used to stimulate laccase production. Among them, the effect of copper on laccase formation is outstanding [9, 14]. For effective laccase expression, it is highly essential to find the critical variables affecting laccase production yield and to establish the optimal set of experimental conditions for the whole solid-state culture process, which further facilitates economic design of the full-scale fermentation operation system.

Conventional optimization method is usually performed by varying the levels of one independent variable while fixing other variables at a certain level. This method is laborious and time consuming, and often interaction effects are overlooked [15]. Statistical experimental designs are powerful tools for searching the key factors rapidly from a multivariable system and to define the optimum settings of these factor levels. Asymmetrical screening design is one such method that has been used for screening multiple factors at a time, taking different level numbers [16–19]. This experimental design is particularly useful for initial screening, as it is used for the estimation of only the main effects. The significant factors obtained from the screening experiments could be further optimized by employing response surface methodology that enables the examination of the combinatory effect of the retained factors and the determination of their optimal levels [20–25]. The application of statistical experimental design techniques in fermentation process development can result in improved product yields, reduced process variability, closer confirmation of the output response to nominal and target requirements, and reduced development time and overall costs [26, 27]. Successful application of RSM to enhance enzyme production in SSF by optimizing the culture media has been reported [28–31]. On the other hand, studies regarding optimization of solid culture medium for the production of laccase are still few in the scientific literature [32].

In this work, laccase production by the white rot fungus *F. fomentarius* in wheat bran-based solid medium has been optimized using experimental designs [19–25]. In a first step, a screening of the most important factors is carried out with an asymmetrical design $2^5 3^3 // 16$. In a second step, Hoke design is applied to determine response surfaces as a function of some of the significant parameters and then to choose the optimal solid state conditions for laccase production.

2. Materials and Methods

2.1. Microorganism. *Fomes fomentarius* (MUCL 35117) used in this study was obtained from the Belgian Coordinated Collections of Microorganisms/Mycothèque de l'Université Catholique de Louvain (BCCM/MUCL) and maintained at 4°C on 2% malt extract agar (MEA). This strain was maintained on 2% malt extract agar (MEA) slants at 4°C and subcultured every three months.

Table 1: Experimental conditions of the asymmetrical screening design.

	Levels		
Factors	1	2	3
M/S ratio (ml/gs) (A)	3	6	—
$MgSO_4$ (mg/gs) (B)	0.3	3	—
$CaCl_2$(mg/gs) (C)	0.3	3	—
Moisturizing agent* (D)	Sodium acetate buffer (SAB)	Citrate phosphate buffer (CPB)	—
Inoculum size (discs**) (E)	4	8	—
Glucose (mg/gs) (F)	0	30	60
Ammonium tartrate (mg/gs) (G)	0	5.5	11
$CuSO_4$ (ml/gs) (H)	0	0.75	1.5

* 20 mM, pH 5; ** diameter, 6 mm.

2.2. Solid Medium Preparation. Wheat bran purchased from a local market was employed as support substrate for SSF. The average particle size of the wheat bran was 1–5 mm. The chemical composition of this substrate was hemicelluloses (notably arabinoxylans, ca. 30%), cellulose (10–15%), starch (10–20%), proteins (15–22%), lignin (4–8%), and other minor components such as cutin and lipids [33, 34].

The experiments were performed in 125-mL flasks with 2.5 g of wheat bran moistened with the required volume of buffer (sodium acetate or citrate phosphate buffer 20 mM, pH 5) containing various nutritional factors according to the experimental designs shown in Tables 1 and 2. The medium was sterilized at 121°C for 20 min. After cooling, the substrate was inoculated with mycelial discs (each 6 mm diameter) obtained from the periphery of 7 days fungal culture grown on MEA plates. The contents were incubated statically in complete darkness at 30°C.

2.3. Enzyme Extraction. The enzyme was extracted with sodium acetate or citrate-phosphate buffer 20 mM, pH 5 (20 mL buffer/g substrate) by shaking for 1 h at 160 rpm at room temperature. The suspension was filtered and centrifuged at 4°C, 8,000 g for 20 min, and the supernatant was used in enzyme assays.

2.4. Enzyme Assays. The laccase activity was measured by monitoring the oxidation of 5 mM 2,6-dimethoxyphenol (DMP) buffered with 0.1 M tartrate buffer (pH 4.5) at 469 nm for 1 min [35]. To calculate the enzyme activity, an absorption coefficient of 27,500 M^{-1} cm^{-1} was used. One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 μM of DMP per minute.

Table 2: Experimental conditions of the screening design and the corresponding responses.

Run no.	M/S ratio (ml/gs)	$MgSO_4$ (mg/gs)	$CaCl_2$ (mg/gs)	Moisturizing —agent	Inoculum size (discs)	Glucose (mg/gs)	Ammonium tartrate (mg/gs)	$CuSO_4$ (mg/gs)	Measured and *estimated* laccase activities (U/gds)				
									4 days	7 days	10 days	13 days	16 days
1	3	0.3	0.3	CPB	4	0	0	0	2.16 *1.88*	37.00 *38.68*	2.88 *9.93*	10.00 *9.54*	6.60 *10.26*
2	3	0.3	0.3	SAB	4	30	5.5	0.75	2.74 *2.65*	19.62 *20.09*	1.56 *−0.04*	1.56 *1.37*	0.52 *−0.41*
3	3	0.3	0.3	CPB	8	60	11	1.5	2.74 *2.65*	39.92 *40.39*	13.60 *11.99*	17.04 *16.85*	5.30 *4.37*
4	3	0.3	0.3	SAB	8	0	0	0	3.66 *4.12*	45.16 *42.52*	9.16 *5.31*	3.78 *4.62*	3.46 *1.66*
5	6	0.3	3	CPB	4	30	11	0	3.60 *3.92*	61.52 *58.14*	63.48 *63.38*	74.60 *73.93*	85.08 *81.99*
6	6	0.3	3	SAB	4	0	0	1.5	4.70 *5.04*	63.48 *61.83*	69.38 *78.32*	96.86 *93.51*	108.64 *107.09*
7	6	0.3	3	CPB	8	0	0	0.75	6.60 *6.08*	64.14 *66.74*	103.40 *91.25*	81.16 *84.12*	101.44 *101.13*
8	6	0.3	3	SAB	8	60	5.5	0	5.16 *5.02*	57.60 *60.02*	61.52 *64.82*	67.40 *68.45*	65.44 *70.38*
9	3	3	3	CPB	4	60	0	0.75	1.50 *1.88*	36.00 *34.83*	7.98 *12.98*	2.34 *1.70*	2.02 *3.28*
10	3	3	3	SAB	4	0	11	0	3.26 *2.80*	37.96 *42.77*	15.96 *8.91*	7.46 *10.46*	0.26 *4.29*
11	3	3	3	CPB	8	0	5.5	0	4.50 *4.78*	51.70 *47.84*	15.44 *19.28*	9.42 *6.04*	9.28 *3.39*
12	3	3	3	SAB	8	30	0	1.5	6.40 *6.20*	40.58 *40.80*	1.56 *−0.24*	1.30 *2.31*	2.16 *2.76*
13	6	3	0.3	CPB	4	0	5.5	1.5	4.90 *4.85*	56.94 *57.90*	99.48 *93.93*	87.70 *90.22*	92.28 *94.16*
14	6	3	0.3	SAB	4	60	0	0	3.60 *3.45*	55.62 *53.89*	70.68 *63.98*	61.52 *61.30*	66.76 *61.49*
15	6	3	0.3	CPB	8	30	0	0	6.60 *6.57*	58.24 *60.92*	64.80 *68.30*	57.60 *57.44*	60.20 *63.61*
16	6	3	0.3	SAB	8	0	11	0.75	5.94 *6.17*	63.48 *61.57*	83.12 *91.87*	82.46 *80.32*	80.50 *80.48*

2.5. Asymmetrical Design and Hoke Design. The optimization of laccase production yield in solid-state medium has been carried out in two steps as described below.

2.5.1. Screening of Important Cultural Factors Using Asymmetrical Design. For systems with a great number of variables, different approaches of experimental factorial designs can be applied to achieve a screening of critical variables and to estimate their main effects on the responses [16, 19, 24].

In this study, a $2^5 3^3//16$ experimental design was used to find out the critical medium components for laccase production by *F. fomentarius* under SSF. It allows the investigation of eight factors in sixteen experiments, five factors A–E each at two levels and three factors F–H each at three levels. Table 1 lists the values given to each factor, the choice was based on previous literature works [5, 11–13] and preliminary experiments. Table 2 shows the $2^5 3^3//16$ experimental design.

From the 16 runs, we can compute, using the least square method [19–24], the "weight" of each factor level. For each factor, the weight of each level is related to the upper level weight, which becomes the "reference state" among each factor [24]. The weight describes the factor effects on the response when changing factor levels with respect to the reference state. The weight of glucose amount (factor F) when fixed at level 1 (F1), for example, corresponds to the differential effect of glucose amount on the response when changing its value from level 3 (60 mg/g) to level 1 (0 mg/g). The obtained results are generally presented as histograms, which graphically illustrate the variable differential weights [24]. At the end of this first step, the variables that did not have a significant effect (checked by applying a t-test) on the

Table 3: Experimental domain for the Hoke design.

Variable	Factor	Unit	Center	Step of variation
X_1	$CaCl_2$	mg/gs	3.0	2.5
X_2	Inoculum size	discs	6.0	3.0
X_3	$CuSO_4$	mg/gs	1.5	1.0
X_4	Incubation time	days	10	6

responses are screened out; the remaining factors affecting the responses are further optimized.

2.5.2. Optimization of Selected Factors Using Response Surface Methodology. The screening data revealed four factors ($CaCl_2$ concentration, inoculum size, $CuSO_4$ concentration and incubation time) influencing the SSF production of laccase by *F. fomentarius*. Optimization of laccase production yield was achieved by using the response surface methodology (RSM). This approach explores the response surfaces covered in the experimental design, thus making the optimization process more efficient and effective [20–23].

The most frequent designs in optimization problems involving three or more factors are central composite designs, Box-Behnken designs, D-optimal designs, and others, such as Hoke designs. [20–25, 36] Central composite designs and Box-Behnken designs are the most appropriate to detect curvatures in a multidimensional space but require a large number of experiments beyond three factors. D-optimal designs are less frequent, but adequate in cases involving linear functions where the factors can only be varied over a restricted area, and thereby creates an irregular experimental domain in which orthogonality cannot be achieved. Hoke designs are economical second-order designs [36] based on irregular fractions of partially balanced type of the 3^k factorial for a number of factors $k \geq 3$. They require fewer experiments than the central composite designs and Box-Behnken designs.

In this work, we consider that the experimental region is a hypercube, thus, to define the optimum settings of the four active factor levels; we applied a four-factor Hoke D6 design [36] in the experimental domain presented in Table 3.

The response (laccase yield) can be described by the following second-order model adequate for predicting the responses in the experimental region:

$$\begin{aligned}\eta = {} & \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_{11} X_1^2 \\ & + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{44} X_4^2 + \beta_{12} X_1 X_2 \\ & + \beta_{13} X_1 X_3 + \beta_{14} X_1 X_4 + \beta_{23} X_2 X_3 + \beta_{24} X_1 X_4 \\ & + \beta_{34} X_3 X_4,\end{aligned} \tag{1}$$

where, η: the theoretical response function, X_j: coded variables of the system, β_0, β_j, β_{jk}, and β_{jj}: true model coefficients.

The observed response y_i for the ith experiment is

$$y_i = \eta_i + e_i \quad (e_i : \text{error}). \tag{2}$$

The model coefficients $\beta_0, \beta_j, \ldots$, and β_{jj} are estimated by a least squares fitting of the model to the experimental results obtained in the 23 design points of the four-variable Hoke D6 design (Table 4). For the estimated values of these coefficients, the symbols $b_0, b_j, \ldots$, and b_{jj} will be used. The computed values of the responses are designated by $\hat{y}_i$

$$\begin{aligned}\hat{y} = {} & b_0 + b_1 X_1 + b_2 X_2 + b_3 X_3 + b_4 X_4 + b_{11} X_1^2 \\ & + b_{22} X_2^2 + b_{33} X_3^2 + b_{44} X_4^2 + b_{12} X_1 X_2 + b_{13} X_1 X_3 \\ & + b_{14} X_1 X_4 + b_{23} X_2 X_3 + b_{24} X_2 X_4 + b_{34} X_3.\end{aligned} \tag{3}$$

The four replicates at the center point (runs n° 20 to 23) are carried out in order to estimate the pure error variance [22–24]. A statistical test of the model fit is made by comparing the variance due to the lack of fit to the pure error variance using the F-test. The fitted model is considered adequate if the variance due to the lack of fit is not significantly different from the pure error variance [22–24]. The adequacy of the model is further tested using four check points [24, 25]. The fitted model was used to study the relative sensitivity of the responses to the variables in the whole domain and to look for the optimal experimental conditions. In this paper, the canonical analysis is used to find out the best experimental conditions, which permitted the maximization of the laccase production yield. It consists of rewriting the fitted second-degree equation in a form in which it can be more readily understood. This is accomplished by a rotation of axes that remove all cross-product terms $b_{jk} X_j X_k$ while keeping the initial origin at the centre point. This step is suitable when the stationary point is outside of the experimental domain [37]. The relationship between the response and the experimental variables is illustrated graphically by plotting the response surfaces and the isoresponse curves [23, 24].

In this study, the generation and the data treatment of the $2^5 3^3 // 16$ screening design and the Hoke design are performed using the experimental design software NemrodW [38].

3. Results and Discussion

3.1. Screening Design. A total of eight variables were analyzed for their effect on laccase production yield using an asymmetrical screening design. Sixteen experiments have been carried out according to the SSF medium preparation method described above and the conditions fixed by the experimental design (Table 2). The obtained responses values related to laccase yields obtained in 4, 7, 10, 13, and 16 days of fungal cultivation are reported in Table 2. As shown in this table, for low moisture to substrate ratios (M/S = 3 : 1 v/w) (experiments N° 1–4 and 9–12), the laccase production increases until 7 days of cultivation, and then, a notable decrease of the enzymatic activity was observed (Figure 1). This result is probably due to reduced solubility of nutrients from the solid substrate, low substrate swelling, and high

Table 4: Experimental conditions of the Hoke design and the corresponding responses.

Run no.	X_1	X_2	X_3	X_4	$CaCl_2$ (mg/gds)	Inoculum size (Discs*)	$CuSO_4$ (mg/gds)	Incubation time (days)	Measured and *estimated* laccase activities (U/gds)	
1	−1.00000	−1.00000	−1.00000	−1.00000	0.5	3	0.5	4	6.72	*5.02*
2	−1.00000	0.00000	0.00000	0.00000	0.5	6	1.5	10	121.00	*119.93*
3	0.00000	−1.00000	0.00000	0.00000	3.0	3	1.5	10	115.90	*122.34*
4	0.00000	0.00000	−1.00000	0.00000	3.0	6	0.5	10	101.12	*110.96*
5	0.00000	0.00000	0.00000	−1.00000	3.0	6	1.5	4	8.56	*5.49*
6	1.00000	1.00000	1.00000	−1.00000	5.5	9	2.5	4	11.60	*14.77*
7	1.00000	1.00000	−1.00000	1.00000	5.5	9	0.5	16	89.32	*86.04*
8	1.00000	−1.00000	1.00000	1.00000	5.5	3	2.5	16	138.24	*136.66*
9	−1.00000	1.00000	1.00000	1.00000	0.5	9	2.5	16	103.90	*106.07*
10	1.00000	1.00000	−1.00000	−1.00000	5.5	9	0.5	4	12.50	*11.84*
11	1.00000	−1.00000	1.00000	−1.00000	5.5	3	2.5	4	6.28	*6.47*
12	1.00000	−1.00000	−1.00000	1.00000	5.5	3	0.5	16	104.16	*101.12*
13	−1.00000	1.00000	1.00000	−1.00000	0.5	9	2.5	4	8.42	*10.48*
14	−1.00000	1.00000	−1.00000	1.00000	0.5	9	0.5	16	80.12	*78.96*
15	−1.00000	−1.00000	1.00000	1.00000	0.5	3	2.5	16	121.84	*121.53*
16	1.00000	1.00000	1.00000	0.00000	5.5	9	2.5	10	139.20	*133.46*
17	1.00000	1.00000	0.00000	1.00000	5.5	9	1.5	16	97.26	*104.42*
18	1.00000	0.00000	1.00000	1.00000	5.5	6	2.5	16	123.88	*127.65*
19	0.00000	1.00000	1.00000	1.00000	3.0	9	2.5	16	113.96	*110.22*
20	0.00000	0.00000	0.00000	0.00000	3.0	6	1.5	10	128.18	*119.84*
21	0.00000	0.00000	0.00000	0.00000	3.0	6	1.5	10	120.36	*119.84*
22	0.00000	0.00000	0.00000	0.00000	3.0	6	1.5	10	117.46	*119.84*
23	0.00000	0.00000	0.00000	0.00000	3.0	6	1.5	10	122.82	*119.84*
24	−0.39528	−0.22822	−0.16137	0.00000	2.0	5	1.3	10	122.28	*118.35*
25	0.39528	−0.22822	−0.16137	0.00000	4.0	5	1.3	10	120.42	*120.51*
26	0.00000	0.45644	−0.16137	0.00000	3.0	7	1.3	10	113.70	*118.07*
27	0.00000	0.00000	0.48412	0.00000	3.0	6	2.0	10	129.30	*123.33*
28	0.00000	0.00000	0.00000	0.50000	3.0	6	1.5	13	125.42	*127.66*

* diameter, 6 mm.

water tension [39, 40]. On the conterary, for the experiments conducted with high moisture to substrate ratios (M/S = 6 : 1 v/w) (experiments N° 5–8 and 13–16), laccase production exhibited a gradual increase, followed by a stabilization phase, where maximal enzyme production was recorded (Figure 1). This stability could likely be due to the cultures are better aerated and clogging problems are avoided [8]. Thus, we chose to estimate the effect of the eight variables on laccase yields obtained in 7 and 16 days of fungal cultivation.

In Table 5 we report coefficient values (the weight associated to each factor level) calculated, as described above, and statistical analyses using *t*-test. These results are illustrated by the histograms shown in Figures 2 and 3, which represent the differential effects of each factor when considering two different levels taken two by two. b6/2-1, for example, defines the weight of factor F (glucose) on the response when changing its level from 1 to 2.

As shown in Table 5 and Figures 2 and 3, moisture to substrate ratio (A) exhibits stronger influence on the laccase production compared to other factors. For SSF, moisture content is a key parameter to control the growth of microorganism and metabolite production [40–42]. In this work, the highest laccase yields were observed for moisture to substrate ratio (v/w) of 6 : 1. A similar effect of moisture to substrate ratio on laccase production, by *Trametes hirsuta* grown on crushed orange peelings, was reported by Rosales et al. [8]. Supplementary experiments, conducted using higher M/S ratios, lead to a very low laccase yields (data not shown). Consequently, we fixed the ratio M/S at 6 : 1 v/w in the optimization step of this study.

$MgSO_4$ concentration (B), $CaCl_2$ concentration (C) moisturizing agent (D), inoculum size (E), and ammonium tartrate concentration (G) seem to have no significant effect on the response. However, we choose to include the factors

Table 5: Estimates of and statistics on the coefficients.

Name	Coefficient	F. inflation	Standard deviation	*t* exp.	Significance (%)
Laccase yield on 7th day of fermentation (U/gds)					
b0	65.420		4.622	14.15	0.0145***
b1A	−21.635	1.00	2.387	−9.06	0.0821***
b2A	−1.510	1.00	2.387	−0.63	56.1
b3A	−4.625	1.00	2.387	−1.94	12.5
b4A	2.745	1.00	2.387	1.15	31.4
b5A	−6.585	1.00	2.387	−2.76	5.1
b6A	5.197	1.50	2.923	1.78	15.0
b6B	−2.295	1.50	3.375	−0.68	53.4
b7A	−0.693	1.50	2.923	−0.24	82.4
b7B	−4.255	1.50	3.375	−1.26	27.6
b8A	0.370	1.50	2.923	0.13	90.5
b8B	−4.420	1.50	3.375	−1.31	26.1
Laccase yield on 16th day of fermentation (U/gds)					
b0	78.366		5.939	13.20	0.0191***
b1A	−78.842	1.00	3.067	−25.71	<0.01***
b2A	7.877	1.00	3.067	2.57	6.2
b3A	−7.337	1.00	3.067	−2.39	7.5
b4A	4.308	1.00	3.067	1.40	23.3
b5A	4.297	1.00	3.067	1.40	23.4
b6A	15.428	1.50	3.756	4.11	1.48*
b6B	2.110	1.50	4.337	0.49	65.2
b7A	1.125	1.50	3.756	0.30	77.9
b7B	−0.905	1.50	4.337	−0.21	84.5
b8A	−14.960	1.50	3.756	−3.98	1.64*
b8B	−5.975	1.50	4.337	−1.38	24.0

* Significant at the level 95%; ** significant at the level 99%; *** significant at the level 99.9%.

Table 6: Analysis of variance of the Hoke design response.

Source of variation	Sum of squares	*df*	Mean square	Ratio	*P*-value (significance)
Regression	52728.2	14	3766.30	76.8852	<.0001 (***)
Residuals	391.889	8	48.9861		
Lack of fit	329.891	5	65.9781	3.1926	0.184 (N.S.)
Error	61.9979	3	20.6660		
Total	53120.1	22			

*** significant at the level 99.9%, N.S.: Non significant at the level 95%.

C and E in the optimization design for two reasons. First, $CaCl_2$ concentration and inoculum size have a relatively high positive effect on the response (Figure 2). Second, the role of calcium in the maintenance of the protein structures and the stabilization of the activities of several enzymes has been well documented [43, 44]. In the same way, many reports have been given about the effect of inoculum size in fungal growth and productivity [40, 41, 45]. Too high or low inoculum concentration cause low growth and productivity. When the inoculum size is small, longer cultivation time is required. A large inoculum size in culture will lead rapidly to crowded and nutritional deficiency. A mycelium mat will soon cover the culture medium causing poor substrate aeration [40].

Table 5 and Figures 2 and 3 show that glucose (F) as carbon supplement exhibits a negative effect on the response. The production is raised more in absence of glucose that with 30 or 60 mg of glucose/g substrate. Such inhibitory effect of glucose on laccase production has also been described by Galhaup et al. [46].

We already mentioned that the ammonium tartrate concentration has no significant effect on the response.

Consequently, it can be used at its low level (0%). Thus, the addition of this nitrogen source is not required.

We can then conclude that wheat bran could be employed without adding any initial amount of carbon and nitrogen supplements in the culture medium. This will help to suppress the overall production cost of culture medium. During cultivation on wheat bran, water soluble cellulose and hemicellulose fractions could serve as carbon source which leads to a carbon: nitrogen ratio sufficient for an effective laccase induction [47, 48].

Finally, the increase in cupper sulphate concentrations (H) (0.75 to 1.5 mg/gds) has resulted in higher laccase production especially at 16 days of cultivation (Figure 3). Many studies have shown that laccase yields in several white-rot species were significantly increased in media containing high concentrations of $CuSO_4$ [46, 49–52].

Results of the screening design pave the way for the next step of the research.

3.2. Hoke D6 Design. Based on the results of asymmetrical design experiments, some factors are fixed at their best levels: A2 B1 D1 F1 G1. In order to look for optimal experimental conditions, a second-order model is built to analyse the relation between the four factors ($CaCl_2$, inoculum size, $CuSO_4$, and incubation time) and the response (laccase yield). Table 4 shows the coded and the real experimental conditions of the Hoke design with the corresponding observed values of the studied response. Results of experiments of the Hoke design are used to estimate the model coefficients (without using the check points). The resulting estimated model, expressed in coded variables is

$$\begin{aligned}\hat{y} = {} & 119.836 + 3.001X_1 - 1.535X_2 + 7.764\ X_3 + 48.547X_4 \\ & + 3.101X_1^2 + 0.969X_2^2 - 1.115X_3^2 - 65.792X_4^2 \\ & - 0.158X_1X_2 + 1.854X_1X_3 + 2.553X_1X_4 - 0.252X_2X_3 \\ & - 6.098X_2X_4 + 7.899X_3X_4.\end{aligned} \tag{4}$$

3.2.1. Statistical Analysis and Validation of the Model. The analysis of variance for the fitted model (Table 6) shows that the regression sum of squares is statistically significant (their P value is less than .05) and the lack of fit is not significant [20–25]. Thus, we can conclude that the models correlate well with the measured data.

In addition, Table 7 shows the check point results used to validate the accuracy of the model. The measured values are very close to those calculated using the model equations. Indeed, the differences between calculated and measured responses are not statistically significant when using the t-test as shown in Table 7. We can then conclude that the second-order models are adequate to describe the response surfaces and can be used as prediction equation in the studied domain.

3.2.2. Interpretation of the Response Surface Model. The second-order polynomial model is a conic function, and it can be analyzed by canonical analysis. This function has a stationary point S, where the partial derivative of predicted response with respect to each of the variables is zero ($\partial y/\partial X_1 = 0$; $\partial y/\partial X_2 = 0$; $\partial y/\partial X_3 = 0$; $\partial y/\partial X_4 = 0$). This point could be a maximum, a minimum, or a saddle point.

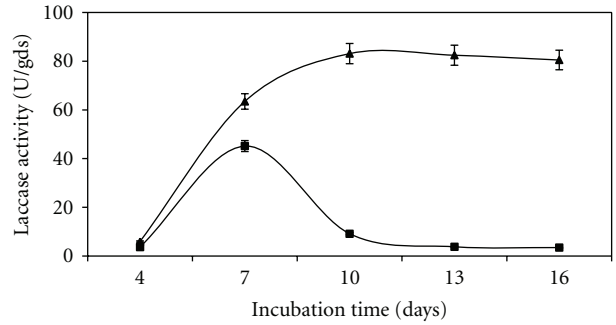

Figure 1: Evolution of laccase production by *F. fomentarius* grown on wheat bran-based solid medium (■) with low moisture to substrate ratio (M/S = 3 : 1 v/w) and (▲) with high moisture to substrate ratio (M/S = 6 : 1 v/w).

In the present study, the coordinates of the saddle point S are $X_1 = -2.032$; $X_2 = 9.876$; $X_3 = 5.419$, and $X_4 = 0.205$. It corresponds to a maximum of $\hat{y}$. This point is situated outside the experimental domain. In this case, the canonical analysis requires only a rotation of the X_j axes in such a way that they become parallel to the principal axes Z_j of the contour system. Under these conditions, the canonical model is of the form

$$\hat{y} = y_s + \sum_{j=1}^{4} b_j Z_j + \sum_{j=1}^{4} \lambda_{jj} Z_j^2. \tag{5}$$

The λ_j ($j = 1, 2, 3, 4$) will describe the curvature of the response, while the linear coefficient b_j will describe the slope of the ridge in the corresponding direction. The constant y_s is the calculated response value at the stationary point. The interpretation is easier by analyzing each the response along every Z_j-axis separately. Using the variable transformation equations:

$$\begin{aligned}X_1 &= 0.971Z_1 + 0.110Z_2 - 0.213Z_3 - 0.018Z_4, \\ X_2 &= -0.024Z_1 + 0.930Z_2 + 0.365Z_3 + 0.043Z_4, \\ X_3 &= 0.237Z_1 - 0.347Z_2 + 0.906Z_3 - 0.057Z_4, \\ X_4 &= 0.032Z_1 - 0.058Z_2 + 0.032Z_3 + 0.997Z_4.\end{aligned} \tag{6}$$

we obtained the following canonical form of the model:

$$\begin{aligned}\hat{y} = {} & 120.111 + 6.351Z_1 - 7.098Z_2 + 7.670Z_3 + 47.701Z_4 \\ & + 3.446Z_1^2 + 0.502Z_2^2 - 0.428Z_3^2 - 66.620Z_4^2.\end{aligned} \tag{7}$$

These data allow us to determine the features of the response surface in each direction of the experimental domain. When analyzing the response surface along each of

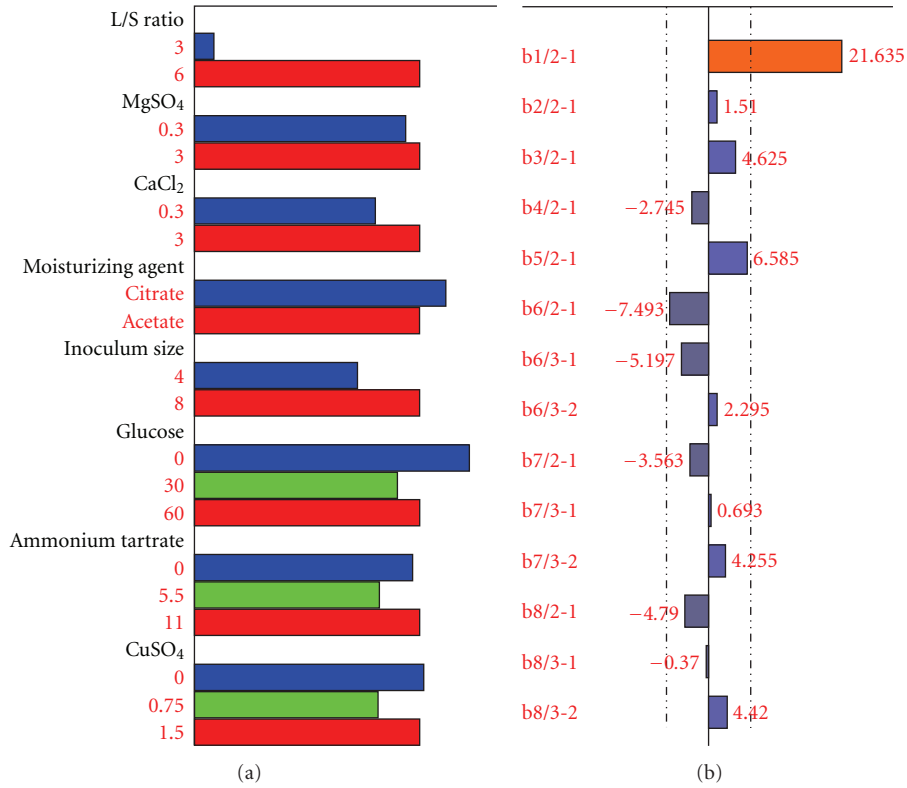

Figure 2: Graphical study of the effects of different operational variables on laccase production at 7 days days of cultivation. (a) Graphical study of the total effects and (b) Differences of the weights of the different levels.

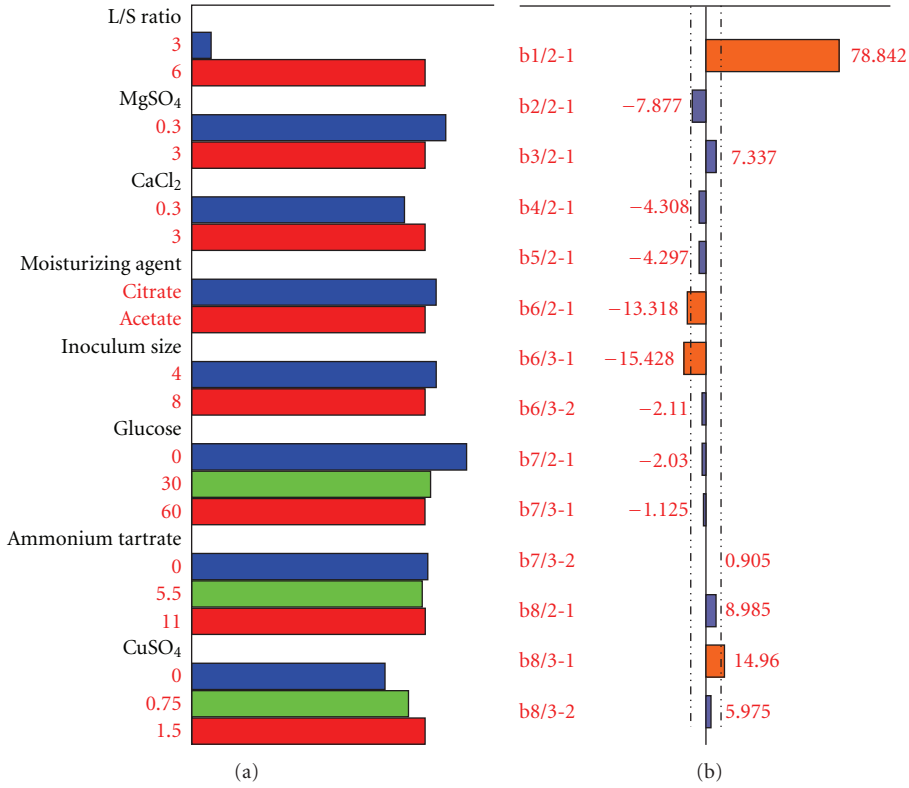

Figure 3: Graphical study of the effects of different operational variables on laccase production at 16 days days of cultivation. (a) Graphical study of the total effects and (b) Differences of the weights of the different levels.

Table 7: The numerical results for check points.

Run	Y_{exp}	Y_{calc}	$Y_{exp} - Y_{calc}$	dU	Ecart type	df	t exp.	Signif %
24	122.280	118.348	3.932	0.162	7.545	8	0.521	61.6
25	120.420	120.512	−0.092	0.194	7.647	8	−0.012	99.1
26	113.700	118.074	−4.374	0.201	7.671	8	−0.570	58.4
27	129.300	123.333	5.967	0.204	7.680	8	0.777	46.0
28	125.420	127.661	−2.241	0.209	7.695	8	−0.291	77.8

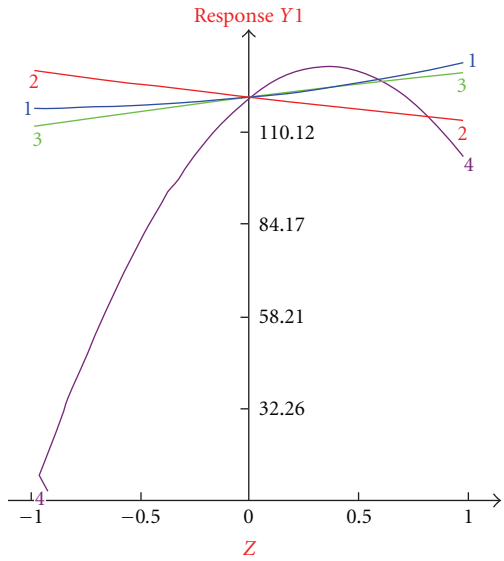

Figure 4: Curvature of laccase yield response versus Z_j ($j = 1, 2, 3$ and 4).

the four directions OZ_1, OZ_2, OZ_3, and OZ_4, the equation of the response is reduced to the following equations, respectively:

$$\begin{aligned}
\hat{y} &= 120.111 + 6.351Z_1 + 3.446Z_1^2,\\
\hat{y} &= 120.111 - 7.098Z_2 + 0.502Z_2^2,\\
\hat{y} &= 120.111 + 7.670Z_3 - 0.428Z_3^2,\\
\hat{y} &= 120.111 + 47.701Z_4 - 66.620Z_4^2.
\end{aligned} \tag{8}$$

The corresponding curves are represented in Figure 4. From these curves and the variable transformation equations, we can conclude that the maximization of the laccase yield requires high level of X_1 ($X_1 = 1$), low level of X_2 ($X_2 = -1$), high level of X_3 ($X_3 = 1$), and the relatively high level of X_4 ($X_4 = 0.5$). This corresponds to the following settings of the natural variables: $CaCl_2$ = 5.5 mg/gds, inoculum size =3 discs, $CuSO_4$ = 2.5 mg/gds and incubation time = 13 days.

Figures 5 and 6 illustrate graphically the evolution of the laccase yield versus two variables, while the other two variables were held constant.

Figure 5 shows that with incubation time of 13 days and $CuSO_4$ concentration of 2.5 mg/gs, the laccase yield can be enhanced from 130 to 150 U/gs by the increase of the concentration of $CaCl_2$ and the decrease of the inoculum size. As reported by many researchers [53–56], an increase of the inoculum size ensures a rapid proliferation of biomass and enzyme synthesis. However, after a certain limit, the enzyme production could decrease because of the depletion of nutrients, which results in decrease in metabolic activity.

From Figure 6, we have observed that the enzyme yield enhances essentially by increasing the incubation time. However, extended cultivation time may cause inhibition of enzyme synthesis. This fact was also reported by other investigators during other laccase production studies [57, 58]. It is also clear from Figure 6 that there is a gradual increase in the enzyme yields upon increasing the concentration of copper sulphate. Thus, it was implied that a high concentration of copper sulphate (2.5 mg/gs) was favourable for the production of laccase by *F. fomentarius*. These results were in agreement with others, for example, Couto and Sanromán [6], who reported an increase in laccase activity by almost 3-fold by adding 2 mM copper sulphate to solid state cultures of *Trametes hirsuta*. Many studies have shown that laccase mRNA levels in several white-rot species were significantly increased in media containing high concentrations of cupric ions [49–51]. Multiple putative cis-acting elements, termed metal responsive elements, were identified in the promoter region of several laccase genes that are transcriptionally activated by copper [51, 52]. A possible explanation for this stimulatory effect of copper on laccase biosynthesis could be a role for this enzyme activity in melanin synthesis [59].

3.2.3. Optimization. As the results of the canonical analysis agree with those of the contour plot study, we can conclude that there is no a masked optimum: the one predicted by few sections of contour plot analysis represents a real optimum for the whole experimental domain. The NemrodW sofware predicted the maximum laccase yield to be 153.3 ± 11.5 U/gds in optimized conditions ($CaCl_2$, 5.5 mg/gs; $CuSO_4$, 2.5 mg/gs; inoculum size, 3 fungal discs (6 mm Ø), and incubation time, 13 days). A supplementary experiment was carried out under the selected optimal conditions. It led to an experimental yield of laccase equal to 151.1 ± 6.0 U/gds which is very close to the expected value (153.3 U/gds). The optimized yield of laccase obtained in this work was higher than those obtained by other high laccase producer fungi; for example, some of the highest records

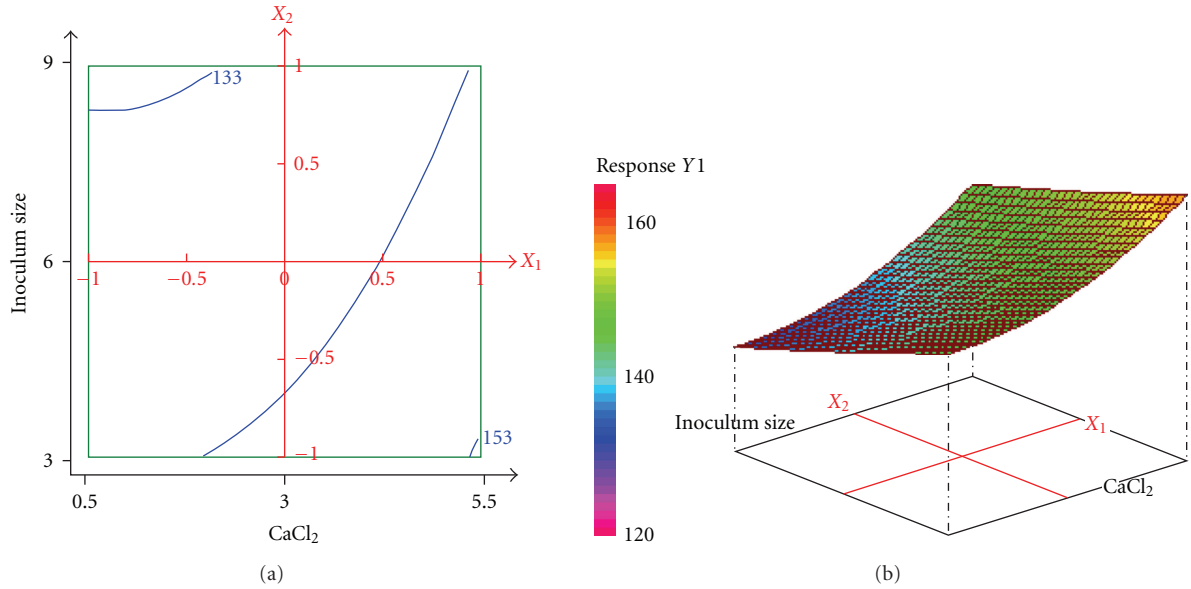

Figure 5: Contour plot and response surface plot showing the effect of $CaCl_2$ and inoculum size on the laccase yield with incubation time, $CuSO_4$ fixed, respectively, at 13 days and 2.5 mg/gs. Laccase activity is expressed in U/gds.

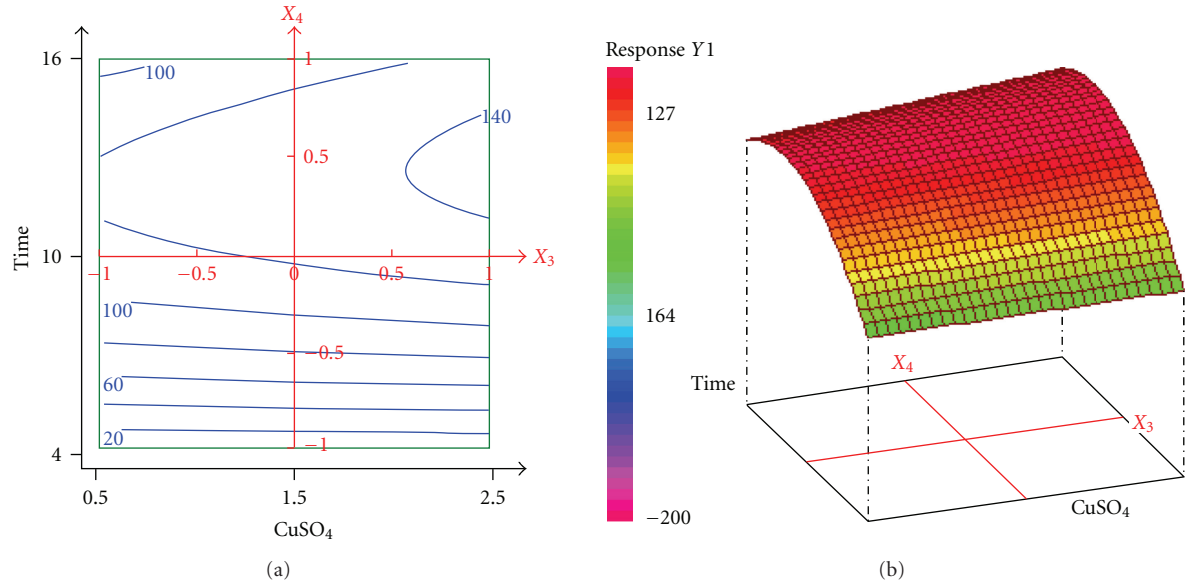

Figure 6: Contour plot and response surface plot showing the effect of incubation time, $CuSO_4$, on the laccase yield with $CaCl_2$ and inoculum size fixed respectively at 3.0 mg/gs and 3 discs. Laccase activity is expressed in U/gds.

of laccase production in SSF were obtained by *Coriolus rigida* (108 U/gs) [5], *Trametes hirsuta* (68.4 U/gs) [60], and *Pleurotus ostreatus* (65.4 U/gs) [13].

4. Conclusion

Statistical optimization of solid state fermentation conditions to obtain a high laccase yield by the white-rot fungus *Fomes fomentarius* has been successfully carried out using asymmetrical and Hoke designs. The optimal conditions for the production of laccase were determined as follows: $CaCl_2$, 5.5 mg/gs, $CuSO_4$, 2.5 mg/gs, inoculum size, 3 fungal discs (6 mm Ø), and incubation time, 13 days. Under these conditions, the experimental yield of laccase was 151.1 U/gds. The strategy adopted in this study was proved to be useful and powerful tool for screening, optimization, and modelling of solid-state fermentation process. Enhanced production of *F. fomentarius* laccase by using the statistical methodology outlined in this paper will help in various biotechnological applications at industrial levels.

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