CHAPTER 2

EXPERIMENTS

2.1 Chemical Reagents and Materials

2.1.1 Chemical Reagents

Names of chemical reagents	Production company
KH_2PO_4	J.T BAKER
$(NH_4)_2SO_4$	MERCK
$MgSO_4.7H_2O$	CARLO ERBA
$CaCl_2.2H_2O$	MERCK
Yeast extract	MERCK
$CuSO_4.5H_2O$	CARLO ERBA
$Fe_2(SO_4)_3 2H_2O$	J.T BAKER
$MnSO_4.H_2O$	J.T BAKER
Poly R-478	SIGMA
Lignin powder	NAKALAI
Glucose	FLUKA
Peptone	DIFCO
2,6- Dimethoxyphenol (DMP)	ALDRICH
Veratryl alcohol	SIGMA
Calibration protein	PHARMACIA
Coomassie Brilliant Blue	AMERSHAM
Acrylamide	PHARMACIA
Bromphenol Blue	AMERSHAM
Ammonium persulfate	PHARMACIA
TEMED (*N,N,N',N'*-tetramethylethylenediamine) -	
2-Mercaptoethanol	PHARMACIA

Names of chemical reagents	Production company
Glacial acetic acid	MERCK
Methanol	MERCK
DEAE-cellulose	PHARMACIA
Sephadex G-100	PHARMACIA
Bisacrylamide	PHARMACIA
Tris-base	AMERSHAM
Sodium dodecyl sulfate	AMERSHAM
Glycerol	-
Glycine	AMERSHAM

2.1.2 Media

1. Components of Pointing's medium (*II*) (Pointing, 1999)

Glucose	10.0	g
KH_2PO_4 $2H_2O$	1.0	g
$MgSO_4.7H_2O$	0.5	g
Urea	0.5	g
$CaCl_2.2H_2O$	0.01	g
Yeast extract	0.01	g
$CuSO_4.5H_2O$	0.01	g
$Fe_2(SO_4)_3$ $.2H_2O$	0.01	g
$MnSO_4.H_2O$	0.01	g
Polymeric dye (Poly R-478)	0.2	g

pH 5.5

2. Components of modified Pointing's medium (*/l*) (Pointing, 1999)

Glucose	10.0	g
KH_2PO_4 $2H_2O$	1.0	g
$MgSO_4.7H_2O$	0.5	g
$CaCl_2.2H_2O$	0.01	g
Yeast extract	0.01	g
$CuSO_4.5H_2O$	0.01	g
$Fe_2(SO_4)_3$. $2H_2O$	0.01	g
$MnSO_4.H_2O$	0.01	g
Polymeric dye (Poly R-478)	0.2	g

pH 5.5

3. Basal medium components (*/l*) (Kantelinen *et al.*, 1989)

Glucose	10	g
Ammonium tartrate	0.22	g
$KH_2PO_4 2H_2O$	1.0	g
$MgSO_4.7H_2O$	0.5	g
$CaCl_2.2H_2O$	0.1	g
$NaCl.2H_2O$	0.1	g
$ZnSO_4.H_2O$	5.5	mg
$FeSO_4.H_2O$	0.54	mg
$CuSO_4.5H_2O$	0.39	mg
$MnSO_4.H_2O$	0.063	mg
H_3BO_3	0.057	mg
Na_2MoO_4	0.05	mg
Biotin	5	*u*g

pH 5.5

2.1.3 Equipment

Names of equipment	Production company
Spectrophotometer Model V-530	JASCO
Colorimeter	HUNTER LAB
Ultra-filtration apparatus	ADVANTEC
Slab gel electrophoresis apparatus and power supplier	BIO-RAD
pH meter	LABINCO
Ion exchange column (4.5x60 cm)	-
Gel filtration column (3.0x120 cm)	-

2.2 Methods

2.2.1 Mycelial induction and isolation of basidiomycetes

Basidiomycete fruiting bodies collected from the decayed and rotted wood in Chiang Mai and Chiang Rai areas were induced to form mycelium by cultivation on Pointing's medium (1999) at 30°C. After incubation, mycelium form was transferred to PDA slant as a stock of pure culture for further study. *Phanerochaete chrysosporium* ATCC 34541 and *Coriolus versicolor* IFO 30388 were used as reference strains.

2.2.2 Screening of basidiomycetes on PDA plate at 37°C

All of the isolates were cultivated on PDA plate and incubated at 37°C for 7 d before the diameter of mycelial growth was measured. The isolates which were able to grow at this condition and showed the colony diameter more than 7 mm (Iimori *et al.*, 1994) were selected for further experiments.

2.2.3 Effects of glucose on growth and decoloration of Poly R-478 and using lignin powder as an indicator

The selected strains of basidiomycetes were cultivated on four types of solid medium. The basal medium was modified Pointing's medium supplemented (w/v) with A 0.02% Poly R-478, B 0.02% Poly R-478 and 1.0% glucose, C 0.025% lignin powder and 0.05% urea, and D 0.025% lignin powder, 0.05% urea, and 1.0% glucose. The cultivated condition was 37°C for 7 d. During incubation, mycelial growth and decoloration of Poly R-478 or lignin powder were observed.

2.2.4. Study of lignin degrading enzyme production in liquid medium

The mycelial seed of basidiomycetes were prepared on PDA plate for four d and the radial edged mycelium was punched by cork borer with 0.5 cm in diameter. One piece was inoculated in liquid medium of Pointing, using 0.025% lignin powder (Kantelinen, 1989) instead of Poly R-478 dye and cultivated on 150-rpm rotary shaker at 37°C for 9 d. Culture broth was taken every 3 d and determined for enzyme activity of laccase, manganese peroxidase, manganese independent peroxidase, and lignin peroxidase.

2.2.5 Enzyme assay

Laccase, manganese peroxidase (MnP) and manganese independent peroxidase (MIP) were determined by oxidation of 2,6- dimethoxyphenol (DMP) (Mester *et al.*,1995). The reaction mixture for the laccase and MnP assay contained 1.0 M sodium tartrate buffer (pH 5.0), 4.0 mM DMP, 1.0 mM $MnSO_4$, and 0.1 ml supernatant in a final volume of 1.0 ml. The MnP reaction were initiated by adding 1.0 mM H_2O_2 and collected for laccase and MIP activity. MIP reaction mixture was the same as MnP excepted 1.0 mM $MnSO_4$. The MIP reaction were initiated by adding 1.0 mM H_2O_2 and collected for laccase activity. The

Lignin peroxidase (LiP) activity was determined by oxidation of veratryl alcohol to veratral aldehyde as described by Leontivesky (1994). All reactions were done at 19±1°C.

2.2.6 Selection of laccase producer on solid culture

Solid state fermentation was performed in Erlenmeyer flask (250 ml) containing 5 g of rubber wood chips and 15 ml of solution mixture of 4% (w/v) glucose and 4% (w/v) peptone and distilled water was used as control. The solid culture was autoclaved at 121°C for 20 min prior to inoculate with 5 dishes cork borer of seed from 42 isolates. The cultures were left in a static condition at 37°C for 9 d. The enzyme was extracted from solid mass with 50 ml of deionized water and the extract was filtrated through the cheesecloth and assay for laccase activity.

2.2.7. Studies of some factors affected on laccase production

a) Effects of veratryl alcohol and Tween-80 on laccase production

The mycelial seed of isolate NP21 was prepared on PDA plate for four days and two pieces of seed were inoculated into Erlemeyer flask (250 ml) containing 50 ml of Kantelinen's mineral basal medium (Kantelinen,1989) supplemented with 0.02%(w/v) Poly R-478. The cultures were grown at 37°C for 12 d with an agitation of 150-rpm rotary shaker. The addition of veratryl alcohol and Tween-80 in each treatment was shown in Table 2.1. The 3.0 ml culture broth was taken daily and determined for laccase activity. The replacement of Poly R-478 by 0.02% (w/v) lignin powder was also studied at the same condition.

The solid state fermentation was performed in 250 ml flask containing 5 g of rubber wood chips as a carbon source and the inducer supplemented with 15 ml of the same basal medium as in liquid culture which was shown in Table 2.2.

Table 2.1 The components containing in each treatment in liquid medium

Treatments	Supplements (w/v)			
	Mineral salts	0.02% Poly R-478	0.025% Veratryl alcohol	0.05% Tween-80
1 (control)	√			
2	√	√		
3	√		√	
4	√			√
5	√	√	√	
6	√	√		√
7	√		√	√
8	√	√	√	√

Table 2.2 The components containing in each treatment on solid culture

Treatments	Supplements (w/v)			
	Distilled water	Mineral salts	0.025% Veratryl alcohol	0.05% Tween-80
1 (control)	√			
2	√		√	
3	√			√
4	√		√	√
5		√		
6		√	√	
7		√		√
8		√	√	√

Those treatments were compared with addition of solution mixture of 4% (w/v) glucose and 4% (w/v) peptone which was used to screen for the best laccase producer in the previous study. The culture was grown in a static condition at 37°C for 12 d. Samples were taken every 3 d and extracted with 50 ml of deionized water. The extract obtained, was filtrated through cheesecloth to remove solid particle and was determined for laccase activity.

b) Effects of glucose and peptone concentration on laccase production

This experiment was studied on solid culture using rubber wood chips containing various concentration of glucose and peptone solution from 0% to 4%(w/v). The total volume of supplements is 15 ml which containing 7.5 ml solution of glucose as a carbon source supplement and 7.5 ml peptone solution as a nitrogen source. The variation of glucose and peptone levels in each treatment was described in Table 2.3. The culture was incubated in static condition at 37°C for 5 d and the extracted enzyme solution was monitored for laccase activity

Table 2.3 Amount of glucose and peptone (w/v) for applying into rubber wood chips

Peptone / Glucose	0%	1%	2%	3%	4%
0%	Trt 1	Trt 2	Trt 3	Trt 4	Trt 5
1%	Trt 6	Trt 7	Trt 8	Trt 9	Trt 10
2%	Trt 11	Trt 12	Trt 13	Trt 14	Trt 15
3%	Trt 16	Trt 17	Trt 18	Trt 19	Trt 20
4%	Trt 21	Trt 22	Trt 23	Trt 24	Trt 25

c) Optimal temperature for laccase production

The culture were grown at 30, 37, and 45°C. The isolate NP21 was cultivated on solid culture under the condition from section 2.2.6. The fermentation was conducted for 9 d. Laccase activity was determined from extracted samples.

d) Optimal initial pH for laccase production

The solid state fermentation condition was used to study on rubber wood chips culture. The pH of culture was adjusted in the range 3.5 to 8.0 by diluted acetic acid. The extracts were determined for laccase activity at the fifth d.

e) Effects of veratryl alcohol and Tween-80 on laccase production at optimal conditions

Solid substrate fermentation condition were used under the condition obtained from section 6.3 and supplemented with 0.025% (w/v) veratryl alcohol, (w/v) 0.05% Tween-80, 0.025% (w/v) veratryl alcohol and 0.05% (w/v) Tween-80 and non-supplemented. Laccase activity was examined over a period of 9 d fermentation. Two culture flasks in each treatment were randomly sampled.

f) Influence of copper induction on laccase production

Solid state fermentation and optimal condition were used for study. The solution of $CuSo_4$ was added to the culture to the final concentration 75 μM, 150 μM, or 300 μM (Palmieri *et al.*, 2000). The extracts were determined for laccase activity at the fifth day.

2.2.8 Purification of the isolate NP21 laccase

The total 2.7 *l* of enzyme solution obtained from the solid culture was simultaneously applied to CM-cellulofine and DEAE-cellulose column (4.5x60 cm), which had previously been equilibrated with 20 mM phosphate buffer (pH 7.5). The bound proteins were eluted by increasing the concentration or linear gradient of NaCl 0-1.0 M in 1.5 *l* of 20 mM phosphate buffer pH 7.0. Laccase containing fractions were pooled and concentrated by ultra-filtration (ADVANTEC) with 10,000 kDa molecular weight cut off membrane and applied to Sephadex G-100 gel filtration chromatography (3x120 cm). The enzyme was eluted by using 20 mM phosphate buffer pH 7.5. The protein was determined by a Spectrophotometer at 280 nm and laccase was examined as described previously.

2.2.9 SDS-PAGE analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using acrylamide gel concentration at 12% for separating gels. The molecular weight standard protein were obtained from PHARMACIA. The protein bands were visualized by Coomassie Brilliant Blue staining. The calibration protein components used for molecular mass determination was shown in appendix 5.

2.2.10 Characterization of laccase from the isolate NP21

a) pH profile

The reaction was performed at room temperature ($19\pm1^{\circ}C$) to determine the optimal pH of enzyme. The activity was measured with 5 mM DMP in 0.1M glycine-HCl buffer in a range of pH 2.5-3.5, 0.1M citrate buffer in a range of pH 3.5-6.0, 0.1M phosphate buffer in a range of 6.0-8.0, and 0.1M Tris- HCl buffer in a range of 8.0-9.0.

b) pH stability

The pH stability was determined in the range 2.5-9.0 using 0.1M of the same buffer as used in pH profile experiment. The enzyme solution was diluted to 10 folds in each buffer and incubated at 4°C for 24 hrs. The enzyme solution was dialyzed against 20mM acetate buffer pH 5.0 and determined for the residual activity of laccase.

c) Thermostability

Thermal stability was determined by incubation the enzyme solution in 0.1 M acetate buffer pH 5.0 in the range of temperature at 25-80°C for 1 hr and the residual activity was determined by using DMP as a substrate. To select the most qualified temperature for variable incubation periods, the samples were taken periodically until the enzyme activity could not be observed.

d) Substrate specificity

The chemicals used as substrates for substrate specificity test for laccase are as follow;

1. DMP (2,6- dimethoxyphenol): The reaction mixture contained 5 mM DMP and the oxidation of DMP was observed by an absorbance increasing at 470 nm (ε_{470} = 49600 $M^{-1}cm^{-1}$) (Eggert *et al.*, 1996).
2. Syringaldazine: The enzyme activity was measured by using 5 mM syringaldazine as substrate (dissolved in 40% ethanol of 0.1 M acetate buffer). Oxidation of syringaldazine was conducted by an absorbance increasing at 525 nm (ε_{525} = 65000 $M^{-1}cm^{-1}$) (Hublic and Schinner, 2001)
3. ABTS [2,2'-azino-di(3-ethylbenzothialozin-6-sulfuric acid)]: The reaction mixture contained 5 mM ABTS and the oxidation of ABTS was performed by

an absorbance increasing at 420 nm (ε_{420} = 36000, $M^{-1}cm^{-1}$) (Hublic and Schinner, 2001).

4. Guaiacol: The assay mixture contained 5 mM guaiacol and its oxidation was determined by an absorbance increasing at 436 nm (ε_{436} = 6400 $M^{-1}cm^{-1}$) (Eggert *et al.*, 1996).

All of these reactions were determined at 18-20°C. Simultaneously, the study of pH profile in range of buffer pH 2.5-9.0 by using the same buffer as described in the pH profile experiment, was also investigated.

e) Effects of metal ions and inhibitors on laccase activity

The effects of metal ions and inhibitors were performed by incubation of the enzyme in the presence of 1 mM (final concentration) of various metal ions or inhibitors for 10 min at 20°C prior to substrate addition (Diamantidis *et al.*, 2000). The activity was measured with 4 mM DMP in 0.1 M acetate buffer pH 5.0. The tested metal ions were $CuSO_4$, LiCl, $CaCl_2$, $ZnSO_4$, $MgSO_4$, NaCl, KCl, $MnCl_2$, $AlCl_3$, $Pb(CH_3COO)_2$, $AgSO_4$, $CdCl_2$, $CoCl_2$, $FeSO_4$, and $HgCl_2$. The tested enzyme inbibitors were, indoleacetic acid (IAA), ethylenediaminetetraacetic acid (EDTA), *o*-Phenanthroline, phenylmethylsulfonyl fluoride (PMSF), 2-Mercaptoethanol, *p*-Chloromercuribenzoate (PCMB), blue copper oxidase, NaN_3 (Eggert *et al.*, 1996), Poly R-478 and Lignin powder.

2.2.11 Preliminary study of pulp biobleaching by laccase

a) Pretreatment of pulp

The pulps used in this study were eucalyptus oxygen-delignified pulp from Phoenix Pulp and Paper Company from Khonkaen. The pulp was extracted with EDTA prior to enzyme treatment for removing of ion presented in the pulps (Moreira *et al.*, 2001). The 0.25 g of pulp was suspended in 25 ml of 3.4 mM EDTA solution, pH 4.0, for 15 hrs with

an agitation of 70-rpm at 25°C. Subsequently, the treated pulp was washed four times with distilled water and air dried for 45 min at 25°C.

b) Pulp treatment by the purified laccase

EDTA-treated pulp 0.2 g was suspended in 20 ml of 0.05 M citrate buffer pH 4.0 containing mediator, HBT, 10 mg/g of pulp and laccase 5 unit/g of pulp, and immediately mixed with the pulp for 1 min. The pulp was shaken at 37°C for 12 hrs. The sample was taken every 2 hrs. The pulp was filtered on the filter paper (Whatman No.2) and washed four times with 40 ml deionized water. The liquid obtained before washing was determined for residual laccase activity. The brightness (%) of pulp was measured by the colorimeter.