CHAPTER 9

GENERAL DISCUSSION AND CONCLUSION

Thailand is an agricultural country and has abundant agricultural residues from different crop residues. Thus, the use of agricultural residue for bio-ethanol can be a method for improving residue value and reducing the effect of the residue pollution in environment. The biological process of fuel ethanol production utilizing lignocelluloses as substrate requires: (1) delignification (pretreatment) to liberate cellulose and hemicelluloses from their complex with lignin, (2) depolymerization (hydrolysis) of the carbohydrate polymers (cellulose and hemicellulose) to produce free sugars and (3) fermentation of mixed hexose and pentose sugars to produce ethanol (Jeewon, 1997).

Pretreatment is an important process step for the biochemical conversion of lignocellulosic biomass into bio-ethanol. Laccase is an important enzyme in fungal ligninolytic systems involved in lignin degradation (Gianfreda *et al*., 1999) and used in pretreatment. White-rot fungi collected from native forest in the northern Thailand were studied for their potential of laccase production with the purpose to apply for biological pretreatment of lignocellulosic biomass in bio-ethanol production. White-rot fungi were primary screened for laccase production by degraded the Poly-R dye and enzyme activity were determined by using 2,6-DMP as substrate. *Coriolus versicolor* strain RC3 (Khanongnuch *et al*., 2004) was used as reference microorganism for laccase production. White-rot fungus which produced the highest

laccase activity was selected from total 31 isolates. Molecular identification with the ITS region of rRNA gene sequence indicated that the selected isolate was a member of genus *Trametes* with 99 % similarity and identified as *Trametes polyzona* strain WR710-1 (accession number JN848329). Mycelial colonies of *T. polyzona* WR 710-1 on PDA media were off-white, showing high density, velvety texture, and abundant aerial hyphae. Completed colonization on the petri dish of *T. polyzona* WR 710-1 took 5 days at 37^{o}C. The fungus produced generative hyphae with clamps connection, thin-walled and 1.5-2.5 µm wide but spore was not found under compound microscope. Moreover, fruiting body formation of white-rot fungus was studied by using orange peel as solid substrate. *Trametes polyzona* WR710-1 formed white global mycelium and polypore structure was also found when using MYG as liquid solution after 40th day, similar to the study of Coleman (2005) that found white-rot fungi produced white fluffy cotton wool-like growth and usually found under humid conditions. In this study, after approximately 85th day, white rot turned to brown color and then wilted, died and decay. No spore or any reproductive structure was observed under compound microscope.

Trametes polyzona is important in the ecosystem as a hard wood decomposer and 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 optimal condition for laccase production was determined. Among 10 agricultural residues, orange peel showed the highest level of laccase at 12-14 day incubation (0.69±0.12 U/gds) indicated that orange peel was the

optimal carbon source for laccase production. Orange (*Citrus* sp.) peel can be used for fungal laccase production in solid state cultivation (Rosales *et al*., 2002; Rosales *et al*., 2007). 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. Among the cultures contained different sources of nitrogen in this experiment, peptone exhibited the highest activity of laccase (1.67 U/gds). This result similarity with the production of laccase by mushroom *Lentinus tuberregium* which laccase activity higher than control when cultured with peptone in 30 day incubation (Manjunathan *et al*., 2010). Peptone, an organic nitrogen source was strongly improved laccase production than inorganic nitrogen, because of organic and inorganic nitrogen sources have different physiological effects on fungi (Dong *et al*., 2005). Optimal carbon to nitrogen sources (C/N) ratio was done by statistical analysis, central composite design (CCD). From the significant equation, with R^2 was 0.970, the result indicated that the fermentation with the interaction C/N ratio at a 15/2 % (w/v) was suitable for laccase production when the predicted activity of laccase was about 1.0 U/gds. Moreover, the solid to liquid (S/L ratio) of solid state cultivation condition was 1:4, initial pH at 6.0, incubated temperature at 37^{o}C and additional of 50 mM $CuSO_4$ promoted the level of laccase, 4.48-folds increase of laccase production.

Although there are some previous studies on laccase production from *T. polyzona*, however steps for purification and characterization of this enzyme have not been studied so far. In the study reported here, laccase activity, molecular weight, substrate specificity and kinetic parameters as well as N-terminal amino acid

sequence were examined. Laccase from *Trametes polyzona* WR710-1, was produced under solid state fermentation using peel from Tangerine orange (*Citrus reticulata*) as a substrate, and was purified to homogeneity. This laccase was found to be a monomeric protein with molecular mass of about 71 kDa estimated by SDS-PAGE. This result is consistent with most laccases which are monomeric glycoproteins having a molecular mass of 50 - 80 kDa (Mario *et al*., 2002; Rosana *et al*., 2007; Nitheranont *et al*., 2011[a]). The spectral characteristics of laccase from *T*. *polyzona* are typical to blue copper proteins containing four copper atoms. A peak at around 600 nm and a shoulder at around 300 nm recorded in the UV-Vis spectra suggested the presence of type-1 and type-3 copper clusters (Maria *et al*., 2000; Mario *et al*., 2002). The optimum pH of the laccase were 2.0 for ABTS and 5.0 for 2,6-DMP substrates. Moreover, the optimum pH was 4.0 for L-DOPA, guaiacol and catechol substrates. Many fungal laccases have been reported to have such a variation in the optimum pH for different substrates (Christiane *et al*., 2002; Nagai *et al*., 2002; Jia and Yi, 2004). The studied laccase was stable between pH 6.0 and 8.0 using ABTS as substrate which is similar to the previous study by Harnándes *et al*. (2006). The *Trametes* laccase was stable up to 40 ^{o}C, with no activity loss. The laccase of *Pleurotus ostreatus* also showed a similar pattern in its thermal stability curve (Okamoto *et al*., 2000). About the highest catalytic efficiency k_{cat}/K_m and the highest affinity (the lowest of K_m value) of *T*. *polyzona* laccase were found for ABTS substrate. The *Trametes* laccase was also found to oxidize typical laccase phenolic substrates like 2,6-DMP, guaiacol, catechol and L-DOPA. These catalytic properties are consistent with those of laccases from many basidiomycetes such as *T*. *pubescens* (Christiane *et al*., 2002), *T*. *trogii* (Héla *et al*., 2006), *Grifola frondosa* (Nitheranont *et al*., 2011[a])

and *Pycnoporus coccineus* (Atef *et al*., 2005). In the present study, the activity of the purified *T. polyzona* laccase and a commercial *T. versicolor* laccase were both inhibited by chloride ions. This result is consistent with previous studies on laccase from different fungi, including those from *Trichophyton rubrun* (Jung *et al*., 2002), *Marasmius quercophilus* (Farnet *et al*., 2008) and *G. frondosa* (Nitheranont *et al*., 2011[a]). An inhibition mechanism of laccase by the chloride ion has been suggested by Naki and Varfolomeev (1981) that chloride ion acts as a competitive inhibitor with the electron donor and blocks the electron pathway at the active site of the laccase. Moreover, the degree of inhibition by chloride or other halide ions seems to be linked to the availability of copper atoms in the active site (Abadulla *et al*., 2000). About the inhibition of laccase activity by metal ions has been reported for some other fungal laccases including those of *Lentinula edodes* (Nagai *et al*., 2002) and *M. quercophilus* (Farnet *et al*., 2008). Similarly, metal ions exhibited inhibitory effects on the activity of *T. polyzona* laccase. Among metal ions tested, Fe^{2+} was the most efficient inhibitor causing more than 95 % inhibition at 5 mM. The laccase of *T. polyzona* was also inhibited by Cu^{2+}, although laccase is a copper-containing enzyme. 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. The effect of inhibitors on the laccase activity was also tested. *Trametes polyzona* laccase was completely inhibited by 0.1 mM NaN_3, 1 mM dithiotheitol and 10 mM L-cysteine. The results showed that laccase in this study more resistant to NaN_3 than laccase from *T. versicolor* that completely inhibited by 0.001 mM NaN_3 (Rosana *et al*., 2007). A strong inhibitory effect of azide on laccase activity has been explained as follows: azide binds to the copper atoms in the protein structure and blocks electron transfer leading to a loss of

catalytic activity (Sugumaran, 1995)**.** In addition, the inhibition by thiol compounds, dithiothreitol and L-cysteine was presumed to be the result of coordination of the thiol to the copper atoms in the enzyme active site (Wells *et al*., 2006). In this study, it is the first report on the N-terminal amino acid sequence of the *T. polyzona* laccase, AVTPVADLQISNAGISPDTF which is highly similar to laccases from other white-rot basidiomycetes, *Rigidoporus microporus* (Liu *et al*., 2003) and *Polyporus brumalis* (Ryu *et al*., 2008) with 75 % identity.

Unless the laccase was used for biological pretreatment of lignocellulosic biomass in bio-ethanol production, it also had high potential for environmental detoxification, bisphenol A degradation and synthetic dye decolorization (Fukuda *et al*., 2001; Cameselle *et al*., 2003; Michizoe *et al*., 2005). Purified laccase from *Trametes polyzona* WR710-1 was used as biocatalyst biodegradation and decolorization. All synthetic dyes used in this experiment, Bromophenol Blue (BRB), Remazol Brilliant Blue R (RBBR), Methyl Orange (MO), Relative Black 5 (RB 5), Congo Red (CR), and Acridine Orange (AO) were decolorized by *Trametes* laccase. More than 80 % of synthetic dyes, BPB, RBBR and MO were decolorized even in the absence of HBT. Moreover, 72, 52 and 27 % decolorization were achieved with CR, RB5 and AO, respectively. The addition of 2 mM 1-hydroxybenzotriazole; HBT as a redox mediator into the reaction mixtures led more decolorization percentages, around 36 and 24 % for RB5 and AO which were significantly increased values when compared to non HBT condition. Laccase may be involved in the oxidation of the phenolic group of azo dye to produce a radical at the carbon bearing the azo linkage (Chivukula and Renganathan, 1995). Each dye was not degraded in the same extent. This can be explained in terms of the structure and size of each dye, a low number of

aromatic rings and the simple molecule is degraded more rapidly than the complex molecule (Cameselle *et al*., 2003). About the degradation of bisphenol A by laccase (0.64 U/ml) with or without redox mediator, HBT was studied. The quantitative analysis by HPLC showed that 12 % of initial bisphenol A was removed within 1 hour and there after the degradation rate was declined in the absence of HBT. The oxidation of bisphenol A can be improved remarkably by the addition of HBT. Bisphenol A was 66.8 % removed within 1 hr and completely removed within 3 hr. Thus, bisphenol A oxidization by laccase was much more effective in the presence of HBT than in laccase alone. In order to identify the reaction product from bisphenol A degradation in the aqueous solution, GC-MS analysis was conducted. The major product from the biodegradation of bisphenol A by laccase from *T. polyzona* was 4-isopropenylphenol. Our results are in agreement with several reports with fungal laccase, *Trametes villosa* (Uchida *et al*., 2001), *Trametes* sp. (Michizoe *et al*., 2005) and *Grifola frondosa* (Nitheranont *et al*., 2011[b]). A portion of bisphenol A in the reaction mixture may be polymerized by laccase to form water-insoluble, high molecular weight compounds, such as oligomers (Uchida *et al*., 2001). This study shown here for the first report on a potential of laccase from *T. polyzona* as an effective enzyme for environmental applications especially decolorization of synthetic dyes and bisphenol A degradation.

In this study, three lignocelluloses (sugarcane bagasse, coffee husk and rice husk) were combined and used as solid substrate for bio-ethanol fermentation. Pretreatment of lignocelluloses were done by biological (*T. polyzona* WR710-1) and chemical method. The surface morphological characterization of lignocelluloses was observed by scanning electron microscopy (SEM). The surface morphology of these

lignocelluloses, showed decreased in cell wall thickness and crystallinity during pretreatment. Some chemical composition analysis showed lignin content was highly removed by 2 % NaOH (73.87 % elimination) followed by enzymatic pretreatment (30.76 %). However, lignin content was unchanged when pretreated by 2 % H_2SO_4. Cellulose content was significant increased closed to 40 % by alkali, 18 % by acid pretreatment, and slightly increased as 5 % by biological pretreatment. Although, the cellulose content in samples treated by biological pretreatment were low, but the surface structure of its was contained a lot of micro-pores, increased in available surface area (pore volume) and led increasing of fungal hydrolysis by *Thermoascus aurantiacus*, total sugar increased 85 % when compared with untreated control. White-rot fungi possess the capabilities to attack lignin in biological pretreatment process before ethanol fermentation (Dashtban *et al*., 2009). Ramos *et al*. (2004) showed that crude ligninolytic enzyme extracted from *Phanerochaete chrysosporium* fungi was successfully used for biological pretreatment of sugarcane bagasse. Moreover, Sasaki *et al*. (2011) suggested that *Ceriporiopsis subvermispora* pretreatment could be beneficial part of the process to produce ethanol from bagasse. Rice husk and coffee husk presented fibres and non-polar matrices of its surface which are mostly hydrophobic. It is the main problem due to high polarity and hydrophilicity in nature of cellulosic fibres. Therefore, pretreatment of the rice husk and coffee husk surface are critically important to increase the hydrophobicity of the fibres, improved on minimizing the surface tension and enhances enzyme hydrolysis. In conclusion, the surface morphological characterization of lignocelluloses was significant to enzyme hydrolysis. In this study, alkali pretreatment caused more cellulose content and cell wall damaged, total sugar was increased by

fungal hydrolysis. Acid pretreatment caused high cellulose content, but surface structure showed a few changed, total sugar was slightly increased when compared with untreated control. Although, the cellulose content in lignocellulose pretreated by fungus was low, but the cell wall contained a lot of micro-pores, increased in available surface area. Biological pretreatment by white-rot fungus *T. polyzona* enhanced fungal hydrolysis by *T. aurantiacus*, led increasing of available sugar for bio-ethanol fermentation.

About hydrolysate enzyme (cellulase and xylanase) production, agricultural residues from different crop residues could be used for these enzymes production. The optimal growth condition and production of cellulase and xylanase by *Thermoascus aurantiacus* SL16W were studied. The fungus *T. aurantiacus* completely grown on PDA plate in 4^{th} day (colony diameters of 9 cm) at the optimal incubation temperature was 45^{o}C. Enzyme production by *T. aurantiacus* under solid state cultivation, carbon and nitrogen sources were selected. Among different residues, coffee husk ensured the highest yield of cellulase (8.72±0.3 U/mg protein) and xylanase (86.6±0.3 U/mg protein). The residues from coffee have been reported in the production of cellulase (Selvankumar *et al.*, 2011) and xylanase (Murthy and Naidu, 2010). Three agricultural residues with high enzyme activity (Rice husk, coffee husk and sugarcane bagasse) were combined in the mixture design experiment. From the significant quadratic model and the response surface overlay plot, indicated that fermentation with 37 % (w/w) rice husk; 6 % (w/w) coffee husk and 57 % (w/w) sugarcane bagasse combination was the optimal combination ratio for both cellulase and xylanase production when the predicted specific activity of cellulase was 20.0 U/mg protein and the predicted specific activity of xylanase was 157 U/mg protein. The mixed

substrate mixture made more nutrients available for mycelia development and the presence of inducer substrates for enzyme production (Papinutti and Forchiassin, 2007). Among the cultures contained different sources of nitrogen in this experiment, cellulase showed high activity within nitrogen sources both organic (peptone and yeast extract) and inorganic (ammonium sulfate, ammonium tartrate and potassium nitrate) around 32-43 U/gds when compared with control (no nitrogen source were added) 13.08 U/ gds. Xylanase activity, 253.92 and 228.37 U/gds were evident when peptone and yeast extract were added, respectively. Optimal components concentration in mineral solution were determined by CCD experiment. From the significant quadratic model at 0.932 and 0.969 of R^2 of cellulase and xylanase experiment, the optimal concentration of mineral solution components were 0.89 % (w/v) of peptone, 0.46 % (w/v) of KH_2PO_4, 0.09 % (w/v) of $MgSO_4.7H_2O$ and 0.07 % (w/v) of $CaCl_2.2H_2O$ when predict activity of cellulase was 47.25 U/gds and xylanase was 333.06 U/gds. The modified mineral solution following CCD experiment presented the increase of cellulase and xylanase activities as 58.03 and 57.86%, respectively when compared with the mineral solution control. Moreover, the S/L ratio of solid state cultivation condition was 1:4, pH 6.0-8.0 and incubated at 45^{o}C for 10-12 days promoted the yield of cellulase (112.3 ± 11.5 U/gds), and xylanase (989.2 ± 61.9 U/gds). Cellulase activity was highly stable in pH range 6.0-8.0 and temperature at 30-50^{o}C while, xylanase activity showed high stability in pH range 4.0-8.0 and temperature at 30-65^{o}C for 1 hr.

Fungal hydrolysis by *T. aurantiacus* showed the highest reducing sugar (0.15 mg/ml) produced at 10th day of incubation. The pretreated substrates showed more reducing sugar production compared with control (unpretreated) substrates, 78.72 %

increasing rate. One of the reasons of increasing hydrolyzed product because pretreatment increased the available surface area (pore volume) for the enzymatic attack (Alvira *et al*., 2010). *Thermoascus aurantiacus* has been proposed as good microorganism for bioconversion of lignocellulosic biomass to sugars (Dashtban *et al*., 2009). The suitable levels of some important factors such as initial pH, the inoculums (yeast) concentration, and the incubation time of ethanol fermentation by *Sacchromycetes cerevisiae* were done by CCD experiment. The optimal values of the test variables were pH 5.04, the concentration of inoculums 1.97 % and incubation time 22.27 hr with the predict ethanol production (0.31 g/L) was observed when initial sugar about 1.0 mg/ml. Based on a theoretical yield of 0.51 g ethanol/g sugars, the ethanol yield from this experiment was calculated to be 0.24 g ethanol/g sugar or 47.06 % of the theoretical yield. The ethanol production from agricultural residues should have more research in the future to enhance the yield of ethanol.