APPENDIX

Appendix 1. Stock solutions preparation for SDS-PAGE analysis

Acrylamide concentrate (30%T, 2.7% C): Dissolve 29.2 g of acrylamide and 0.8 g of bisacrylamide in 70 ml of deionized water. When the acrylamide is completely dissolved, add water to a final volume of 100 ml. Filter the solution under vacuum though a 0.45 μm membrane. Store stock acrylamide at 4°C in dark bottle for no more 1 month.

1.5 M Tris-Cl, pH 8.8, concentrated resolving gel buffer: Dissolve 19.2 g Tris base in ≈ 80 ml of water, adjust to pH 8.8 with HCl and add water to a final volume of 100 ml. Store at 4°C.

0.5 M Tris-Cl, pH 6.8, concentrated stacking gel buffer: Dissolve 6.1 g Tris-base in ≈ 80 ml of water, adjust to pH 6.8 with HCl and add water to a final volume of 100 ml.

10% Sodium dodecyl sulfate (SDS) : Dissolve 10 g SDS in ≈ 60 ml of water and add water to a final volume of 100 ml.

Catalysts

10% ammonium persulfate (APS): Dissolve 100 mg APS in 1 ml of water. Make solution fresh daily.

TEMED (*N,N,N',N'*-tetramethylethylenediamine): Use TEMED undiluted from the bottle. Store cool, dry, and protect from light.

Electrolyte buffer

Electrolyte buffer: 0.025 M Tris, 0.192 M glycine, 0.1%(w/v) SDS pH 8.3 (0.3 g Tris base, 1.4 g glycine, 1 ml 10% SDS/100 electrolyte buffer). Do not adjust the pH of electrolyte

buffer; just mix the reagents together and confirm that the pH is near 8.3 (±0.2). Electrode buffer can be made as 5X concentrate consisting of 15 g Tris base, 72 g glycine, and 5 g SDS/*l*. 5X electrde buffer concentrate, dilute it with four parts of water.

Staining buffer

(0.06 M Tris-Cl, pH 6.8, 2% SDS, 10% glycerol, 0.025% bromphenol blue):

Water	4.8 ml
0.5 M Tris-Cl, pH 6.8	1.2 ml
10% SDS	2.0 ml
Glycerol	1.0 ml
0.5% Bromphenol Blue	0.5 ml

Store at room temperature. SDS-reducing buffer is prepared by adding 50 µl of 2-mercaptoethanol to each 0.95 ml of stock sample buffer before use.

Appendix 2. Formulations of SDS-PAGE resolving gel

Component	7.5%T	12%T
Water	4.85 ml	3.35 ml
1.5 M Tris-Cl, pH 8.8	2.5 ml	2.5 ml
10% SDS	0.1 ml	0.1 ml
Acrylamide(30%T, 2.7%C)	2.5 ml	4.0 ml
10% ammonium persulfate	50 µl	50 µl
TEMED	5 µl	4 µl

Appendix 3. Formulations of SDS-PAGE stacking gel

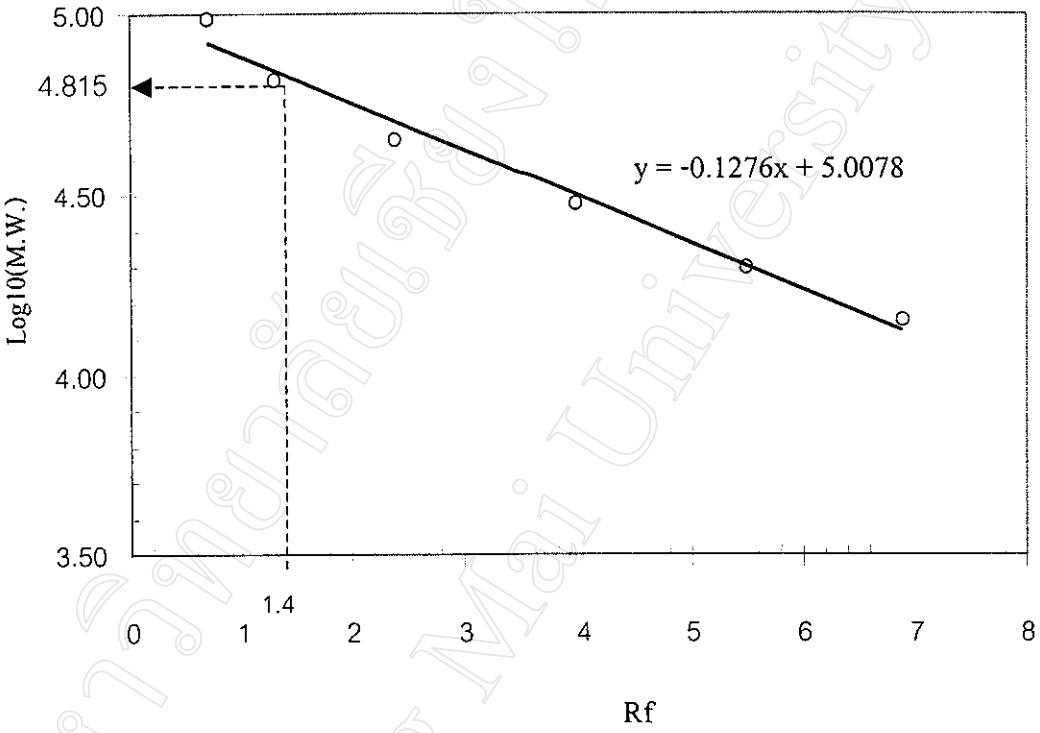
Component	4%T
Water	6.1 ml
1.5 M Tris-Cl, pH 8.8	2.5 ml
10% SDS	0.1 ml
Acrylamide/Bisacrylamide (30%T,2.7%C)	0.96/0.34 ml
10% ammonium persulfate	50 µl
TEMED	4 µl

Appendix 4. Calibration protein mixture

Protein	Molecular weight (Mr)	Source
Phosphorylasr b	97,000	Rabbit muscle
Albumin	66,000	Bovine serum
Ovalbumin	45,000	Chicken egg white
Carbonic anhydrase	30,000	Bovine erythrocyte
Trypsin inhibitor	20,100	Soybean
α-Lactalbumin	14,400	Bovine milk

The total amount of each protein has been chosen to give bands of equal intensity when stained with Coomassie Brilliant Blue following Laemmli-type gel electrophoresis. The intensities may vary when using other staining methods.

Appendix 5. Estimation of molecular weight of laccase from *Lenzites* sp. NP21 based on standard proteins calibration curve



Calibration curve constructed using the results show in Fig 3.20

Calculation of laccase from calibration curve of standard proteins.

$$\text{Log}_{10}(\text{M.W.}) = 4.815$$

$$\text{M.W} = 65313$$

Thus, molecular weight of purified laccase from NP21 is around 65 Kda.

Appendix 6. Beer's law and enzymatic activity calculation

Beer's law

Beer's law is the relationships between the absorbance of the solution which directly increase with the concentration of the absorbing substrate and the path length passed through by the beam. Therefore, Beer's law relates these quantities as follow (Douglas, 1965):

$$\varepsilon bc = A$$

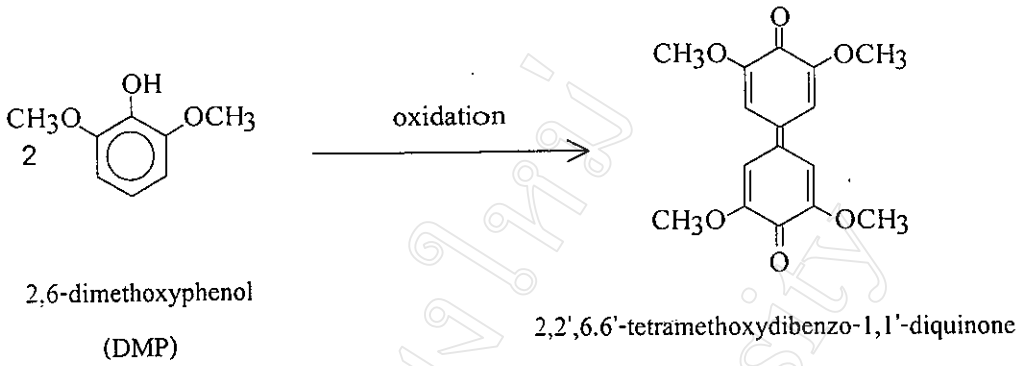
In this equation composts of many symbols and their definitions are as follow :

- ε is a constant number called molar adsorptivity or molar extinction coefficient
- b is path length of radiation in cm
- A is absorbance
- c is concentration of an adsorbing substance in mole/l

Enzymatic activity calculation

The activity of enzyme is the oxidation of substrate to oxidized the product that could be observed under the different wave length which depends on substrate. According to the definition of enzyme activity that is " one unit was defined as the amount of enzyme producing a one unit change in absorbance per min" (Harkin and Obst, 1973; Leonowicz and Grzywnowicz, 1981).

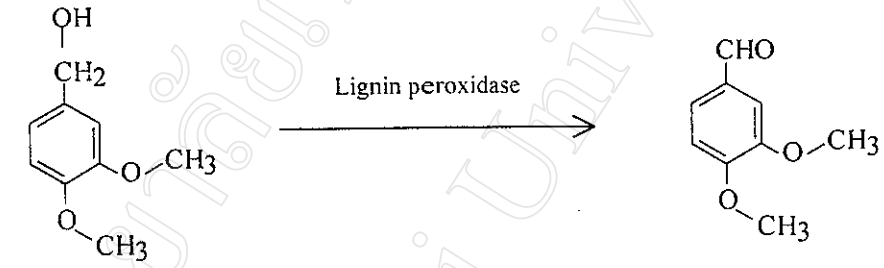
Two chemicals, DMP and veratryl alcohol were used as standard substrates for the assay of manganese peroxidase, manganease independent peroxidase, laccase and lignin peroxidase, respectively. The oxidizing mechanism of DMP by manganese peroxidase, manganease independent peroxidase and laccase, and the oxidation of veratryl alcohol to be veratraldehyde by lignin peroxidase as Fig. a) and b), respectively.



2,6-dimethoxyphenol (DMP)

2,2',6.6'-tetramethoxydibenzo-1,1'-diquinone

Fig. a) Oxidation of DMP by oxidative enzymes



Veratryl alcohol

Veratryl aldehyde

Fig. b) Oxidation of veratryl alcohol by lignin peroxidase

According to Beer's law and enzyme activity in IU leads to modify the equation for enzyme activity calculation as follow:

$$C = (A_1 - A_0) / d.\varepsilon b.t$$

In the above equation:

c is enzyme activity in μmol /ml/min or unit/ml.

A is the difference of absorbance from t_0 to t_1

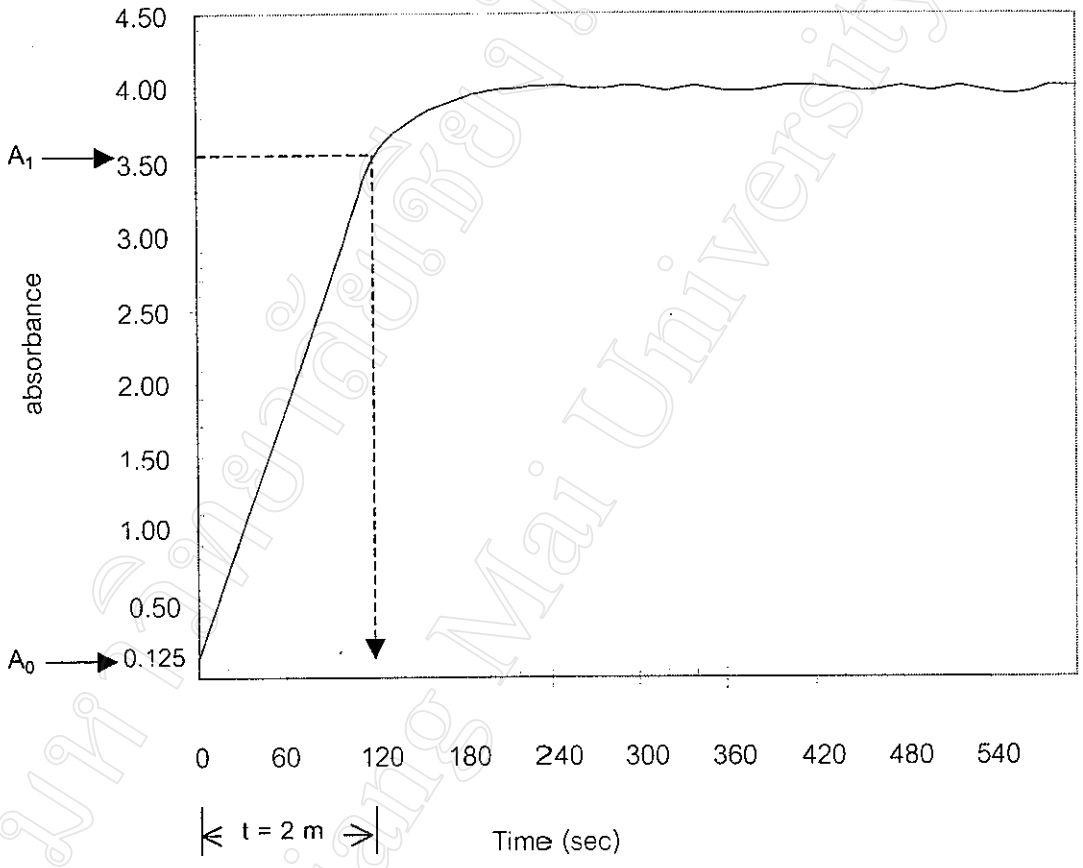
ε is molar extinction coefficient in $mM^{-1}cm^{-1}$

b is path length of radiation in cm

t is the suitable period of reaction in minute

d is dilution fold

Example for laccase activity calculation by oxidizing DMP based on Beer's law



DMP oxidation by laccase, MnP, and MiP

When the suitable volume of enzyme solution was used 50 µl in 2000 µl total volume of reaction mixture and ε_{470} of DMP oxidation is 49.6 $mM^{-1}cm^{-1}$

According to calculation equation

$$C = (A_t - A_0) / d.\varepsilon b.t$$
$$= (3.50 - 0.125)/ [(50/2000) \times 49.6\ mM^{-1}cm^{-1} \times 1cm \times 2min]$$
$$= 1.36\ mM/min$$
$$= 1.36 \times 10^{-3} \times 10^{6}\ [\mu mole/(10^{3}\ ml)/min]$$
$$= 1.36 \times 10^{-3} \times 10^{6} \times 10^{-3}\ [\mu mole/ml/min]$$
$$= 1.36\ \mu mole/min/m$$
$$= 1.36\ unit/ml$$

Therefore, the activity of laccase is equal 1.36 unit/ml.

Enzymatic activity calculation in dimension of unit/g substrate

The enzyme was produced on 5 g of rubber wood chips and extracted by 50 ml distilled water. The total volume of enzyme solution obtained 50 ml. Assumption that the enzyme activity was calculated to be 1.36 unit/ml.

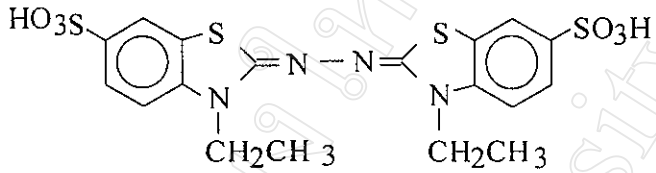
total activity	=	1.36	unit/ml X 50 ml
	=	68	unit (from 5 g of rubber wood chips)

Rubber wood chips 5 g obtained the total of enzyme activity 68 unit.

Thus, rubber wood chips 1 g obtained the enzyme (68/5) 13.6 unit.

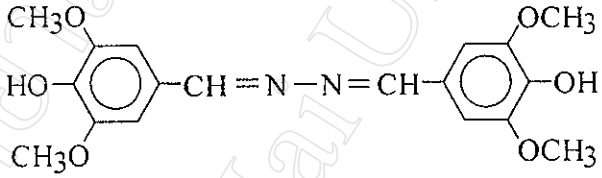
It could be concluded that the enzyme activity in this example was 13.6 unit/ g substrate.

Appendix 7. Structures of aromatic compounds



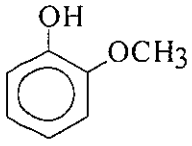
2,2'-azino-bis(3-ethylbenzithiazoline-6-sulfonic acid)

(ABTS)



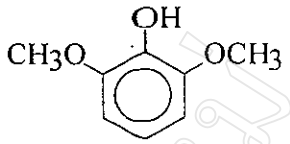
4-hydroxy-3,5-dimethoxybenzaldehyde azine

(Syringaldazine)



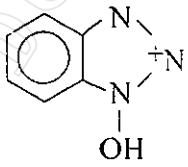
2-methoxyphenol

(Guaiacol)



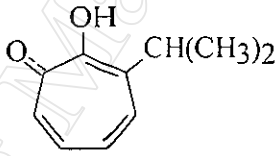
2,6-dimethoxyphenol

(DMP)

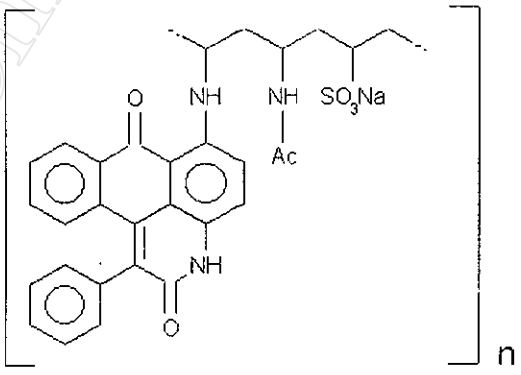


Hydroxybenzotriazole

(HBT)

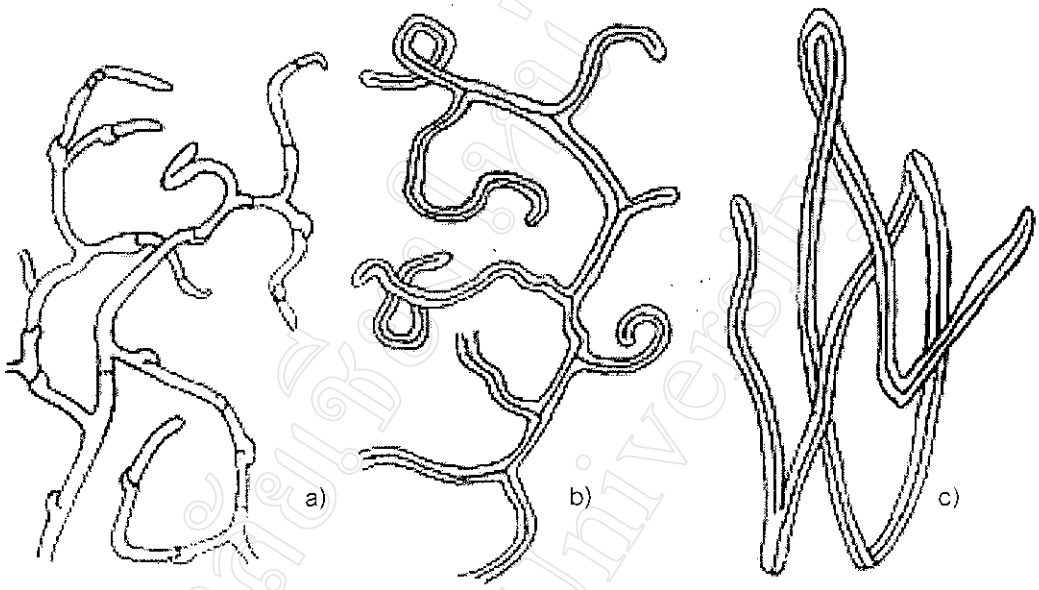


Tropolone



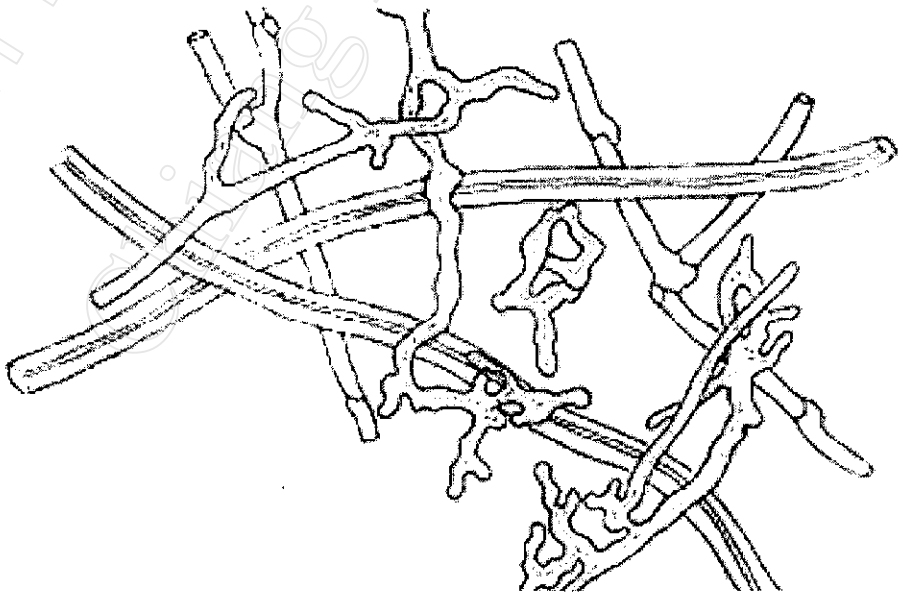
Poly R-478

Appendix 8. Structure of hypha



Hyphal structrure a); generative hyphae, b); binding hyphae, and c); skeletal hyphae

Based on data from Sa-ad-sud, 1998



Hyphal tructure of *C. hirsutus* which is member of hard basidiocarp

Based on data from Sa-as-sud, 1998

CIRRICULUM VITAE

Name Miss Nittaya Wanphrut

Date of Birth March 12, 1976

Place of birth Phetchaboon

Academic background Finished high school from Lomsak Witthayakom School, Lomsak, Phetchaboon in 1996.
B.S. (Biotechnology in Agro-Industry) from Chiang Mai University in 1999

Scholarships : Graduate School Foundation of Chiang Mai University for student in 2000
Graduate School Foundation of Chiang Mai University for research work in 2000
Government Scholarships in 2000

Publication :

Wanphrut, N., Lumyong, S., Watanabe, T. and Khanongnuch, C. (2000) Isolation and screening of basidiomycetes fungi for using in enzymatic pulp and paper bleaching. *Proceeding of the 1st National Conference on Graduate Research, 10th-11th June 2000, Chiang Mai, Thailand.*

Khanongnuch, C., Wanphrut, N., Lumyong, S., Honda, Y., Kuwahara, M., and Watanabe, T. (2000) Thermotolerant wood rotting basidiomycetes, *Lenzites sp.*, newly isolated from tropical rain forest in Thailand. *Proceedings of the third international wood science symposium, November 1st-2nd, 2000, Uji, Kyoto, Japan*

Khanongnuch, C., Wanphrut, N., Lumyong, S., Honda, Y., Kuwahara, M., and Watanabe, T. (2001) A new thermotolerant wood rotting fungi, *Coriolus versicolor,* isolated from northern Thailand and its potential in lignin degrading applications. *Presymposium on recent advances in lignin biodegradation and biosynthesis, June 3rd-4th, 2001, Viikki Biocenter, University of Helsinki, Finland*

Khanongnuch, C., Wanphrut, N., Lumyong, S., Honda, Y., Kuwahara, M., and Watanabe, T. (2001) Production and purification of laccase from white rot fungus, *Lenzites sp* NP21. *Proceedings of the 46th international lignin symposium, November 1st-2nd, 2001, Kyoto University, Kyoto, Japan*