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Effect of Supplements Mn^{2+}, Cu^{2+}, and Aromatic Compounds and *Penicillium decumbens* on Lignocellulosic Enzyme Activity and Productivity of *Catathelasma ventricosum*

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This is the first report on using *Catathelasma ventricosum* for production of fruiting body and lignocellulosic enzymes. To improve the laccase activity and productivity of mushroom, the substrate was added with different supplements (eight aromatic compounds, Mn^{2+}, and Cu^{2+}). Based on the results, all these supplements can improve the laccase activity and productivity of *C. ventricosum*, and it seems that there is a critical value of laccase activity that affects the productivity of *C. ventricosum*. In addition, when *Penicillium decumbens* was inoculated into the substrate that had been cultivated with *C. ventricosum* for 20 days, the highest values of laccase activity, FPA activity, and productivity of *C. ventricosum* were obtained. Moreover, the laccase activity showed a positive correlation with the productivity of *C. ventricosum*. Finally, the effect of Mn^{2+}, Cu^{2+}, and *P. decumbens* on laccase activity was investigated by response surface methodology (RSM).

Key words: Agro-industrial residues, cultivation, edible mushroom, laccase

In recent years, great interest has been shown to the efficient use of agro-industrial residues. Sugarcane is one of the most widely consumed fruits in the world, and China is one of the largest producers among the world with a cultivated area of 1.11×10^5 hectares and an output of 11.53×10^6 tons per year [13]. It will generate about 7×10^6 tons of bagasse [29]. Rice bran, which contains plenty of lignocellulose, protein, and other nutrients, is another important agricultural waste [21].

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Lignocellulose is the richest renewable natural biological resource in the world [1]. A large quantity of such biomass accumulates every year, which not only results in deteriorating the environment but also wasting the valuable materials [31]. Therefore, producing biological products and bioenergy from these less costly renewable lignocellulosic materials is of great significance to the sustainable development of human beings [25].

The mushroom industry extends worldwide with an annual output of 5×10^6 tons of fresh mushroom [9]. In general, different lignocellulosic materials from the industrial and agricultural wastes are usually adopted to cultivate mushrooms [24]. Some mushrooms (especially white-rot fungi) can utilize these lignocellulosic materials to produce lignocellulosic enzymes that are widely applied in industries of food, medicine, textile, and printing and dyeing [4, 6, 22, 27]. These enzymes are crucial for the colonization as well as yield of the fruit body. Optimization of the substrate and cultivation techniques is one of the most important ways to enhance yield and reduce cost [14].

Catathelasma ventricosum is a kind of delicious and nutritious edible fungus that is also recognized as a source of various medicinal compounds [15]. So far, almost all the *C. ventricosum* are obtained in the wild environment, and its consumption is mainly confined to rural communities. There is still not a suitable method to cultivate this mushroom that has been reported. Bagasse and rice bran are low-cost materials and easier acquired than other agro-industrial residues for use. Therefore, this study aims at utilizing agricultural waste (bagasse and rice bran) as the cultivation substrate to realize the artificial cultivation of *C. ventricosum* and simultaneously obtain by-products like lignocellulosic enzymes.

Materials and Methods

Microorganism and Its Maintenance

Samples of *C. ventricosum* were collected under *Pinus massoniana* in the Mianyang mountainous area (Southwest China) in April 2010 (in general, the growth season of this mushroom is from March to October). The identification of macrofungi materials was confirmed by Prof. Douxi Zhu, a taxonomist from Mianyang Edible Fungi Research Institute in Sichuan. *Penicillium decumbens* ML-017 (GenBank Accession No. FJ458446) was kindly provided by Prof. Zhong Hu from Microorganism Laboratory of Shantou University. These two strains were maintained on potato dextrose agar (PDA) comprehensive medium slants at 4°C with periodic passage.

Cultivation Methods

Spawn was prepared by putting the boiled wheat grains in plastic bags (100 g/bag), and the moisture content was adjusted to 60% (w/w); that is, 100 g wheat grains plus 150 ml double-distilled water. These bags were autoclaved for 1 h at 121°C. After cooling, they were inoculated with 1 cm^2 of *C. ventricosum* mycelia and then cultivated at 20°C for 15–20 days in total darkness, until the mycelia completely covered the wheat grains.

For producing fruiting bodies, bagasse and rice bran obtained from Shantou, China were milled. The milled rice bran and bagasse particles would be 100 meshes on average. The substrate contained 78% of bagasse, 20% of rice bran, 0.4% of calcium carbonate, and 1.6% of calcium sulfate, and the moisture content was adjusted to 70% (w/w). Each plastic bag was filled with 300 g of this substrate and then sterilized at 121°C, 1.1 kg/cm^2 for 1 h. Once cooled, the substrate was inoculated with spawn of 5% (w/w) *C. ventricosum*. The bags were incubated in the dark at 22°C until primordia appeared (after about 15 days) and then the bags were transferred to favorable conditions with natural light (12 h light/12 h darkness per day) and artificial ventilation (the air in the cultivation room was renewed six times per hour). In this way, the content of CO_2 was kept at a low level (<1,200 ppm), the relative humidity was 90% (v/v), and the temperature was 20°C. The mushrooms were harvested when the pileus was fully extended [7].

Effects of aromatic compounds, Mn^{2+}, and Cu^{2+} on the lignocellulosic enzyme activity and productivity of *C. ventricosum* were investigated by adding these test compounds separately (final concentration: 0.05–3.0 mM) into bagged substrate, which had incubated *C. ventricosum* for 15 days [5].

P. decumbens mycelia were inoculated in the bagged substrate at different times (0, 15th, 20th, 25th, 30th, 40th, and 45th day from the inoculation of *C. ventricosum*), and *P. decumbens* was also inoculated at the final harvesting time.. The productivity of *C. ventricosum* was expressed by biological efficiency (BE, fresh weight of harvested mushrooms/dry weight of substrate used×100%) and production rate (PR, biological efficiency/days elapsed between inoculation and final harvest). All experiments were carried out in triplicate.

Enzyme Extraction and Activity Determination

Crude enzyme was obtained by adding 10-times distilled water (at this ratio, the highest extraction will be obtained) into the samples of substrate to be tested. Then, the mixed substrate was stirred for 1 h, and filtrated and centrifugated to wipe out impurities. All the steps for crude enzyme extraction were performed at room temperature. The clarified supernatant was used as the crude enzyme and stored at −20°C. All experiments and measurements were carried out in triplicate.

Laccase activity was determined at 420 nm ($\varepsilon_{420} = 3.6 \times 10^4$ $mol^{-1}cm^{-1}$) by using 0.03% (w/v) 2,2'-azino-*bis*-ethylbenthiazoline (ABTS) as substrate. Measurements were made in 0.1 M sodium acetate buffer (pH 5.0) and at 32°C [2]. Lignin peroxidase (LiP) activity and manganese peroxidase (MnP) activity were determined following the procedure previously described by Homma *et al*. [8]. To determine the filter paper activity (FPA), the methodology of Miller was adopted [17]. Three replicates were prepared for each case.

Polyacrylamide Gel Electrophoresis (PAGE) and Activity Staining of Gels

Native PAGE [12% (w/v) gel] was used to determine the existence of laccase in the crude enzyme and to reflect laccase activity indirectly. If the crude enzyme has laccase activity, it will reveal a green protein band after being stained with 0.125 M acetate buffer (pH 5.0) with ABTS [0.03% (w/v)] [5].

Statistical Analysis

All assays were carried out in triplicate. The results were expressed as mean values and standard deviation (SD). The results were analyzed through one-way analysis of variance (ANOVA) followed by Tukey's test with $\alpha = 0.05$. This test was carried out by use of the SPSS ver. 16.0 program.

Results and Discussion

Fruiting Body Production

The reports about artificial cultivation of *C. ventricosum* are really scarce, and this study is the first report on the artificial cultivation of *C. ventricosum* using bagasse and rice bran as substrate. Compared with straw, bagasse has

Table 1. Distribution of the total fresh mushroom production and productivity (biological efficiency, BE, and production rate, PR) of the strains of *C. ventricosum*.

IP[a] (days)	PP[b] (days)	Production distribution[c], g (%[d])			Total weight[c] (g)	BE (%)	PR (%)
		1st	2nd	3rd			
15	35	848.51 (63.20)	409.35 (30.49)	84.72 (6.31)	1,342.58	74.59	1.49

[a]Incubation period (days required for the formation of the primordia).
[b]Production period, starting with the formation of primordia and ending with the third harvest.
[c]Weight of mushrooms obtained in 20 plastic bags cultivated with *C. ventricosum*.
[d]The percentage of mushroom production during each harvest.

lower pesticide residues and a higher degree of lignification, properties that are important for the cultivation of mushroom [26]. Table 1 showed primordia can be formed after incubating for 15 days. Gaitán-Hernández and Salmones [7] indicated that the time of forming primordia had an inversely proportional correlation with BE. Primordia appearing early did not imply high yield. In Table 1, the production period (starting from the formation of primordia and ending with the final harvest) lasted for 35 days. Gaitán-Hernández and Salmones [7] and Philippoussis *et al.* [20] reported that the production period of *Pleurotus* species lasted for 32 and 34 days, respectively, whereas the production period of *Lentinus tigrinus* was about 95 days [12].

Compared with biomass or yield, productivity (BE and PR) is more comprehensive, as it not only focuses on the yield but also involves the production cycle. Table 1 shows that BE and PR were 74.59% and 1.49%, respectively. Different values were observed in other researches [7, 12, 32] and ranged from 62.20% to 98.94% and 1.28% to 2.21%, respectively. In general, more than 90% of the total production of mushroom was produced in the first two harvests, where 63.20% of the total production was obtained in the first harvest, 30.49% in the second harvest, and only 6.31% in the third harvest.

Producing Lignocellulosic Enzymes of *C. ventricosum*

Fig. 1 shows the laccase activity was detectable after incubating for 15 days, and the peak value of 49.70 IU/g was obtained after incubating for 35 days. Then, the laccase activity gradually declined by approximately 80% after 40 days. However, the laccase activity increased again (up to 32.27 IU/g) after 45 days. Xing *et al*. [30] reported the similar situation and Reddy *et al.* [23] indicated that enzyme activity is in association with morphogenesis of the mushroom. In Fig. 1, the laccase activity would increase after the appearing primordial and the first two harvests. It could be predicted that the formation of the primordial and fruit bodies will help *C. ventricosum* to increase the production of laccase.

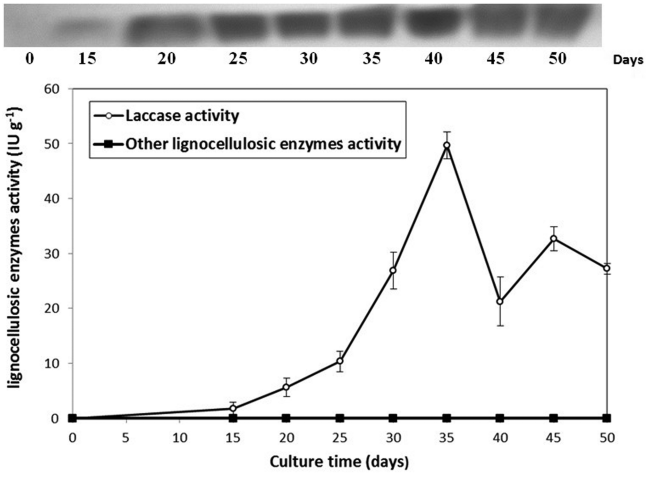

Fig. 1. Time course of lignocellulosic enzymes production by *C. ventricosum* grown on bagged substrate.

Other lignocellulosic enzymes contain lignin peroxidase (LiP) activity, manganese peroxidase (MnP) and FPA activity. Values are the mean of triplicate determinations; error bars represent standard deviation (SD). Bands representing laccase proteins were separated by Native-PAGE and visualized by activity staining with ABTS.

The result of native-PAGE analysis of laccase activity staining is shown in Fig. 1. It can be seen that the variations of laccase activity are consistent with the above experiments (the color of protein band is darker, the laccase activity is higher). There is only one protein band in each lane and the bands of protein are very thick. It can be inferred that there might be more than one laccase isozyme in each lane and the molecular weight of laccase isozymes produced by *C. ventricosum* is very close. Lu and Ding [16] have demonstrated that *Coprinus comatus* has at least six protein bands exhibiting laccase activity.

It is well known that lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase are the main enzymes that are involved in lignin degradation; moreover, their activities are related with the production of mushroom [11]. However, *C. ventricosum* lacks LiP, MnP, and FPA. Some research reported the same situation [8] and indicated that the low FPA activity will reduce the efficiency of degrading native cellulose by white-rot fungi [23].

Effect of Supplements on the Laccase Activity and Productivity of *C. ventricosum*

Eight aromatic compounds, Mn^{2+}, and Cu^{2+} were evaluated for their effects on laccase activity from *C. ventricosum*.

The highest level of laccase activity was found in supplementing with 2.0 mM Mn^{2+}, and it was 216.17 IU/g, ANOVA $p < 0.01$ (increased 4.3-fold when compared with control of 49.70 IU/g). Lu and Ding [16] reported that laccase activity increased 4.5-fold in supplementing with 0.8 mM $MnSO_4$ and indicated that manganese was a highly effective inducer of laccase in mushroom.

Fig. 2 shows that Cu^{2+} and aromatic compounds all can stimulate laccase activity. Following Mn^{2+}, Cu^{2+} at a concentration of 1.0 mM got the second highest laccase activity (208.74 IU/g, ANOVA $p < 0.01$). Since laccase is a copper-containing protein, supplementing copper in the culture medium to enhance enzyme production is widely reported [16, 30, 31]. Baldrian and Gabriel [3] indicated that Cu^{2+} not only induced laccase by the expression of laccase genes, but also positively affected the activity and stability of laccase. Palmieri *et al*. [19] inferred that the effect of Cu^{2+} on enzyme stability may be due to the extracellular protease, which is responsible for the degradation of laccase and is inhibited by Cu^{2+}. However, the concentration of copper (1.0 mM) is higher than a

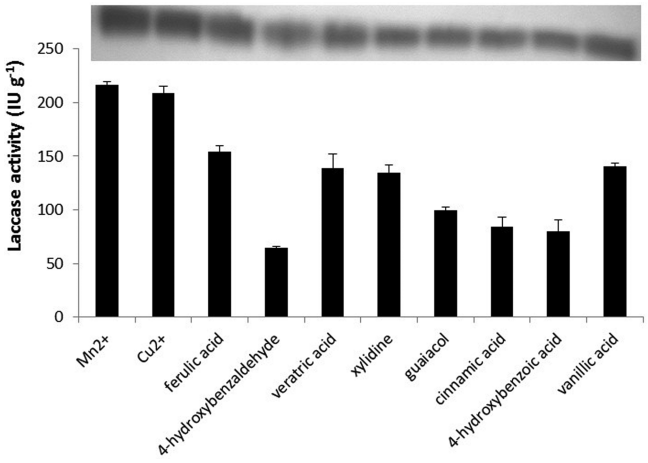

Fig. 2. Effect of different aromatic compounds, Mn^{2+}, and Cu^{2+} supplementation on laccase production by *C. ventricosum*.
Values are the mean value of triplicate determinations, and error bars represent the standard deviation (SD). Bands representing laccase proteins were separated by native-PAGE and visualized by activity staining with ABTS. Each lane corresponds to the aromatic compound shown on the bar graph.

normal toxic concentration (20 μM) and all supplements were added in culture media after *C. ventricosum* had being cultivated for 15 days (at this time, primordia just appeared). It implies that the primordia of *C. ventricosum* are able to withstand Cu^{2+} of high concentration. Lu and Ding [16] reported the similar result.

Stimulation of laccase production in fungi by aromatic compounds had been widely reported, and the eight aromatic compounds tested in this study had positive effects on the production of laccase in *C. ventricosum*, although they were not as powerful as Mn^{2+} and Cu^{2+}. In the test of increasing laccase activity by adding supplements, xylidine (0.8 mM) ranked six (134.19 IU/g) among all the supplements, but xylidine did the best in improving the productivity of *C. ventricosum*. Table 2 shows that after adding xylidine, the productivity (BE and PR) reached 94.39% and 2.05% (ranked first among all the supplements, ANOVA $p < 0.01$), respectively, which is much higher than the previous (74.59% and 1.49% in Table 1). Moreover, Fig. 2 and Table 2 shows that there might be a critical value that influences the correlation between laccase activity and productivity. That is, when laccase activity was lower than 134.19 IU/g (adding with xylidine), laccase activity showed a positive correlation with productivity, whereas when laccase activity was higher than that value, laccase activity showed no correlation with productivity. This result could infer the amount of degradation products by laccase that can be utilized by *C. ventricosum* is limited. Thus, superfluous laccase will produce plenty of degradation products that cannot be utilized by *C. ventricosum.*

Native-PAGE analysis of laccase activity staining proves the above result (Fig. 2). It just had one protein band, which was similar to that in Fig. 1, which indicated that all these supplemented compounds might have no impact on the kinds of laccase isozyme that *C. ventricosum* expressed.

Effects of *P. decumbens* on Laccase Activity and Productivity of *C. ventricosum*

P. decumbens ML-017 is a cellulose-decomposing strain, and can produce cellulase of high activity. The effects of *P. decumbens* on laccase activity of *C. ventricosum* are shown in Fig. 3. Laccase activity was not observed in the samples inoculated only with *P. decumbens* or nothing, which suggested that *P. decumbens* was unable to produce laccase. Laccase production was influenced by the adding time of *P. decumbens*. When *P. decumbens* was inoculated in the culture media that had cultivated *C. ventricosum* for 20 days, the highest laccase activity and FPA was obtained (265.36 and 11.39 IU/g, ANOVA $p < 0.01$). FPA increased

Table 2. Effect of different aromatic compounds, Mn^{2+} and Cu^{2+} supplementation on fresh mushroom production and productivity (BE and PR) of *C. ventricosum.*

Supplements	Cultivation times (days)	Total Weight[a] (g)	BE (%)	PR (%)
Mn^{2+}	48	1,655.28	91.96	1.92
Cu^{2+}	47	1,661.40	92.30	1.96
Ferulic acid	48	1,656.36	92.02	1.92
4-Hydroxybenzaldehyde	50	1,372.14	76.23	1.52
Veratric Acid	47	1,645.92	91.44	1.95
Xylidine	46	1,699.02	94.39	2.05
Guaiacol	48	1,587.06	88.17	1.84
Cinnamic acid	48	1,561.86	86.77	1.80
4-Hydroxybenzoic acid	49	1,499.22	83.29	1.70
Vanillic acid	47	1,643.76	91.32	1.94

[a]Weight of mushrooms obtained in 20 plastic bags cultivated with C. ventricosum.

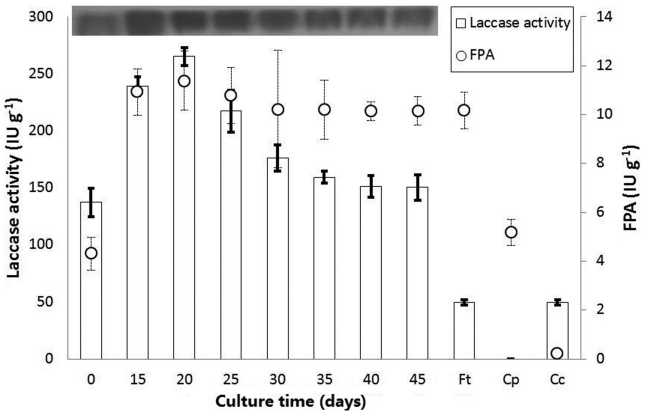

Fig. 3. Effects of inoculating *P. decumbens* on lignocellulosic enzymes activity.
(0–45, the days when *P. decumbens* were placed in substrate which had been inoculated with *C. ventricosum*; Ft, inoculation with *P. decumbens* at final harvest time; Cp, the contrast when inoculated only with *P. decumbens*; Cc, the contrast when inoculated only with *C. ventricosum*). Values are the mean of triplicate determinations, error bars represent standard deviation (SD). Bands representing laccase proteins were separated by Native-PAGE and visualized by activity staining with ABTS. Each lane corresponds to the cultivating time shown on bar graph.

Table 3. Effects of inoculating *P. decumbens* on the productivity (BE and PR) of *C. ventricosum.*

Inoculation times[a] (days)	Cultivation times (days)	Total weight[b] (g)	BE (%)	PR (%)
0	65	1,140.12	63.34	0.97
15	43	1,710.54	95.03	2.21
20	41	1,757.88	97.66	2.38
25	44	1,658.88	92.16	2.09
30	44	1,555.38	86.41	1.96
35	46	1,477.26	82.07	1.78
40	48	1,446.66	80.37	1.67
45	49	1,398.42	77.69	1.59

[a]Inoculation time (days when *P. decumbens* was inoculated into bagged substrate that had been cultivated with *C. ventricosum*).
[b]Weight of mushrooms obtained in 20 plastic bags cultivated with *C. ventricosum*.

more than 2-fold when compared with the control inoculated only with *P. decumbens* (5.17 IU/g). Previous researches had been reported that *P. decumbens* ML-017 utilized bagasse and rice bran as the substrate for producing cellulase (FPA), and the maximum cellulase (FPA) were obtained with 3.65 and 5.76 IU/g [16]. In Fig. 3, excepting inoculation of *P. decumbens* at the same time as *C. ventricosum*, the others had positive increase of FPA activity. Lignin in lignocelluloses is the biggest obstacle, as it hinders cellulase from degrading lignocelluloses. Laccase can degrade lignin with high efficiency. It could be seen from Fig. 3 that the higher the laccase activity is, the higher the cellulase activity is. Table 3 shows this situation too, excepting inoculation of *P. decumbens* at the initial time of cultivation. Distinctly laccase activity is positively related to the productivity of *C. ventricosum*, and the critical value that was mentioned above did not appear. There is a possible reason; that is, when cellulose-decomposing strain *P. decumbens* was added in culture media, it can degrade lignocelluloses that had been treated by laccase and produce reducing sugar and carbon source for *C. ventricosum* growth. When laccase activity becomes higher, more lignocelluloses will be treated by laccase, and these treated lignocelluloses will be degraded by cellulase, and in the end *C. ventricosum* will utilize the products of reducing sugar for its growth. However, some previous studies demonstrated that adding other strains like cellulose-decomposing strain into culture media that had been inoculated with mushroom could improve the lignocellulosic enzymes activity and productivity of mushroom [10, 18, 28], but there was no report on the comprehensive analysis of relevance among laccase activity, cellulase activity, and productivity of edible fungus when cellulose-decomposing strain was added to culture media at different times. In addition, when *C. ventricosum* and *P. decumbens* were inoculated at the same time, the laccase activity, cellulase activity, and productivity of *C. ventricosum* all declined. The reason might be that the strains need plenty of nutrition at the initial stage of growth, but their system of lignocelluloses enzyme is not complete enough to degrade enough substrate at the initial time. The two strains will compete with each other for nutrition, which will delay their growth.

When *P. decumbens* was inoculated in the culture media that had cultivated *C. ventricosum* for 20 days, the highest productivity (BE and PR) was obtained at 97.66% and 2.38% (ANOVA $p < 0.01$).

Fig. 3 shows the native-PAGE analysis of laccase activity staining. It proves the above results again. It shows that *P. decumbens* has no impact on the kinds of laccase isozyme that *C. ventricosum* expressed, which is the same as Figs. 1 and 2.

Optimizing Conditions of Solid-State Fermentation for Improving Laccase Activity

When *P. decumbens* was inoculated in the culture media that had cultivated *C. ventricosum*, there was no effect of Mn^{2+}, Cu^{2+}, and aromatic compounds on the productivity of *C. ventricosum* (ANOVA $p > 0.05$). Thus, the solid-state fermentation conditions were only optimized for improving laccase activity. Mn^{2+} (X_1), Cu^{2+} (X_2), and *P. decumbens* (X_3) are the major factors that affect the production of laccase. Therefore, these parameters were further investigated by response surface methodology (RSM) [14]. A three-level and three variable Box–Behnken design (BBD) was applied to evaluate the best condition for production of

Table 4. Box–Behnken design matrix and the response of the laccase activity[a].

Experiment order	Independent variables			Laccase activity IU/g
	X_1 (Mn^{2+})	X_2 (Cu^{2+})	X_3 (*P. decumbens*)	
1	0[b]	0	0	306.74
2	1	–1	0	211.39
3	–1	0	–1	252.11
4	0	1	–1	257.34
5	0	1	1	228.79
6	0	–1	1	217.84
7	0	–1	–1	249.38
8	1	0	–1	211.54
9	–1	0	1	237.66
10	0	0	0	302.75
11	–1	–1	0	268.47
12	1	1	0	214.82
13	–1	1	0	281.76
14	0	0	0	301.48
15	1	0	1	198.65

[a]Experimental laccase activity was averages of triplicates.
[b]Coded levels: Mn^{2+} (–1:1.5 mM, 0:2 mM, 1:2.5 mM), Cu^{2+} (–1:0.8 mM, 0:1 mM, 1:1.2 mM) and *P. decumbens* (–1:15 days, 0:20 days, 1:25 days).

laccase. The average laccase activity was taken as the response variable.

Regression analysis (using the software Minitab, Version 16.1.0) was performed, based on the experimental data, and was fitted into an empirical second-order polynomial model, as shown below in the following equation:

$$Y = \sum A_0 + \sum_{i=1}^{3} A_i X_i + \sum_{i=1}^{3} A_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} A_{ij} X_i X_j \quad (1)$$

where Y was the response variable; A_0, A_i, Aii, and A_{ij} were the regression coefficients of variables for intercept, linear, quadratic, and interaction terms, respectively; and X_i and X_j were independent variables.

The data (Table 4) obtained was scrutinized by applying the multiple regression analysis based on Eq. (1). The predicted response Y for the laccase activity Eq. (2) was obtained as follows:

$$Y = 303.657 - 25.45X_1 + 4.454X_2 - 10.929X_3 - 36.447X_1^2 - 23.1X_2^2 - 42.22X_3^2 \quad (2)$$

The R^2 value of the two models was 0.9401, which showed that the regression model defined well the true behavior of the system. The *P* value of the models was 0.002 ($p < 0.05$, a value below 0.05 indicated significance level and vice versa), which indicated that the model fitness was significant. According to the *p* value (data not show), X_1, X_2, X_3, $X1^2$, $X2^2$, and $X3^2$ were significant ($p < 0.05$), but X_1 and X_2, X_1 and X_3, as well as X_2 and X_3 interactions were not significant ($p > 0.05$). To further validate the optimal culture condition, the impact on the laccase activity by the optimal values of the variables was calculated in the equation through the software, and the results are as follows: Mn^{2+} (1.81 mM), Cu^{2+} (1.02 mM), and *P. decumbens* were inoculated into the substrate that had been cultivated with *C. ventricosum* for 19 days. Under these conditions, the predicted value of Y (laccase activity) from the model was 309.12 IU/g. When *C. ventricosum* was cultivated under the optimal condition, the laccase activity was found to be 289.74 IU/g (increased by 9.19%). The maximum value was very close to the predicted value, which indicated the validity of the RSM.

Acknowledgments

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