Optimisation of Laccase Production using White Rot Fungi and Agriculture Wastes in Solid State Fermentation

Hendro Risdianto[1,2], Elis Sofianti[1], Sri Harjati Suhardi[3] & Tjandra Setiadi[1]

[1] Department of Chemical Engineering, Institut Teknologi Bandung
Labtek X, Jl. Ganesa 10 Bandung 40132, Indonesia
[2]Center for Pulp and Paper, Ministry of Industry
Jl. Raya Dayeuhkolot 132, Bandung 40258, Indonesia
[3] School of Life Sciences and Technology, Institut Teknologi Bandung
Labtek XI, Jl. Ganesha 10, Bandung 40132, Indonesia
Email: hendrorisdianto@yahoo.com

Abstract. Laccase has been produced in a solid state fermentation (SSF) using white rot fungi and various lignocellulosic based substrates. White rot fungi used were *Marasmius* sp, *Trametes hirsuta*, *Trametes versicolor* and *Phanerochaete crysosporium*. The solid substrates employed in this research were collected from agriculture waste which were empty fruit bunches (EFB), rice straw, corn cob, and rice husk. 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 higher laccase activity than that of any other strain of white rot fungi. *Marasmius* sp. and 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 response surface method on laccase production using *Marasmius* sp and rice straw. The optimized conditions were 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 agriculture industries in Indonesia are generating an enormous amount of waste in form of biomass, such as as Empty Fruit Bunches (EFBs), rice straw, rice husk and corn cob. In 2010, Indonesia produced Crude Palm Oil (CPO), rice and corn at 21.5 million tonnes, 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 husk and corn cob were at 22.5 million tonnes, 99 million tons, 11 million tons and 5.3 million tons, respectively [2]. These agriculture wastes are relatively inexpensive and contain abundant nutrient such as hemicellulose, cellulose and lignin and act as inducer to stimulate an enzyme

Received June 22nd, 2011, Revised August 25th, 2011, 2nd Revision October 4th, 2011, Accepted for publication February 2nd, 2012.

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 a solid material [4]. SSF processes have proved 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 solid state fermentation is very important, as it has a strong influence on the process [3].

Laccases have been the subjects of an intensive research in the last decades, because they have the following properties, such as broad substrate specificity, do not need the addition or synthesis of a low molecular weight cofactor, as their co-substrate (oxygen) is usually present in their environment. Most laccases are extracellular 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 the application in several bioprocesses technology such as biopulping, biobleaching, and the industrial wastewater treatment [5].

Currently, it is very interesting to find new methods in laccase production with high activities at lower cost due to the huge potential for the development of efficient biotechnology processes [5]. It has been a 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 optimise significant process parameters. Application of statistical methods such as Response Surface Method (RSM) is very useful in defining the effects and interactions of physiological factors that play an important role in laccase production. An RSM consists of empirical modelling 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 the several factors influencing the responses by varying several significant parameters simultaneously with limited number of experiments [7].

The objectives of this research were at first to select the most promising fungal species and the best lignocellulosic support substrate for the production of laccase. In the second step the optimum conditions for SSF had to be determined using 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 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 the Petri dish and incubated for 7 days at 28ºC. The seven days old culture served as stock and was stored at 4ºC until it was used. All strains are re-plated with the same medium every three months.

2.2 Lignocellulosic Materials

Empty Fruit Bunches (EFB), rice straw, rice husk and corn cob were used as support-substrate of Solid State Fermentation (SSF). EFBs were collected from palm plantation in South Sumatera Province. Rice straw, rice husk and corn cob were collected from farm near Bandung City. Prior to use in fermentation, EFB and rice straw were chopped in length of 3 cm, while corn cob was cut into cube size of 1 x 1 x 1 cm. No pre-treatment was used for rice husk. Cellulose, hemicelluloses and lignin in each agriculture wastes were analysed using TAPPI standard method 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 Center for Pulp and Paper, Ministry of Industry, Bandung, Indonesia.

2.3 Experimental

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 conical flask of 250 mL and supplemented by 20 mL of the 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 inoculums from agar plate in size of 1.5 x 1.5 cm were then transferred aseptically into the flask. All cultures were incubated under static condition at room temperature (±28ºC) for 12 days fermentation time. Laccase activity was analyzed every day in duplicates. Substrate, microorganism and the day of incubation that gave maximum laccase activity were used in optimisation of laccase production.

2.3.2 Optimisation of Laccase Production

The selected substrate and microorganism were used to optimize the process parameters of temperature, pH and yeast extract concentration. Effects of each parameter were investigated using Central Composite Design (CCD). A set of 20 experiments including six center points was performed, in duplicates. The effect of each variable on enzyme production was studied at five different levels viz; -α, -1, 0, +1, + α. The level of temperature, pH and yeast extract used were at the range of 20 – 35°C, 4 – 6, and 0.2 – 0.8 g/L, respectively. The actual values of each variable for the optimisation are presented in Table 1. In order to build a convenience model, all analysis is based on the actual value.

Table 1 The value of each variables for the 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 sample was extracted by adding 50 mL of sodium acetate buffer (pH 4.5) and thoroughly shaking at 100 rpm for 2 hours and finally grounded in a mortar. Ten milliliters 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 Spectrophotometer. One unit of enzyme activity (U) was defined as the amount of enzyme required to oxidize 1 μmol of ABTS per minute.

3 Results and Discussion

3.1 Screening of White Rot Fungi and Lignocellulosic Materials

The main component of agriculture 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%) is contained in rice straw, rice husk and empty fruit bunches, respectively. The lignocelluloses can be utilized by fungi as support, nutrient and also as inducer for the laccase production.

Table 2 Chemical composition of agriculture wastes.

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

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

When EFB 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 a support substrate, the fungal cultures displayed a lower activity than on any other substrate. As shown in Fig. 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 corn cobs were used (Fig. 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 (Fig. 1 B). This study showed that the laccase production depends on species of white rot fungi used. The enzyme activity was higher than the activities we observed in a previous study in 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 and the best fungal species for the 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 a higher hydrophobicity and surface charge of rice straw in comparison with other lignocellulosic materials. Osma, et al. [10] revealed that the most important characteristics that influence adhesive behaviour of filamentous fungi to the support are the hydrophobicity and the surface charge of the substrates.

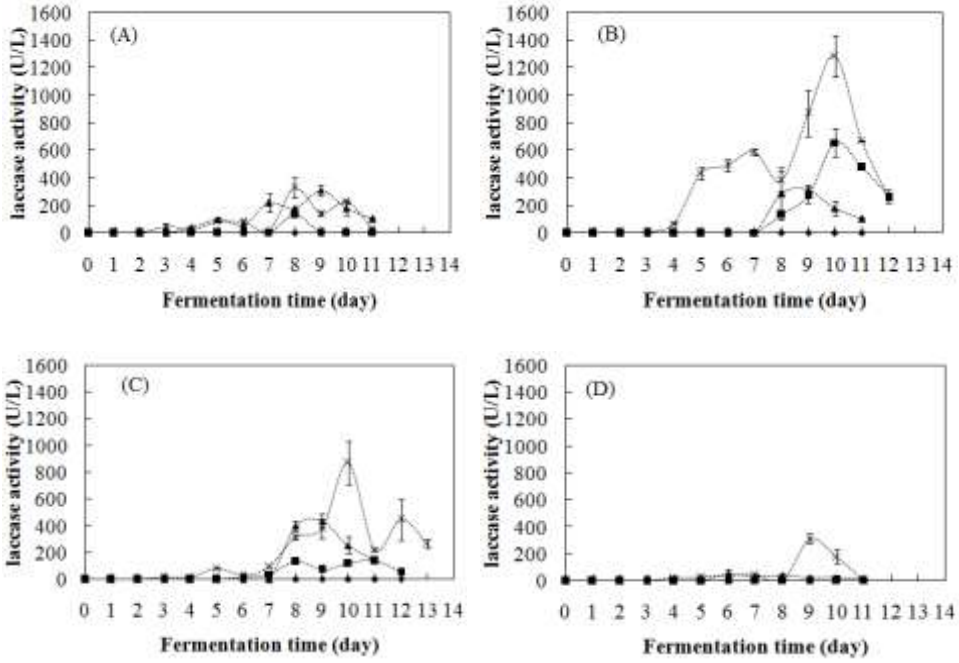

Figure 1 Time course of laccase activity from white rot fungi on agriculture wastes in SSF, (A) empty fruit bunches; (B) rice straw; (C) corn cob; (D) rice husk (---◆--- *P. crysosporium*, ---■--- *T. versicolor*, ---▲--- *T. hirsuta*, ---×--- *Marasmius* sp.)

3.2 Optimisation of Laccase Production

The selection of the white rot fungus and the substrate for the laccase production by SSF was basically using optimisation of One Factor at a Time (OFAT). The OFAT method is very useful for categorical factor such as type of substrate and fungus but can not investigate the interaction between those factors. *Marasmius* sp. and rice straw, produce the highest laccase activity so this fungus and substrate, were used in the optimisation of the laccase production. Laccase production was optimised by Response Surface

Methodology (RSM) and coupled by Central Composite Design (CCD) consisting of 20 runs, including six center points. The variables used for this optimisation were temperature, pH, and concentration of yeast extract. Those variables were chosen due to their strong influence on the fungal growth and laccase production.

The results for 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 experiments in duplicate. Analysis of Variance (ANOVA) is presented in Table 3. Fit analysis summary of the model suggested that the data model is a quadratic model.

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 95% confidence level. While the p-value (Prob>F) is less than 0.05 (<0.0001) indicating that the model is significant. This means that the variables have significant effects on the response. In the analysis of variance, the variables *A, B, C,* A^2, B^2, C^2, *AB,*

AC are significant variables with p value (Prob>F) less than 0.05 and only *BC* is not significant. The mathematical model resulting from the analysis of the data in Table 4 is expressed in Eq. 1.

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

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 the 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 the laccase production at 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 curve shown in Figure 4, Figure 5 and Figure 6. Figure 4 shows the curve of three-dimensional effect of the relationship between temperature and pH, while Figure 5 shows the curve of the three-dimensional effect of pH and concentration of yeast extract. Figure 6 shows the curve of the three-dimensional effect of temperature and concentration of yeast extract.

Laccase activity increased with increasing temperature until it reached a maximum and then decreased. Laccase production revealed an optimum

temperature around 30-31ºC. Based on the results of ANOVA analysis, the temperature is a variable that has a very significant effect on laccase production. These 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]. They reported that laccase production is optimal at a temperature of 25ºC in the presence of light. But without light the ideal temperature is 30ºC. In general, laccase production has an optimum in the temperature range from 25-30ºC. According to Krishna [12], the temperature is influencing the growth in SSF, the production of enzymes and metabolites. In the development of biological processes, the temperature has an important role since it determines several other factors such as protein denaturation, acceleration and inhibition of the enzyme production [12].

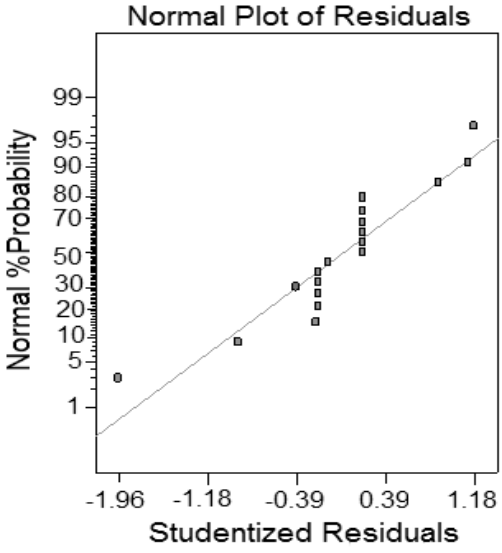

Figure 2 Normal probability plot of laccase activity.

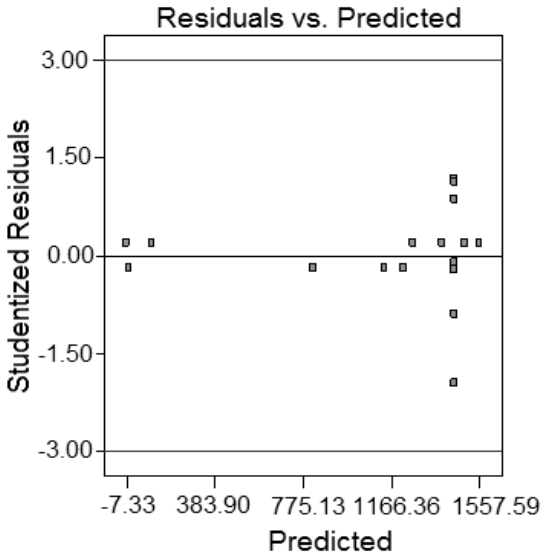

Figure 3 Plot of residual and predicted for laccase activity.

Effect of pH and yeast extract concentration on laccase production can be seen in Figure 5. Laccase production is optimum at a pH around 5. These results confirm the findings of Kunamneni, et al. [11]. Filamentous fungi grow well on wide pH, the optimum range is 2-9 [12]. For yeast extract, an optimum concentration is 0.2 g/L. Figure 5 shows that the concentration of yeast extract has a negative effect on laccase production [13]. A higher concentration of yeast extract causes a decrease of the laccase production.

In order to check the accuracy of the optimum conditions generated from the software, there is an additional experiment presented in Table 5 and compared to the value predicted by the software. It shows that there is a good agreement between predicted and experimental value which indicated that the model had been validated.

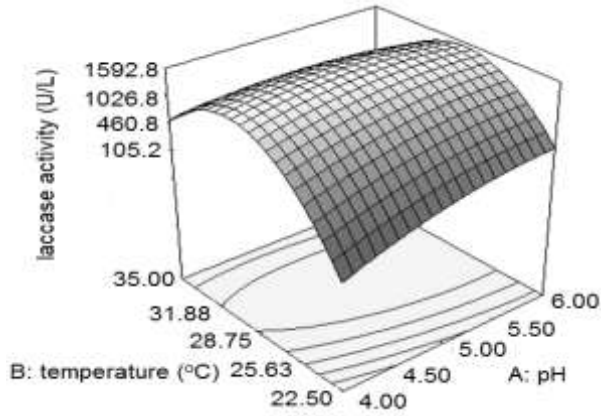

Figure 4 Effect of pH and temperature on laccase activity at yeast extract concentration 0.5 g/L.

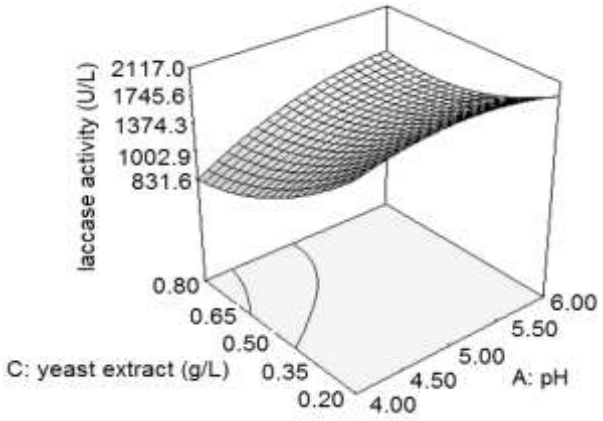

Figure 5 Effect of pH and yeast extract concentration at temperature 27.5°C.

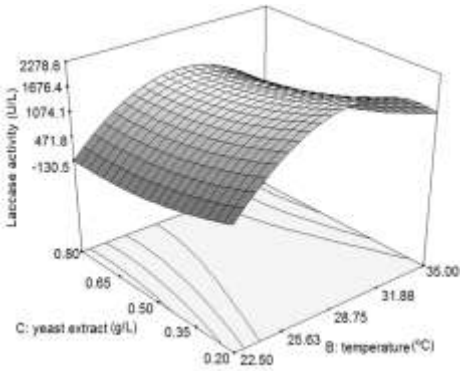

Figure 6 Effect of temperature and yeast extract concentration at pH 5.

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

The laccase production by white rot fungi growing on agricultural wastes using SSF has been carried out by several researchers as shown in Table 6. This study showed that the use of rice straw can be very promising for laccase production since it might contain substances i.e lignin, cellulose and hemicellulose acting as nutrient for fungal and inducers for laccase. Therefore, 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 agriculture wastes in SSF.

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

4 Conclusions

Laccase can be excellently produced with white rot fungi in Solid State Fermentation. The highest activity of 1116.11 U/L had 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 production. The optimisation of significant parameter using Response Surface Methodology for enhancing laccase production by *Marasmius* sp. grown on rice straw was successfully evaluated. The optimum conditions for laccase production from *Marasmius* sp. grown on rice straw in SSF method were at temperature, pH and yeast extract concentration of 31°C, 4.9 and 0.36 g/L, respectively.

Acknowledgment

This work was funded by Ministry of Research and Technology, Republic of Indonesia, Riset Insentif, Fiscal Year of 2009.

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