CHAPTER 3

RESULTS AND DISCUSSION

3.1 Isolation of basidiomycetes

One hundred and sixty-four samples of the fruiting bodies of basidiomycetes were collected from decayed and rotted wood in diverse environment in Chiang Mai and Chiang Rai Provinces. Each sample was induced to form mycelium on modified Pointing's medium. The collected place and their members are as following;

Agro–industry (CMU); (AI) Isolates AI1 and AI6.

Chiang Mai university; (CMU) Isolates CUM2, CUM4 and CUM7 from 7 samples.

Doi Suthep ; (ST) Isolates ST3, ST4, ST7, ST9, ST10, ST16, ST21, ST25, ST40 and ST41 from 33 samples.

Huay Hong Krai; (H) Isiates 32H, 34H and 36H.

Mae Ka Charn; (MK) Isolates 8MK, 20MK, 23MK, 25MK, 26MK, 30MK, 32MK, 33MK and 35MK.

Mae Sa ; (M) Isolates 01M, 7M, 11M, 13M and 15M.

Nongplaman; (NP) Isolates NP1, NP3, NP4, NP5, NP7, NP8, NP9, NP10, NP11, NP12, NP13, NP14, NP15, NP18, NP19, NP20, NP21, NP22, NP23, NP24, NP25, NP26, NP27, NP28, NP29, NP30 and NP32 from 32 samples.

Nong Pla Man ; (NPM) Isolates 5NPM, 7NPM and 8NPM.

Rukachart park; (RC) Isolates RC2, RC3, RC4, RC5, RC8, RC9, RC10, RC11, RC12 and RC13 from 14 samples.

Tupoo; (TP) Isolates TP3, TP4, TP6, TP7, TP8, TP11, TP12, TP16, TP18 and TP21 from 21 samples.

Others; Isolates BK1, BK2, NO.U1, NO.9, NO.12, NO.15, DS3, DS7, MS1 and UNK1.

Finally, 113 isolates were obtained and could be isolated as pure culture. The remaining 51 samples were assumed to be the mixed culture of fungi. All of samples were classified into two groups of hard and soft fruiting bodies. Most succeeded mycelium forming isolates belonged to hard fruiting body group. This might be due to the strong structure of fruiting body, which was harder to decay compared to soft fruiting body group. On the other hand, soft fruiting bodies were easily rotted. In the case of hard basidiocarps, they were classified to be trimitic mycelium, which contained three types of hypha as generative, binding and skeletal hyphae, which could form harder and stronger mycelium compared to monomitic and dimitic mycelium found in soft fruiting body group (Sa-ad-sud, 1998). The structures of three types of hypha were shown in Appendix 8.

The decoloration Poly R-478 medium with the original Pointing's medium could not be observed, even though with the reference strains, *P. chrysosporium* ATCC 34541 and *C. versicolor* IFO 30388. After an omission of urea, the standard strains and 21 of new isolates showed the clear halo of Poly R-478 decoloration as shown in Fig. 3.1. This phenomenon is corresponded with Kirk and Fern's report which described that high nitrogen concentration suppressed ligninolytic enzymes production (Kirk and Fenn, 1987).

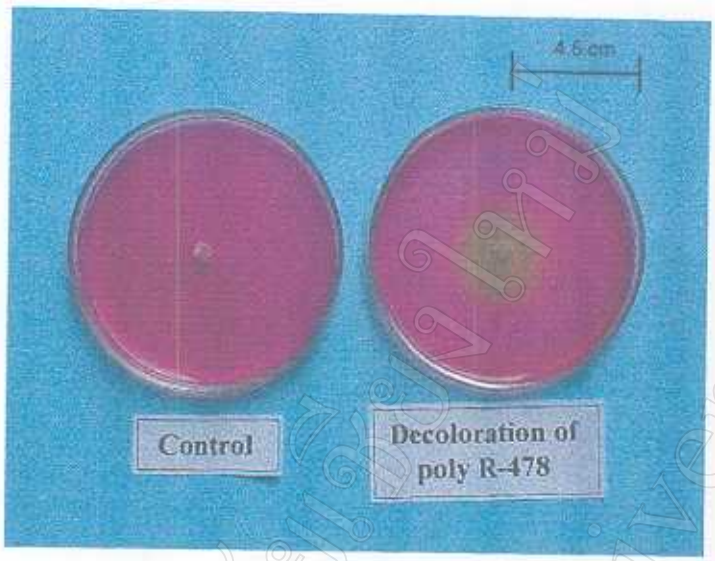

Fig. 3.1 The typical pattern of Poly R-478 decoloration (right) compared to control (left).

Nitrogen limiting medium could induce ligninolytic enzymes production rather than abundant of nitrogen because under natural lignin degrading condition, which was limited nitrogen but sufficient carbon sources. In the presence of urea as a nitrogen source in medium, the strains could uptake nitrogen easily in the culture. Without nitrogen source, the strains had to find nitrogen from other sources for their living and growth. Considering to the structure of Poly R-478 (Appendix 7), it consisted of C, H, O, N, S, and Na, thus, the strains had to produce the enzyme to degrade this dye for a nitrogen source. Accordingly, when the screening culture containing Poly R-478 was limited nitrogen source, the halo formation could be observed.

3.2 Screening of basidiomycete fungi on PDA at 37°C

In primary screening of 113 isolates on PDA plate at 37°C, we obtained 42 isolates with the diameter of mycelial growth more than 7 mm were obtained; ST9, ST40, ST41, TP6, TP7, TP11, TP16, NP1, NP3, NP7, NP8, NP9, NP10, NP11, NP14, NP15, NP18, NP21, NP22, NP23, NP25, NP26, NP27, NP28, NP31, RC2, RC3, RC5, CMU2, CMU7, 34H, 26MK, 30MK, 01M, 7M, 13M, 15M, 19M, 8NPM, BK1, BK2 and UNK1. The distribution of growth capability was shown in Fig.3.2.

From their abilities to grow at 37°C, they were classified to be thermotolerant basidiomycete fungi because the majority of basidiomycete fungi have optimal temperature for growth between 20 and 30°C (Hudson, 1972). Moreover, the obtained isolate RC3 was able to grow at 42-45°C.

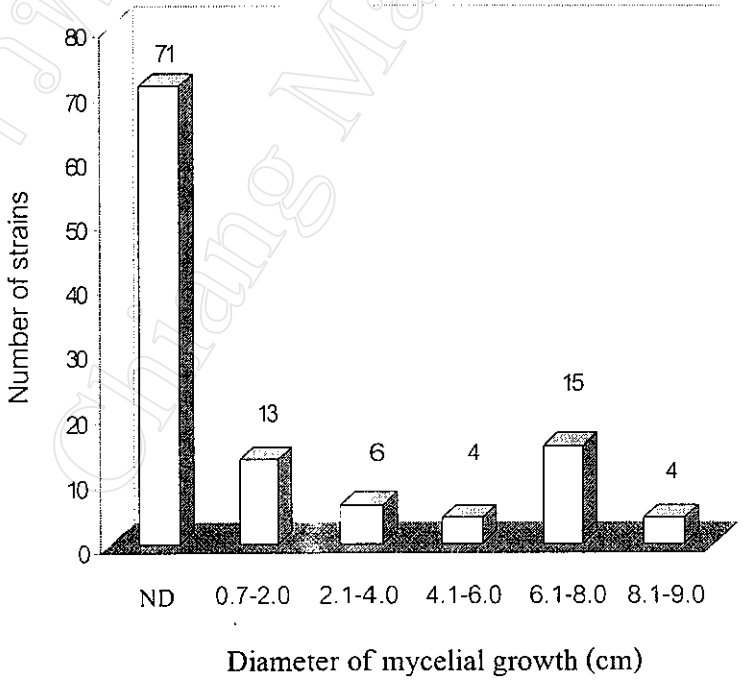

Fig. 3.2 Distribution of 42 isolates separated by growth capability at 37°C on PDA plate.

Note : NG = No growth detected

3.3 Influences of glucose on growth and decoloration of Poly R-478 and utilization of lignin powder as indicator for screening of ligninolytic fungi

All of 42 isolates were cultivated on 4 types of modified Pointing's medium supplemented with glucose and lignin. 21 isolates showed capability to form halo on Poly R-478 medium (Table 3.1). There were 9 isolates which were able to degrade Poly R-478 as well as *P. chrysosporium* ATCC 34541 as follow: ST40, TP7, TP16, NP14, NP18, NP21, NP26, NP27 and RC3 (Fig. 3.3).

Addition of 1% (w/v) glucose derived little effect on mycelial growth when Poly R-478 was present in the supplement but it showed growth enhancement when Poly R-478 was replaced by 0.025% (w/v) lignin powder.

However, the decoloration of the brown color of lignin powder could not be observed (Fig. 3.4). There was no report about the utilization of lignin powder as an indicator for screening ligninolytic strains although it was more economic than Poly R-478.

In case of the decoloration of Poly R-478 in A and B supplements, the presence of 1.0%(w/v) glucose increased the Poly R-478 degradation especially the standard strain *P. chrysosporium* ATCC 34541 and the new isolate NP11 (Fig. 3.5). The isolate NP11, non-halo forming basidiomyctes on the glucose-absented medium, could be stimulated by addition of glucose on Poly R-478 degradation. However, addition of 1.0%(w/v) glucose did not affect on the halo formation of the isolate NP10.

It could be suggested that the natural lignin degradation in wood could be degraded by white rot fungi, which most of their components were sufficient polysaccharide including cellulose and hemicellulose. In laboratory study, it was necessary to add glucose as a carbon source for ligninolytic fungi. In conclusion, the addition of 1.0% (w/v) of glucose into Poly R-478 containing medium was more suitable than lignin powder for screening of lignin degrading fungi.

Table 3.1 Effects of glucose on mycelial growth and decoloration of Poly R-478 and lignin powder at 37°C after 4 d cultivation

Isolate	Growth				Clear zone			
	R	R+G	L	L+G	R	R+G	L	L+G
PC*	++++	+++++	+++	++++	+++	+++++	-	-
CV**	+	+	+	+	+	+	-	-
BK1	+	+	++	++	-	-	-	-
BK2	+	+	+	+	-	-	-	-
CMU2	++	++++	+++	+++++	-	-	-	-
CMU7	++	++	++	++	++	++	-	-
34H	+++	+++	+++	++++	+++	+++	-	-
01M	++++	++++	+++	++++	++	+++	-	-
7M	++++	++++	+++	++++	+++	+++	-	-
13M	++++	+++++	++++	+++++	-	-	-	-
15M	++++	+++++	++++	+++++	-	-	-	-
19M	++++	++++	+++	++++	+++	+++	-	-
26MK	+++	++++	+++	++++	-	-	-	-
30MK	+	+	+	+	-	-	-	-
NP1	+	+	+	+	+	+	-	-
NP3	+	+	+	++	-	-	-	-
NP7	+	+	++	++	-	-	-	-
NP8	++	++	++	++	+	+	-	-
NP9	++	++	++	++	++	++	-	-
NP10	++++	++++	+++	+++	-	-	-	-
NP11	+++	+++	+++	+++	-	+++	-	-
NP14	++++	++++	+++	++++	+++	++++	-	-
NP15	++	++	++	++	-	-	-	-
NP18	+++	++++	+++	++++	+++	++++	-	-
NP21	+++	+++	++	+++	+++	++++	-	-
NP22	+	+	+	++	-	-	-	-
NP23	+	+	+	++	-	-	-	-
NP25	++++	++++	+++	+++	-	-	-	-

Table 3.1 (continoued).

Isolate	Growth				Clear zone			
	R	R+G	L	L+G	R	R+G	L	L
NP26	++++	++++	+++	++++	+++	++++	-	-
NP27	++++	++++	+++	+++	++++	++++	-	-
NP28	+	+	++	++	-	-	-	-
NP31	++	++	++	++	-	-	-	-
8NMF	++++	+++++	+++	+++++	-	-	-	-
RC2	+	+	+	+	+	+	-	-
RC3	++++	++++	++++	++++	+++	++++	-	-
RC5	+++	+++	+++	+++++	-	-	-	-
ST9	+	+	+	+	+	+	-	-
ST40	++++	++++	+++	++++	++++	++++	-	-
ST41	++++	+++++	++++	+++++	-	-	-	-
TP6	+	+	+	+	+	+	-	-
TP7	+++	++++	++	++	+++	++++	-	-
TP11	++	++	++	++	++	++	-	-
TP16	++++	++++	+++	+++	+++	++++	-	-
UNK1	+	+	+	+	-	-	-	-

Note : symbol

- : No detected
+ : diameter size 0.7 - 2.0 cm.
++ : diameter size 2.1 - 4.0 cm.
+++ : diameter size 4.1 – 6.0 cm.
++++ : diameter size 6.1 – 8.0 cm.
+++++ : diameter size 8.1 – 9.0 cm.

G, R, and L : glucose, Poly R-478, and lignin powder, respectively

* : *P. chrysosporium* ATCC 34541

** : *C. versicolor* IFO 30388

Fig. 3.3 Decoloration of Poly R-478 by NP21 and RC3 compared to the reference strains, *P. chrysosporium* ATCC 34541 and *C. versicolor* IFO 30388, after cultivation on modified Pointing's medium at 37°C for 4 d.

Fig. 3.4 Decoloration of lignin powder containing in modified Pointing's medium by the isolate NP21 and RC3 compared to the reference strains, *P. chrysosporium* ATCC 34541 and *C. versicolor* IFO 30388, which could not be observed halo forming after cultivation at 37°C for 4 d.

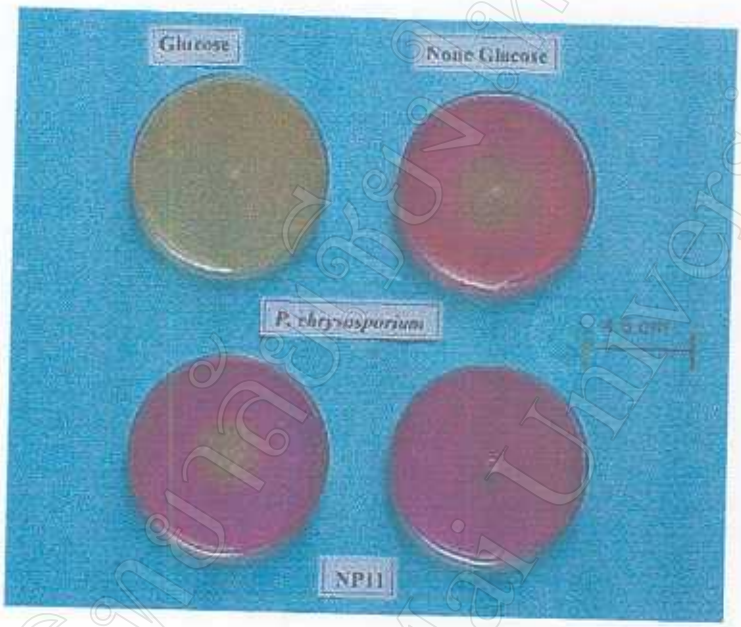

Fig. 3.5 Effects of glucose on decoloration of Poly R-478 by the reference strain, *P. chrysosporium* ATCC 34541 and the new isolate NP11 after cultivation on modified Pointing's medium at 37°C for 4 d.

3.4 Screening of lignin degrading enzyme producer by direct determination of enzyme activity

To confirm the screening method on Poly R-478 agar, the enzyme production was necessary to be studied and the result of lignin degrading enzymes production by all 42 isolates in liquid culture were shown in Table 3.2. It was found that the halo forming isolates were capable to produce different kinds and amount of ligninolytic enzymes, especially, the isolate NP10, which its halo could not be observed, but when cultivation in liquid medium, it could produce 3 types of enzymes including; MnP, LiP and laccase. The strain showed the total enzymes activity up to 18.32 units/*l*. Therefore, the efficiency of screening procedure should be confirmed by directly investigated for enzymes production which was corresponding with the reports of Shinmen *et al.*(1986) and Assavning *et al.*(1992).

The efficiency of screening method by decoloration of Poly R-478 dye was shown in Table 3.3. The Poly R-478 degrading positive strains showed the positive production ratio at 95, 76, 29, and 71 for LiP, laccase, MIP, and MnP, respectively. While negative strains showed missing ratio at 86, 14, 29, and 71, respectively.

The decoloration of Poly R-478 screening method was reasonable for only laccase producing strains only because it showed high level of positive strains ratio up to 76% and low level of missing ratio down to 14%. But in case of other enzymes, this screening method showed low relationship between enzymes production and Poly R-478 decoloration. Thus, the primary screening method by using Poly R-478 decoloration should be more investigated by direct determination of enzyme activity. Otherwise, some isolates which could show highly lignin degrading enzymes production might be loss. However, this was a primary screening method which was popular for ligninolytic fungi screening. The potential screening method was not considered by observation of Poly R-478 halo forming only, but also should be directly investigated by enzyme production.

Table 3.2 Enzyme activity (U/l) of MnP, MIP, Laccase and LiP, and total enzyme activity in culture broth

Day / Isolate	MnP			MIP			Laccase			LiP			Total activity
	3	6	9	3	6	9	3	6	9	3	6	9	
PC*	-	-	-	-	-	-	-	-	-	9.4	15.3	12.6	12.6
CV**	-	1.69	3.93	-	-	-	10.2	8	6	13.9	20	12.2	22.13
7M	3.75	-	3.6	-	21.6	-	12.5	40	137	8.8	8.6	11.2	151.8
TP16	-	-	-	-	-	-	54.3	19.6	86.7	11.4	11.8	16.1	102.8
NP27	0.24	2.54	0.12	-	-	6.3	15.3	57.3	26.5	15.2	6	15	47.92
01M	12.1	3.2	1.12	-	6.4	6.12	12.31	2.01	-	13.01	9.24	26.58	34.3
19M	0.6	-	-	6.32	0.56	18.25	18.63	-	2.75	17.89	11.69	8.6	29.6
8NPM	-	-	-	-	-	-	-	14.36	-		6.12	28.5	28.5
NP1	0.98	6.32	1.12	-	-	-	8.45	10.01	5.88	3.21	6.12	20.23	27.23
NP22	-	-	1.11	-	-	-	-	-	-	6.25	2.14	23.2	24.31
BK1	-	1.05	7.25	-	0.87	1.75	-	-	-	8.28	4.89	14.21	23.21
NP9	1.02	1.36	2.69	-	-	-	1.12	2.09	5.42	5.36	8.56	13.24	21.35
NP25	-	-	-	-	-	0.24	4.84	5.93	7.63	-	11.83	12.68	20.25
NP31	1.01	1.93	0.48	0.24	0.724	-	-	-	-	10.32	-	19.52	19.52
ST40	8.48	-	-	-	3.39	-	6.1	6	7.9	14.2	12	11.2	19.17
NP10	0.12	1.33	0.12	-	-	-	12.5	8.6	3.15	5.37	2.36	15.05	18.32
RC2	-	-	-	-	-	-	-	-	-	10.32	3.62	18.21	18.21
NP18	-	0.48	0.48	-	-	-	16.1	20.1	9.5	8.6	16.9	7.5	17.48
NP26	2.42	0.72	0.6	-	-	-	12.3	1.3	3.5	11.2	20.6	13.1	17.21
NP14	-	0.6	0.24	-	-	-	5.2	5.4	7.4	9	20	9.5	17.14
15M	-	0.36	0.83	-	-	0.7	-	-	-	8.38	4.51	15.26	16.09
NP15	0.63	-	-	-	-	-	-	-	-	7.52	3.22	15.05	15.05
RC3	-	-	1.33	-	-	-	16.8	7.5	12.9	12.7	18	-	14.23
NP23	-	1.09	4.8	-	-	-	-	-	-	5.17	1.29	9.03	13.83
UNK1	-	-	-	-	-	-	-	-	-	6.4	19.6	13.29	13.29
NP3	1.21	0.48	0.36	-	-	0.74	-	-	-	-	4.51	11.18	12.28
34H	-	-	-	-	-	-	-	-	-	13.9	25.2	12.32	12.32
TP7	-	0.12	-	-	1.45	-	19.5	1.7	2.8	12.2	23.8	9.4	12.2

Table 3.2 (continued).

Day / Isolate	MnP (U/l)			MIP(U/l)			laccase(U/l)			LiP(U/l)			Total activity
	3	6	9	3	6	9	3	6	9	3	6	9	
26MK	-	-	-	-	-	-	-	-	-	17.32	6.25	9.63	9.63
13M	-	1.23	1.25	-	1.59	0.75	-	-	-	12.3	8.60	7.42	9.42
BK2	-	1.05	1.23	-	-	-	-	-	-	1.26	2.35	7.02	8.25
TP11	-	-	-	-	-	6	-	-	-	6.59	0.98	1.56	7.56
CMU7	6.91	0.84	3.15	-	-	-	2.1	25.6	3.75	4.3	1.72	-	6.9
NP8	-	-	-	-	-	-	-	-	-	5.39	1.23	6.64	6.64
NP11	-	1.02	1.25	-	2.14	-	5.26	1.11	1.75	4.02	4.96	3.24	6.24
NP21	-	-	-	-	-	-	16.54	15.3	3.6	9.9	11.8	1.5	5.1
RC5	-	0.98	1.01	-	-	-	-	-	-	-	-	3.57	4.58
ST41	-	6.21	4.68	-	-	-	-	-	-	21.23	7.45	-	4.68
TP6	4.12	-	0.91	-	-	-	-	-	-	4.56	6.32	0.2	1.1
CMU2	1.98	1.12	-	-	-	-	-	-	-	1.97	6.25	1.03	1.03
ST9	0.025	-	-	0.59	-	-	-	0.21	-	-	-	-	0
NP7	-	-	-	-	-	-	-	-	-	0.003	-	-	0
NP28	-	-	-	-	-	-	-	-	-	0.013	-	-	0
30MK	-	0.001	-	-	-	-	-	-	-	0.001	-	-	0

Note : PC* : *P. chrysosporium* ATCC 34541 ; CV** : *C. versicolor* IFO 30388

Table 3.3 Comparison the efficiency of lignin degrading basidiomycetes screening method by using decoloration of Poly R-478

	Decoloration on Poly R-478medium							
	+				-			
	MnP	MIP	Laccase	LiP	MnP	MIP	Laccase	LiP
Fungi (A)	21	21	21	21	21	21	21	21
Lignin degrading Fungi (B)	15	6	16	20	15	6	3	18
Positive strain ratio (% B/A)	71	29	76	95				
Missing ratio (% B/A)					71	29	14	86

3.5 Selection of laccase producing strain on solid culture

All of 42 isolates were grown on rubber wood chips containing 4% (w/v) glucose and 4% (w/v) peptone solution mixture compared with control. In the combination of 4% (w/v) glucose and 4% (w/v) peptone supplementation, the isolates could show higher laccase activity than the distilled water addition (Table 3.4). Thus, this condition was used to select laccase producing strains. The obtained three isolates of 7M, TP16, and NP21 showed high level of laccase activity up to 3.12, 3.05, and 2.96 unit/g substrate, respectively. These were almost 30 folds higher than the activity found in *C. versicolor* IFO 30388.

Considering in morphological characteristics of three newly isolated basidiomycete fungi, 7M and TP16, were of the Genus *Pycnoporous*, which was recently reported to be an extremely laccase producing strain. Its mechanism of the enzyme production in molecular level was already studied (Eggert *et al.*, 1996). The isolate NP21 was identified to be *Lenzites* sp. Its laccase activity production was not quite different from the isolate 7M and TP16. The fruiting body of the isolate NP21 collected from Nongplaman in Ampher Maerim was clearly studied (Table 3.5, Fig 3.6, and 3.7). From those data, the isolate NP21 was classified to be *Lenzites* sp. (Dickinson and Lucus, 1979) as was concluded in Table 3.6. The enzyme solution of NP21 was found xylanase, β-mannanase, and cellulase to be 8.1, 4.6, and 1.9 unit/g substrate, respectively. Recently, there was no report on laccase production from this Genus of white rot fungi. Therefore, the isolate NP21 was selected for further studies.

Table 3.4 Laccase production by 42 isolates when cultivated on rubber wood chips at 37°C for 9 d

Isolate	Activity of laccase (unit/g substrate)	
	Distilled water	4% glucose–4%peptone
PV*	-	-
CV**	0.012	0.114
7M	0.111	3.116
TP16	0.123	3.053
NP21	0.082	2.964
NP14	0.013	2.570
NP18	0.012	1.990
NP9	0.257	1.354
01M	0.345	1.342
NP10	0.030	1.262
TP11	0.055	1.052
RC3	0.023	0.956
NP27	0.067	0.745
NP26	0.015	0.532
19M	0.065	0.382
ST40	0.058	0.321
NP11	0.069	0.164
NP25	0.035	0.121
8NPM	0.003	0.118
NP1	0.001	0.115
NP8	0.012	0.098
NP23	0.005	0.034
NP22	0.008	0.024
34H	-	0.019

Table 3.4 (continued)

Samples	Activity of laccase (unit/g substrate)	
	Distilled water	4% glucose+4%peptone
NP28	0.008	0.019
NP31	0.004	0.019
RC2	0.002	0.018
26MK	0.001	0.016
15M	0.003	0.015
NP15	-	0.015
BK1	-	0.014
UNK1	-	0.013
NP3	-	0.011
TP7	-	0.010
BK2	0.005	0.010
13M	-	0.007
CMU7	-	0.005
RC5	-	0.003
ST41	-	0.003
TP6	-	0.002
CMU2	-	0.001
ST9	-	-
NP7	-	-
30MK	-	-

Note: PV*; *P. chrysosporium* ATCC 34541

CV**; *C. versicolor* IFO 30388

Table.3.5 Characteristic identification of NP21

Description	Gill like plates undersurface. Forming fruiting bodies yearly, the mycelium continues growing on the same stump or log for many years.
Cap	Annual, 5.5-8.8 cm broad, white to yellowish with brown, thin walls, semi-circular, flattened zone, the brownish zone. Margin sometimes wavy. Fresh yellowish soft at first. becoming grayish or pale and hard when drying.
Gills	cream to yellowish, elongated to form gill like plates, often branched, thin walls.
Stalk	Absent
Spores	White to yellowish in color, cylindrical, smooth, average size 2.0-3.0 μm.
Habitat	Growth on dried dead trees, stump or log.

Table 3.6 Classification of the new isolate NP21 (*Lenzites* sp.)

Kingdom *Fungi*

Phylum *Dikaryomycota*

Subphylum *Basidiomycotina*

Class *Holobasidiomycetes*

Genus *Lenzite* sp.

Fig. 3.6 Fruiting bodies of the isolate NP21

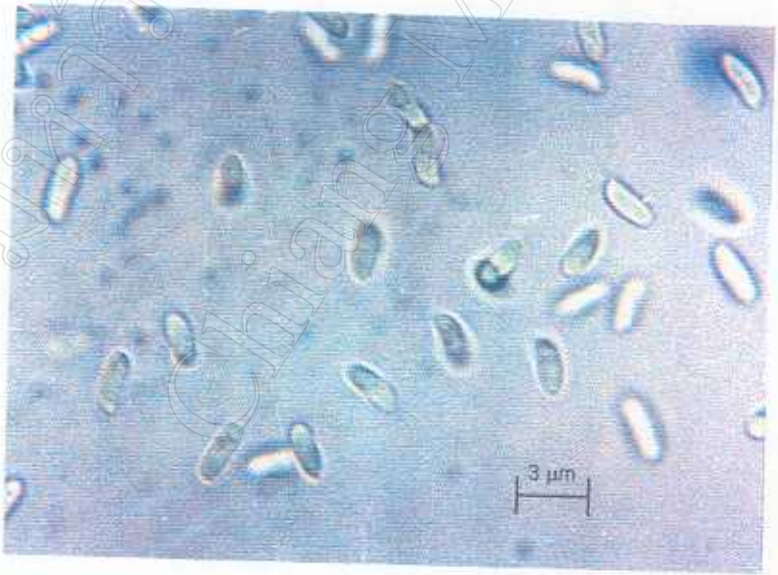

Fig. 3.7 Spore shape of the isolate NP21(x100)

3.6 Some effects on laccase production from *Lenzites* sp. NP21

3.6.1) Effect s of veratryl alcohol and Tween-80 on laccase production

Addition of Tween-80 showed the enhancing effect on laccase production which was found up to 11 unit/*l* when compared with very low laccase activity found in control treatment (Fig. 3.8). This suggested that Tween-80 could activate the excretion of laccase into culture broth. Addition of veratryl alcohol also increased laccase production. Veratryl alcohol was the secondary metabolite of white rot fungi which may be used as an inducer (Barbosa, 1996) and this was the simplicity of enzyme production and versatility of laccase-catalyzed mechanism of substrate (Thurston, 1994).

Addition of Poly R-478 enhanced the enzyme production when compared with control and Tween-80 addition. The results was corresponding with using of 0.02% (w/v) lignin powder replacement of Poly R-478 (Fig. 3.9). Both aromatic compounds, Poly R-478 and lignin powder seemed to be repressors or inhibitors to reduce the enhancing effects of veratryl alcohol and Tween-80 on laccase production. Laccase activity was decreased in the presence of both compounds. There was a report (Diamantidis *et al.*, 2000) that laccase activity was inhibited by tropolone which was also aromatic compound (Appendix 7). Tropolone was a specific inhibitor of metal containing enzyme such as laccase. This evidence was corresponding with our result because all of tropolone, Poly R-478 and lignin powder structures were also aromatic compounds so it was possible if laccase activity was inhibited by Poly R-478 and lignin powder in the similar fashion to tropolone. However the highest laccase production from this strain was found up to 102 unit/*l* in basal medium contained 0.02% (w/v) veratryl alcohol and 0.05% (w/v) Tween-80 (Fig. 3.8).

Laccase production was also studied on solid state fermentation by using rubber wood chips as a solid substrate, supplemented with basal medium, 0.02%(w/v) veratryl alcohol, and 0.05% (w/v) Tween-80 as liquid culture. Almost the same results when compared to those from the liquid medium (Fig. 3.10). Mineral salt showed enhancing

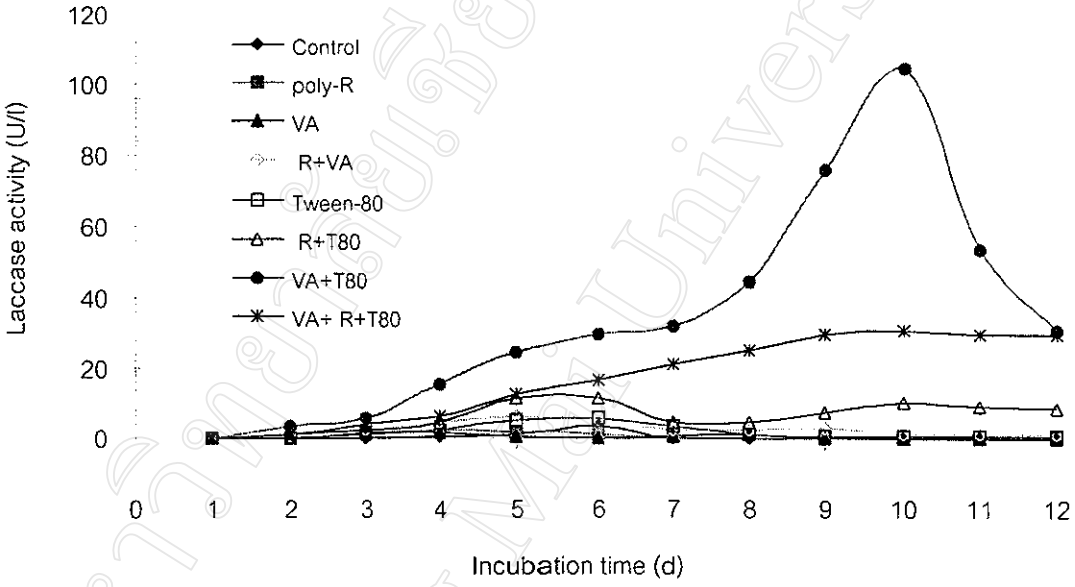

Fig. 3.8 Effects of veratryl alcohol and Tween-80 on laccase production in liquid culture containing 0.02% (w/v) Poly R-478 by *Lenzites sp.* NP21 at 37°C.

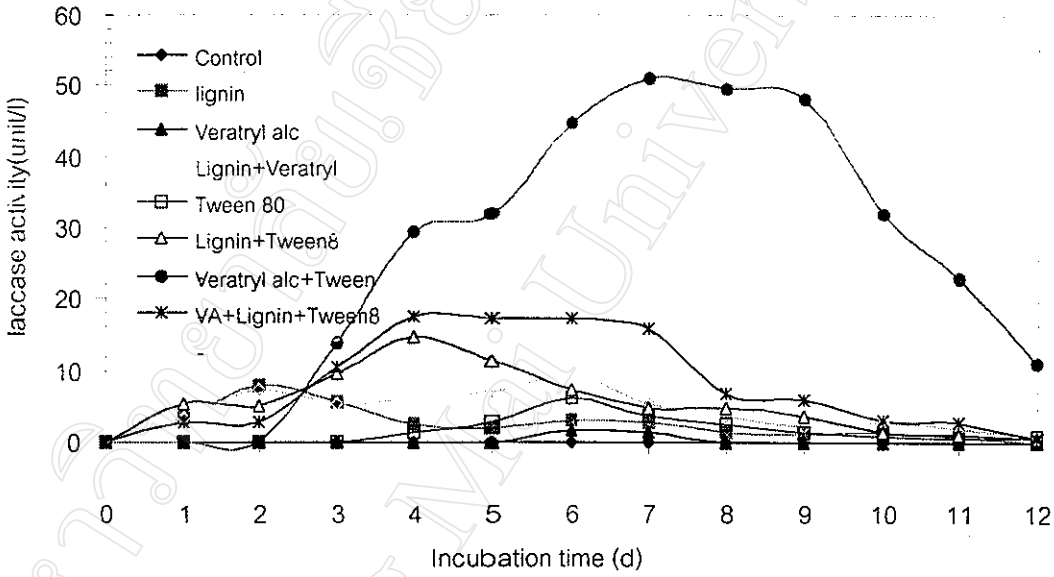

Fig. 3.9 Effects of veratryl alcohol and Tween-80 on laccase production in liquid medium containing 0.02% (w/v) lignin powder.

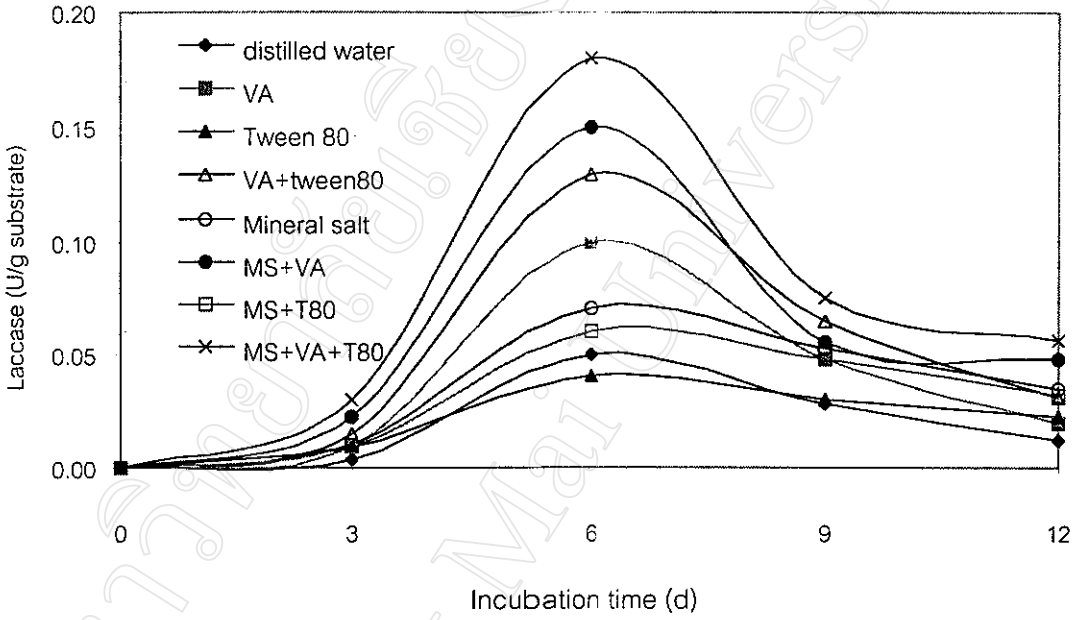

Fig. 3.10 Effects of veratryl alcohol and Tween-80 on laccase production by *Lenzites* sp. NP21 when cultivated from rubber wood chips at 37°C.

effects on laccase production which corresponding with Kumaran's results (Kumaran *et al.*, 1997), which *Sago hampas* from Sago palm was used as substrate for laccase production by *Pleurotus ostreatus.* Hudson (1972) reported an importance of minerals that fungi also required a rang of mineral, such as potassium and phosphorus, in large quantity. In case of manganese and zinc were required in trace, which were very similar to those of other organisms. However, the presence of 0.025% (w/v) veratryl alcohol and 0.05% (w/v) Tween-80 in basal medium was still the most efficient way to stimulate laccase production, which was found laccase activity up to 0.18 unit/g substrate. Therefore, this condition was selected to compare with combination of 4% (w/v) glucose and 4% (w/v) peptone addition which was used in the previous studies for selection of the best laccase producing strain experiment.

Furthermore, the comparison of combined glucose and peptone solution addition to study laccase production and distilled water was added as control. The addition of combined 0.025% (w/v) veratryl alcohol and 0.05% (w/v) Tween-80 in the presence of mineral salt was not so effective on laccase production compared with the addition of 4%(w/v) glucose and 4% (w/v) peptone solution (Fig. 3.11). This result indicated that the excess carbon or nitrogen sources were more effective than veratryl alcohol and Tween-80 in the presence of mineral salt. The laccase activity was increased to 2.12 unit/g substrate when added the combined 4% (w/v) glucose and 4% (w/v) peptone solution.

All of the highest laccase production in each treatment were concluded in Table 3.7. The results showed that the addition of solution mixture of 4% (w/v) glucose and 4% (w/v)peptone on rubber wood chips was selected as the best treatment for further studies. Although their dimensions could not be compared together. The solid state fermentation was simplify for culture preparation and more economic when compared with other conditions.

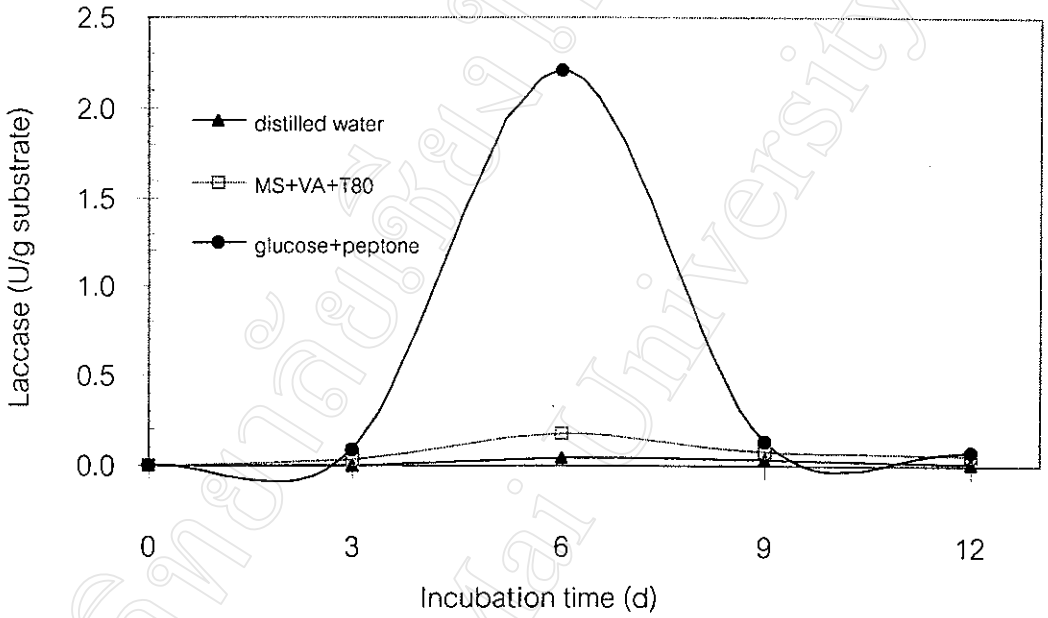

Fig. 3.11 Comparison between effects of veratryl alcohol and Tween-80, and 4% (w/v) glucose and 4% (w/v) peptone supplemented on laccase production by *Lenzites* sp. NP21 on rubber wood chips at 37°C.

Table 3.7 Comparison of the highest laccase activity in each treatment

Types of culture	Supplements	Laccase activity
Liquid -	Veratryl alcohol[1] and Tween-80[2]	0.104 unit/ml
- Poly R-478[3]	Veratryl alcohol and Tween-80	0.036 unit/ml
- lignin powder[3]	Veratryl alcohol and Tween-80	0.018 unit/ml
Solid - rubber wood chips	Veratryl alcohol and Tween-80	0.18[4] unit/g. sub.
rubber wood chips	4% glucose and 4% peptone	2.12 unit/g. sub.

Note : 1 : 0.025% (w/v)

2 : 0.05% (w/v)

3 : 0.02% (w/v),

4 : presence of basal medium

3.6.2) Effects of glucose and peptone concentration on laccase production

Considering the amount of glucose and peptone addition in the previous experiment, it seemed to be highly consumed of nutrient. Thus, the optimal quantity of the nutrient added was studied and the result was shown in Fig. 3.12. Addition of peptone showed more enhancing effects on laccase production than glucose. Moreover, at the low level or non-glucose addition showed the higher level of enzyme production than those of the higher level. This corresponding with the result by Jeffries *et al.* (1981) which reported that carbohydrate limitation may induce secondary metabolism and lignin degradation when studied in *P. chrysosporium, C. versicolor* and *Pleurotus ostereatus* (Ander and Eriksson, 1975). Addition of 4% (w/v) peptone without glucose was the best treatment which the strain could produce laccase up to 5.69 unit/g substrate. This result was corresponding with many previous reports which explained that nitrogen repression of lignin degradation in white rot fungi was common but might not always be the rule, on the other hand, nitrogen sources in fungal medium might increase an efficiency in degradation of lignin or lignin related compounds. Such natural fungi were probably found in the environments of high

nitrogen concentrations such as cattle dung, although most of fungi that growed in wood usually encounter low nitrogen concentration (Eriksson *et al.*, 1990). Thus, the obtained result from this study was one of such rare case.

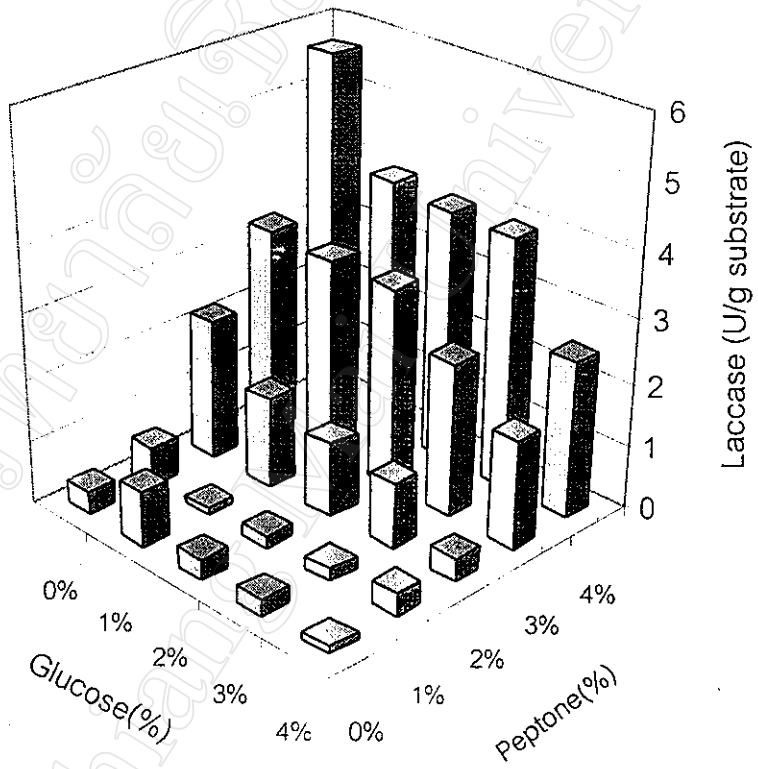

Fig. 3.12 Effects of glucose and peptone concentration on laccase production by *Lenzites* sp. NP21 from rubber wood chips at 37°C.

3.6.3) Optimal temperature for laccase production

Three levels of temperature including 30, 37, and 45°C were used for laccase production study. The result showed that 37°C was the most suitable for enzyme producing condition which was detected activity up to 5.69 unit/g substrate (Fig. 3.13). Although at 45°C, the isolate NP21 could produce the enzyme with almost the same level. After cultivation at 30°C more than 6 d, laccase activity was found higher than that from 37 and 45°C cultivation. Considering in moisture content by visually observed the stream obtained within flasks containing medium, the stream still occurred at 30°C while at 37 and 45°C could not be visualized after cultivation more than 6 d. From this phenomenon, it could be suggested that the moisture content might be an important factor for the enzyme activity obtained.

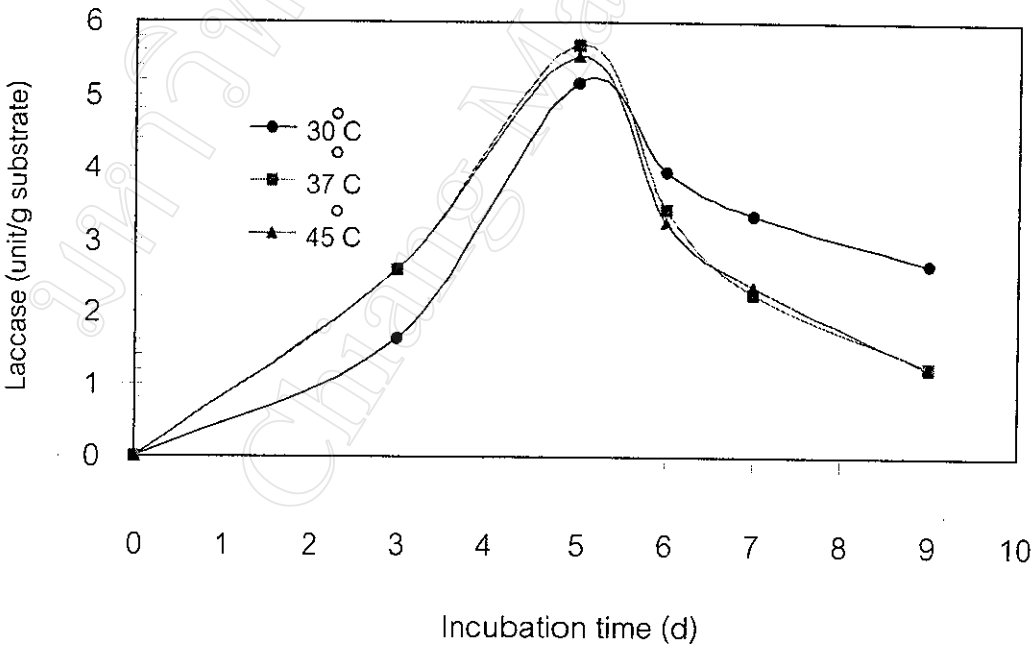

Fig. 3.13 Optimal temperature for laccase production on rubber wood chips by *Lenzites* sp. NP21 at 37°C.

3.6.4) Effect of initial pH on laccase production

The initial pH of 5.0 to 8.0 is satisfactory for laccase production (Fig. 3.14). The optimal initial pH was found to be around 7 from the culture preparation or at control condition. At the optimal initial pH, the strain produced the highest laccase activity up to 5.69 unit/g substrate which was used as control condition. However at the initial pH range from 3.5 to 4.0, the strain was not only unable to produce laccase, but also unable to grow The mycelial growth could not be visualized. The obtained result was corresponded to the report of Emerson and Cantino (1948) which reported that an initial pH of 5.0 to 6.0 was satisfactory for the majority of the fungi. Moreover, Wolpert (1924) found that the optimum pH of various basidiomycetes was to be around 5.5.

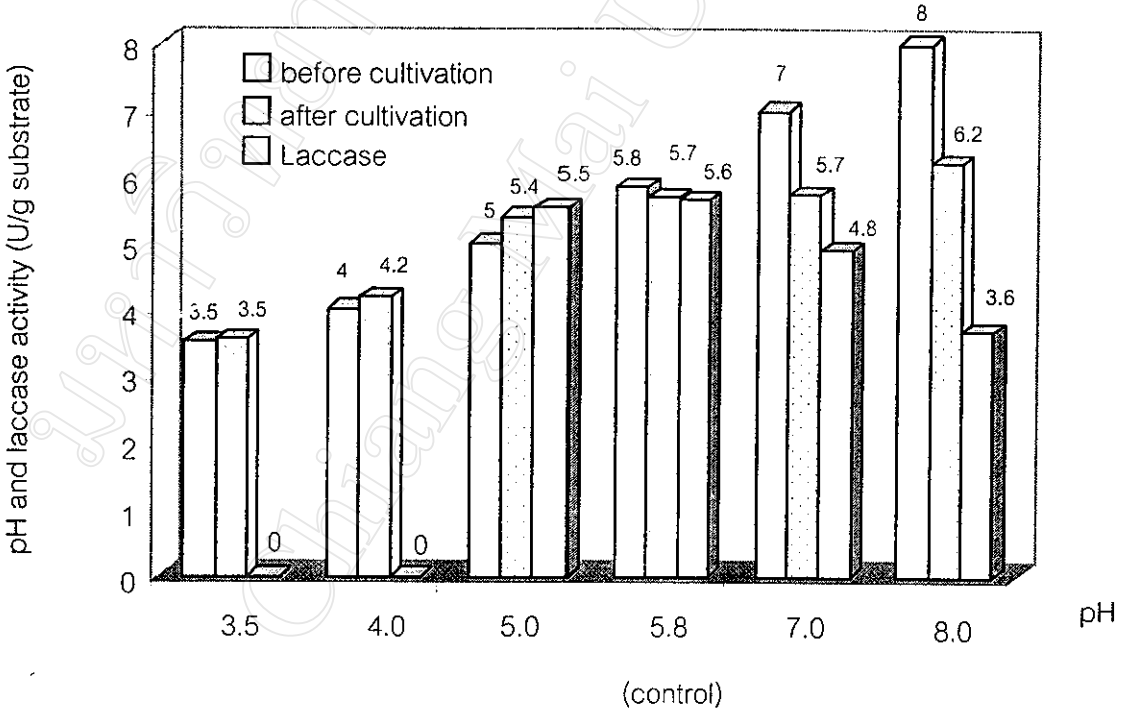

Fig. 3.14 Effects of initial pH on laccase production by *Lenzites* sp. NP21 on solid culture at 37°C.

3.6.5) Effects of veratryl alcohol and Tween-80 on laccase production at optimal condition

The main purpose in this experiments was attempt to induce the ability of laccase production by using veratryl alcohol and Tween-80 again. Cultivation on rubber wood chips in the presence of 0.025% (w/v) veratryl alcohol showed slightly higher laccase activity (5.81 unit/g substrate) than those obtained in the presence of both 0.025% (w/v) veratryl alcohol and 0.05% (w/v) Tween-80 (5.63 unit/g substrate), Tween-80 only (5.37 unit/g substrate), and 4% (w/v) peptone supplement solely (5.68 unit/g substrate) was shown in Fig. 3.15. Considering the cost of production, the addition of veratryl alcohol was not suitable for enzyme production due to the increasing cost. Thus, the addition of 4%(w/v) peptone on rubber wood chips was selected to produce the enzyme for the purification step.

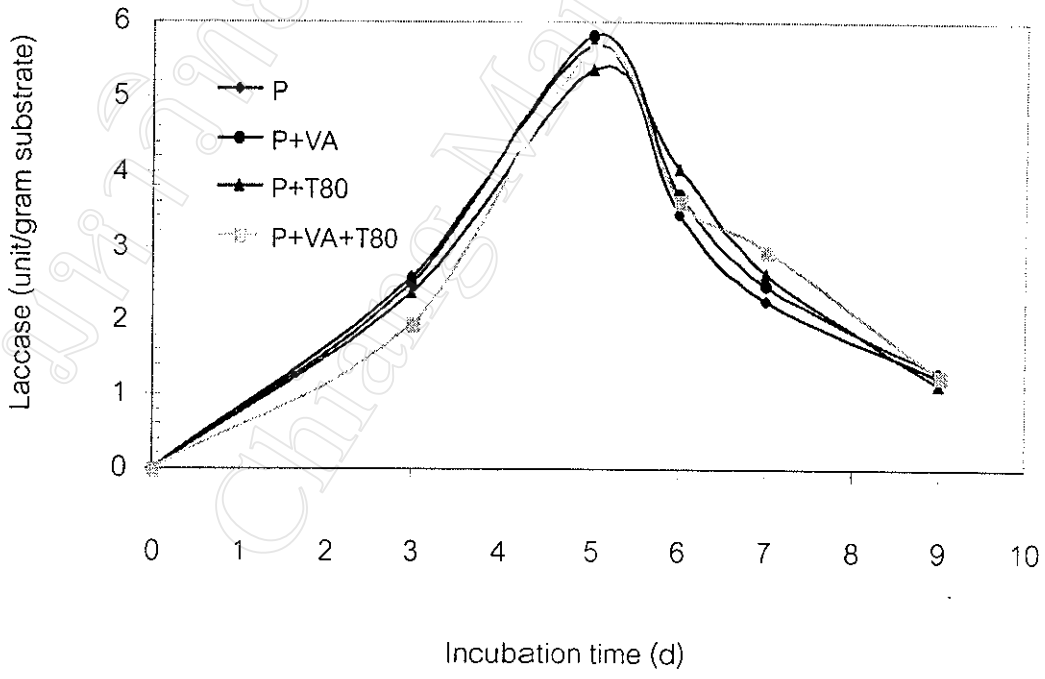

Fig. 3.15 Effects of veratryl alcohol and Tween-80 on laccase production on rubber wood chips by *Lenzites* sp. NP21 at 37°C.

3.6.6) Effect of copper induction on laccase production

To study an induction effect of copper on laccase production in the presence of 75, 150, and 300 μM $CuSO_4$ at the optimal conditions. The maximal induction effect was obtained at 150 μM of $CuSO_4$ concentration which the maximal activity was found to be 9.43 unit/g substrate (Fig. 3.16). Under this condition, laccase activity was increased almost twice as much as with none of $CuSO_4$ addition. The increase of laccase production was directly proportional to the amount of $CuSO_4$ supplemented, which coincided with the report of Palmeiri about a significant increase for laccase production in copper supplemented culture of *Pleurotus ostreatus* (Palmeiri *et al.*, 2000). Furthermore, copper had been reported to be a strong laccase inducer in white rot fungi, *Trametes versicolor* and *Phanerocheacte chrysosporium* (Coilins and Dohsan, 1997 and Dittmer *et al.*, 1997).

However, the excess of $CuSO_4$ might be toxic or repressed laccase production because of free copper ions, which could result in oxidative stress at late transcriptional induction (Fernandez and Staht, 1996). This suggestion was corresponded with this study that the addition of 300 μM of $CuSO_4$ into the cultures showed the lower enhancing effect than that of 150 μM.

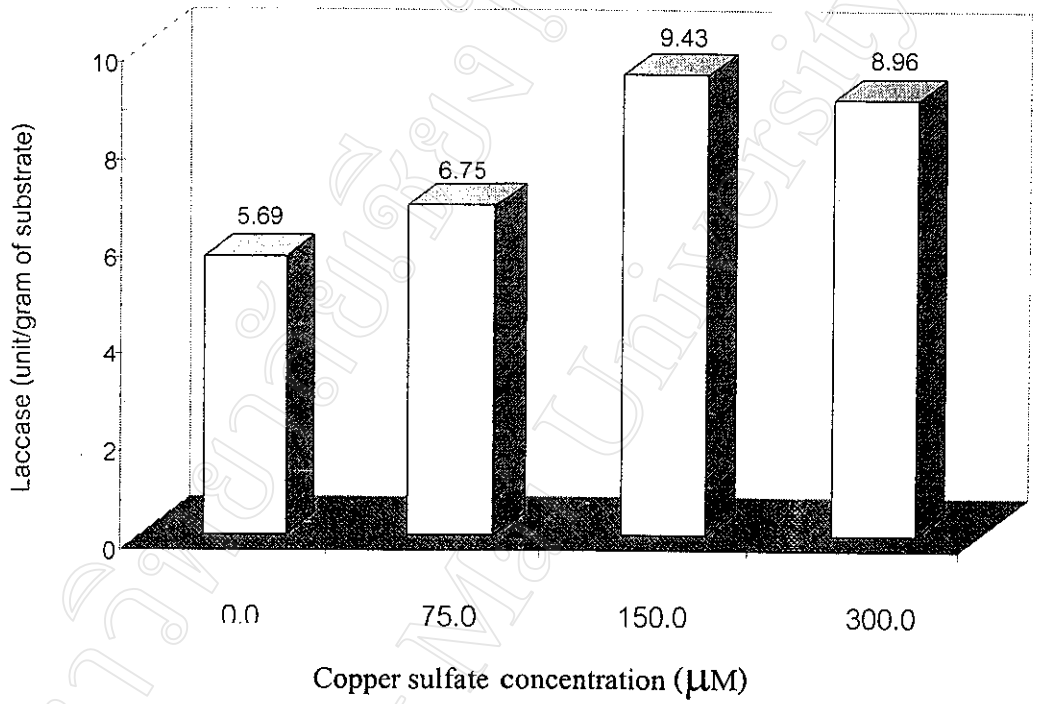

Fig. 3.16 Effect of copper induction on laccase production from rubber wood chips by *Lenzites* sp. NP21 at 37°C.

3.7. Purification of laccase of *Lenzites* sp. NP21 Laccase

After 5 d of cultivation, the enzyme was extracted from solid substrate and applied to CM-cellulofine and DEAE-cellulose column chromatography, respectively. Unbound and bound proteins were eluted by 20 mM phosphate buffer (pH 7.5) and linear gradient of 0-1.0 M NaCl, respectively. The result was shown in Fig. 3.17. The active fractions which could be detected laccase activity were combined and concentrated by ultra-filtration using 10,000 MW cut off membrane. The 250 ml enzyme solution was concentrated to 11.0 ml. Subsequently, applied to Sephadex G-100 gel filtration column which was eluted by 20 mM phosphate buffer pH 7.5. The chromatogram was shown in Fig. 3.18.

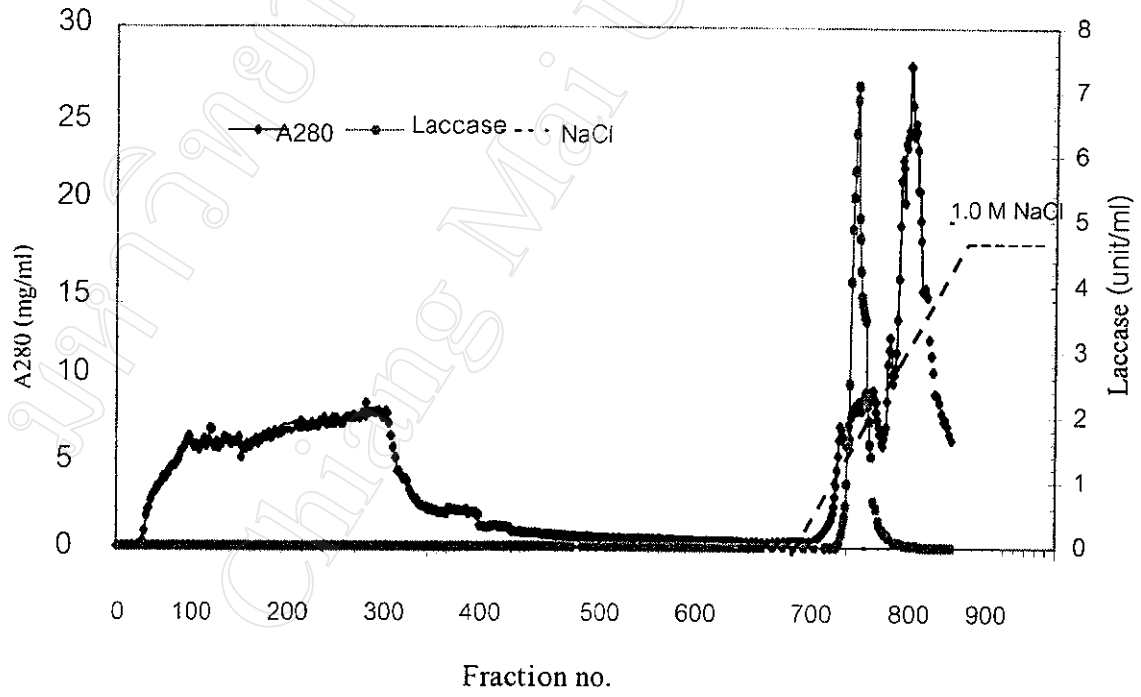

Fig. 3.17 DEAE-cellulose column chromatography of laccase from *Lenzites* sp. NP21.

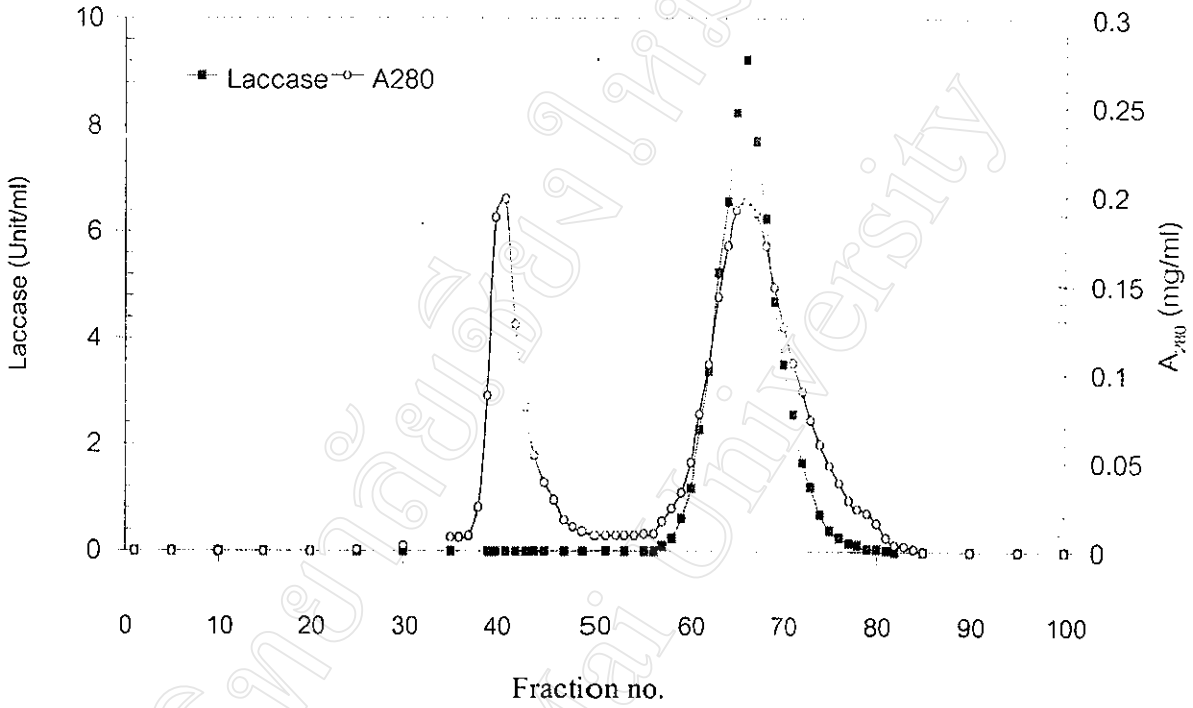

Fig. 3.18 Sephadex G-100 gel filtration column chromatography of the purified laccase from *Lenzites* sp. NP21.

3.8. SDS-PAGE analysis

The enzyme protein from single sharp peak of laccase activity was analyzed by SDS-PAGE. The result from Fig.3.19 showed that the enzyme was purified to homogeneity and the purifying result were summarized in the Table 3.8. The enzyme protein was analyzed by SDS-PAGE and showed a single band with the molecular weight of 65 kDa when compared with the proteins marker mobilities from PHARMACIA (Appendix 5).

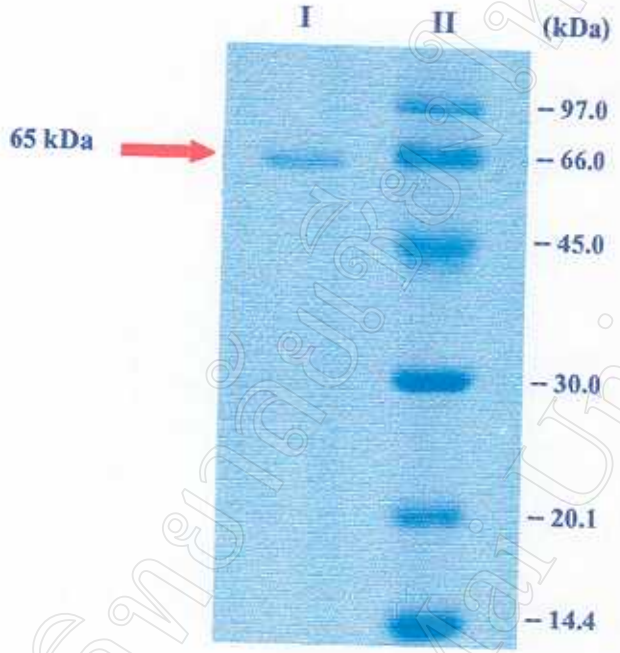

Fig. 3. 19 Sodium dodecyl sulfate polyacrylamide gel electrophoresis of purified laccase from *Lenzites* sp. NP21 (Lane I). The calibration protein used for molecular weight determination from PHARMACIA were phosphoryrase b from rabbit muscle (97 kDa), albumin from bovine serum (66 kDa), ovalbumin from chicken egg white (45 kDa), carbonic anhydrase from bovine erythrocyte (30 kDa), trypsin inhibitor from soybean (20.1 kDa), and α-lactalbumin from bovine milk (14.4 kDa) (Lane II).

Table 3.8 Purification of laccase from *Lenzites* sp. NP21

Purification step	Total activity (unit)	Total protein (mg)	Specific activity (unit/mg protein)	Yield (%)	Fold
Crude enzyme	799.2	25083.0	0.03	100.00	1.0
CM-cellulofine column	967.9	20517.5	0.05	121.1	1.5
DEAE-cellulose column	784.3	845.0	0.93	98.1	29.1
Sephadex G-100 column	396.2	12.91	28.44	49.8	892.5

The purification table showed that laccase obtained from the extract of *Lenzites* sp. NP21 was purified with CM-cellulofine, DEAE-cellulose and Sephadex G-100 gel filtration column, respectively. The purification of laccase procedure could show that laccase from *Lenzites* sp. NP21 was purified with 49.6% recovery yield and 893 folds of purification factor. Surprisingly, when considering with the total activity or activity yield value from crude enzyme compared to these of DEAE-cellulose column, we found the increase of activity yield at CM-cellulofine column. This matter seemed to be unusual from this observation, we have focused on lignin content, derived from rubber, in crude extract solution. Lignin may be caused inhibitory effect on laccase during determination of activity by using DMP and CM-cellulofine column may be removed lignin content from crude enzyme which leaded to increase in activity yield when the enzyme was purified through the CM-cellulofine column. However, this suggestion will be confirmed again for inhibitory effect of lignin on laccase activity in the characterization of purified laccase experiment.

3.9 Characterization of purified laccase

3.9.1) pH profile

The purified laccase from *Lenzites* sp. NP21 was studied in the range of pH from 2.5 to 9.0 and were initially active from pH 3.0 to 4.5. The highest activity was found to be at pH 4.0 with DMP as a substrate (Fig. 3.20). Other fungal laccase were also generally active at low pH: *Botrytis cinerea,* optimal pH 3.5; *Ceriporiopsis subvermispora,* optimal pH 3.0-5.0; *Trametes sanguinea,* optimal pH 5.0; *Pleurotus ostreatus,* optimal pH 3.8-4.2; *Pycnoporous cinnabarinus,* optimal pH 4.0 (Bollage and Leonowicz, 1984; Slomezynski *et al.*, 1995; Eggert *et al.*, 1996).

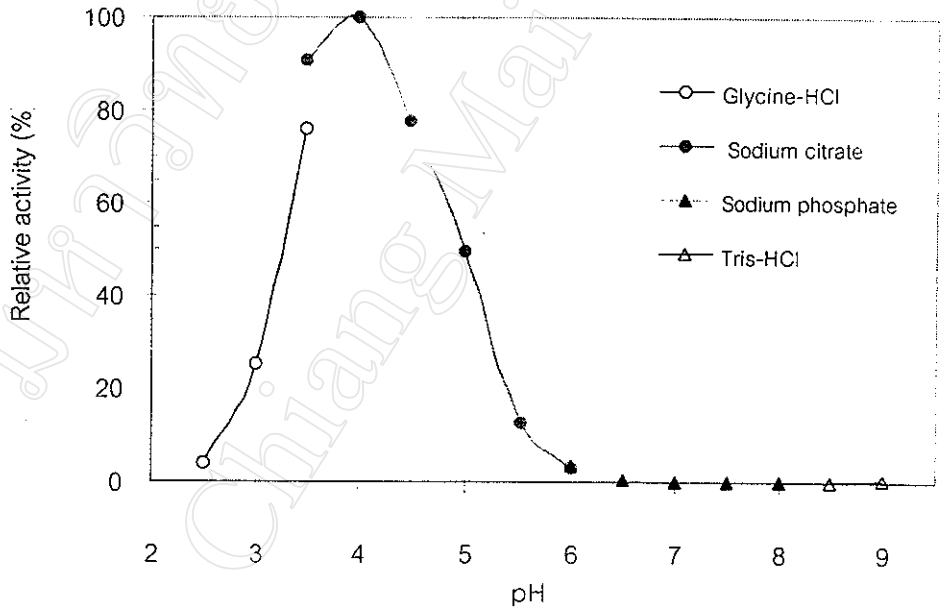

Fig. 3.20 Optimal pH of laccase activity from *Lenzites* sp.NP21.

3.9.2) Thermal stability

The laccase activity was studied for thermal stability be incubated at 30-80°C for 1 hr in order to test the thermal stability. Laccase was initially and clearly inactivated from 45°C and was completely inactivated in the range 75-80°C (Fig. 3.21). The range of temperature from 55-65°C was selected to study for half-life of enzyme. The half-life of purified laccase at 55, 60, and 65°C were found to be 130, 70, and 33 min, respectively, (Fig. 3.22). The activity at 65°C was almost completely inhibited within the half-life period of 55°C.

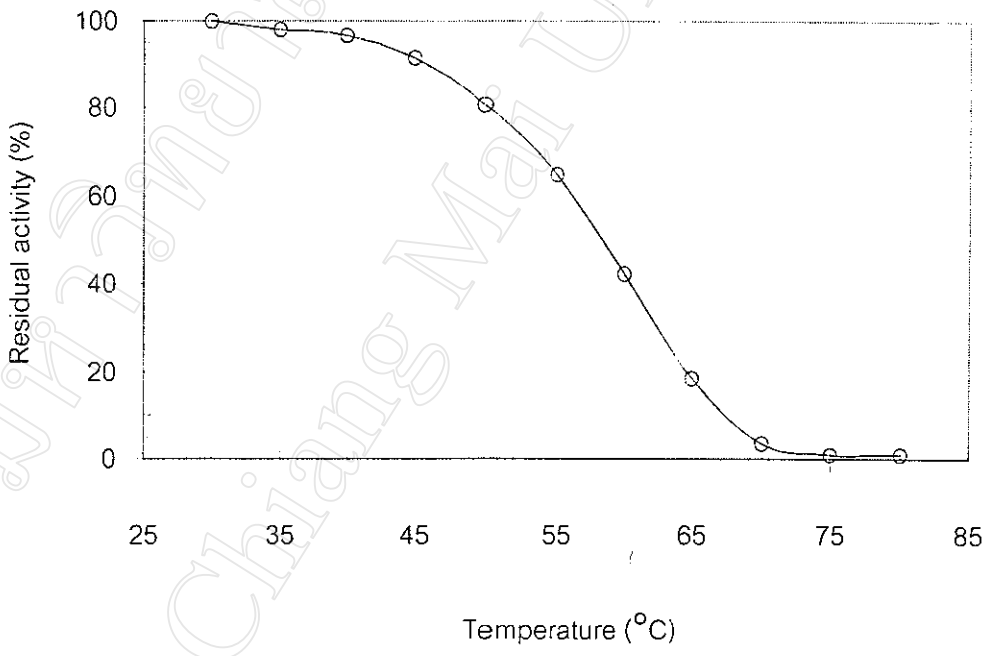

Fig. 3.21 Thermostability of purified laccase after incubation at various temperature for 1 hr.

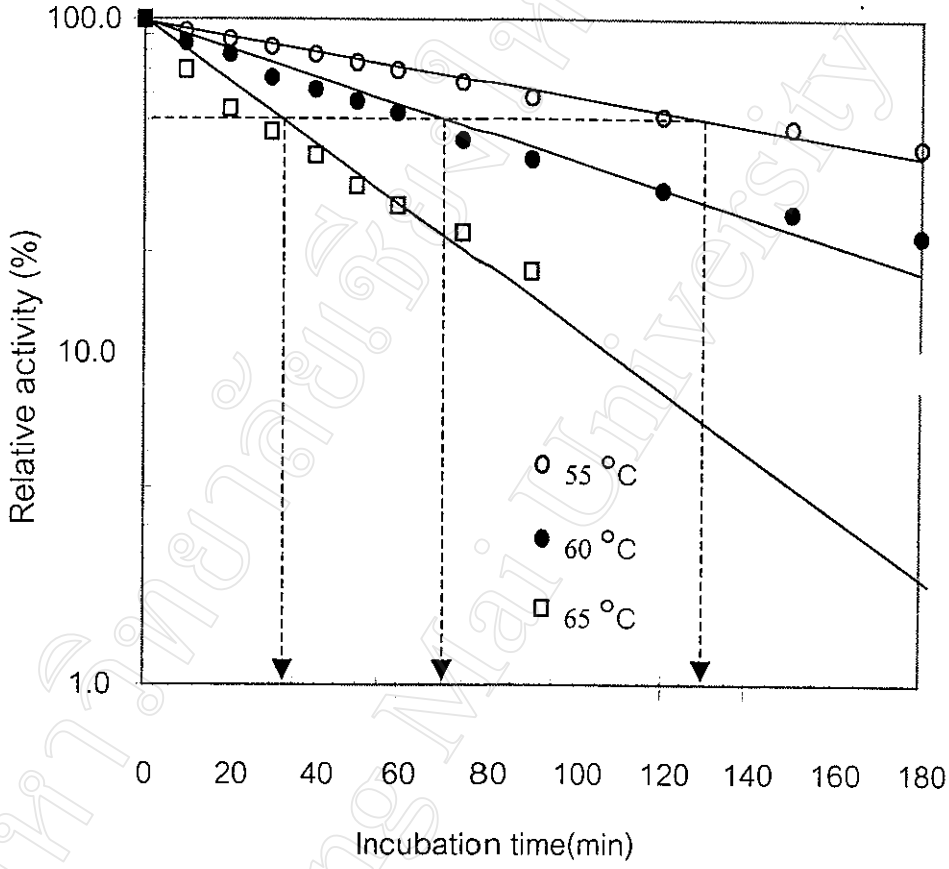

Fig. 3.22 Time course of heat inactivation of the purified laccase at 55, 60, and 65°C.

3.9.3 pH stability

The purified laccase was studied for pH stability in the range of pH 2.5-9.0 at 4°C for 24 hrs. Laccase was slightly inactivated from pH 2.5-3.0, whereas in the range of pH 3.5-9.0, the enzyme showed a high stability in a broad range of pH including acidic, neutralized, and alkali conditions was shown in Fig. 3.23. This was the great feature of laccase from *Lenzites* sp. NP21 because it was stable in a broad range of pH which led to be an attractive property for application in pulp and paper bleaching that need to be treated through multi-stage at the different pH, especially in alkali condition by using sodium hydroxide as lignin degrader.

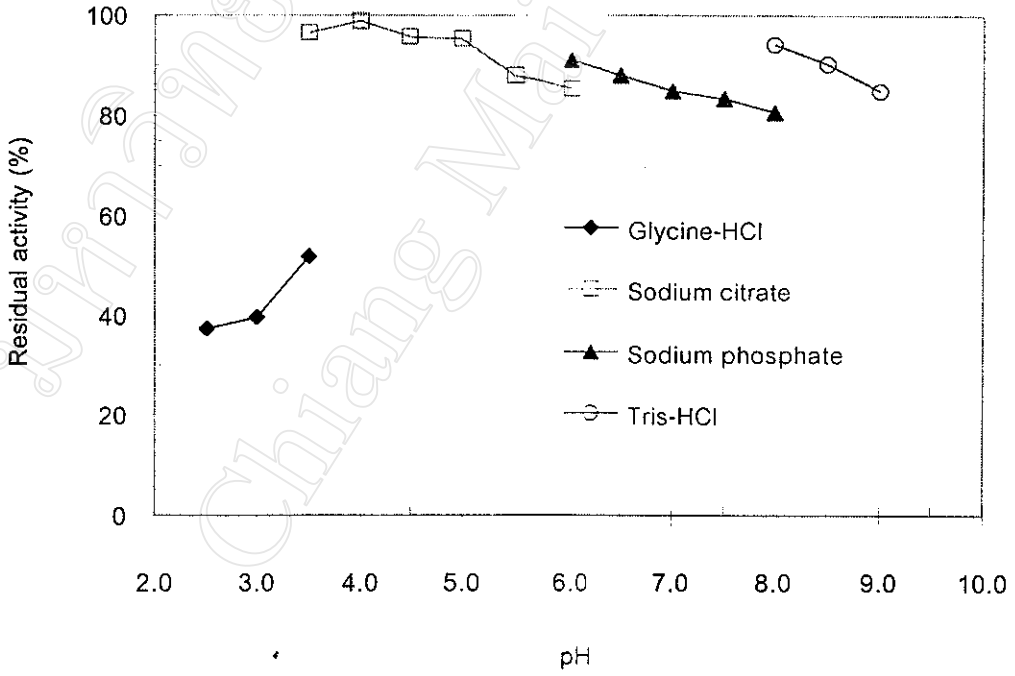

Fig. 3.23 pH stability of purified laccase from *Lenzites* sp. NP21 after incubation at various pH at 4°C for 24 hrs.

3.9.4 Substrate specificity

A series of different substituted phenol were examined for potential substrate specificity of laccase. Fig. 3.24 showed the pH profile of laccase in oxidation of DMP, ABTS, syringualdazine, and guaiacol. Under the same condition, the peak of optimal pH in oxidizing both ABTS and guaiacol were the same value at pH 3.0-4.5 which were showed a specific activity at pH 3.5 of 10% and 46%, respectively, base on 100% of DMP at pH 4.0. For syringaldazine oxidation, the optimal pH was in the range of 4.5- 5.0 with a specific activity of 12% when compared with oxidizing DMP at its optimal pH. However the difference of specific activity value of laccase from the *Lenzites* sp. NP21 depended on the optimal pH range in each compound. Therefore, it could be concluded that DMP was the most suitable substrate for laccase production from *Lenzites* sp. NP21 among four representatives used for enzyme assay in this experiment.

Considering the structure of four substrate compounds were classified into two groups. The first group was phenolic compound included DMP and guaiacol (Cai *et al.*, 1993). The second group which comprised of ABTS and syringaldazine was nonphenolic compound (Thurston, 1994). From the study, the phenolic compounds were oxidized and showed higher laccase activity than nonphenolic compounds which coincided with the result of Geiger *et al.* (1986) reported that the physiological function of fungal laccase was thought to be primarily related to its role in lignin decomposition. Its major function was not only the oxidation of lignin structure, but also including polymerization of their oxidative products. From the reports of Geiger *et al.* (1986), Suflita and Bollage (1981), and Youn *et al.* (1995) were coincided which reported for the studies relating to lignin degradation, laccase has been found to be capable of catalyzing polymerization rather than depolymerization. According to the results of Bell and coworker (1986) and Stevenson (1994)'s reports, they explained that mostly laccase effected on lignin product such as quinonic subunit may be polymerized into large molecule (Bell *et al.*, 1986) and condensed

to the stable molecules (Stevenson, 1994) which could be visibly observed during the enzyme assay.

Therefore, laccase activity mechanism was classified to be the polymerization of substrate to be a larger molecule of product. These supported the results obtained from this study that the purified laccase from *Lenzites* sp. NP21 showed higher laccase activity in oxidation of DMP and gauiacol than syringaldazine and ABTS oxidation. The structures of phenolic and nonphenolic compounds were shown in Appendix 7.

However, there are several works which reported that laccase from *Pycnoporous cinnabarius* and *Trametes versicolor* could show the highest laccase activity when ABTS was used as a substrate (Eggert *et al.*, 1996 and Bourbonnais *et al.*, 1995). In contrast, oxidization of syringaldazine showed the highest laccase activity when was oxidized by laccase from a thermophillic composted municipal solid waste (CMSW) (Chefetz *et al.*,1998). Therefore, laccases from various fungi belonged to their own specific substrates, which depended on their features or properties and source of producing strains.

Table. 3.9 Relative activity of oxidation of various substrates[a] by laccase from NP21

Substrates	Concentration (mM)	ε^{b} ($mM^{-1}x\ cm^{-1}$)	Wavelength (nm)	Laccase activity[c] (unit/ml)
DMP	5.0	49.6	470	100
Gauiacol	5.0	6.4	436	46
Syringaldazine	5.0	65.0	525	12
ABTS	5.0	36.0	420	10

[a] Assay were done in 0.1 M buffer as descriped in pH profile experiment in the range from 2.5-9.0 at 20°C.

[b]Increase of absorbance (product formation) (Eggert *et al.*, 1996 and Paszcynski *et al.*, 1988)

[c]Laccase activity at optimal pH in each substrate.

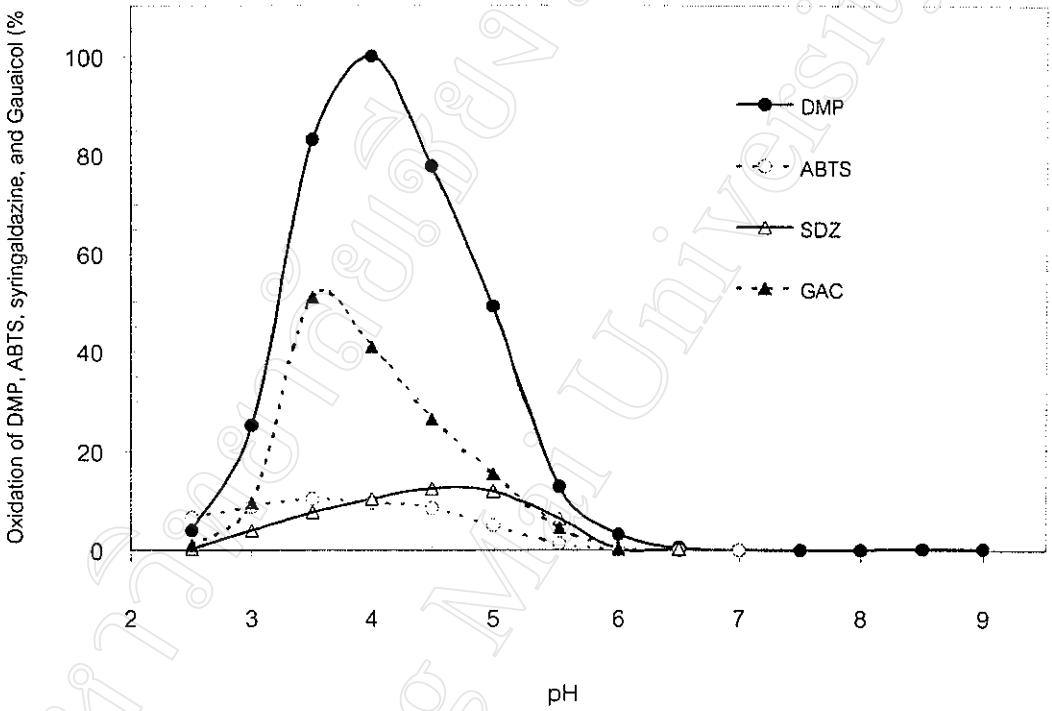

Fig. 3.24 Substrate specificity and pH profile of various substrates of laccase from *Lenzites* sp. NP21.

3.9.5 Effects of metal ions on laccase activity.

The laccase activity was almost and completely inhibited by $FeSO_4$ and $HgCl_2$, respectively (Table 3.10) Laccase was slightly inactivated by $CdCl_2$ and $Pb(CH_3COO)_2$ but it was significantly inhibited to be around 35% by $AgSO_4$. The copper ion (Cu^{2+}) from $CuSO_4$ markedly activated laccase due to its being stimulated by Cu^{2+} as other copper containg protien (Walter, 1965). For $CaCl_2$, $ZnSO_4$, and LiCl showed slightly activating effect or no effect on laccase activity. NaCl, $MgSO_4$, KCl, $CoCl_2$, and $AlCl_3$ had no effect on laccase activity.

Table 3.10 Effects of various metal ions on the laccase activity

Metals (1 mM)	Residual activity (%)
Control	100
$CuSO_4$	108
LiCl	105
$CaCl_2$	104
$ZnSO_4$	103
$MgSO_4$	102
NaCl	101
KCl	101
$MnCl_2$	98
$AlCl_3$	98
$Pb(CH_3COO)_2$	98
$AgSO_4$	90
$CdCl_2$	84
$CoCl_2$	65
$FeSO_4$	8
$HgCl_2$	0

3.9.6 Effects of inhibitors on laccase activity

The sensitivity of laccase from NP21 towards several laccase inhibitors was similar to laccase from *Coriolus hirsutus* and *Pycnoporous cinnabarinus* (Eggert *at el.*, 1996). All three laccases were completely inhibited by 1.0 mM NaN_3. 2-Mercaptoetanol, indoleacetic acid (IAA), *o*-phenanthroline, phenylmethylsulfonyl fluoride, and *p*-chloromercuribenzoate, which were general inhibitors for various enzymes, showed the inhibitory effect on laccase activity (Table 3.11). The indoleacetic acid showed some inhibitory effect on laccase from *Lenzites* sp. NP21 as well as on laccase from *P. cinnabarinus* and *C. hirsutus* (Eggert *et al.*, 1996).

Table 3.11 Effects of inhibitors on oxidation of DMP by the purified laccase

Inhibitors (1 mM)	Residual activity (%)
Control	100
Indoleacetic acid (IAA)	98
Ethylenediaminetetraacetic acid (EDTA)	92
o-Phenanthroline	91
Phenylmethylsulfonyl fluoride (PMSF)	90
2-Mercaptoethanol	78
p-Chloromercuribenzoate (PCMB)	73
NaN_3	0
Poly-R 478	93
Lignin powder	81

EDTA, a metal chelator, showed no inhibitory effect on laccase activity at the concentration 10.0 mM (data not shown), whereas other extracellular laccase were inhibited by more than 50% at 3 mM (Bollage and Leonowicz, 1984). From these results was corresponded with laccase from *P. cinnabarinus* and *C. hirsutus* at the concentration of EDTA at 4.0 mM was not inhibitory effect on laccase (Eggert *et al.*, 1996). It also showed only slightly inhibited laccase B and C from thermophilic composted municipal solid waste to be around 6% and 2% inhibition, respectively. eventhough at high concentration of 8.15 mM EDTA was tested (Chefetz *et al.*, 1998).

In the case of lignin powder and Poly R-478, to confirm their inhibitory effects from medium optimization for laccase production in liquid culture and this matter was observed again in the purification stage. It was found that lignin present in crude extracted solution showed the inhibitory effect on laccase activity. Its inhibitory effect was clearly decreased when lignin was removed by CM-cellulofine column. These supported the result that Poly R-478 and the synthesized lignin powder or natural lignin were also possible to be the inhibitors for laccase activity. The inhibitory mechanism of laccase by those compounds are similar to tropolone or other aromatic compounds which show the inhibitory effects on laccase of *P. cinnabarinus* and *C. hirsutus* when the reaction mixture is present of tropolone (Eggert *et al.*, 1996). Lignin powder and Poly R-478 could possibly be inhibitors for laccase activity. The effect on laccase production should be further more investigated.

3.10 Preliminary study of pulp biobleaching by laccase

The results in Fig. 3.25 showed the brightness of bleached pulp and residual activity of laccase during 12 hrs treatment of oxygen-delignified eucalyptus kraft pulp (OKP). To compare the potential of laccase only and laccase with HBT supplemented for pulp bleaching compared with control, the brightness of laccase adding was slightly different from control which was absent from laccase. In the case of HBT addition, the brightness was more markedly increased than other condition especially when increased the incubation time. These results were corresponding with Higuchi, who reported that laccase only had a limited effect on pulp bleaching due to its specificity for phenolic subunit in lignin (Higuchi *et al,* 1989) and its large molecule (M.W. around 70,000 Da). Therefore, it could not enter the fiber secondary wall to attact the lignin subunit directly. From these reasons, there are several works report on the potential of mediator, such as ABTS, on efficient demethylation and delignified kraft pulp (Bourbonnais, 1992) which coincided with our results that HBT or mediator was still important requirement for laccase bleaching, although its residual activity of laccase remained less than none HBT adding condition. From this matter, it could be suggested that HBT may inhibit laccase activity due to its structure which is also aromatic compound (Appendix 7) like DMP behaves as tropolone or lignin powder which this phenomenon has been occurred when there was lignin contained in enzyme solution at purification step.

From these results. the brightening increase was not marked increased when compared with control when using of EDTA-treated pulp for this experiment and the brightness obtained was 78.8% which was different from non EDTA-treated pulp that which obtained 74.2% of brightness. From this high brightness value of EDTA-treated pulp, the increase of brightness was not high when compared with control at 0 hr. However, results from this study were not in the same way with Bourbonnais's work which reported that HBT increasingly showed brightness of kraft pulp bleaching from 10.8% to 37.8% when compared with control (Bourbonnais, 1997). This might be due to an

insufficient of oxygen. In this study, oxygen was not applied in the enzymatic bleaching with HBT as Bourbonnais's experiment. Thus, the oxygen consumption might be strictly important in pulp biobleaching with laccase and mediator.

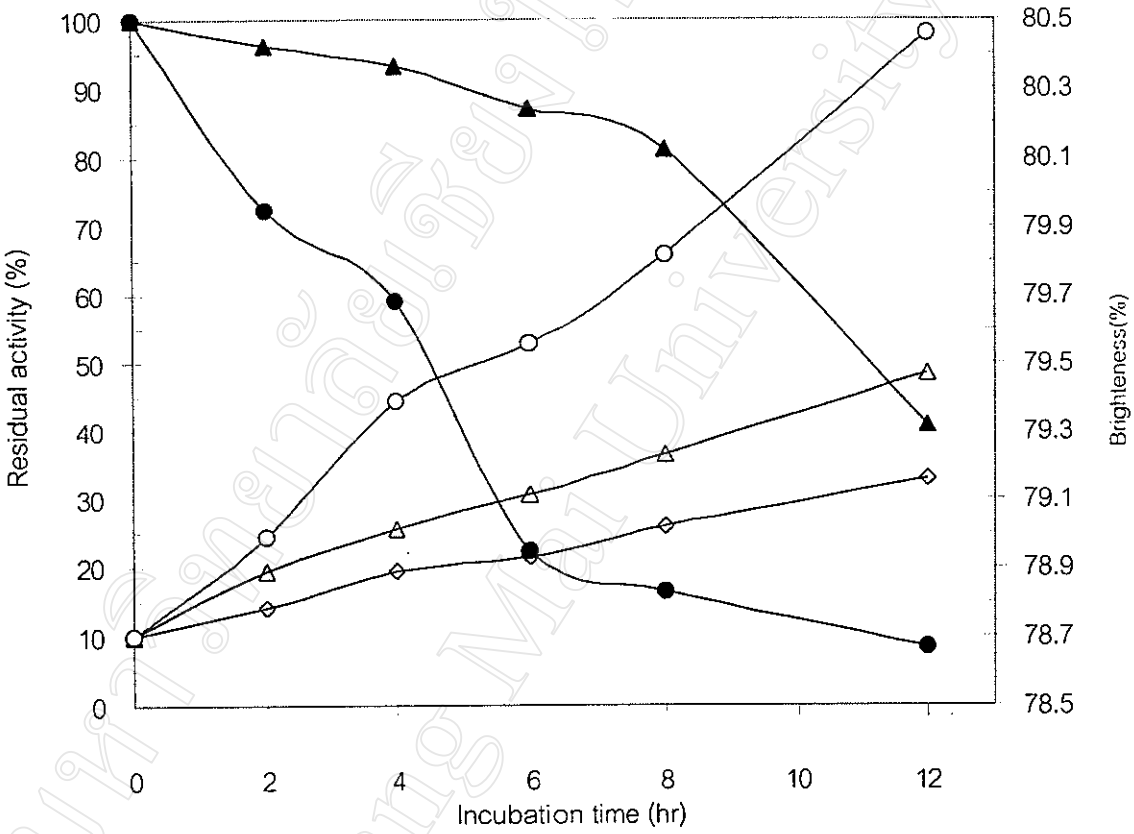

Fig. 3.25 Bleaching of eucalyptus oxygen-delignified kraft pulp with the purified laccase from *Lenzites* sp. NP21.

Note: ◇ ;The brightness of control

△ ;The brightness of enzyme added

○ ;The brightness of enzyme and HBT added treatment

▲ ;Residual enzyme in enzyme added

● ; Residual enzyme in enzyme and HBT added treatment