Optimization of Laccase Production using White Rot Fungi and Agricultural Wastes in Solid-State Fermentation

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Abstract. Laccase has been produced in a solid-state fermentation (SSF) process using white rot fungi and various lignocellulose-based substrates. The white rot fungi used were *Marasmius* sp., *Trametes hirsuta*, *Trametes versicolor* and *Phanerochaete crysosporium*. The solid substrates employed in this research were collected from agricultural waste, specifically empty fruit bunches (EFB), rice straw, corncobs and rice husks. The objective of this research was to determine the most promising fungus, the best solid substrate and the optimal conditions for the production of laccase. The results showed that *Marasmius* sp. on all solid substrates displayed a higher laccase activity than that of any other strain of white rot fungi. *Marasmius* sp. and a solid substrate of rice straw demonstrated the highest laccase activity of 1116.11 U/L on day 10. Three significant factors, *i.e.* pH, temperature and yeast extract concentration, were studied by the response surface method on laccase production using *Marasmius* sp. and rice straw. The optimized conditions were a pH, temperature and yeast extract concentration of 4.9, 31ºC and 0.36 g/L, respectively. The fermentation of *Marasmius* sp. in SSF on agricultural waste shows a great potential for the production of laccase.

Keywords: *agriculture waste; laccase; Marasmius sp.; optimization; solid-state fermentation.*

1 Introduction

Various agricultural industries in Indonesia are generating an enormous amount of waste in the form of biomass, such as empty fruit bunches (EFB), rice straw, rice husks and corncobs. In 2010, Indonesia produced crude palm oil (CPO), rice and corn at 21.5 million tons, 66 million tons, and 17.8 million tons, respectively [1]. From that production, solid wastes in the form of empty fruit bunches, rice straw, rice husks and corncobs were at 22.5 million tons, 99

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million tons, 11 million tons and 5.3 million tons, respectively [2]. These agricultural wastes are relatively inexpensive and contain abundant nutrients, such as hemicellulose, cellulose and lignin, and act as an inducer enzyme production [3]. Therefore, these materials can be utilized as support-substrate for fermentative processes, especially to produce ligninolytic enzymes in solid-state fermentation (SSF).

SSF is a fermentation process conducted in the absence of free flowing water, using either a natural support or an inert support as solid material [4]. SSF processes have proven to be particularly suitable for the production of enzymes by filamentous fungi, due to the fact that they reproduce the natural living conditions of filamentous fungi. The selection of an appropriate solid material for performing SFF is very important, as it has a strong influence on the process [3].

Laccases have been the subject of intensive research during the last decades, because they have broad substrate specificity and do not need the addition or synthesis of a low molecular weight cofactor since their co-substrate (oxygen) is usually present in their environment. Most laccases are extra-cellular enzymes, making the purification stages very easy and they generally show a considerable level of stability. Due to these characteristics, laccases are very suitable for application in several bioprocess technologies, such as bio-pulping, bio-bleaching, and industrial wastewater treatment [5].

Currently, it is very interesting to discover new methods in laccase production with a higher activitiy at a lower cost, due to the huge potential for the development of efficient biotechnology processes [5]. It has been the practice to use lignocellulosic agro-industrial wastes for the production of ligninolytic enzymes such as laccases [5, 6]. For an effective laccase production, it is highly essential to optimize the most significant process parameters. Application of statistical methods such as the response surface method (RSM) is very useful in defining the effects and interactions of the physiological factors that play an important role in laccase production. The RSM consists of an empirical modeling system that evaluates the relationship between a group of variables that can be controlled experimentally and an observed response. It is the most widely used method to study the effects of several factors influencing the responses by varying significant parameters simultaneously with a limited number of experiments [7].

The objectives of this research were, firstly, to select the most promising fungal species and the best lignocellulosic support substrate for the production of laccase, and secondly to determine the optimum conditions for SSF using the response surface method (RSM) with the selected fungus and substrate.

2 Materials and Method

2.1 Microorganisms

Four strains of white rot fungi, *Phanerocaete chrysosporium*, *Trametes hirsuta*, *Trametes versicolor* and *Marasmius* sp., were provided by the Laboratory of Microbiology and Bioprocess Technology, Department of Chemical Engineering, Institut Teknologi Bandung, Indonesia. All strains were maintained on potato dextrose agar (PDA) medium in a Petri dish and incubated for 7 days at 28ºC. The seven-day old culture served as stock, and was stored at 4ºC until use. All strains were re-plated with the same medium every three months.

2.2 Lignocellulosic Materials

Empty fruit bunches, rice straw, rice husks and corncobs were used as support-substrate in the solid-state fermentation process. Empty fruit bunches were collected from a palm plantation in the province of South Sumatra. Rice straw, rice husks and corncobs were collected from a farm near Bandung City. Prior to use in the fermentation process, the empty fruit bunches and rice straw were chopped to a length of 3 cm, while the corncobs were cut to a cubical size of 1 x 1 x 1 cm. No pre-treatment was used for the rice husks. Cellulose, hemicelluloses and lignin in each agricultural waste were analyzed using TAPPI standard methods T 17 wd-70 – Cellulose in Wood, T 223 – Pentosans in Wood and Pulp, and T222 – Acid-Insoluble Lignin in Wood and Pulp, respectively. All analyses were conducted at the Center for Pulp and Paper, Ministry of Industry, Bandung, Indonesia.

2.3 Experiment

2.3.1 Screening of White Rot Fungi and Lignocellulosic Material

To identify the suitable substrate and microorganism, seven grams of lignocellulosic material was put into a conical flask of 250 mL and supplemented by 20 mL of modified Kirk medium [8]. The medium contained glucose 4.3 g/L, KH_2PO_4 1.7 g/L, $MgSO_4.7H_2O$ 0.4 g/L, $CaCl_2$ 0,09 g/L, sodium acetate 2.3 g/L, diammonium tartrate 0.4 g/L, $MnCl_2$ 0.02 g/L, yeast extract 0.3 g/L, $CuSO_4.7H_2O$ 0.01 g/L, H_2MoO_4 0.007 g/L, $MnSO_4.4H_2O$ 0.01 g/L, $ZnSO_4.7H_2O$ 0.006 g/L and $Fe_2(SO4)_3$ 0.007 g/L. The flask with substrate and medium were steam-sterilized at 120ºC for 20 minutes prior to inoculation. The medium was then allowed to cool down. Two pieces of inoculum from the agar plate with a size of 1.5 x 1.5 cm were then transferred aseptically into the flask. All cultures were incubated under static conditions at room temperature (±28ºC) for 12 days fermentation time. Laccase activity was analyzed every day in

duplicate. Substrate, microorganism and day of incubation giving the maximum laccase activity were used to optimize the laccase production.

2.3.2 Optimization of Laccase Production

The selected substrates and microorganisms were used to optimize the process parameters of temperature, pH and yeast extract concentration. Effects on each parameter were investigated using central composite design (CCD). A set of 20 experiments, including six center points, was performed in duplicate. The effect of each variable on the enzyme production was studied at five different levels, *viz*. –α, –1, 0, +1, + α. The level of temperature, pH and yeast extract used were in the range of 20-35°C, 4-6, and 0.2-0.8 g/L, respectively. The actual values of each variable used for the optimization are presented in Table 1. In order to build a convenient model, all analyses were based on the actual values.

Table 1 Value of each variable used for optimization.

	(–α)	**(–)**	**(0)**	**(+)**	**(+α)**
pH	4.0	6.0	3.3	6.7	5.0
temperature, °C	25.0	35.0	22.5	40.0	27.5
yeast extract , g/L	0.2	0.8	0.0	1.0	0.5

2.3.3 Enzyme Extraction

Samples were collected daily to determine the laccase activity. First, the samples were extracted by adding 50 mL of sodium acetate buffer (pH 4.5) and thoroughly shaken at 100 rpm for 2 hours, and finally ground in a mortar. Ten mL of slurry was centrifuged at 5000 rpm for 10 minutes. The filtered supernatant was then used for the determination of the laccase activity.

2.3.4 Laccase Assay

Laccase activity was determined with 2,2'-azinobis (3-ethylbenzthiazoline 6 sulphonic acid) (ABTS) in 0.4 mM sodium acetate buffer (pH 4.5). Oxidation of ABTS was determined by the increase in A_{420} (ε_{420} = 36 (mM cm)$^{-1}$) using a spectrophotometer. One unit of enzyme activity (U) was defined as the amount of enzyme required in order to oxidize 1 μmol of ABTS per minute.

3 Results and Discussion

3.1 Screening of White Rot Fungi and Lignocellulosic Materials

The main components of the agricultural wastes as listed in Table 2 are lignin, cellulose and hemicellulose. The highest content of lignin (31.97%), cellulose

(41.05%) and hemicelluloses (25.55%) were found in the rice straw, rice husks and empty fruit bunches, respectively. Lignocelluloses can be utilized by fungi as support nutrient and also as an inducer for laccase production.

Table 2 Chemical composition of agriculture wastes.

Agriculture waste	Content (%)		
	Lignin	α-cellulose	hemicellulose
Rice husks	18.82	41.05	17.63
Rice straw	31.97	31.10	18.35
Corncobs	15.48	16.21	17.65
Empty fruit bunches	25.79	29.30	25.55

The results show that *Marasmius* sp. and *Trametes hirsuta* grew on all substrates. *Trametes versicolor* also grew on all substrates, except on the rice husks. However, growth of *Phanerocaete chrysosporium* was only observed on the rice husks. Laccase production was associated with fungal growth on the support-substrate. The time course of the laccase activity is shown in Figure 1.

When empty fruit bunches were used as support substrate, all strains produced laccase, except for *Phanerocaete chrysosporium*. As shown in Figure 1(A), laccase production in the cultures of *Marasmius* sp. began on day 3 (51.04 U/L) and reached maximum activity at day 8 (330.07 U/L). The maximum production in the cultures of *Trametes hirsuta* and *Trametes versicolor* were 220.14 U/L (on day 7) and 134.02 U/L (on day 8) respectively.

When rice husks were used as support substrate, the fungal cultures displayed a lower activity than on any other substrate. As shown in Figure 1(D), only the cultures of *Marasmius* sp. and *Trametes hirsuta* produced laccase, at a maximum level of 182.64 U/L (on day 10^{th}) and 51.53 U/L (on day 4^{th}), respectively.

A higher activity was obtained when corncobs were used (Figure 1 C). *Marasmius* sp. reached peak activity at 872.09 U/L, followed by *T. hirsuta* and *T. versicolor* with a peak activity of 400.56 U/L (on day 8) and 134.03 U/L (on day 8), respectively.

The culture of *Marasmius* sp. grown on rice straw produced the highest laccase activity (1116.11 U/L), on day 10 (Figure 1 B). This study showed that laccase production depends on the species of white rot fungi used. Enzyme activity was higher than the activity we observed during a previous study in a submerged culture (457.6 U/L, data not shown). González, *et al.* [9] also reported that they

were able to generate higher enzyme activities in SSF than in submerged fermentation.

Obviously the best support-substrate/fungal species combination for laccase production was *Marasmius* sp. grown on rice straw. Moreover, rice straw served as an efficient matrix for the attachment of *Marasmius* sp. This might be due to the higher hydrophobicity and surface charge of rice straw in comparison to other lignocellulosic materials. Osma, *et al.* [10] revealed that the most important characteristics that influence adhesive behavior of filamentous fungi towards the support-substrate are the hydrophobicity and the surface charge of the substrate.

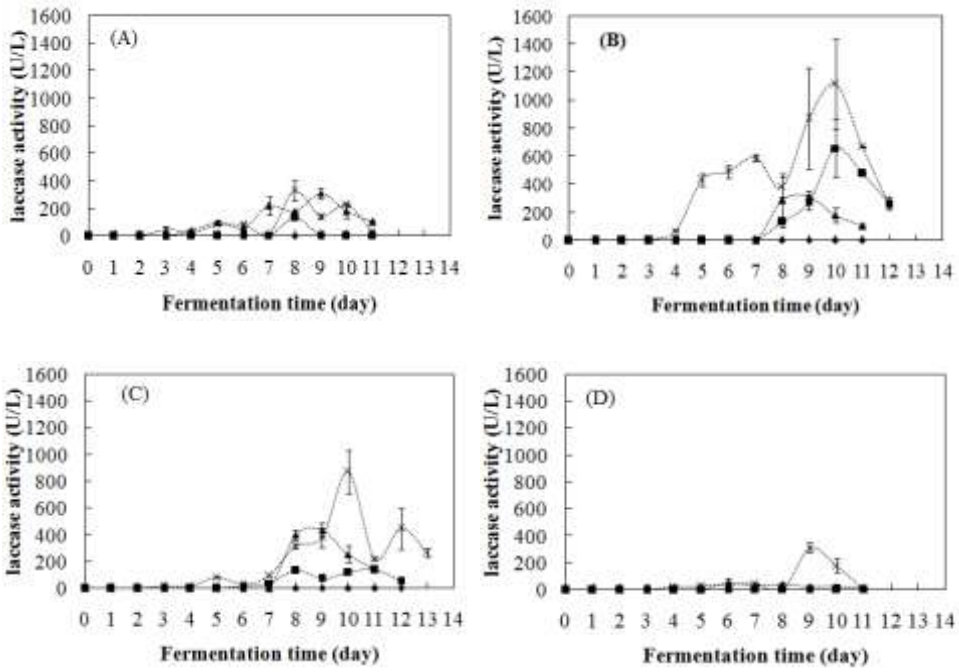

Figure 1 Time course of laccase activity from white rot fungi on agricultural wastes in SSF, (A) empty fruit bunches; (B) rice straw; (C) corncobs; (D) rice husks (---◆--- *P. crysosporium*, ---■--- *T. versicolor*, ---▲--- *T. hirsuta*, ---×--- *Marasmius* sp.)

3.2 Optimization of Laccase Production

The selection of the best white rot fungus and substrate for laccase production by SSF was basically done by using the optimization by one-factor-at-a-time (OFAT) method. This method is very useful for categorical factors, such as type

of substrate and fungus, but cannot investigate the interaction between those factors. *Marasmius* sp. and rice straw produced the highest laccase activity, so this fungus and substrate were used for the optimization of the laccase production. Laccase production was optimized by the response surface method (RSM) coupled with central composite design (CCD), consisting of 20 runs, including six center points. The variables used for this optimization were: temperature, pH, and concentration of yeast extract. These variables were chosen due to their strong influence on fungal growth and laccase production.

The results of the 20 runs are randomly presented in Table 3. Laccase activity as a response is presented in the right column, and shows the average value of the experiment in duplicate. The analysis of variance (ANOVA) is presented in Table 3. Fit analysis summary of the model suggests that the data model is quadratic.

Table 3 Results for laccase production in SSF.

Run	A: pH	B: Temperature (°C)	C: Yeast extract (g/L)	Laccase Activity (U/L)
1	5.0	27.5	0.5	1565.28±95.07
2	5.0	27.5	0.5	1249.79±59.91
3	6.0	35.0	0.2	815.69±49.69
4	4.0	25.0	0.2	1215.28±47.92
5	5.0	27.5	0.5	1534.17±71.94
6	6.0	35.0	0.8	108.40±9.25
7	4.0	35.0	0.8	ND
8	5.0	22.5	0.5	ND
9	5.0	27.5	0.0	1356.25±70.71
10	3.3	27.5	0.5	103.74±1.52
11	4.0	25.0	0.8	1170.56±56.17
12	6.0	25.0	0.2	1492.36±52.05
13	4.0	35.0	0.2	1563.33±63.24
14	5.0	27.5	0.5	1425.28±24.36
15	5.0	40.0	0.5	270.67±0.27
16	5.0	27.5	0.5	1435.97±48.12
17	5.0	27.5	0.5	1560.42±46.55
18	5.0	27.5	1.0	1399.51±67.72
19	6.0	25.0	0.8	1122.92±84.26
20	6.7	27.5	0.5	1271.18±53.03

ND – Not detected

From Table 4, the F value shows that the model is significant at a 95% confidence level. The p-value (Prob>F) being less than 0.05 (<0.0001) indicates that the model is significant. This means that the variables had a significant effect on the response. According to the analysis of variance, the variables *A, B, C, A^2, B^2, C^2, AB, AC* are significant with a p value (Prob>F) less than 0.05. Only *BC* is not significant. The mathematical model resulting from the analysis of the data in Table 4 is expressed in Eq. 1.

$$\begin{aligned} &Laccase\ activity = \\ &-37230.1 + 3179.8A + 2200.7B - 5927.0C - 192.2A^2 \\ &- 32.5B^2 - 2642.2C^2 - 50.5AB + 698.0AC - 59.9BC \end{aligned} \quad (1)$$

Table 4 Analysis of variance (ANOVA) for quadratic model with laccase activity as a response.

Variable	Sum of Squares	DF	Mean Square	F Value	Prob > F
Model	4816515.0	9	535168.3	45.34	< 0.0001
A	127094.0	1	127094.0	10.77	0.0135
B	1179180.0	1	1179180.0	99.91	< 0.0001
C	702820.1	1	702820.1	59.55	0.0001
A^2	128507.9	1	128507.9	10.89	0.0131
B^2	2840175.0	1	2840175.0	240.65	< 0.0001
C^2	345546.1	1	345546.1	29.28	0.0010
AB	294784.7	1	294784.7	24.98	0.0016
AC	216909.2	1	216909.2	18.39	0.0036
BC	43372.8	1	43372.8	3.67	0.0968
Residual	82615.9	7	11802.3		

The model was examined by model adequacy checking, including the normal probability plots and residual plots. Figure 2 is the normal probability plot of residuals and shows that the residuals are generally located on a straight line, which means that errors are normally distributed, while Figure 3 shows that the residuals do not display a specific pattern. This indicates that the model is accurate to predict laccase production for the variables pH, temperature and concentration of yeast extract. The effects of pH, temperature and yeast extract concentration on laccase production can be seen from the three-dimensional profile curves shown in Figures 4, 5 and 6. Figure 4 shows the curve of the relationship between temperature and pH, while Figure 5 shows the curve of the relationship between pH and concentration of yeast extract, and Figure 6 shows

the curve of the relationship between temperature and concentration of yeast extract.

Laccase activity increased as the temperature increased until it reached a maximum and then decreased. Laccase production revealed an optimal temperature around 30-31ºC. Based on the results of the ANOVA, the temperature is a variable that has a very significant effect on laccase production. The results indicate that *Marasmius* sp. produces laccase in accordance with its growth at room temperature (±28°C). These results are in agreement with Kunamneni, *et al.* [11], who reported that laccase production is optimal at a temperature of 25ºC in the presence of light. However, without light the ideal temperature is 30ºC. In general, laccase production has an optimum within a temperature range of 25-30ºC. According to Krishna [12], the temperature influences the growth in SSF, the production of enzymes and metabolites. In the development of biological processes, the temperature plays an important role since it determines several other factors, such as protein denaturation, and acceleration and inhibition of enzyme production [12].

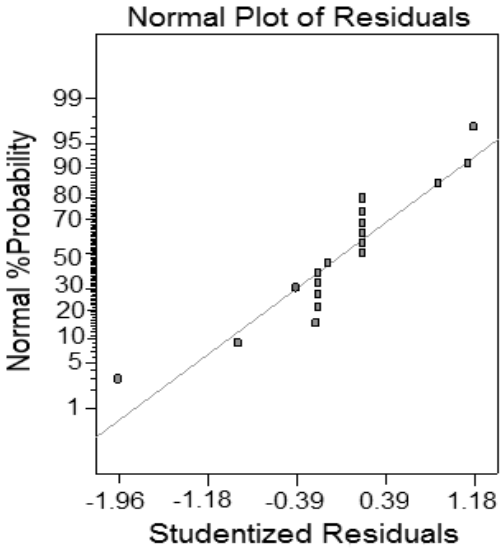

Figure 2 Normal probability plot of laccase activity.

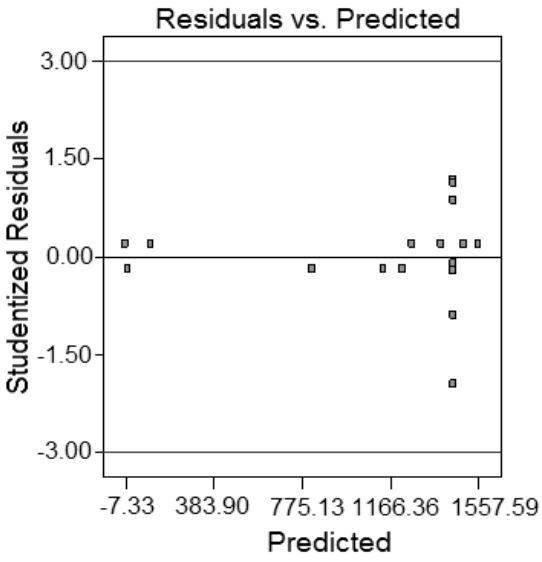

Figure 3 Plot of residual and predicted laccase activity.

The effects of pH and yeast extract concentration on laccase production can be seen in Figure 5. Laccase production is optimal at a pH level of around 5. The results confirm the findings of Kunamneni, *et al.* [11]. Filamentous fungi grow

well over a wide range of pH levels, the optimum range being 2-9 [12]. For yeast extract, the optimum concentration is 0.2 g/L. Figure 5 shows that the concentration of yeast extract had a negative effect on laccase production [13]. A higher concentration of yeast extract caused a decrease in laccase production.

In order to check the accuracy of the optimum conditions generated by the software, there was an additional experiment, presented in Table 5, in order to compare the values predicted by the software with the experimental values. It shows that there is a good agreement between the predicted and the experimental values, which validates the model.

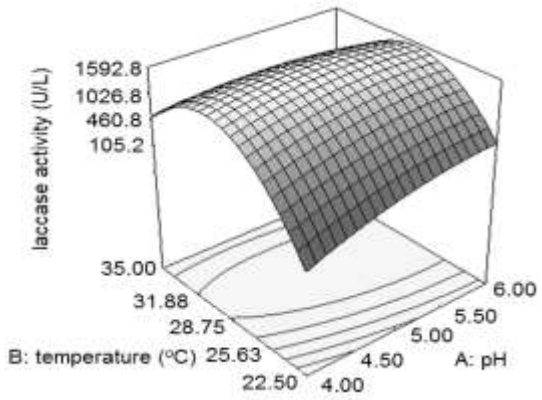

Figure 4 Effects of pH and temperature on laccase activity at a yeast extract concentration of 0.5 g/L.

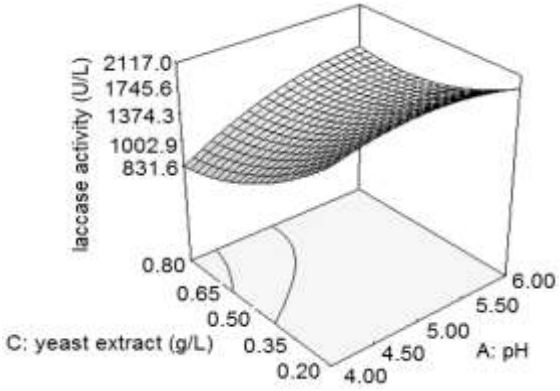

Figure 5 Effects of pH and yeast extract concentration at a temperature of 27.5°C.

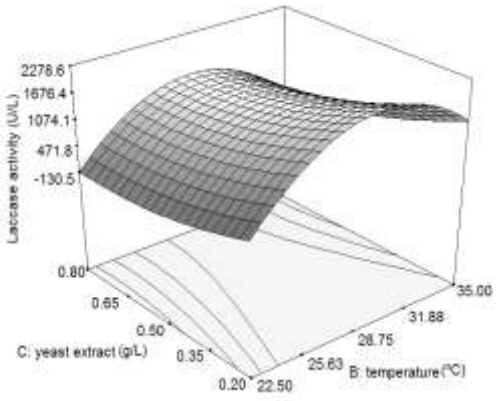

Figure 6 Effects of temperature and yeast extract concentration at a pH of 5.

Table 5 Validation of model.

pH	Temperature (°C)	Yeast extract (g/L)	Laccase activity (U/L)	
			Predicted	Validation
4.9	31	0.36	1676	1564

Several researchers have researched laccase production by white rot fungi growing on agricultural wastes in SSF, as shown in Table 6. This study shows that the use of rice straw is very promising for laccase production since it may contain substances, i.e lignin, cellulose and hemicellulose, that act as nutrients for the fungi and inducers for the production of laccase. The fermentation of *Marasmius* sp. on rice straw revealed a great potential for the production of laccase on a large scale.

Table 6 Laccase from various agricultural wastes in SSF.

Microorganisms	Solid substrate	activity (U/L)	Researcher
Phanerochaete crysosporium	grape seeds, wheat straw, and wood shavings	1620	[14]
Trametes hirsuta	wheat straw mixed with apple and orange peelings or potato skins	5000	[15]
Trametes versicolor	wheat husks	160	[16]
	wheat straw	662	[13]
Trametes pubescens	banana skins	1570	[10]
Marasmius sp.	lignite granules	67	[17]
Marasmius sp.	rice straw	1564	This study

4 Conclusions

Laccase can be produced excellently with white rot fungi in solid-state fermentation. The highest activity (1116.11 U/L) has been achieved in the culture of *Marasmius* sp. grown on rice straw. This can be proposed as a strategy for low-cost enzyme production on a large scale. The optimization of significant parameters using the response surface method for enhancing laccase production by *Marasmius* sp. grown on rice straw was successfully evaluated. The optimal conditions for laccase production by *Marasmius* sp. grown on rice straw in SSF were at a temperature, pH and yeast extract concentration of 31°C, 4.9 and 0.36 g/L, respectively.

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