Wood Degradation and Optimized Laccase Production by Resupinate White-Rot Fungi in Northern Thailand

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One hundred and thirty samples of resupinate white-rot fungi were collected from natural sites in Northern Thailand during the dry season (October-December) as a bioresource for lignin-degrading enzymes (laccase (Lac), lignin peroxidase (LiP), and manganese peroxidase (MnP)). All 130 isolated fungal strains grew well in potato dextrose broth and produced lignin-degrading enzymes at different levels after 7 days of incubation. The selected resupinate fungi, RCK783S, produced maximum Lac at 4,218 U/L, whereas MnP and LiP activities were detected at relatively low levels in all selected fungal strains. The RCK783S was further identified as a new record of *Fibrodontia* sp. in Thailand. Response surface methodology (RSM) was applied to evaluate the effect of medium composition, *i.e.*, peptone, glycerol, L-asparagine, and $CuSO_4$, on Lac production by *Fibrodontia* sp. RCK783S. The experiments showed optimum concentrations of peptone, glycerol, L-asparagine, and $CuSO_4$ at 0.625, 15.00, 2.188, and 0.003 g/L, respectively, to produce the highest Lac concentration of 6,086.01 U/L, a 1.44-fold increase from that in the original medium. In addition, the degradation of *Eucalyptus camaldulensis* was investigated during the solid-state cultivation of *Fibrodontia* sp. RCK783S. The results showed that lignin was degraded, with lignin loss being 18% after 30 days, coinciding with the highest released Lac activity.

Keywords: Laccase; Resupinate fungi; Fibrodontia; Wood degradation

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INTRODUCTION

Resupinate fungi are characterized by growth habits that flatten on the substrate with a hymelial layer on the outer side (Kirk *et al*. 2008). These fungi are separated primarily into two groups, brown-rot and white-rot fungi, following the physical characteristics of degraded wood or wood residue. Resupinate white-rot fungi represent wood-rotting microorganisms as lignified material-degrading fungi that use wood as their substrate. Several strains of them have been reported, including various species of *Pleurotus*, *Coriolus*, *Trametes*, *Polyporus*, *Lentinus*, and *Pycnoporus* (Das *et al*. 2000; Kahraman and Yesilada 1999; Leonowicz *et al*. 2001; Sani *et al*. 1998; Zilly *et al*. 2002). Some species of basidiomycetes designated as white-rot fungi are able to break down all components of lignocellulose, including lignin, which is generally regarded as the

polymer most resistant to microbial attack. Non-resupinate white-rot fungi, such as *Trametes versicolor* (Jönsson *et al*. 1989), *Ganoderma* spp. (Silva *et al*. 2005), *Pleurotus ostreatus* (Santoyo *et al*. 2008), *Coriolus versicolor*, and *Phlebia radiata* (Martinez *et al*. 2005), and resupinate white-rot fungi, such as *Phanerochaete chrysosporium* (Kersten and Cullen 2007) and *Dichomitus squalens* (Eichlerová *et al*. 2006), are the most active ligninolytic organisms described to date. These white-rot fungi produce three major lignin-degrading enzymes: lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase (Lac), all of which are responsible for the biodegradation of lignin (Vicuna 2000). Some white-rot fungi produce all three enzymes, while others produce only one or two of them (Zilly *et al*. 2002). The mechanisms of lignin biodegradation have not been completely elucidated. The enzymes LiP and MnP, have been found from *Phanerochaete chrysosporium*, and a vast amount of information on these enzymes has accumulated during the last 10 years (Kersten *et al*. 2007; Singh and Chen 2008; Sharyl *et al*. 2008). However, Lac is also presumed to be involved in lignin degradation. An expanded role of Lac in lignin degradation has been proposed (Bourbonnais *et al*. 1990). Studies on lignin biodegradation are restricted to several dozens of fungi, and most of the knowledge on the enzymatic mechanisms of lignin degradation focuses on *P. chrysosporium*. Previous screening of basidiomycetes for the presence of lignin-degrading enzymes has been reported (de Jong *et al*. 1992; Esposito *et al*. 1991).

The expression of lignin-degrading enzymes can be optimized by providing a better understanding of the interactions among factors on the outcome of the fermentation (Revankar and Lele 2006a). Statistical techniques such as response surface methodology (RSM) have gained broad acceptance in fermentation optimization because they reduce the total number of experiments needed for the optimization and provide a better understanding of the interactions among factors on the outcome of the fermentation. Many previous studies have used RSM for the rapid construction of accurate models for optimization.

This study aimed to find potential fungal strains, with their bioremediation ability, from resupinate fungi (Basidiomycota) collected from northern Thailand. The lignin-degrading activities (Lac, LiP, MnP) were screened from the cultured liquid media. RSM was also conducted to optimize the culture medium for Lac production. The degradation of *Eucalyptus camaldulensis* was also investigated during the solid-state cultivation of *Fibrodontia* sp. RCK783S.

EXPERIMENTAL

Screening of Lignin-Degrading Enzymes Producing Fungi

Approximately 200 resupinate fungi were collected on decaying monocotyledonous and dicotyledonous trees from six sites in the dry deciduous and deciduous community forests of Northern Thailand during the dry season in year 2010. One hundred and thirty fungal strains were isolated for lignin-degrading enzymes while their basidiocarps were preserved as herbaria for taxonomic study. Axenic cultures were preserved in the BIOTEC Culture Collection, while herbaria were stored at the BIOTEC Bangkok Herbarium for future study. Fungal herbaria were preserved and decontaminated (Bridson *et al*. 1998).

Due to the large number of fungal strains studied, only a single set of growth conditions was used for preliminary screening, *i.e.*, for each sample, a 250-mL

Erlenmeyer flask with 100 mL of potato dextrose broth (PDB) (200 g/L potato, 20 g/L dextrose) was inoculated with 10 agar plugs (Ø 0.75 cm) cut out from a colony grown on potato dextrose agar. Incubation was carried out at room temperature (25 to 30 °C) under shaking conditions. After 7 days, the culture supernatants were used as enzyme sources for the activities (Lac, MnP, and LiP) assay.

Fungal Taxonomic Studies

Macromorphological features were recorded from fresh material, documenting all aspects of basidiome (mushroom) size, shape, color, surface, texture, and odor following Hemmes and Desjardin (2002). The colors of basidiomes were described in daylight using the color guide of Kornerup and Wanscher (1963). Basidiomes were illustrated in pencil, and digital photographs were taken when possible. Dried specimens were used for the analyses of micromorphological features, wherein all cell types and tissue types were described in detail. The head, stipe, and basae were dissected using a razor blade. The dissecting portion was rehydrated in 95% ethanol followed by distilled water, 3% KOH or Melzer's reagent (Largent *et al*. 1977). Microscopic structures were measured with an ocular micrometer and illustrated with the aid of a drawing tube attached to an Olympus BX51 compound microscope. The size, shape, location, organization, and chemical reaction of each cell type and tissue were documented (Dai 2010).

Experimental Design for Enzyme Production

Effect of carbon and nitrogen sources on Lac production

To investigate the effect of the carbon source on Lac production, enzyme production was carried out in 250-mL flasks containing 100 mL of Minimal Medium (MM, g/L: yeast extract 1.250, K_2HPO_4 1, $MgSO_4 \cdot 7H_2O$ 0.500, KCl 0.500, $FeSO_4 \cdot 7H_2O$ 0.100, $MnSO_4 \cdot 4H_2O$ 0.008, $Zn(CH_3COO)_2$ 0.003, $Ca(NO_3)_2 \cdot 4H_2O$ 0.006, and $CuSO_4 \cdot 5H_2O$ 0.003). Eight sources of carbon at concentrations of 10 g/L were used: glucose, fructose, sucrose, maltose, lactose, cellobiose, soluble starch, and glycerol. Yeast extract at a concentration of 1.25 g/L was fixed as the nitrogen source. The reactions were incubated at room temperature (30 °C) with shaking at 150 rpm. Samples were periodically taken for the analysis of cell dry weight and Lac activity.

Similarly, the effect of the nitrogen source on Lac production was investigated in 250-mL flasks containing 100 mL MM. Yeast extract, beef extract, peptone, L-asparagine, NH_4NO_3, $(NH_3)_2SO_4$, and urea at concentrations of 1.25 g/L were applied as the nitrogen sources. Glycerol at a concentration of 10 g/L was fixed as the carbon source. The reaction mixtures were incubated at room temperature (30 °C) with shaking at 150 rpm. Samples were periodically taken for the analysis of cell dry weight and Lac activity.

Optimization of Lac production by RSM

In this study, response surface methodology (RSM) was applied to investigate the effects of four factors at three levels of variation (Table 1) on the response of Lac production by selected species of white-rot fungus. The factors optimized included the concentrations of glycerol, peptone, and L-asparagine. The concentration of copper sulfate ($CuSO_4$) was included in the optimization because the Cu^{2+} ion has been reported to affect the production of white-rot fungi Lac and MnP (Chen *et al*. 2003). All treatment combinations were performed in 100-mL Erlenmeyer flasks according to the central composite design (CCD; Table 2). Thirty treatment combinations were generated. To set

up a statistical model, five levels for each variable were chosen. The upper and lower limits of each variable were chosen to encompass the range in the literature and to reflect what was done in practice after a preliminary investigation of the limits. The codes of $\pm\alpha$ ($\pm$2.0) were designed at a distance of 2.0 ($2n/4 = 2.0$ for $n = 4$) from the design center. The remaining levels were identified using CCD (5, 10, 15, 20, and 25 g/L glycerol; 0.313, 0.469, 0.625, 0.781, and 0.938 g/L peptone; 0.938, 1.563, 2.188, 2.813, and 3.438 g/L L-asparagine; and 0.001, 0.002, 0.003, 0.004, and 0.005 g/L $CuSO_4$). Table 2 shows the treatment combinations and mean response. From the experimental data using this design, a second-order polynomial regression model was determined (Equation 1),

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X^2_1 + b_{22}X^2_2 + b_{33}X^2_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + £ \quad (1)$$

where Y is the Lac activity (U/L), b_i values are the linear coefficients, b_{ii} are the quadratic coefficients, b_{ij} are the cross product coefficients, and £ is the model constant.

Table 1. Experimental Factors and Levels for Optimizing Lac Production by RSM

Factor	Code[a]	Actual factor level at coded factor levels				
		-α	-1	0	1	α
Initial concentration of glycerol (g/L)	X_1	5	10	15	20	25
Initial concentration of peptone (g/L)	X_2	0.313	0.469	0.625	0.781	0.938
Initial concentration of $CuSO_4$ (g/L)	X_3	0.001	0.002	0.003	0.004	0.005
Initial concentration of L-Asparagine (g/L)	X_4	0.938	1.563	2.188	2.813	3.438
[a]Code level limits are based on preliminary investigations and also reflect what was done in practice.						
Levels based on the central composite design						

Degradation of *Eucalyptus camaldulensis* by the Selected White-Rot Fungus

Eucalyptus camaldulensis chips were used in this study. The chips were dried and ground to particles 20 to 60 mesh in size. The degradation of *E. camaldulensis* by the selected white-rot fungus was carried out under semi-solid-state conditions. The fungus was grown in 250-mL Erlenmeyer flasks containing 36 mL of optimized media from the previous experiment and 12 g of *E. camaldulensis* sawdust. Cultures were maintained at room temperature for 4 months. Non-inoculated sterilized *E. camaldulensis* sawdust served as a control. All experiments were performed in triplicate. Samples were collected every month.

Each collected sample was extracted with 20 mM sodium acetate buffer (pH 4.5) under shaking (150 rpm) at room temperature (30 °C) for 30 min. The extracts were filtered through filter paper (Whatman No.4), centrifuged at 8,000x*g* at 4 °C for 20 min, and the filtrate was then assayed for Lac activity. After extraction, the *E. camaldulensis* sawdust was washed with water to remove residual mycelia and dried at 60 °C. A part of the extraction was used to determine the lignin content according to the TAPPI (1996). Another part was subjected to study under a scanning electron microscope (SEM). Prior to the study, cover glasses with individual samples were glued to aluminum stubs with adhesive tape and kept in a desiccator. After that, samples were directly sputtered with gold in a sputter coater (Eiko Engineer; IB-2) according to Junipur *et al.* (1970) and

examined using an SEM (JSM-5600 LV, manufactured by JEOL, Japan) with a magnification of 1,000x, operated at 10 kV.

Enzyme Assays

Lac activity was measured with 2,2'-azino bis(3-ethylbenzthiazole-6-sulphonic acid) (ABTS) in 1.0 M sodium acetate buffer (pH 4.5). Oxidation of ABTS was determined by the increase in A420 (ε420 = 36,000 M/ L.CM) (Eggert *et al*. 1996). All measurements were performed in triplicate. The average value of one unit of Lac activity was defined as the amount of enzyme that oxidizes one µmol ABTS per minute under given conditions.

MnP activity was measured with 3-methyl-2-benzothiazolinone hydrazone (MBTH) in 1.0 mL sodium acetate buffer (pH 4.5) with 4.95 mM DMAB and 0.25 mM hydrogen peroxide. Oxidation of MBTH was determined by the increase in A590 (ε590 = 53,000 M/ L.CM) (Castillo *et al*. 1994). All measurements were performed in triplicate. The average value of one unit of MnP activity was defined as the amount of enzyme catalyzing the production of 1 µmol of green or purple product per minute under given conditions.

LiP activity was measured with veratryl alcohol (Ming and Kent 1984) in 200 mM succinate buffer (pH 3.0) with 2 mM hydrogen peroxide. Oxidation of LiP was determined by the increase in A310 (ε310 = 9,300 M/ L.CM). All measurements were performed in triplicate. The average value of one unit of MnP activity was defined as the amount of enzyme catalyzing the formation of 1 µmol of veratraldehyde per minute under given conditions.

RESULTS AND DISCUSSION

Screening of Lignin-Degrading Enzyme-Producing Fungi

The present study represents a broad screening for lignin-degrading enzymes. Most of the fungal strains studied showed abundant growth under the culture conditions used. The levels of Lac, MnP, and LiP activities in the studied species, estimated after 7 days of incubation, are shown in Fig. 1. The enzyme activities were determined from the culture filtrates of the fungal strains. Figures 1a, b, and c show detected activities of Lac, MnP, and LiP, respectively, in the culture filtrates of the observed strains. The highest Lac activity was 4,218 U/L on day 7 of incubation from RCK783S, whereas MnP and LiP activities were detected at very low levels. The maximum MnP and LiP activities were 227.9 and 25.56 U/L, respectively, on day 7 of incubation. Lignin-degrading enzymes have been widely researched in aphyllophoraceous fungi (*Basidiomycota*), but not in resupinate fungi. The comparison of lignin-degrading enzymes from fungal strains in this study with previously reported enzymes in white-rot fungi showed that both white-rot fungi and our resupinate fungal strains are good Lac producers. MnP and LiP activities were detected in fewer fungal strains than was Lac activity, and the activity levels estimated were also comparatively lower. Figure 2 demonstrates the effect of time on Lac activity and cell dry weight when RCK783S was grown in MM at room temperature for 7 days. This result indicates that Lac was produced during the secondary metabolism, which corresponds with previous studies (Tuomela *et al*. 2000; Wesenberg *et al*. 2003; Teerapatsakul *et al*. 2007).

Fungal Taxonomic Studies

Approximately 20% of the 130 fungal specimens were resupinate fungi and were identified as being in the families Corticiaceae *sensu lato* and Hymenochaetaceae. The RCK783S resupinate fungal strain was identified as *Fibrodontia* sp. in the family Hydnodontaceae. It was characterized by: odontoid hymenophore, dimitic or pseudodimitic hyphal system, clamp connection present, thick-walled pseudoskeletal hyphae, cystidia absent, clavate basidia, 4-spore basidia with basal clamp, ellipsoid basidiospores, smooth, thin-walled, hyaline, IKI-. This is a new record of this species in Thailand because it produced a few characteristics different from *Fibrodontia gossypina* Parmasto, which is the only *Fibrodontia* species previously reported in Thailand (Hjortstam and Ryvarden 1982; Chandrasrikul *et al*. 2011). Thus, in the interim, the best solution is to include it as a member of the genus *Fibrodontia* without a species identification until more information is obtained.

Experimental Design for Enzyme Production

Effect of carbon and nitrogen sources on Lac production

The effects of carbon and nitrogen sources on Lac production by *Fibrodontia* sp. RCK783S are shown in Figs. 3a and b, respectively. Among all the tested carbon sources, glycerol produced the highest Lac activity, at 2,506.81 U/L on day 10 of cultivation. The advantage of glycerol for the production of fungal Lac was supported by studies of *Ganoderma* sp. KU-Alk4 (Teerapatsakul *et al*. 2007) and *Fomes fomentarius* (Songulashvili *et al.* 2007). Other carbon sources reported to promote fungal Lac production included cellobiose (Galhaup *et al*. 2002; Vaithanomsat *et al.* 2012), soluble starch (Revankar and Lele 2006a), and glucose (Revankar and Lele 2006b). A sequential addition of different carbon sources was also reported to yield high Lac production. For example, the addition of fructose followed by glycerol led to an increase in Lac production in *Trametes hirsuta* relative to glucose alone (Rodriguez *et al*. 2006).

The type of nitrogen source also clearly affected Lac production by *Fibrodontia* sp. RCK783S. Figure 3b demonstrates the top three highest Lac activities (2,243, 1,927, and 1517 U/L) on day 8 from the MM containing peptone, beef extract, and yeast extract, respectively. Supplementation with organic nitrogen sources such as yeast extract, beef extract, and peptone clearly showed higher production of Lac than those from inorganic nitrogen sources such as NH_4NO_3, $(NH_4)_2SO_4$, and urea. Many scientific publications have reported the promotion of Lac production by peptone in species such as *Trametes pubescens* MB 89 (Galhaup *et al*. 2002), *Lentinus edodes* (Hatvani and Mecs 2002), *Pleurotus dryinus* (Elisashvili *et al*. 2006), *P. ostreatus* strain 32 (Hou *et al*. 2004), and *P. ostreatus* (Stajic *et al*. 2006). Furthermore, Colao *et al*. (2003) showed that peptone promoted Lac production from *T. trogii* by increasing a laccase-encoding gene. Revankar and Lele (2006a) reported that an organic nitrogen source was essential for Lac production by the white-rot fungus *WR-1* and that an organic nitrogen source could not be substituted. In some cases, higher Lac production can be obtained from cultivation in the presence of inorganic nitrogen sources. For example, Lac activity in *Cerrena unicolor* IBB62 depends on the nitrogen source, and the highest Lac activity was observed in a medium containing $(NH_4)_2SO_4$ (Elisashvili *et al*. 2001). When *T. versicolor* 145 was cultivated, the highest Lac production was obtained in a medium containing $(NH_4)_3PO_4$, compared with those containing casein hydrolysate or peptone (Mikiashvili *et al.* 2005). These findings imply that carbon and nitrogen sources regulate the expression of Lac in fungi; thus, Lac activity can be increased by the choice of carbon and nitrogen source.

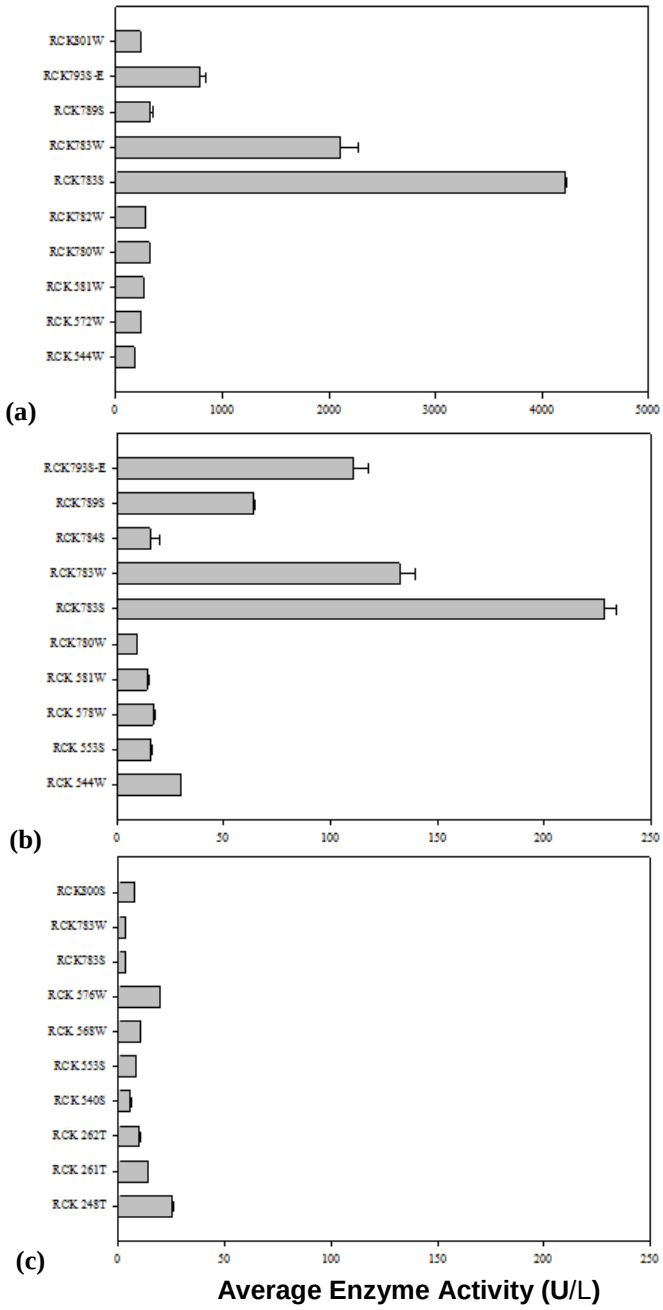


Fig. 1. Production of lignin-degrading enzymes from 10 potential strains grown in PDB at room temperature with shaking at 150 rpm for 7 days; a) Lac, b) MnP, and c) LiP

Optimization of Lac production by RSM

As mentioned above, glycerol and peptone were shown to be the best carbon and nitrogen sources, respectively, for Lac production from *Fibrodontia* sp. RCK783S. Thus, RSM was employed to determine their optimal concentrations to obtain the highest Lac production. In addition, the concentrations of $CuSO_4$ and L-asparagine were also varied because they have been reported to induce the production of Lac in various fungi (Galhaup and Haltrich 2001; Baldrian and Gabriel 2002; Chen *et al*. 2003).

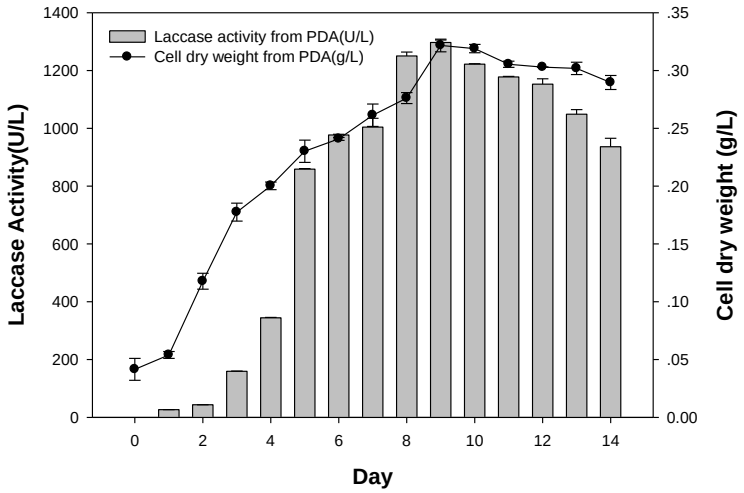


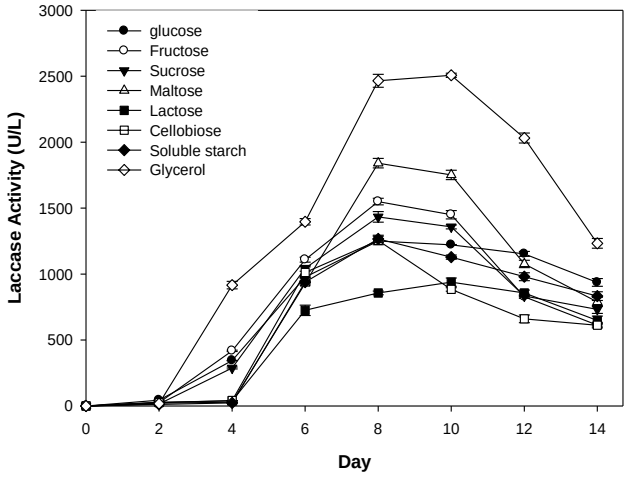
Fig. 2. Effect of time on Lac activity with cell dry weight when *Fibrodontia* sp. RCK783S was grown in MM at room temperature for 7 days. The vertical bars represent the standard deviation range for mean values.

Data from all experimental treatment combinations were analyzed by multiple regression analysis using the Design-Expert 8.0 program. The predicted and experimental responses are presented in Table 2. The final response equation representing a suitable model for Lac production is given in Equation 2,

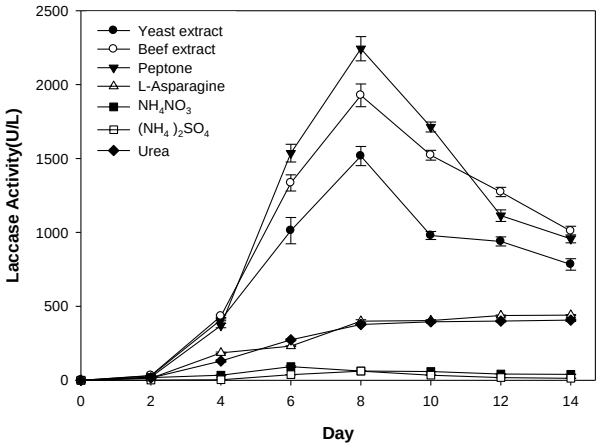
$$Y = 6385.15 + 245.00X_1 + 328.24X_2 - 27.60X_3 + 290.62X_4 - 180.60X_1X_2 - 246.37X_1X_3 - 95.79X_1X_4 + 454.75X_2X_3 + 56.18X_2X_4 - 184.42\ X_3X_4 - 1062.50X_1X_1 - 990.64X_2X_2 - 827.37X_3X_3 - 1020.01X_4X_4 \quad (2)$$

where Y is the Lac activity (U/L), X_1 is the initial concentration of glycerol (g/L), X_2 is the initial concentration of peptone (g/L), X_3 is the initial concentration of $CuSO_4$ (g/L), and X_4 is the initial concentration of L-asparagine (g/L).

The ANOVA for the response surface quadratic model is summarized in Table 3. The model p-value was very low ($p < 0.0001$), indicating the very high significance of the quadratic model compared with linear, two-level factorial (2FI), and cubic models. The correlation coefficient (R^2) value for the model, being an indicator of the accuracy of the model, was 0.8337, indicating that 83.37% of the variability in the observed response could be explained by the statistical model. The presented R^2 value reflected a good fit between the experimental and predicted responses; thus, the model was considered reasonable to use to analyze the response trends. The lack of fit p-value of 0.0680 implied that the quadratic model fit was not significant ($p > 0.05$) and suitable for the prediction of Lac production by *Fibrodontia* sp. RCK783S. The regression coefficients and corresponding p-values for the model are presented in Table 4. The smaller the p-value is, the bigger is the significance of the corresponding coefficient (Chen *et al*. 2009). The results show that none of the independent variables or their interactions had a significant effect on Lac production. The quadratic terms of the four variables, however, did show significant effects.



(a)



(b)

Fig. 3. Effects of (a) carbon and (b) nitrogen sources on Lac activity when *Fibrodontia* sp. RCK783S was grown in MM at room temperature for 14 days. The vertical bars represent the standard deviation range for mean values.

The interaction of various variables on Lac production was studied by plotting three-dimensional response curves against any two independent variables while keeping the other independent variable at its "0" level, as shown in Fig. 4. The three-dimensional curves facilitated the visual interpretation of the interaction between two variables and the location of the optimum experimental conditions (Chen *et al*. 2009). Figure 5a shows the three-dimensional curve of the calculated response surface from the interaction between the concentrations of L-asparagine and peptone, indicating maximum Lac activity when the L-asparagine and peptone concentrations were around 2.0 to 2.4 and 0.6 to 0.7 g/L, respectively. However, a further increase in the concentrations of L-asparagine and peptone beyond 2.4 and 0.7 g/L, respectively, negatively affected Lac production. Thus, this result implied the important role of nitrogen sources, as well as their concentrations, in Lac production by *Fibrodontia* sp. RCK783S.

Table 2. Treatment (T) Combinations and Mean Response for Lac Activity by *Fibrodontia* sp. RCK783S

T	Glycerol (g/L)	Peptone (g/L)	$CuSO_4$ (g/L)	L-Asparagine (g/L)	Lac activity (U/L)	
					Experiment	Prediction
1	10	0.469	0.002	1.563	2078	1452
2	20	0.469	0.002	1.563	2604	2988
3	10	0.781	0.002	1.563	1047	1448
4	20	0.781	0.002	1.563	2468	2261
5	10	0.469	0.004	1.563	1745	1349
6	20	0.469	0.004	1.563	1794	1899
7	10	0.781	0.004	1.563	3084	3164
8	20	0.781	0.004	1.563	4388	2991
9	10	0.469	0.002	2.813	2210	2481
10	20	0.469	0.002	2.813	4053	3634
11	10	0.781	0.002	2.813	3145	2702
12	20	0.781	0.002	2.813	3862	3132
13	10	0.469	0.004	2.813	1772	1641
14	20	0.469	0.004	2.813	3334	1808
15	10	0.781	0.004	2.813	5189	3680
16	20	0.781	0.004	2.813	2838	3125
17	5	0.625	0.003	2.188	1200	1645
18	25	0.625	0.003	2.188	1606	2625
19	15	0.313	0.003	2.188	1329	1766
20	15	0.938	0.003	2.188	2052	3079
21	15	0.625	0.001	2.188	3178	3131
22	15	0.625	0.005	2.188	1509	3020
23	15	0.625	0.003	0.938	1628	1724
24	15	0.625	0.003	3.438	1518	2886
25	15	0.625	0.003	2.188	6074	6385
26	15	0.625	0.003	2.188	5888	6385
27	15	0.625	0.003	2.188	6646	6385
28	15	0.625	0.003	2.188	7438	6385
29	15	0.625	0.003	2.188	6471	6385
30	15	0.625	0.003	2.188	5794	6385

Table 3. ANOVA for the Second-Order Polynomial Model

Source	SS	DF	MS	*F*-value	*p*-value
Mean	319753242.5	1	319753242.5		
Linear	6071723.749	4	1517930.937	0.392557657	0.8120
2FI	5543320.713	6	923886.7855	0.192632763	0.9751
Quadratic	74042738.61	4	18510684.65	16.25337352	< 0.0001
Cubic	8155368.095	8	1019421.012	0.799288754	0.6229
Residual	8927871.24	7	1275410.177		
Total	422494264.9	30	14083142.16		
R^2 = 0.8337; SS, sum of squares; DF, degrees of freedom; MS, mean square.					

Table 4. Regression Results of the Central Composite Design

Factor	Coefficient	*p*-value
Constant	6385.15	0.0013
Initial concentration of glycerol (X_1)	245.00	0.2784
Initial concentration of peptone (X_2)	328.24	0.1526
Initial concentration of $CuSO_4$ (X_3)	-27.60	0.9009
Initial concentration of L-asparagine (X_4)	290.62	0.2021
X_1X_2	-180.60	0.5088
X_1X_3	-246.37	0.3704
X_1X_4	-95.79	0.7246
X_2X_3	454.75	0.1089
X_2X_4	56.18	0.8360
X_3X_4	-184.42	0.5000
X_1^2	-1062.50	0.0001
X_2^2	-990.64	0.0002
X_3^2	-827.37	0.0010
X_4^2	-1020.01	0.0002

Figures 4b, c, d, and f also support the effect of nitrogen sources in the forms of L-asparagine and peptone on Lac production by *Fibrodontia* sp. RCK783S. Wu *et al*. (2005) reported that an increase in the concentration of ammonium tartrate led to a decrease in ligninolytic enzyme production by *P. ostreatus*. A study by Stajic *et al*. (2006) reported that the cultivation of *Pleurotus eryngii* in higher concentrations of $(NH_4)_2SO_4$ resulted in a decrease in Lac production. This was consistent with Vaithanomsat *et al*. (2012), who reported the effect of L-asparagine concentration on Lac production by *Lentinus strigosus*.

The concentration of glycerol as a carbon source also affected Lac production by *Fibrodontia* sp. RCK783S, as shown in Figs. 4b, d, and e. Interestingly, an increase in the $CuSO_4$ concentration resulted in higher Lac activity, but after a certain point, any subsequent increase resulted in a decrease in Lac activity, as shown in Figs. 4c, e, and f.

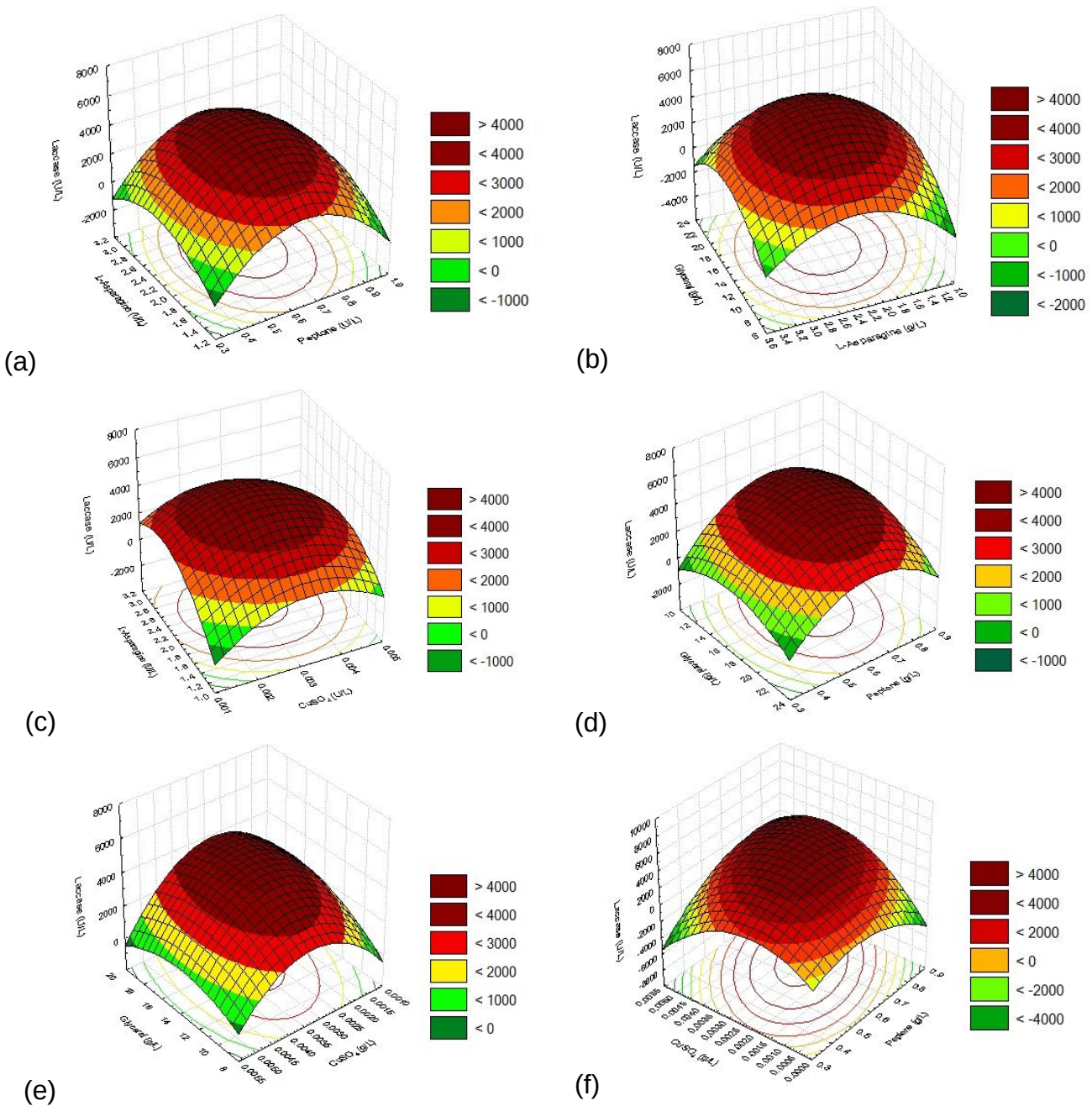


Fig. 4. Response surface for the interactive effects of (a) L-asparagine and peptone, (b) glycerol and L-asparagine, (c) L-asparagine and $CuSO_4$, (d) glycerol and peptone, (e) glycerol and $CuSO_4$, and (f) $CuSO_4$ and peptone on Lac activity after 9 days of *Fibrodontia* sp. RCK783S incubation at room temperature

It has been reported that the production of ligninolytic enzymes by fungi is influenced by heavy metals that in turn affect the levels of transcriptional and translational regulation, and a strong positive effect of Cu^{2+} addition on the production of Lac has been observed in several species, such as *P. crysosporium* (Dittmer *et al*. 1997), *T. hirsute* (Soden and Dobson 2001), and *L. strigosus* (Vaithanomsat *et al*. 2012). Cu^{2+} serves as a cofactor in the catalytic core of Lac; thus, a minimum concentration (in the milli mole range) of Cu^{2+} is necessary for the production of active Lac.

Excess Cu^{2+} may have a toxic effect on the fungal biomass and thus decrease Lac production (Majeau *et al*. 2010). However, a decrease in the fungal growth rate accompanied by a prolonged lag phase has sometimes been observed in the presence of Cu^{2+}. For example, a concentration of less than 1 mM Cu^{2+} inhibited the growth of *Ganoderma lucidum* (Baldrian 2003), while 1.6 mM Cu^{2+} decreased the growth rate of *T. trogii* (Levin *et al*. 2002).

A confirmation test was conducted for the production of Lac using the above identified optimum process parameters. The mean value of Lac activity from the experimental validation (6,086 U/L) was found to be in excellent correlation with the predicted value (6,385 U/L); thus, the model proved to be adequate. The final medium composition after RSM optimization was (g/L): glycerol, 15; peptone, 0.625; L-asparagine, 2.188; and $CuSO_4$, 0.003. This condition favored the preferential production of Lac and therefore maximized its production, with an overall 1.44-fold increase in Lac activity compared with that before the optimization.

Degradation of *Eucalyptus camaldulensis* by *Fibrodontia* sp. RCK783S

The degradation of *Eucalyptus camaldulensis* by *Fibrodontia* sp. RCK783S was studied. *Fibrodontia* sp. RCK783S was grown on *E. camaldulensis* sawdust under solid state fermentation at room temperature for 4 months. Samples were collected for further analysis every month.

SEM analysis of untreated and treated *E. camaldulensis* sawdust by *Fibrodontia* sp. RCK783S is shown in Figs. 5a-e. Degradation of cellulose in *E. camaldulensis* was clearly observed starting from the second month of cultivation (Fig. 5c) and corresponded with the removal percentage of lignin. The extracellular enzymes (Lac, MnP, LiP, and cellulase) produced during the solid state fermentation were also extracted from the cultures and assayed for their activities. *Fibrodontia* sp. RCK783S produced Lac during the entire fermentation. Its level reached a maximum (115.0 U/L) on the 30^{th} day of biodegradation and constantly decreased after that (12.47, 2.71, and 2.12 U/L on the 60^{th}, 90^{th}, and 120^{th} day of biodegradation, respectively). The activities of LiP, MnP, and cellulase were not detected.

Analysis of the lignin content in the untreated and treated *E. camaldulensis* sawdust revealed that this compound was degraded during the process. The untreated *E. camaldulensis* sawdust contained 25.94% lignin. After biodegradation by *Fibrodontia* sp. RCK783S for 30, 60, 90, and 120 days, the levels of lignin in the raw material decreased to 25.03$\pm$0.53, 23.75$\pm$0.26, 23.97$\pm$0.55, and 22.39$\pm$0.47%, respectively, equivalent to 17.91$\pm$0.42, 21.28$\pm$0.44, 21.57$\pm$0.48, and 22.56$\pm$0.46% lignin degradation.

Neither of the enzyme activity peaks in either the treated or untreated sawdust corresponded well with the highest lignin removal; thus, the direct correlation between Lac activity and removal of lignin was not clear. The biotransformation of organic matter is a very complex process influenced by different factors (Vargas-Garcia *et al.* 2007). The results described here suggested the contribution of fungal inoculation to the degradation process of lignin under appropriate conditions. For the highest benefit, the capabilities of this fungal strain should be further studied to establish the most efficient inoculation pattern.

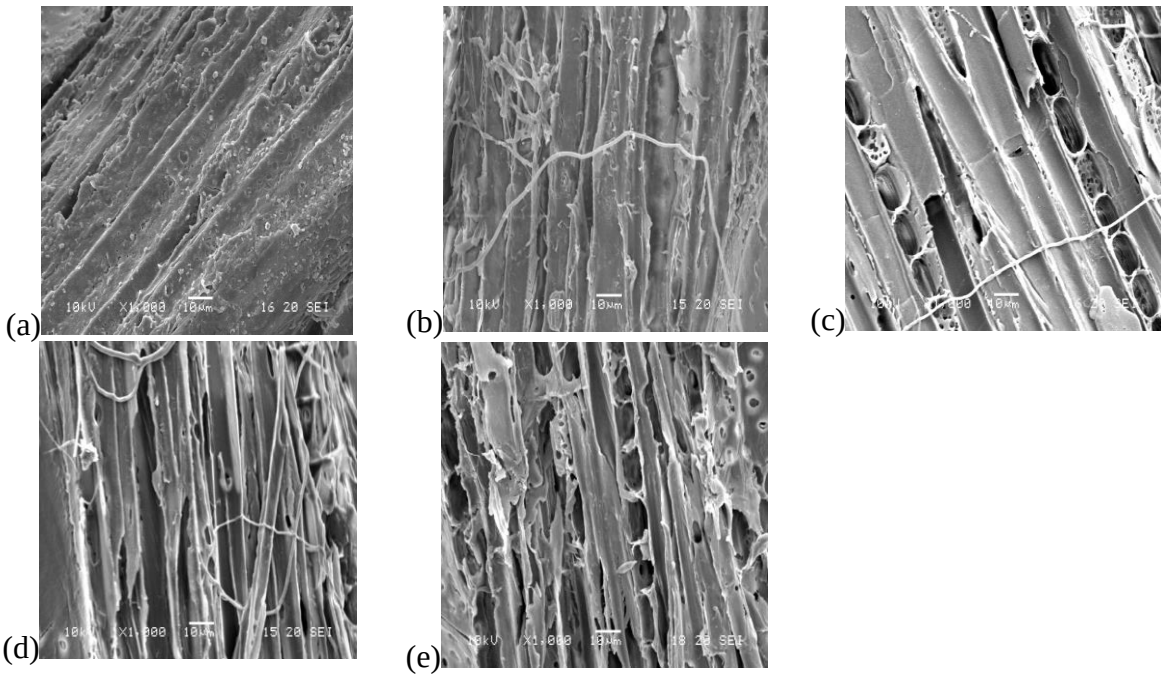


Fig. 5. SEM (1000x) of *Eucalyptus camaldulensis*; (a) untreated, (b) treated with *Fibrodontia* sp. RCK783S under solid state fermentation at room temperature for 1 month, (c) 2 months, (d) 3 months, (e) and 4 months

The results from this study were consistent with other studies. Machuca and Ferraz (2001) applied *Trametes versicolor*, *Ganoderma lucidum* BK2, *G. brownie* KH2, and *G. gibbosum* LP2 in the solid state fermentation of *Eucalyptus grandis*. The highest lignin degradation (54%) of *E. grandis* was observed from *T. versicolor* after 30 days of fermentation, whereas those treated with *G. lucidum* BK2, *G. brownie* KH2, and *G. gibbosum* LP2 showed lignin degradation within the range 16.65 to 19.87%. Ferraz *et al.* (2003) grew *Ceriporiopsis subvermispora* on *E. grandis* wood chips under solid state fermentation. They observed the highest loss of wood weight and lignin at 11.7 and 27%, respectively, after 90 days of incubation. The variation of the detected Lac activity was observed during the fermentation. It reached the maximum level at 63.0 IU/L (30 days of incubation) and then clearly decreased to 12.6 and 14.0 U/L at 60 and 90 days, respectively. Rodríguez Couto *et al.* (2003) studied the degradation of grape cluster stems during the semi-solid-state cultivation of *Phanerochaete chrysosporium* BKM-F-1767 (ATCC 24725). The results showed that about 20% lignin, 48% hemicellulose, and 5% cellulose were degraded during the cultivation; these values were assumed to be related to the ligninolytic enzymes (MnP and LiP) produced. Xu *et al.* (2009) incubated *Irpex lacteus* CD2 on corn stover under solid-state fermentation for 120 days. After 15 days, 63% of hemicellulose was degraded, whereas 17.2% of cellulose was degraded after 10 days. The activities of cellulase and hemicellulase peaked at days 5 and 30, respectively. Lignin was significantly degraded, by nearly 80%, after 60 days; however, no ligninase activity was detected during the lignin removal stage. Ji *et al.* (2012) studied the degradation of *Populus euramericana* sawdust during enzyme production by *Trametes trogii* MT. The results demonstrated high production of Lac and MnP, as well as the ability of this strain to simultaneously degrade the lignin, cellulose, and hemicellulose of *P. euramericana* sawdust.

CONCLUSIONS

1. This paper reports a new record of *Fibrodontia* sp. in Thailand. The designated *Fibrodontia* sp. RCK783S is an efficient Lac producer, with very high activity compared to most of the reported strains under similar conditions.
2. Lac production by *Fibrodontia* sp. RCK783S was successfully optimized using response surface methodology. High production levels of Lac from this strain could be advantageous with respect to synergistic reactions in bioremediation and biotransformation processes. This strain could therefore be an attractive source for industrial applications.
3. Solid state fermentation of *Eucalyptus camaldulensis* by *Fibrodontia* sp. RCK783S showed degradation of lignin during the process, indicating its potential for the pretreatment of lignocellulose prior to further utilization.

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