CHAPTER 4

OPTIMIZATION CONDITION FOR LACCASE PRODUCTION

4.1 Introduction

The production of laccase is dependent on difference factors, which included the strain of microorganism, the composition of culture medium (compounds that provide a nitrogen and carbon sources), the cultivation method (solid substrate or submerged), and the culture conditions (pH and temperature). Laccase is generally produced in appreciable concentrations during the idiophase, where growth remains static due to a decrease in available substrate. In order to provide laccase in the quantities required and at a low cost, it is vital that yields are increased or that production costs are reduced (Strong and Claus, 2011).

Lignocelluloses are the most abundant biopolymer in nature, comprising the polysaccharides cellulose and hemicelluloses, plus polyphenolic heteropolymer, and lignin (Eaton and Hale, 1993). Lignocellulosic biomass includes materials such as agricultural residues (*e.g.* corn stover and rice straw), forestry residues (*e.g.* sawdust and mill wastes), portions of municipal solid waste (*e.g.* waste paper), woody and various industrial residue (*e.g.* fruit residues) (Palonen, 2004). The attraction of lignocelluloses comes from its simplicity and closeness to the natural way of life for many microorganisms, high volumetric productivity and as an alternative in preventing environmental pollution (Pandy, 1994).

White-rot fungi attack the lignin component in lignocellulosic material, action of lignin-degrading enzyme such as laccase (Lee *et al*., 2007) and leave the cellulose and hemicelluloses less affected. Evaluation of the white-rot fungal enzyme activity showed that the fruit residues such as orange peel and banana residue are also appropriate growth substrates for laccase production (Rosales *et al*., 2007 and Elisashvili *et al*., 2008). Orange (*Citrus* sp.) peel can be used for fungal laccase production in solid state cultivation (Rosales *et al*., 2002; Rosales *et al*., 2007). Moreover, the agricultural residues such as rice bran and wheat straw are reported as the materials for laccase production (Durán *et al*., 2002, Sathish *et al*., 2008). This enzyme has a great potential in application to various industries especially in pulp and paper industry (Bajpai, 1999). In addition, beverages industries such as fruit juices, wine and beer, also use this enzyme as phenol derivatives remover (Durán *et al*., 2002).

Trametes polyzona is important in the ecosystem as a hard wood decomposer. It has not been associated to any plant diseases, therefore it is not considered to be pathogenic. Many species of *Trametes* have been reported as laccase producers for example, *T. versicolor* (Jing *et al*., 2007; Tišma *et al*., 2012), *T. hirsuta* (Rosales *et al*., 2002; Couto *et al*., 2006) and *T. trogii* (Héla *et al*., 2006; Kocyigit *et al*., 2012). However, previous studies about laccase production from *T. polyzona* was limited. In this study, the white-rot basidiomycetous fungus *T. polyzona* WR710-1 was used for laccase production on many agricultural residues under solid state cultivation and the optimal conditions for enzyme production were determined. *T. polyzona* WR710-1 and its laccase will be used for lignocellulosic biomass pretreatment for bio-ethanol production in the next experiment.

4.2 Materials and Methods

4.2.1 Lignocellulose substrates preparation

Lignocelluloses, agricultural residues including rice husk, coffee husk, rice straw, corncob, sugarcane bagasse, rice bran, orange peel, banana peel, bamboo pulp, and corn stover were used as substrates in this research. All substrates were collected in the north of Thailand; Chiang Rai, and Chiang Mai provinces. Substrates were washed 2-3 times with water and dried in oven at 50-60^{o}C for 24 hr. Dried substrates were ground to a particle size of about 2-5 mm. Each sample was transferred into a zip locked plastic bag and stored in desiccators until used.

4.2.2 Total protein determination

Total protein determination in this experiment using Bradford method (Bradford, 1976). Bovine serum albumin (BSA) was used as standard protein.

4.2.3 Optimization condition for laccase production

The selected white-rot fungus, *T. polyzona* WR710-1 was improved for laccase production by optimization of carbon and nitrogen sources, initial pH, temperature and effect of metal ions such as, $CuSO_4$.

4.2.3.1 Optimization of carbon source for laccase production

Lignocellulosic biomass were used as carbon source for laccase production, 10 agricultural residues; rice husk, coffee husk, rice straw, corncob, sugarcane bagasse, rice bran, orange peel, banana peel, bamboo pulp, and corn stover were used as solid substrate for *T. polyzona* cultivation. Five gram of solid substrates were added into 15 ml of basal media (Mikiashvili *et al*., 2006) and incubated at 37^{o}C, darkness and static condition for 7 day. The relative laccase activity (%) was determined. Agricultural residues that showed the highest laccase activity was further used as carbon source in

solid state fermentation and incubated at 37°C under shaking for 24 day. The time-course of relatively laccase activity (%) were collected every 2 days.

4.2.3.2 Optimization of nitrogen source for laccase production

The effect of various nitrogen sources, ammonium nitrate, NH_4NO_3; ammonium tartrate, $(NH_4)_2C_4H_4O_6$; ammonium sulfate, $(NH_4)_2SO_4$; potassium nitrate, KNO_3; peptone and yeast extract (0.2% w/v) was investigated. Different nitrogen sources were added in 15 ml basal medium, adjusted initial pH 6.0. Orange peel 4% (w/v) was added in basal medium and incubated at 37°C for 7 days. The cell-free supernatant was assayed for laccase activity.

4.2.3.3 Effect of solid to liquid ratio on laccase production

The effect of solid (orange peel) and liquid (basal solution) ratio (S/L) on laccase production was studied. The ratios of 1:1 to 1:7 used to determine the optimal solid to liquid ratio for solid state fermentation by *T. aurantiacus* and the enzyme activities were measured by laccase assay.

4.2.3.4 Optimization level of carbon and nitrogen sources on laccase production using statistical experimental design

Experimental design for optimization of the correlation between carbon and nitrogen sources for laccase production was done using central composite design (CCD). Orange peel and peptone were used as carbon and nitrogen sources, respectively. The different levels of their coded and actual values for CCD, octagon design are shown in Table 4.1. All trials were performed in triplicate, and the mean was determined. The data were analysed for significant difference between treatments using analysis of variance (ANOVA). To establish if differences between individual trails were significant ($p < 0.05$) and multiple linear regression analysis was

performed using software Design-expert 6.0.2. The maximum laccase production was taken as the dependent variable of response (Y). The observed values were analyzed and fitted to the second-order model equation is as follows;

$$Y = \beta_0 + \Sigma\beta_i x_i + \Sigma\beta_{ii} x_i^2 + \Sigma\beta_{ij} x_i x_j \quad (4.1)$$

Where, Y is the variable response (enzyme activity) and β is the regression coefficients given by the model and x_i and x_j are the independent factors of the experiment (Akay, 2007). If the curve shape of the response surface plot is elliptical or circular then it is presumed that the correlation between the variables is most significant.

Table 4.1 Codes and actual levels of the independent variables for design experiment

Variables	Code levels				
	-2	-1	0	1	2
Orange peel (% w/v)	0	10	30	50	70
Peptone (% w/v)	0	0.5	2.75	5	7.25

4.2.3.5 Optimization of initial pH for laccase production

Twenty ml of basal medium (Mikiashvili *et al*., 2006) was adjusted initial pH range from 3.0 to 9.0. Under solid state fermentation, the culture containing 0.8 g orange peel as solid substrate and basal medium were added in 125 ml Erlenmeyer flask and sterilized. Mycelium plugs of white rot fungus were tranfered in each flask and incubated at 37^{o}C, static condition for 7 days. The culture broth was centrifuged and supernatant was used for laccase activity assays.

4.2.3.6 Optimization temperature for laccase production

The test flask 250 ml contained 5 g of ground orange peel and 15 ml of basal media and incubated at different temperature, 25, 30, 37 and 45^{o}C for 7 days. The cultures were extracted with distilled water and centrifuged, the supernatant was assayed for laccase activity. The cell-free supernatant was assayed for laccase activity.

4.2.3.7 Effect of copper on laccase production

The effect of copper on laccase production was investigated in this study. Different concentration 0, 50, 100, 200, 300, 400 and 500 mM of $CuSO_4$ was added in basal medium (Mikiashvili *et al.*, 2006). White rot fungus WR710-1 was grown on orange peel substrates supplemented with basal medium under solid state fermentation. The fermentation flasks were incubated at 37^{o}C for 7 days. The cultures were extracted with distilled water and centrifuged, the supernatant was assayed for laccase activity.

4.2.4 Analysis of chemical composition in orange peel

Orange peel was washed 2-3 times with sterile water and dried in oven at 50-60^{o}C. Dried orange peel was ground to a particle size of about 2-5 mm. Each sample was transferred into a zip locked plastic bag and stored in desiccator. Dried sample were analyzed for chemical composition including; cellulose, hemicelluloses and lignin by using the TAPPI procedure (Appendix B).

4.3 Results and discussion

4.3.1 Optimal carbon and nitrogen sources for laccase production

Lignocellulose (agricultural residues) is well known to be used as carbon sources for laccase production by many researchers (Reddy *et al*., 2003; Chawachart *et al*., 2004; Elisashvili *et al*., 2008). In this study the best substrate for laccase production by *T. polyzona* WR710-1 was shown in Fig. 4.1; the levels of extracellular enzyme activities produced during cultivation of different plant raw materials varied. Tangerine orange peels (*Citrus reticulata*) ensured the highest yield of laccase (0.69±0.12 U/gds) and the activities were 0.17±0.03 and 0.16±0.04 U/gds when grown on corncob and coffee husk, respectively. This experiment showed that fruit residue such as orange peel was a good substrate for laccase production by *T. polyzona* WR710-1. This observation agrees with the recently reported finding of Rosales *et al*., 2007 and Elisashvili *et al*., 2008 exhibited that orange peel was suitable for laccase production. The high laccase activities detected in orange peel medium are likely due to essential oils in orange peel. The major aromatic compounds of essential oils are phenolic and similar molecule (Kamal *et al*., 2011). Laccase is a nonspecific oxidative system to degrade lignin and other aromatic compounds in orange peel. Thus, aromatic oil in orange peel could be induced laccase activity when used orange peel as solid substrate for laccase production.

Moreover, the agricultural residue such as rice bran and wheat straw are reported as the materials for laccase production (Durán *et al*., 2002; Sathish *et al*., 2008). Recently many publications reported that wheat bran and rice bran were suitable carbon sources for laccase production. Solid wheat bran substrate induced high laccase activity while, bran extract induced higher protein content in culture

filtrates and dry biomass of *Pleurotus ostreatus* culture media (Moharib *et al*., 2011). Rice bran was an efficient substrate for laccase production by thermotolerant basidiomycete *Coriolus versicolor* strain RC3 (Chawachart *et al*., 2004).

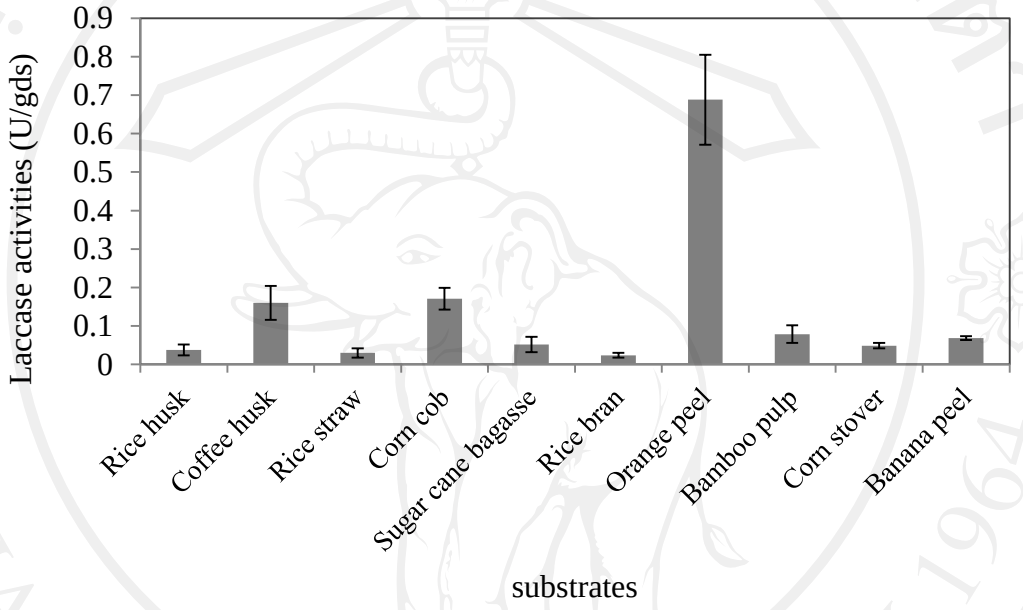

Fig. 4.1 Laccase production from various substrates by *T. polyzona* WR710-1 under solid state cultivation, incubated at 37^{o}C, darkness and static condition for 7 days. Error bars represent the standard deviation from the mean of three replications.

When compared solid substrate for laccase production between glucose (general carbon source for microorganism growth), common lignocelluloses for laccase production (wheat bran and rice bran) and orange peel (in this study), the result showed in Fig. 4.2, orange peel presented the best substrates for laccase production in this experiment. White-rot fungus produced the high level of laccase at 12-14 day

incubation and the enzyme activity was unchanged after 16 day. Orange peel can be used for fungal laccase production in solid state cultivation (Rosales *et al*., 2002; Rosales *et al*., 2007). Some chemical composition of orange peel substrate in this study was analyzed and found similarity with the component analysis of orange peel by Ververis *et al*. (2007) that it contained 13.6% cellulose, 6.10% hemicelluloses, and 2.10% lignin (Table 4.2).

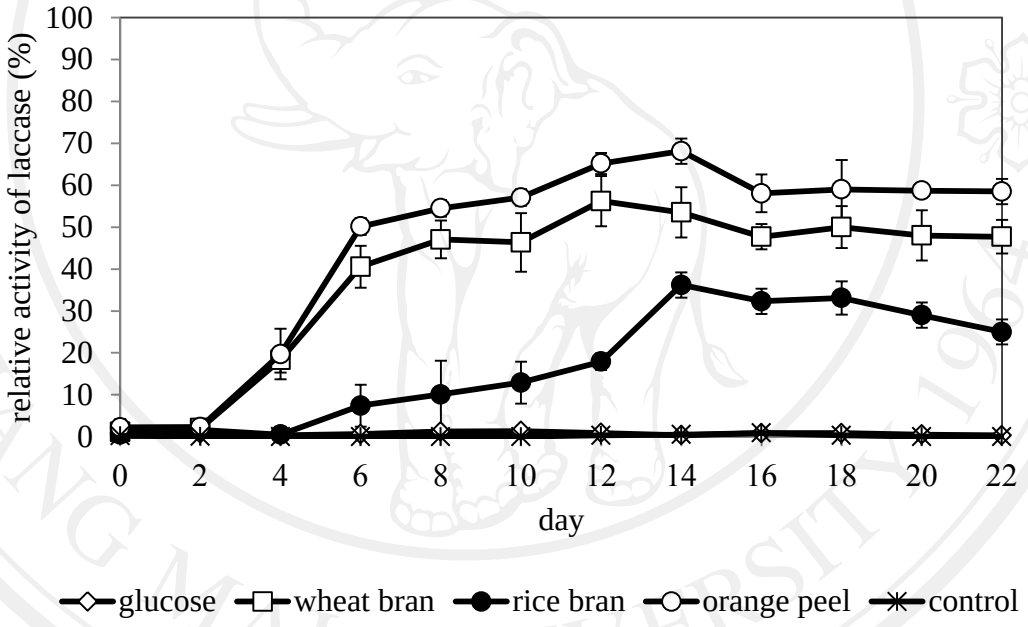

Fig. 4.2 Time-course of laccase production by *T. polyzona* WR710-1 in various carbon sources under solid state cultivation.

Table 4.2 Some chemical composition of orange peel

Sources	Chemical composition (%)			References
	Cellulose	hemicellulose	lignin	
Orange peel	16.5 ± 0.6	9.31± 0.1	8.99 ± 0.9	This study
Orange peel	13.6 ± 0.6	6.10 ± 0.2	2.10 ± 0.3	Ververis *et al*., 2007

Among the cultures contained different sources of nitrogen in this experiment, peptone exhibited the highest activity of laccase at 1.67 U/gds (Fig. 4.3). This result similar with the production of laccase by mushroom *Lentinus tuberregium* which laccase activity higher than control (without nitrogen sources) when cultured with peptone, while ammonium molybdate (1 mM) and urea (1 mM) completely inhibited laccase activity in 30 day incubation (Manjunathan *et al*., 2010). Peptone, an organic nitrogen source was strongly improved laccase production, because of organic and inorganic nitrogen sources have different physiological effects on fungi (Dong *et al*., 2005).

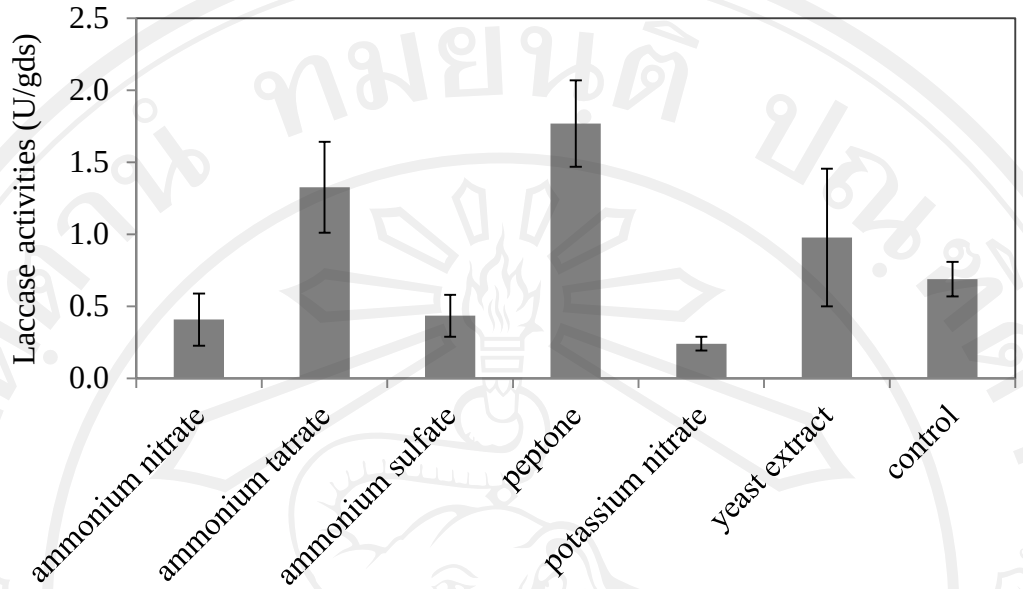

Fig. 4.3 Laccase production by *T. polyzona* WR710-1 in various nitrogen sources under solid state cultivation. Error bars represent the standard deviation from the mean of three replications.

4.3.2 Optimal level of carbon and nitrogen sources on laccase production using statistical experimental design

Statistical testing of the model was performed with the Fisher's statistical test for analysis of variance. The results of the ANOVA for laccase production were shown in Table 4.3. The quadratic regression showed the model was significant because the value of F-test (the ratio of mean square due to regression to mean squares to real error) less than 0.05 indicated the significance of the model terms. The non significant value 0.2049 lack of fit showed that the quadratic model was valid for the present study and the value of correlation was 0.970.

Table 4.3 ANOVA for nitrogen and carbon sources for laccase production fitted to the response surface quadratic model

sources	Mean Square	F value	Prob > F	
model	0.20	25.92	0.0038	significant
x_1	0.01	1.51	0.2862	
x_2	0.47	60.82	0.0015	
x_1^2	0.45	57.54	0.0016	
x_2^2	0.10	12.41	0.0244	
x_1x_2	0.02	2.44	0.1933	
Residual	0.03			
Lack of Fit	0.01	2.61	0.2049	non significant
Pure Error	5.53×10^{-3}			

x_1 = peptone and x_2 = orange peel

R^2 = 0.970; Coefficient of Variance (C.V.%) = 18.05

Values of Prob > F is less than 0.05 for x_2 , x_1^2 and x_2^2 terms indicate model terms are significant in table 4.3. On the basis of quadratic polynomial equation of response surface model (Eq. 4.1), the effect of independent factors; peptone as nitrogen source and orange peel as carbon source on the laccase activity were analyzed in Eq. (4.2)

$$\textit{Laccase activity}\ (\text{U/gds}) = +0.74175 - 0.39636\,x_2 - 0.57825\,x_1^2 - 0.26860\,x_2^2 \quad (4.2)$$

Equation (2) was performed using software Design-expert 6.0.2 and showed the second-order model equation for two factors where x_1 and x_2, were peptone and

orange peel amount, respectively, x_1x_2 is the correlation between peptone and orange peel and R^2 is the Coefficient of Determination. The non significant value of x_1 and x_1x_2 were not showed in the equation. From the model, the result indicated that the fermentation with the interaction between orange peel and peptone at 15/2% was showed the optimal laccase production when the predicted activity of laccase was about 1.0 U/gds. The response surface plot was shown in Fig. 4.4, the curve of the plot was close to circular at around the optimal ratio of carbon and nitrogen sources.

The white-rot fungi are extremly efficient in their use of nitrogen. Some reports suggested that a limited concentration of nitrogen in the culture media favored the production of laccase by basidiomycetous fungi (Buswell *et al*., 1995; Calvo *et al*. 1998). However, some reports promoted that laccases were produced at high nitrogen concentrations, although it is generally accepted that a high carbon to nitrogen ratio is required for laccase production (Buswell *et al*., 1995).

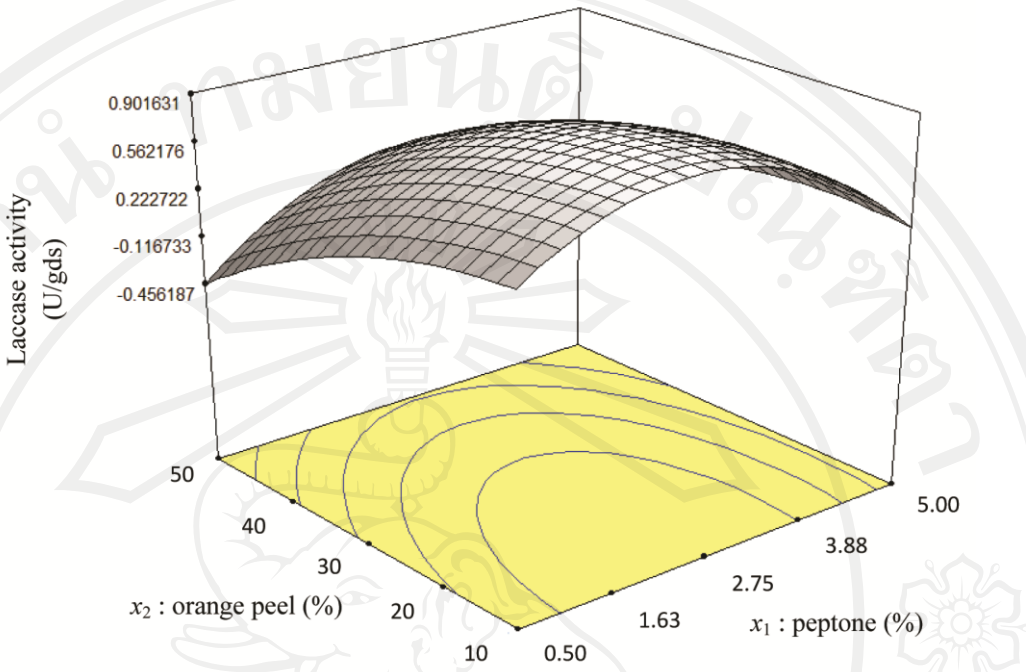

Fig. 4.4 Response surface plot of the correlation between carbon source (orange peel) and nitrogen source (peptone) for laccase production by *T. polyzona* WR710-1 under solid state cultivation.

4.3.3 Solid to liquid ratio in solid state cultivation for laccase production

Water is one of the most important factors in solid state cultivation. Solid to liquid ratio (S/L ratio) affects not only the growth and metabolism of microorganisms, but also effect to oxygen transferation which significant to aerobic growth (Qui and Chen, 2008). As shown in Fig. 4.5, it was found that S/L ratio as 1:3 to 1:4 promoted the laccase activity produced from orange peel by *T. polyzona* under solid state cultivation for 12 day. When the S/L ratio was 1:1which lack of necessary water, the laccase production were completely inhibited and the S/L ratio was up to 1:7, high water content would also harm the aeration of microbial growth.

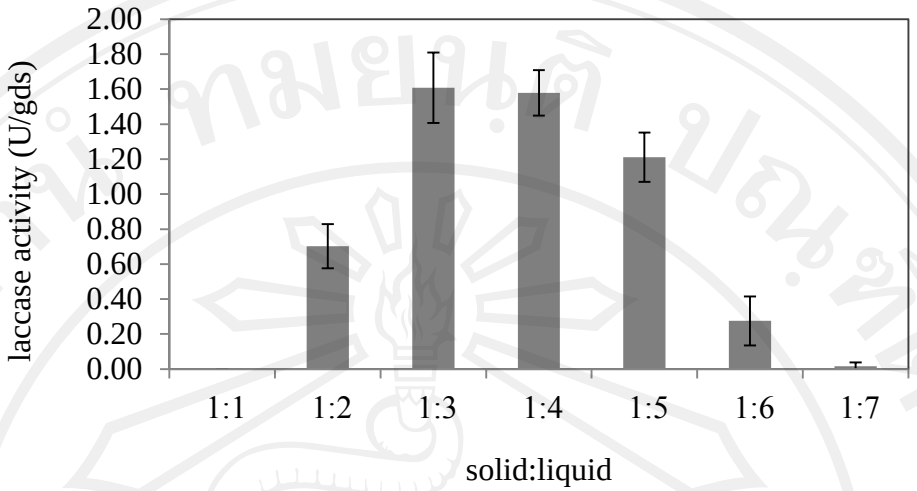

Fig. 4.5 Solid to liquid ratio of solid state cultivation for laccase production from orange peel by *Tremetes polyzona* WR710-1. Error bars represent the standard deviation from the mean of three replications.

4.3.4 Optimal pH for laccase production

There is not much information available on the influence of pH on laccase production, but when fungi were grown in a medium of which the pH is optimal for growth (pH 5) the laccase will be produced in excess (Thurston, 1994). In this study, the optimal initial pH for laccase production was 6.0 (Fig. 4.6) when incubated at 37^{o}C for 7 days. Dhouib *et al*., 2005 showed that *T. trogii* CTM 10156 produced laccase at wide pH, 5.0-6.5.

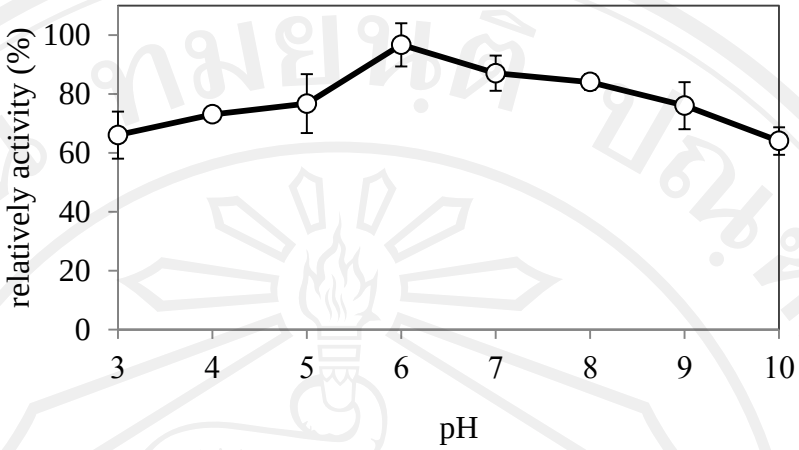

Fig. 4.6 Laccase production by *Trametes polyzona* WR710-1 at various initial pH. Error bars represent the standard deviation from the mean of three replications.

4.3.4 Optimal temperature for laccase production

Trametes polyzona WR710-1 produced the highest activity of laccase when grew on orange peel under solid substrate cultivation and incubated at 37^{o}C, while no activity of laccase was found when cultured at 45^{o}C (Fig 4.7). Many fungi generally grew between 30°C to 37°C, and for some isolates enzymatic activities were detected at this temperature (Schliephake *et al*., 2000; Asgher *et al*., 2008). However, many fungi were cultivated at temperatures between 25 °C and 30 °C, for optimal laccase production (Dhouib *et al*., 2005; Qiu and Chen, 2008). When cultivated at temperatures higher than 30°C the activity of ligninolytic enzymes was reduced (Zadrazil *et al*., 1999). It has been found that the optimal temperature for fruiting body formation and laccase production is 25 °C in the presence of light, but 30 °C for laccase production when the cultures are incubated in the dark (Thurston, 1994).

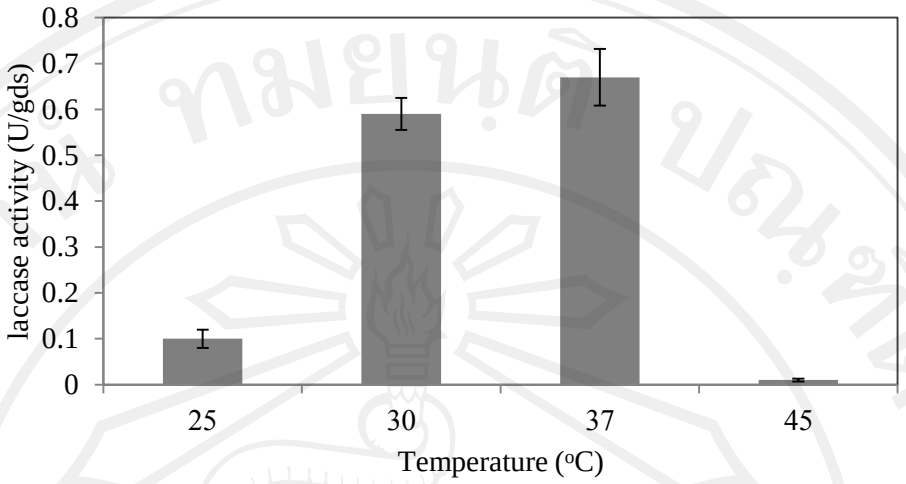

Fig. 4.7 Laccase production by *Trametes polyzona* WR710-1 at various temperatures. Error bars represent the standard deviation from the mean of three replications.

4.3.6 Effect of copper ion on laccase production

In comparison to relative activity of laccase production in control cultivation (without $CuSO_4$), the culture with 50 mM of $CuSO_4$ by *T. polyzona* WR710-1 led to 4.48-folds increased of laccase production (Fig 4.8). Gnanamani *et al*. (2006) found that $CuSO_4$ alone at 30 mM concentration accelerated the laccase production at 3.5-fold increase compared to control. Moreover, many studies suggested that Cu^{2+} promoted laccase production (Dhouib et al., 2005; Tychanowicz et al., 2006; Cordi et al., 2007). Copper is an essential nutrient for most living organisms, and copper requirements by microorganisms are usually satisfied by very low concentrations of the metal (Labbé and Thiele, 1997). Laccase production by *T. polyzona* in this study was inhibited by high concentration (300 mM) of Cu^{2+} (Fig 4.8). Copper present in higher concentration is extremely toxic to microbial cells (Labbé and Thiele, 1997). Okamoto *et al*. (2000) suggested that an excess supply of Cu^{2+} ions might cause a

change in the structure of laccase leading to a loss of activity, although laccase is a copper-containing enzyme.

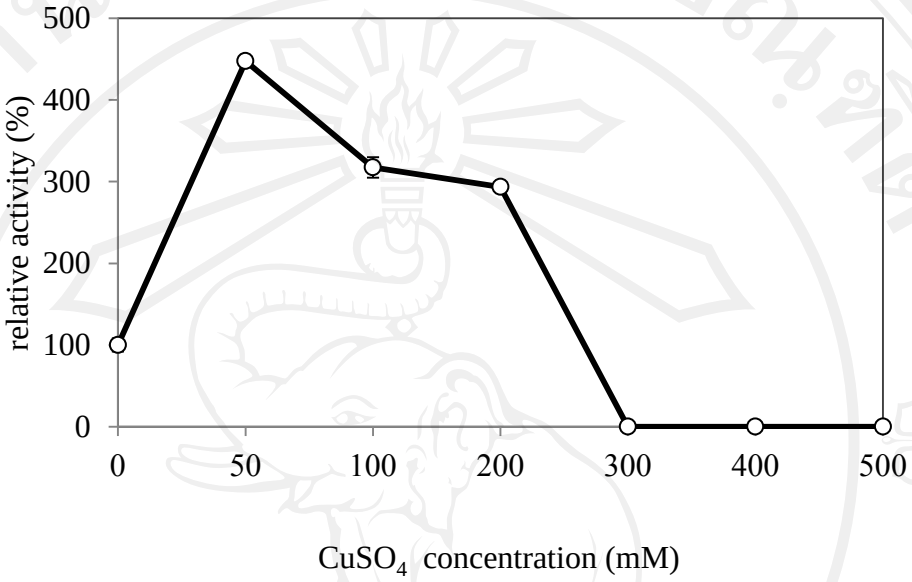

Fig. 4.8 Relative activity of laccase with various Cu^{2+} concentration compared with the culture without $CuSO_4$, defined as 100% (control). Error bars represent the standard deviation from the mean of three replications.

In view of the encouraging results described above, the optimal condition of laccase production under solid state cultivation are as follows; orange peel as carbon source, peptone as nitrogen source, carbon to nitrogen source ratio 15:2%, S/L ratio 1:3-1:4, pH 6.0 and incubated at 37°C for 14 days. Following the optimum condition for laccase production, the maximum enzyme activity was 0.75 U/ml enhanced laccase activity to 8-folds from initial condition (0.1 U/ml) compared with the study of laccase production from agricultural residues, Chawachart *et al*. (2004) showed the maximum laccase activity (0.22U/ml) was produced from rice bran substrate by *Coriolus versicolor* strain RC3. While laccase production under submerge

fermentation by white-rot fungus *Rigidoporus lignosus* showed the highest activity of laccase around 0.37 U/ml (Cambria *et al*., 2000).

In this study, the optimization for various variables was done by the single-variable optimization method. It was easy to set the experiment and simple to analyze the result. However, this method was lead to misinterpretation of results because the interaction between different variables was overlooked (Wenster-Botz, 2000). Thus, the multi-variable optimization methods such as statistical experimental design, response surface methodology (RSM) should be used in the future study.