CONTENTS

	Page
Acknowledgement	iii
Abstract	v
Contents	ix
List of illustrations	xii
List of tables	xvi
Abbreviations and symbols	xviii
Chapter 1 Introduction	1
Literature review	3
Pulp and paper industry and impacts on environment	3
Lignin	12
The nature of residual lignin in kraft pulp	16
Relation of white rot fungi and basidiomycetes	20
Role of lignin degrading enzymes in kraft pulp bleaching	23
Lignin peroxidase	23
Manganese peroxidase	24
Laccase	25
Role of Laccase-mediator system on the paper pulp bleaching	29
Obstacle to implementing bleaching with oxidative enzymes	32
Application of white rot fungi on pulp and paper industry	33
Evolution of paper pulp biobleaching by microorganisms	43
Chapter 2 Experiments	48
2.1 Chemical reagents and materials	48
2.1.1 Chemical reagents	48
2.1.2 Media	49

	2.1.3	Equipment	51
2.2	Method	s . \Diamond	51
	2.2.1	Mycelial induction and isolation of basidiomycetes	51
	2.2.2	Screening of basidiomycetes on PDA plate at 37°C	51
	2.2.3	Effect of glucose on growth and decoloration of Poly	
		R-478 and study of using lignin powder as indicator	52
	2.2.4	Study of lignin degrading enzyme production	
		in liquid medium	52
	2.2.5	Enzyme assay	52
	2.2.6	Selection of laccase producer on solid culture	53
	2.2.7	Studies of factors affected on laccase production	53
	a) Effects of veratryl alcohol and Tween-80 on	
		laccase production	53
	b) Effects of glucose and peptone concentration	
		on laccase production	55
	c) Optimal temperature for laccase production	56
	d) Optimal initial pH for laccase production	56
	e) Effects of veratryl alcohol and Tween-80 on	
		laccase production at optimal condition	56
	f	Influence of copper induction on laccase production	56
	2.2.8	Purification of the isolate NP21 laccase	57
	2.2.9	SDS-PAGE analysis	57
	2.2.10	Characterization of laccase from the isolate NP21	57
	a)	pH profile	57
	b	pH stability	58
	c)	Thermostability	58
	d)	Substrate specificity	58
	e)	Effects of metal ions and inhibitors on laccase activity	58

		2.2.11 Preliminary of pulp biobleaching by laccase	59
		a) Pretreatment of pulp	59
		b) Pulp treatment by purified laccase	60
	_		
Chapter 3		alts and Discussions	61
	3.1	Isolation of basidiomycetes	61
	64		
	3.3	Influences of glucose on growth and decoloration of	
		Poly R-478 and utilization of lignin powder using as	
		indicator for screening of ligninolytic fungi	65
	3.4	Screening of lignin degrading enzymes producer by	
		direct determination of enzyme activity	71
	3.5	Selection of laccase producing strain on solid culture	75
	3.6	Some effects on laccase production from Lenzites sp. NP21	80
		3.6.1) Effect s of veratryl alcohol and Tween-80 on	
		laccase production	80
		3.6.2) Effects of glucose and peptone concentration	
		on laccase production	86
		3.6.3) Optimal temperature for laccase production	88
		3.6.4) Effect of initial pH on laccase production at optimal	89
		3.6.5) Effects of veratryl alcohol and Tween-80 on laccase	
		production at optimal condition	90
		3.6.6) Effect of copper induction on laccase production	91
	3.7.	Purification of laccase of Lenzites sp. NP21 Laccase	93
	3.8	SDS-PAGE analysis	94
	3.9	Characterization of purified laccase	97
		3.9.1 pH profile	97
		3.9.2 Thermal stability	98
		3.9.3 pH stability	100

	3.9.4	Substrate specificity	10
	3.9.5	Effects of metal ions on laccase activity	104
	3.9.6	Effects of inhibitors on laccase activity	105
3	.10 Prelim	inary study of pulp biobleaching by laccase	107
Chapter 4 Co	nclusions		109
References			111
Appendix			124
A	ppendix 1.	Stock solutions preparation for SDS-PAGE analysis	124
A	ppendix 2.	Formulations of SDS-PAGE resolving gel	125
A	ppendix 3.	Formulations of SDS-PAGE stacking gel	126
A	ppendix 4.	Calibration protein mixture	126
A	ppendix 5.	Estimation of molecular weigh of laccase from Lenzites	
		sp. NP21 based on standard proteins calibration curve	127
A	ppendix 6.	Beer's law and enzymatic activity calculation	128
() A	ppendix 7.	Structures of aromatic compounds	132
A	ppendix 8.	Structure of hypha	134
Curriculum Vi	tae [©]		135

LIST OF ILLUSTRATIONS

Figure		Page
Figure 1.1	The distribution of wood components and lignin within cell wall. A;	
	tracheids, B; cell wall layers and C; ultrastructural arragement of	13
Figure 1.2	The lignin precursors p-coumaryl alcohol, couniferyl alcohol and	
	sinnapyl alcohol.	14
Figure 1.3	The structure of spurce lignin.	15
Figure 1.4	Dominant substructures in the residual lignin of kraft pulps. I. β -O-4'	
	alkyl-aryl ether. II. α -ether linkage to carbohydrate. III. β -5'	17
Figure 1.5	Demethylation and bleaching of hard wood kraft pulp by manganese	
	peroxidase.	19
Figure 1.6	Hypha of basidiomycete with clamp connection of Coriolus versicolor,	,
	a white rot fungus (A) and Poria placenta, a brown rot fungus (B).	21
Figure 1.7	Classification of basidiomycete fungi.	22
Figure 1.8	Hypothetical scheme for lignin degradation by lignin peroxidase with	
	veratryl alcohol as mediator.	23
Figure 1.9	Oxidative pathway for catalytic action of manganese peroxidase on	
	lignin.	24
Figure 1.10	Oxidative pathway for catalytic action of laccase on lignin.	25
Figure 1.11	Oxidation of hydroquinone by laccase to give a phenoxy radical which	
	proportionate into hydroquinone and p-benzoquinone.	26
Figure 1.12	Laccase mechanism acts on a phenolic β -1 syringyl lignin substructure	
	by laccase from C. versicolor.	27
Figure 1.13	Three dimentional structure of a copper amine oxidase (laccase)	
	showing the two monomers of the homodimer color coded in blue	28
Figure 1.14	Schematic representation of lignin degradation by laccase and mediator	
6	system. Structure of two mediators used in LMS for kraft pulp	30

Figure 1.15	Computer simulation of a cross-section through the secondary wall		
	of an unbleached kraft fiber in proportional comparison with putative	31	
Figure 1.16	Schematic of proposed biopulping process.		
Figure 3.1	The typical pattern of Poly R-478 decoloration (right) compared to		
	control (left).	63	
Figure 3.2	Distribution of 42 isolates separated by growth capability at 37°C		
	on PDA plate.	64	
Figure 3.3	Decoloration of Poly R-478 by NP21 and RC3 compared with the		
	reference strains, P. chrysosporium ATCC 34541 and C. versicolor	68	
Figure 3.4	Decoloration of lignin powder containing in modified Pointing's		
	medium by the isolate NP21 and RC3 compared to the reference	69	
Figure 3.5	Effects of glucose on decoloration of Poly R-478 by the reference		
	strain, P. chrysosporium ATCC 34541 and the new isolate NP11	70	
Figure 3.6	Fruiting bodies of the isolate NP21.	79	
Figure 3.7	Spore shape of the isolate NP21(x100).	79	
Figure 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	81	
Figure 3.9	Effects of veratryl alcohol and Tween-80 on laccase production in		
	liquid medium containing 0.02% (w v) lignin powder.	82	
Figure 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.	83	
Figure 3.11	Comparison between effects of veratryl alcohol and Tween-80, and		
	4% (w/v) glucose and 4% (w/v) peptone supplemented on laccase	85	
Figure 3.12	Effects of glucose and peptone concentration on laccase production by		
	Lenzites sp. NP21 from rubber wood chips at 37°C.	87	
Figure 3.13	Optimal temperature for laccase production on rubber wood chips by		
	Lenzites sp. NP21 at 37°C.	88	

Figure 3.14	Effects of initial pH on laccase production by Lenzites sp. NP21 on	
	solid culture at 37°C.	89
Figure 3.15	Effects of veratryl alcohol and Tween-80 on laccase production on	
	rubber wood chips by <i>Lenzites</i> sp. NP21 at 37°C.	90
Figure 3.16	Effect of copper induction on laccase production from rubber wood	
	chips by Lenzites sp. NP21 at 37°C.	92
Figure 3.17	DEAE-cellulose column chromatography of laccase from Lenzites sp.	
	NP21.	93
Figure 3.18	Sephadex G-100 gel filtration column chromatography of the purified	
	laccase from Lenzites sp. NP21.	94
Figure 3.19	Sodium dodecyl sulfate polyacrylamide gel electrophoresis of purified	
	laccase from Lenzites sp. NP21 (Lane I). The calibration protein	95
Figure 3.20	Optimal pH of laccase activity from Lenzites sp.NP21.	97
Figure 3.21	Thermostability of purified laccase after incubation at various	
	temperature for 1 hr.	98
Figure 3.22	Time course of heat inactivation of the purified laccase at 55, 60,	
	and 65°C.	99
Figure 3.23	pH stability of purified laccase from Lenzites sp. NP21 after	
	incubation at various pH at 4°C for 24 hrs.	100
Figure 3.24	Substrate specificity and pH profile of various substrates of laccase	
	from Lenzites sp. NP21.	103
igure 3.25	Bleaching of eucalyptus oxygen-delignified kraft pulp with the purified	
	laccase from Lenzites sp. NP21.	108
	•	

LIST OF TABLES

Table		Page
Table 1.1	Amount of chemicals uses of pulp bleaching per ton of pulp	4
Table 1.2	Characteristics of bleached Chithermomechanical pulping (CTMP)	7
14010 1.2	wastewater wastewater	_
Table 13		6
Table 1.3	Characteristics of bleach plant extraction-stage effluent in India	7
Table 1.4	Regulatory emission limits for pulp mills in Sweden	8
Table 1.5	Regulatory emission limits for pulp and paper mills in Germany	8
Table 1.6	Discharge limits for pulp and integrated paper mills in Portugal	9
Table 1.7	AOX discharge limits in Canada	9
Table 1.8	Discharge limits for pulp and paper mills in Japan	10
Table 1.9	Effluent standards for pulp and paper industry in India	10
Table 1.10	Effluent limitation of pulp mill in China	10
Table 1.11	Effluent discharge standard in South-East Asia	11
Table 1.12	Energy saving from biomechanical pulping of loblolly pine chips with	
	different white rot fungi (4-week incubation)	35
Table 1.13	Bleaching conditions and optical properties of conventionally	
	bleached and biobleached commercial SWKP	36
Table 1.14	Differences in xylanase and laccase/mediator treatment	37
Table 1.15	Effect of Rhizopus oryzae treatment on chlorophenols and chloroaldehydes	i
	in extraction-stage effluent	37
Table 1.16	Current status of biotechnology in the pulp and paper industry	42
Table 2.1	The components containing in each treatment in liquid medium	54
Table 2.2	The components containing in each treatment on solid culture	·54
Table 2.3	Amount of glucose and peptone (w/v) for applying into rubber wood chips	56
Γable 3.1	Effects of glucose on mycelial growth and decoloration of Poly R-478	
	and lignin powder at 37°C after 4 d cultivation	66

Table 3.2	Enzyme activity (U/I) of MnP, MIP, Laccase and LiP, and total enzyme activity		
	in culture broth	72	
Table 3.3	Comparison the efficiency of lignin degrading basidiomycetes screening		
	method by using decoloration of Poly R-478	74	
Table 3.4	Laccase production by 42 isolates when cultivated on rubber wood chips		
	at 37°C for 9 d	76	
Table 3.5	Characteristic identification of NP21	78	
Table 3.6	Classification of the new isolate NP21 (Lenzites sp.)	78	
Table 3.7	Comparison of the highest laccase activity in each treatment	86	
Table 3.8	Purification of laccase from Lenzites sp. NP21	96	
Table 3.9	Relative activity of oxidation of various substrates by laccase from NP21	102	
Table 3.10	Effects of various metal ions on the laccase activity	104	
Table 3.11	Effects of inhibitors on oxidation of DMP by the purified laccase	105	

ABBREVIATIONS OF SYMBOLS

Fig. figure

g gram

w/v weight by volume

M molar

l liter

μm micrometer

nm nanometer

A absorbance

kDa kilodalton

M_w molecular weight

MiP manganese independent peroxidase

LiP lignin peroxidase

MnP manganese peroxidase

d day

hr hour

sec second

min minute

C degree Celsius

% percent

~ approximately

R&D research and development

Trt treatment

DMP 2,6- dimethoxyphenol

ABTS 2,2'-azono-di

(3-ethylbenzothialozin-6-sulfuric acid)