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Solid-state Fermentation for Enhanced Production of Laccase using Indigenously Isolated *Ganoderma* sp.

Madhavi S. Revankar · Kiran M. Desai · S. S. Lele

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Abstract Laccase production by solid-state fermentation (SSF) using an indigenously isolated white rot basidiomycete *Ganoderma* sp. was studied. Among the various agricultural wastes tested, wheat bran was found to be the best substrate for laccase production. Solid-state fermentation parameters such as optimum substrate, initial moisture content, and inoculum size were optimized using the one-factor-at-a-time method. A maximum laccase yield of 2,400 U/g dry substrate (U/gds) was obtained using wheat bran as substrate with 70% initial moisture content at 25°C and the seven agar plugs as the inoculum. Further enhancement in laccase production was achieved by supplementing the solid-state medium with additional carbon and nitrogen source such as starch and yeast extract. This medium was optimized by response surface methodology, and a fourfold increase in laccase activity (10,050 U/g dry substrate) was achieved. Thus, the indigenous isolate seems to be a potential laccase producer using SSF. The process also promises economic utilization and value addition of agro-residues.

Keywords Laccase · *Ganoderma* sp. · Solid-state fermentation · Response surface methodology

Introduction

Laccases (EC 1.10.3.2) are copper-based polyphenol oxidases that catalyze the one-electron oxidation of a wide variety of organic and inorganic substrates, including mono-, di-, and polyphenols, aminophenols, methoxyphenols, aromatic amines, and ascorbate with the concomitant four-electron reduction of oxygen to water [2, 22]. Due to their broad substrate specificity, this group of enzyme has great biotechnological functions in diverse fields of industrial applications such as in pulp delignification [3, 5], textile dye bleaching [1, 11], wastewater detoxification [6, 20], and xenobiotic detoxification [16, 17]. Laccase expression in fungi is influenced by culture conditions such as nature and concentration

M. S. Revankar · K. M. Desai · S. S. Lele (✉)
Food Engineering and Technology Department, Institute of Chemical Technology,
University of Mumbai, Matunga, Mumbai 400019, India
e-mail: sslele@vsnl.net

of carbon and nitrogen sources, media composition, pH, temperature, presence of inducers and lignocellulosic materials, etc. The nutritive substances employed in the culture medium constitute significantly to the total production costs. Hence, it has been a matter of concern to find environmentally sound and economically feasible media constituents for laccase production.

Lignocellulosic residues comprising a broad range of wastes from agricultural and forest industries are known to stimulate laccase production. These agricultural wastes provide cheap source of nutrients to the fungi, which reduces the production costs considerably [7]. In recent years, there has been an increasing trend toward the exploitation of agro-industrial wastes in solid-state cultivation. Utilization of these wastes in bioprocesses not only provides alternative, cheap, and renewable substrates but also helps in solving pollution problems. In addition, the microorganisms in solid-state cultures grow under conditions closely resembling to their natural habitat. This results in higher yields of certain enzymes and metabolites than their yields in submerged cultures [15].

For an effective laccase production, it is highly essential to optimize the composition of medium and various culture conditions. However, practically, to optimize all the parameters and to establish the best possible conditions by interrelating all the parameters, numerous experiments have to be carried out which is not economical and practical. For large number of variables, the conventional "one-factor-at-a-time" approach is laborious and time consuming. Moreover, it seldom guarantees the determination of optimal conditions. Application of statistical methods such as response surface method (RSM) is helpful in defining the effects and interactions of physiological factors that play an important role in laccase production. An RSM consists of empirical modeling system that evaluates the relationship between a group of variables that can be controlled experimentally and an observed response [23]. It is the most widely used method to study the effects of the several factors influencing the responses by varying them simultaneously with limited number of experiments [4, 21, 23].

Recently, Revankar and Lele [18] have reported a new isolate of white rot fungus, the *Ganoderma* sp. (*WR-1*), which has a very high laccase producing ability. To evaluate the efficiency of this indigenous isolate *Ganoderma* sp. for laccase production under solid-state cultivation, the present work was undertaken. The objective of this work was the optimization of fermentation parameters for solid-state fermentation (SSF) by using the one-factor-at-a-time approach and by enhancing the laccase yield through supplementing the medium with additional carbon and nitrogen source using RSM.

Materials and Methods

Solid Substrates

All the agro-industrial residues, including wheat bran, were obtained locally in Mumbai, India, and were used without any pretreatment.

Fungal Strain and Culture Conditions

Indigenously isolated white rot basidiomycete *Ganoderma* sp. (*WR-1*) was used in the present work [18]. The organism was maintained through fortnightly transfer at 25°C on potato dextrose agar plates.

Preparation of SSF Medium for Inoculation

Five grams of dry substrate was taken in a 250-ml Erlenmeyer flask. The contents of the flask were autoclaved at 121°C for 20 min. A salt solution with 0.0004% $CuSO_4$, 0.005% Na_2HPO_4, 0.1% KH_2PO_4, 0.05% $MgSO_4$, 0.001% $CaCl_2$, 0.0001% $MnSO_4$, 0.0001% $ZnSO_4$, and 0.0001% $FeSO_4$ was used as the moistening medium. Two milliliters of sterile moistening medium and an appropriate amount of sterile distilled water were added aseptically in the flask, containing 5 g of sterile substrate, such that the initial moisture content was adjusted to 70%. Five agar plugs (0.8 mm in diameter) cut from actively growing fungal mycelium were used as inoculum. The contents of the flask were mixed thoroughly and incubated in a controlled humidity atmosphere at 25°C for 7 days.

To select the best substrate for laccase production, different agro-industrial wastes such as sugarcane bagasse, wheat bran, rice bran, wood shavings, peanut hull, and oat bran were used. Five grams of substrate was separately added to 250 ml Erlenmeyer flask to which a sterile moistening medium (2 ml) and an adequate quantity of sterile distilled water were added aseptically to adjust the initial moisture content to 70%.

To optimize the moisture content, 5 g of optimized substrate was added to 250 ml Erlenmeyer flask. A moistening medium (2 ml) and varying amounts of distilled water were added under sterile conditions to the substrate to adjust the initial moisture content from 40 to 80%.

To optimize the inoculum, a varying number of agar plugs were inoculated in 5 g of substrate moistened with 2 ml of moistening medium. An adequate amount of distilled water was added to achieve the optimized initial moisture content.

Response Surface Methodology: Experiment Design and Statistical Analysis

An RSM consists of an empirical modeling system that evaluates the relationship between a group of independent variables and observed responses [23]. In RSM, multiple regression analysis is employed on the quantitative data obtained from properly designed experiments. A fractional factorial central composite rotatable design (CCRD) for five independent variables was used in the present study. The experimental design comprising of concentration of different components are shown in Table 1. All the experiments were carried out in triplicates, which was necessary to estimate the experimental variability of measurements.

Five grams of wheat bran supplemented with starch and yeast extract was added in 250 ml Erlenmeyer flask. The contents of the flask were autoclaved at 121°C for 20 min. Two milliliters of sterile moistening medium and varying amounts of distilled water were aseptically added to the substrate to adjust the required moisture content. Seven agar plugs cut from actively growing fungal mycelium of *Ganoderma* sp. (*WR-1*) that was grown on potato dextrose agar plates was inoculated in each flask. The contents of the flask were mixed thoroughly and incubated in a controlled humidity atmosphere at 25°C for 7 days.

Table 1 represents the experimental design with respective laccase yields. The relationship of the independent variables and the response was quantified by the second-order polynomial equation. The Design Expert (Version 2.05, Stat-Ease Inc., Minneapolis, MN, USA) software package was used to estimate the response of dependent variables and optimized conditions.

Table 1 Response surface central composite design and experimental laccase yield.

Number	A: wheat bran (g)	B: $CuSO_4$ (mg)	C: moisture (%)	D: starch (g)	E: yeast extract (g)	Laccase activity (U gds^{-1} min^{-1})
1	2	2	50	0.5	0.13	804.95±0.54
2	5	2	50	0.5	0.06	1,706.25±0.35
3	2	5	50	0.5	0.06	1,048.05±0.76
4	5	5	50	0.5	0.13	2,228.4±0.38
5	2	2	70	0.5	0.06	1,340.99±0.82
6	5	2	70	0.5	0.13	6,383.04±0.51
7	2	5	70	0.5	0.13	3,156.56±0.29
8	5	5	70	0.5	0.06	6,492.06±0.42
9	2	2	50	1	0.06	810.055±0.37
10	5	2	50	1	0.13	2,283.06±0.29
11	2	5	50	1	0.13	1,209.82±0.51
12	5	5	50	1	0.06	2,509.37±0.73
13	2	2	70	1	0.13	1,636.57±0.47
14	5	2	70	1	0.06	6,858.98±0.28
15	2	5	70	1	0.06	4,682.55±0.37
16	5	5	70	1	0.13	8,974.67±0.19
17	0.5	3.5	60	0.75	0.09	275.92±0.29
18	6.5	3.5	60	0.75	0.09	3,136.63±0.74
19	3.5	0.5	60	0.75	0.09	1,934.91±0.58
20	3.5	6.5	60	0.75	0.09	1,070.32±0.69
21	3.5	3.5	40	0.75	0.09	1,459.79±0.72
22	3.5	3.5	80	0.75	0.09	2,102.55±0.55
23	3.5	3.5	60	0.25	0.09	3,213.12±0.98
24	3.5	3.5	60	1.25	0.09	3,736.58±0.37
25	3.5	3.5	60	0.75	0.03	3,199.33±0.63
26	3.5	3.5	60	0.75	0.16	3,143.3±0.58
27	3.5	3.5	60	0.75	0.09	3,443.69±0.94
28	3.5	3.5	60	0.75	0.09	3,220.45±0.39
29	3.5	3.5	60	0.75	0.09	3,304.65±0.82
30	3.5	3.5	60	0.75	0.09	3,534.26±0.39
31	3.5	3.5	60	0.75	0.09	3,476.77±0.84
32	3.5	3.5	60	0.75	0.09	3,354.72±0.74

Laccase Assay

The fermented substrates were mixed thoroughly by keeping the flasks on a rotary shaker at 150 rpm for 10 min after adding 50 ml of distilled water. Crude enzyme from the fermented matter was extracted by direct filtration using Whatman #1 filter paper. The filtrate was collected, and the laccase activity was determined.

The laccase activity was determined by measuring the oxidation of 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) at 420 nm ($\varepsilon=3.6\times10^4$ cm^{-1} M^{-1}) [10]. The reaction mixture contained 0.4 ml of 1 mM ABTS, 1.2 ml of 0.1 M glycine–HCl buffer (pH 3), and 0.8 ml aliquots of appropriately diluted culture fluid. One activity unit was defined as the amount of enzyme that oxidized 1 μmol ABTS/min. Laccase yield was expressed as units per gram dry substrate (U/gds). All enzyme analysis has been carried out in triplicates.

Results and Discussion

Optimization of Different Parameters for SSF

Different agro-industrial wastes were screened to select the best substrate for maximum laccase production. Figure 1 depicts the yield of laccase for various solid-state substrates. Minimum laccase yield of 142 U/gds was obtained using sugarcane bagasse as the substrate. Wheat bran was found to be the best substrate and gave maximum laccase activity of 974 U/gds. This may be because wheat bran is rich in growth factors, vitamins, and proteins [8]. The higher yields with wheat bran are consistent with earlier reports on SSF using wheat bran as substrate [8, 12]. Hence, it was selected as the best substrate for laccase production.

The screening of substrate was followed by optimization of the initial moisture content for laccase production. Moisture is a critical factor in SSF for new cell synthesis, growth, and enzyme production [13]. Optimization of initial moisture content is essential in SSF as it affects the substrate utilization and laccase production. Figure 2 shows the effect of varying moisture contents on laccase production. It can be seen that with increasing initial moisture content from 40 to 70%, a considerable increase in laccase activity was observed. Maximum laccase activity of 1,387 U/gds was obtained at 70% moisture content. However, when the moisture content was increased beyond 70%, a substantial decrease in laccase activity was observed. This may be because the increase in water content at constant substrate volume reduces the air content of the substrate (air occupied within the interparticle space), thereby affecting laccase activity. In addition, at the lower and the higher water contents beyond 70%, the decomposition rate of the total organic matter decreases and this in turn affects laccase production [14]. Thus, a 70% initial moisture content was selected for the further studies.

Inoculum plays a significant role in enzyme production in SSF. Lower level of inoculum may not be sufficient for initiating the growth [19], whereas higher level may cause competitive inhibition. Thus, determining optimum inoculum size becomes a crucial step in SSF. To optimize the inoculum size for laccase production, a varying number (one to nine) of agar plugs (0.8 mm in diameter) cut from actively growing fungal mycelium were inoculated. Figure 3 shows the effect of inoculum size on laccase production. It was found that laccase activity increases with the increase in the number of agar plugs. Maximum

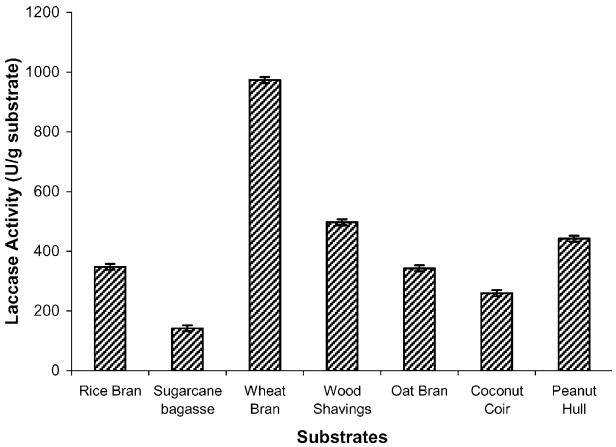

Fig. 1 Effect of different substrates on laccase production by *Ganoderma* sp.

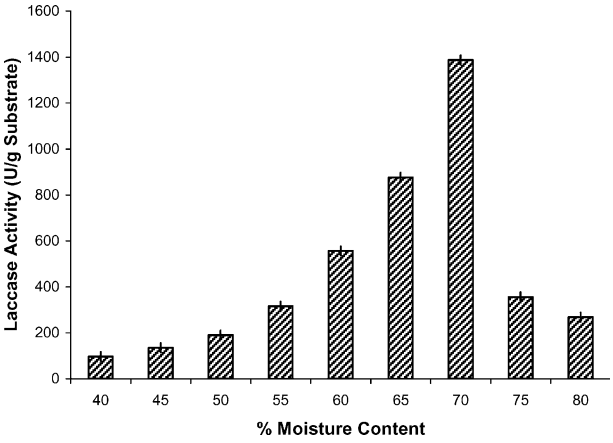

Fig. 2 Effect of varying moisture content on laccase production by *Ganoderma* sp.

laccase activity of 2,399 U/gds was obtained with seven agar plugs. An increase in inoculum size enhanced the utilization of the solid substrate, thereby improving laccase activity. However, with further increase in inoculum above seven agar plugs, laccase production was found to decrease because of the depletion of the nutrients, resulting in a decrease in metabolic activity.

Thus, all the parameters for solid-state fermentative production of laccase were optimized. Wheat bran with 70% initial moisture content and an inoculum size of seven agar plugs were found to be optimum and gave a maximum laccase yield of 2,400 U/gds using *Ganoderma* sp. Earlier reports suggest that supplementation of solid-state medium with additional carbon and nitrogen sources enhance enzyme production [19]. Previous studies in our lab suggested that starch and yeast extract were the best carbon and nitrogen sources for laccase production in submerged fermentation [18]. Hence, in the present work, the SSF medium was supplemented with starch and yeast extract and was optimized using RSM.

Response Surface Methodology

Response surface methodology is an empirical statistical modeling technique. In the present study, a fractional factorial CCRD, which consists of 32 trials, including six center-point

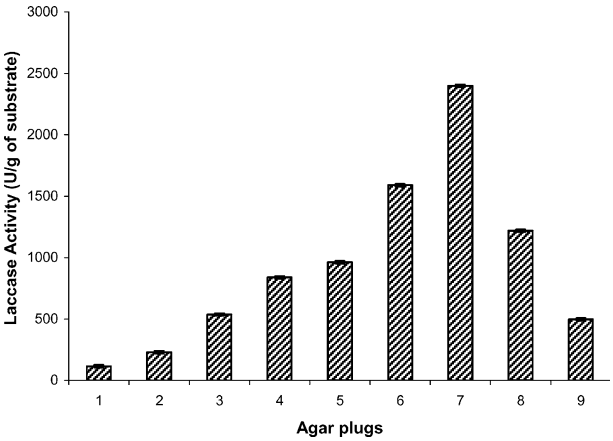

Fig. 3 Effect of varying inoculum size on laccase production by *Ganoderma* sp.

replicates, was used to develop a second-order polynomial model form only [9]. Earlier work that was carried out indicated that $CuSO_4$, starch, and yeast extract have the most prominent effect on laccase production [18]; hence, these variables were selected. Preliminary experiments were carried out to select the concentration range for RSM. Table 1 documents the experimental design and the concentration of different components used. The last column of the table documents the experimental laccase yield obtained. Second-order polynomial equation was used to correlate the independent process variables, X_i, with laccase yield. The second-order polynomial coefficient for each term of the equation was determined through multiple regression analysis using the Design Expert.

The analysis of variance (ANOVA) summary is shown in Table 2. Analysis of variance is important in determining the adequacy and significance of the quadratic model. Values of "Prob>*F*" less than 0.0500 indicate that the model terms are significant. The model *F* value of 19.73 implies that the model is significant and there is only a 0.01% chance that a "model *F* value" this large could occur due to noise. In this system, A (wheat bran), B ($CuSO_4$), C (moisture), D (starch), B^2 $(CuSO_4)^2$, AC (wheat bran × moisture), and BC ($CuSO_4$ × moisture) are significant model terms.

Various statistical parameters are shown in Table 3. The Cook's distance and the outlier *t* indicate the position of the points corresponding to the normalized values, whereas leverage is a measure of the amount of influence a given data value has on the fitted linear regression. The residual represents the difference between the observed value of a response measurement and the value that is fitted under the hypothesized model. Here, the residual values are small, which shows that the model prediction is accurate. As can be seen from the table, outlier values are negligible which means that errors in the recording of experimental values are negligible.

Table 2 ANOVA for response surface quadratic model.

Source	*DF*	*F* value	Prob>*F*
Model Significant	20	19.73	<0.0001 (significant)
A	1	133.51	<0.001
B	1	11.75	0.0057
C	1	153.53	<0.001
D	1	9.52	0.0104
E	1	0.19	0.6687
A^2	1	2.48	0.1438
B^2	1	5.29	0.0420
C^2	1	1.89	0.1962
D^2	1	3.83	0.0762
E^2	1	3.69	0.0811
AB	1	0.43	0.5240
AC	1	45.12	<0.0001
AD	1	0.63	0.4454
AE	1	3.60	0.0842
BC	1	8.21	0.0153
BD	1	3.60	0.0843
BE	1	1.768	0.9672
CD	1	3.45	0.0902
CE	1	0.12	0.7324
DE	1	2.35	0.1539
Lack of fit			0.0008 (significant)

Table 3 Calculation of various statistical element.

Run order	Residual	Leverage	Cook's distance	Outlier *t*
1	−0.901	0.598	0.058	−0.892
2	−0.959	0.598	0.065	−0.955
3	−0.462	0.598	0.015	−0.445
4	1.525	0.879	0.803	1.638
5	−0.042	0.879	0.001	−0.040
6	−0.485	0.879	0.081	−0.468
7	0.183	0.598	0.002	0.175
8	−2.623	0.598	0.488	−4.087
9	0.729	0.598	0.038	0.712
10	−1.546	0.598	0.170	−1.666
11	0.495	0.879	0.085	0.477
12	0.442	0.879	0.068	0.425
13	0.403	0.159	0.001	0.387
14	−1.525	0.879	0.802	−1.637
15	−0.404	0.598	0.012	−0.388
16	0.997	0.879	0.343	0.997
17	−0.102	0.159	0.000	−0.098
18	0.545	0.879	0.103	0.527
19	0.162	0.159	0.000	0.155
20	0.011	0.879	0	0.010
21	−2.091	0.598	0.310	−2.569
22	1.029	0.879	0.366	1.032
23	−0.102	0.159	0.000	−0.098
24	−0.538	0.879	0.100	−0.520
25	1.261	0.598	0.113	1.299
26	0.517	0.159	0.002	0.499
27	2.565	0.879	2.271	3.857
28	−1.072	0.879	0.397	−1.080
29	1.978	0.879	1.350	2.349
30	0.064	0.159	0.000	0.061
31	1.481	0.879	0.758	1.579
32	2.512	0.879	2.178	3.668

Various model parameters are shown in Table 4. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Here, the ratio of 19.147 obtained in this model indicates an adequate signal. The mathematical model is reliable with a correlation coefficient of 0.9729. It suggests that the model was unable to explain only 2.71% of the total variations. The adjusted R^2 value of 0.9236 suggests that the model was significant. A very low value of coefficient of the variation (C.V.) (17.46) clearly indicates a very high degree of precision and a good deal of reliability of the experimental values.

Table 4 Model fitting for laccase production.

Model terms	Values
C.V.	17.46
R^2	0.9729
Adj. R^2	0.9236
Adeq. Precision	19.147

The application of RSM resulted in the following empirical relationship between laccase yield and media components in actual factors:

$$\begin{aligned}\text{Laccase yield} = {} & 10,248 - 2,897 * \text{wheat bran} - 990 * CuSO_4 - 64 * \text{moisture} - 10,736 \\ & * \text{starch} - 20,147 * \text{yeast extract} - 71.42 * \text{wheat bran}^2 - 104.38 \\ & * CuSo_4^2 - 1.40 * \text{moisture}^2 + 3,197 * \text{starch}^2 + 1.6 * \text{yeast extract}^2 \\ & - 40.0 * \text{wheat bran} * CuSO_4 + 61.9 * \text{wheat bran} * \text{moisture} + 291.81 \\ & * \text{wheat bran} * \text{starch} + 4,999.8 * \text{wheat bran} * \text{yeast extract} + 26.41 \\ & * CuSO_4 * \text{moisture} + 699.61 * CuSO_4 * \text{starch} + 110.73 * CuSO_4 \\ & * \text{yeast extract} + 102.71 * \text{moisture} * \text{starch} - 138.57 * \text{moisture} \\ & * \text{yeast extract} - 24,199 * \text{starch} * \text{yeast extract}\end{aligned}$$

Figure 4 represents the response surface plots showing the effect of interactions of $CuSO_4$ and moisture on the yield of laccase, whereas Fig. 5 elaborates the laccase yield as a function of moisture and wheat bran. The critical analysis of the response surface represents that laccase yield is a function of concentrations of two medium components, with all other nutrients being at a fixed level.

Thus, the optimal concentrations of individual media components for laccase production by solid surface fermentation obtained were found to be 6 g of wheat bran, 6 mg of $CuSO_4$, 72% of moisture, 0.7 g of starch, and 0.16 g of yeast extract. The model predicted a value

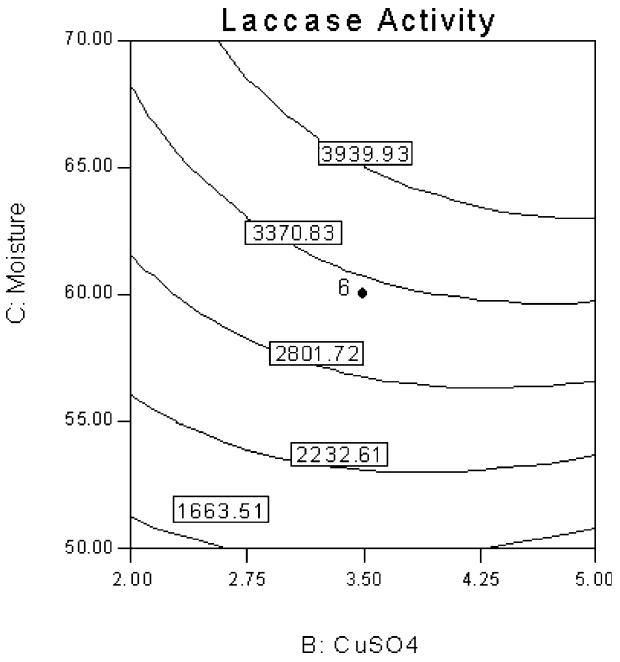

Fig. 4 Response surface plots showing the effect of interaction between $CuSO_4$ and moisture on the yield of laccase

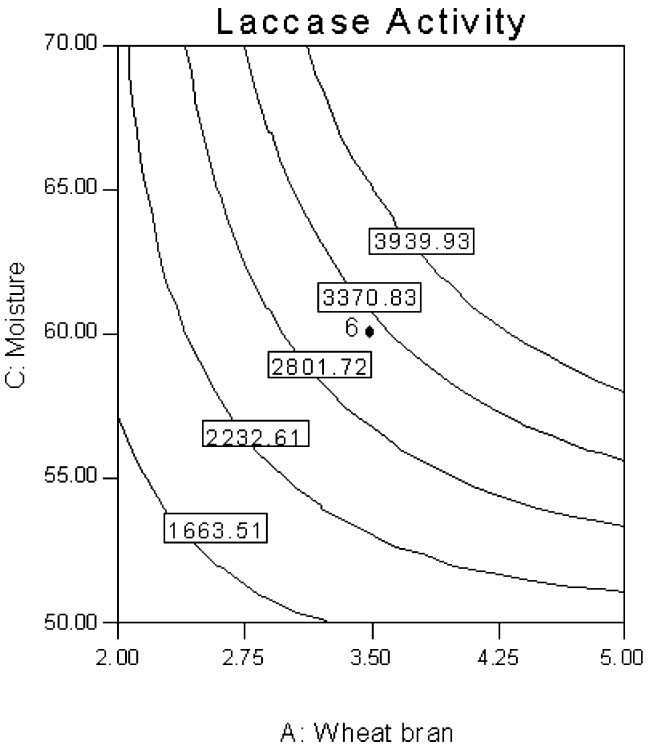

Fig. 5 Response surface plots showing the effect of interaction between wheat bran and moisture on the yield of laccase

of 10,125 U/gds for laccase production using optimized media composition. On experimental verification, a laccase activity of 10,050 U/gds was obtained which was in close agreement with the RSM-model-predicted yield. Thus, a fourfold increase in laccase yield from 2,400 to 10,050 U/gds was obtained by statistical optimization of supplemented media. Thus, an optimum medium for laccase production by *Ganoderma* sp. using SSF was determined. Laccase yield by SSF, which is reported by this strain, is very high. Earlier studies in our lab reported that this strain is a potential laccase producer for submerged fermentation [18]. Similar results were obtained for SSF. Thus, the indigenous isolate *Ganoderma* sp. seems to be potentially competitive as a commercial laccase producer.

Conclusions

The indigenous isolate *Ganoderma* sp. isolated from the bark of a dead tree was found to be an excellent laccase producer using SSF. The solid-state medium was optimized using the one-factor-at-a-time approach. Wheat bran with 70% initial moisture, inoculated with seven agar plugs, was found to be optimum and gave a laccase yield of 2,400 U/gds. Supplementation of medium with starch and yeast extract enhanced laccase activity. The supplemented medium was optimized by RSM, and a fourfold increase in laccase activity (10,050 U/gds) was achieved. Thus, it can be said that the indigenous isolate *Ganoderma* sp. can be effectively used for large-scale production of laccase, an important enzyme for various industrial applications.

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