MATERIALS AND METHODS

1. Physiological studies on the production of lignin-degrading enzymes by a macrofungus, *Ganoderma* sp. KU-Alk4

1.1 Production of the enzymes in liquid medium

1.1.1 Fungal strain

Ganoderma sp. designated as KU-Alk4, a new isolate from a living tree, *Terminalia bellerica* Roxb., at Kasetsart University, Thailand, was used. The fungus was maintained on a potato dextrose agar (PDA) slants and stored at 4ºC. Longer storage was the solid state cultivation on rice grain solid state at room temperature.

1.1.2 Culture conditions for lignin-degrading enzymes production

The fungus was grown on PDA plates at 30ºC for 4 days. Fifteen plugs of 5-mm diameter from the growing edge of mycelia were used as inoculum in 250 mL Erlenmeyer flasks containing 50 mL Kirk's liquid medium (Tien and Kirk, 1988). The original medium contained 1% glucose and 0.02% $CuSO_4$. Initial pH of the media was 7.0. Where was no pH control during the cultivation. The culture was grown unshaken at 30ºC for 3 days. Then, an inducer, 0.85 mM of veratryl alcohol was added to the medium. The culture was continued incubated shaking on rotary shaker at 140 rpm until its peak of enzyme activity. A 1 mL of sample was taken from the flask every day and centrifuged at 10,000 × g for 15 min at 4ºC. Supernatant was used to determine the enzymatic activities of laccase, manganese peroxidase (MnP) and lignin peroxidase (LiP).

Preliminary study on the best condition for production of lignin-degrading enzymes was determined in 3 different conditions which were static culture, 3-day static culture then shaken, and culture shaken throughout the experiment.

1.1.3 Enzyme activities assay

A modified method of Kondo *et al*. (1994) was used for the lignin-degrading enzymes, laccase, MnP and LiP, assays. Laccase activity was assayed by monitoring the oxidation of 2,6-dimethoxyphenol (DMP) at 469 nm. The reaction mixtures contained 1 mL of 3.0 mM DMP in 50 mM malonate buffer pH 4.5, 1.5 mL of 50 mM malonate buffer pH 4.5 and 0.5 mL of supernatant.

MnP activity was assayed by monitoring the oxidation of DMP at 469 nm. The reaction mixture contained 1 mL of 3.0 mM DMP, 1 mL of 3.0 mM $MnSO_4$, 100 µl of 6 mM H_2O_2 and 0.8 mL of 50 mM malonate buffer pH 4.5. All chemicals were dissolved in 50 mM malonate buffer pH 4.5. The reaction was initiated by the addition of 100 µl of supernatant.

LiP activity was assayed by monitoring the oxidation of veratryl alcohol at 310 nm. The reaction mixture contained 2 mL of 1.5 mM veratryl alcohol, 100 µL of 0.2 mM H_2O_2 and 0.8 mL of 50 mM malonate buffer pH 4.5. All chemicals were dissolved in 50 mM malonate buffer pH 4.5. The reaction was initiated by the addition of 100 µl of supernatant.

All of the reaction mixtures were 3 mL and the reactions were performed at 25ºC. Enzyme activity was assayed by following the absorbancy change within the initial 3 min. One unit (U) of either laccase, MnP or LiP was defined as the amount of enzyme that caused 1.0 increase in the absorbancy per minute.

1.2 Physiological aspects of the regulation of lignin-degrading enzymes synthesis

Regulation of the enzymes production was investigated in liquid medium. The fungus was grown on PDA plates at 30ºC for 4 days. Fifteen plugs of 5-mm diameter from the growing edge of mycelia were used as inoculum in 250 mL Erlenmeyer flasks containing 50 mL Kirk's medium. The cultures were incubated at 30ºC during cultivation for 9 days, the time at which the maximal enzyme activities were observed. The conditions were varied in each experiment. One mL of the contents of three flasks, as replicates, were randomly taken everyday.

1.2.1 Regulation of lignin-degrading enzymes production by pH

The initial pH of media was 4.5, 7.0 or 8.0. pH was not controlled during the cultivation. The culture was grown unshaken for 3 days. Then, an inducer, 0.85 mM of veratryl alcohol was added to the medium. The culture was continued incubated, now shaken on rotary shaker at 140 rpm for 6 days.

1.2.2 Regulation of laccase production by induction control

Induction controlled regulation was studied in pH 8.0-medium with and without addition of 0.85 mM veratryl alcohol either at the beginning of the cultivation or on the 3rd day of culture. The culture was grown unshaken for 3 days and continued shaken at 140 rpm for 6 days.

1.2.3 Induction of laccase production by different phenolic compounds

Low molecular weight phenolic compounds, veratryl alcohol, guaiacol and 2,6-dimethoxy phenol (DMP) were tested as inducers of laccase production. The fungus was grown in pH 8.0-medium unshaken for 3 days. Then, each of above inducers was added to the final concentration of 0.85 mM. The culture was continued incubated shaken at 140 rpm for 6 days.

1.2.4 Effect of carbon sources

The fungus was grown in pH 8.0-medium. Glucose, lactose and carboxymethylcellulose (CMC) at 1% were compared as carbon sources. The culture was grown unshaken for 3 days, and then an inducer, 0.85 mM of veratryl alcohol, was added to the medium. The culture was continued incubated shaken at 140 rpm for 6 days.

1.2.5 Regulation of laccase production by catabolite repression control

The fungus was grown in pH 8.0-medium. Effect of glucose concentration at 0.1, 0.5, 1, 2, 3 or 4% (w/v) was examined. The culture condition was as described in 1.2.4.

1.2.6 Effect of metal ions

Effects of metal ions such as Cu^{2+} and Mn^{2+} ions were observed at different concentrations. The fungus was grown in pH 8.0-medium with $CuSO_4$ at final concentration either of 0, 0.02, 0.2 or 2.0 mM. Effect of Mn^{2+} ion was studied by adding $MnSO_4$ to the medium at final concentration either of 0, 0.5, 1.0 or 2.0 mM. The culture condition was as described in 1.2.4.

1.2.7 Effect of aeration on laccase production

The fungus was grown in pH 8.0-medium containing 1% glucose and 0.2% $CuSO_4$. The culture was grown unshaken for 3 days. Then an inducer, 0.85 mM of veratryl alcohol was added to the medium. The culture was continued incubated, shaken at 100, 120 or 140 rpm for 6 days.

1.2.8 Analytical determinations

Cell dry weights were determined by filtering the cultures through filter paper (Whatman no. 4). The filters containing the mycelial mass from 3 replicates were rinsed with 100 mL of distilled water and dried at 60ºC to a constant weight to obtain the cell dry weight. The supernatant was treated as crude lignin-degrading enzymes.

Lignin-degrading enzymes activities were assayed as previous described in 1.1.3.

Protein concentration was measured following Lowry *et al*. (1951) with bovine serum albumin as a standard.

Laccase isozymes were detected on zymograms. The 16 µg protein of crude enzymes from the 9-day-culture were separated using active polyacrylamide gel electrophoresis (PAGE) (Laemmli *et al*., 1970) which was performed under nondenaturing conditions. The separating gel contained 10% acrylamide, and the buffer solution was 300 mM Tris-HCl, pH 8.8. The stacking gel contained 5% acrylamide and the buffer solution was 125 mM Tris-HCl, pH 6.8. The electrode buffer solution contained 50 mM Tris-HCl and 380 mM glycine, pH 8.3. The gel was previously equilibrated in 100 mL of 50 mM malonate buffer, pH 4.5 for 10 min at 25ºC, washed with the same buffer. Active band of the enzymes was visualized using 150 mL of 1 mM DMP in 50 mM malonate buffer, pH 4.5. The gel was incubated on reciprocal shaker at 50 rpm, 25ºC, for 15 min.

1.3 Production of the enzymes on solid substrate

1.3.1 Various kinds of solid substrate

Grains of corn, sweet sorghum and rice were boiled at 100ºC for 3 minutes and dried at 45ºC for 3 h. After that, 130 g of each kind of grains was packed into glass bottle, size 4 x 8 x 18 cm, then, sterilized at 121ºC for 15 minutes. Initial pH of the solid substrate was 7.0 or 8.0. Where was no pH controlling during the cultivation. Inoculum was the fungus grown on PDA plates at 30ºC for 4 days. Fifteen plugs of 5-mm diameter from the growing edge of mycelia were inoculated in the glass bottle containing grain. The culture was grown in static condition at 30ºC for 9 days. Lignin-degrading enzymes were extracted by adding 495 µL of toluene dissolved in 150 mL of 50 mM malonate buffer, pH 4.5, and incubated at 4ºC for 12 h before solution extraction. The extracted solution was separated from culture by filtration through filter paper Whatman no. 4. The filtrate was treated as crude enzyme to determine enzyme activities.

1.3.2 Effect of 0.85 mM veratryl alcohol on enzyme production on solid substrate

Induction controlled lignin-degrading enzymes production on solid substrate was studied with and without addition of an inducer, veratryl alcohol. Addition of veratryl alcohol was either at the beginning of the cultivation or on the 3rd day of culture. Amount of veratryl alcohol was 1 mL of 0.85 mM, dissolved in distilled water. The culture was incubated at 30º for 9 days.

2. Improvement of lignin-degrading enzymes production from *Ganoderma* sp. KU-Alk4 by medium engineering

2.1 Chemicals

Veratryl alcohol was purchased from Fluka (Buchs, CH). Other chemicals were analytical grade and purchased from Sigma (Poole, UK) unless otherwise indicated.

2.2 Media design and composition

The medium design was based on Kirk's liquid medium. Non-optimized medium that was used as control, contained 10 g/L glucose and 0.22 g/L ammonium tartrate as C- and N-sources with 0.85 mM veratryl alcohol as an inducer. The pH of control medium was not controlled through the culture period. Medium composition was statistical design and experimental modified by changing the nature and concentration of carbon and nitrogen sources, pH and inducers. The pH of the modified media was controlled throughout the culture period with 0.1 M citrate-phosphate buffer.

The design was confirmed with the fungal culture of fifteen plugs of 5-mm diameter grown on PDA at 30ºC, 4 days, inoculated in 50 mL medium. The cultures were incubated at 30ºC in static condition for 3 days. On the 3rd day, an inducer was added and the culture continued, grown with shaking at 30ºC. Samples were taken from the flask everyday and centrifuged at 10,000 rpm, 4ºC, for 15 min. Supernatant was used for the determination of laccase activity.

2.3 Enzyme assay

Laccase activity was determined spectrophotometrically (Perkins Elmer Lambda 29) by the oxidation of the 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) at 415 nm (ε =3.6 × 10^4 M^{-1} cm^{-1}). The assay mixture contained 50 µL of supernatant, 200 µL of 2.5 mM ABTS in 0.1 M sodium tartrate buffer, pH 4.5, and 950 µL of 0.1 M sodium tartrate buffer, pH 4.5. One unit of laccase activity, so-called 1 International Unit (IU), is defined as the amount of enzyme required to oxidize 1 µmol of ABTS per min at 25°C.

2.4 Experimental design and evaluation

Box-Behnken design was used in the medium engineering. Effects of seven factors such as pH, C-source, concentration of C-source, N-source, concentration of N-source, inducer type and concentration were statistically tested for the best combination of these factors on laccase produced by *Ganoderma* sp. KU-Alk4. The optimization was based on Box–Behnken design with 66 combinations and ten replications of the centre point. (Box, 1965).

Table 12 shows the factor codes and natural values used in this experiment. The factors are prescribed into three levels, coded -1, 0, and +1 for low, middle and high concentration (or value). Factors at 3 different levels were selected based on results of previous study in 50 mL medium without any pH controlled. The pH levels were 4.0, 6.0, and 8.0, and the coded values were -1, 0, and +1, respectively. Similarly, types of C-source were glucose, lactose, and glycerol. Types of N-source were ammonium tartrate, yeast extract, and malt extract. The concentrations for C-source were set at 10, 25, and 40, g/L and for N-source, at 0, 0.22, and 0.44, g/L. Inducer types were veratryl alcohol, guaiacol, and ferulic acid. The inducer concentrations were set at 0, 0.85, and 1.7 mM.

Table 13 represents the design matrix of the 66 trials experiment. For predicting the optimal point, a second order polynomial function was fitted to correlate relationship between variables and response laccase activity.

2.5 Statistical analysis

For optimizing purposes, various medium components and culture parameters have been evaluated. A mathematical model describing the relationships between laccase produced and the medium component contents in second-order equation was developed.

The laccase activity produced by *Ganoderma* sp. KU-Alk4 was multiply regressed with respect to the medium component contents by the least squares method as follows:

$$Y = A_0 + \sum A_i X_i + \sum A_{ii} X_i^2 + \sum A_{ij} X_i X_j \qquad \text{(Eq. 1)}$$

Where Y is the predicted response variable (laccase produced); A_0, A_i, A_{ii}, A_{ij} are constant regression coefficients of the model and *Xi, Xj* (*i = 1, 2,..7*; *j = 1, 2,..7*). The coefficients represent the independent variables (medium composition) in the form of coded values. The accuracy and general ability of the above polynomial model could be evaluated by the coefficient of determination R^2. All experimental designs were randomized to exclude any bias.

The analysis of regression and variance (ANOVA) was carried out using the experimental design of the Statistica 7 (StatSoft Inc, USA).

Table 12 Experimental design combinations for the factors and levels used for the optimization of laccase production based on substrates, inducers and concentrations.

Key	Factor	Levels		
		Low	Medium	High
		-1	0	+1
X_1	pH[a]	4[a]	6[a]	8[a]
X_2	Carbon source[a, h]	Glucose[a, b]	Lactose[a, c]	Glycerol[a, c]
X_3	Carbon source concentration[a, h]	10[a, b] g/L	25 g/L	40[a] g/L
X_4	Nitrogen source[c, d, h]	Ammonium tartrate[b]	Yeast extract[c, e]	Malt extract
X_5	Nitrogen source concentration[f, g]	0 g/L	0.22[b] g/L	0.44 g/L
X_6	Inducer type[a, h, k]	Veratryl alcohol[a, i]	Guaiacol[a, c, j, k]	Ferulic acid[c, j, k]
X_7	Inducer concentration[a, c, i]	0 mM	0.85 mM[a]	1.7 mM

[a] previous study by our group, [b] original composition of Kirk's medium,
[c] Revankar and Lele (2006), [d] Stajic *et al*. (2006), [e] Nyanhongo *et al*. (2002)
[f] D'souza *et al*. (1999), [g] Vasconcelos *et al*. (2000), [h] Galhaup *et al*. (2002)
[i] Dekker and Barbosa (2001), [j] Herpoël *et al*. (2000), [k] Arora and Gill (2001)

Table 13 Seven factors in three levels Box–Behnken design, ten replications of the centre point used to design the best medium for *Ganoderma* sp. KU-Alk4.

Runs	X_1	X_2	X_3	X_4	X_5	X_6	X_7	Runs	X_1	X_2	X_3	X_4	X_5	X_6	X_7
1	0	0	0	-1	-1	-1	0	30	+1	-1	0	+1	0	0	0
2	0	0	0	+1	-1	-1	0	31	-1	+1	0	+1	0	0	0
3	0	0	0	-1	+1	-1	0	32	+1	+1	0	+1	0	0	0
4	0	0	0	+1	+1	-1	0	33	0	0	-1	-1	0	0	-1
5	0	0	0	-1	-1	+1	0	34	0	0	+1	-1	0	0	-1
6	0	0	0	+1	-1	+1	0	35	0	0	-1	+1	0	0	-1
7	0	0	0	-1	+1	+1	0	36	0	0	+1	+1	0	0	-1
8	0	0	0	+1	+1	+1	0	37	0	0	-1	-1	0	0	+1
9	-1	0	0	0	0	-1	-1	38	0	0	+1	-1	0	0	+1
10	+1	0	0	0	0	-1	-1	39	0	0	-1	+1	0	0	+1
11	-1	0	0	0	0	+1	-1	40	0	0	+1	+1	0	0	+1
12	+1	0	0	0	0	+1	-1	41	-1	0	-1	0	-1	0	0
13	-1	0	0	0	0	-1	+1	42	+1	0	-1	0	-1	0	0
14	+1	0	0	0	0	-1	+1	43	-1	0	+1	0	-1	0	0
15	-1	0	0	0	0	+1	+1	44	+1	0	+1	0	-1	0	0
16	+1	0	0	0	0	+1	+1	45	-1	0	-1	0	+1	0	0
17	0	-1	0	0	-1	0	-1	46	+1	0	-1	0	+1	0	0
18	0	+1	0	0	-1	0	-1	47	-1	0	+1	0	+1	0	0
19	0	-1	0	0	+1	0	-1	48	+1	0	+1	0	+1	0	0
20	0	+1	0	0	+1	0	-1	49	0	-1	-1	0	0	-1	0
21	0	-1	0	0	-1	0	+1	50	0	+1	-1	0	0	-1	0
22	0	+1	0	0	-1	0	+1	51	0	-1	+1	0	0	-1	0
23	0	-1	0	0	+1	0	+1	52	0	+1	+1	0	0	-1	0
24	0	+1	0	0	+1	0	+1	53	0	-1	-1	0	0	+1	0
25	-1	-1	0	-1	0	0	0	54	0	+1	-1	0	0	+1	0
26	+1	-1	0	-1	0	0	0	55	0	-1	+1	0	0	+1	0
27	-1	+1	0	-1	0	0	0	56	0	+1	+1	0	0	+1	0
28	+1	+1	0	-1	0	0	0	57-66	0	0	0	0	0	0	0
29	-1	-1	0	+1	0	0	0								

3. Properties of the crude enzymes

Crude laccase from *Ganoderma* sp. KU-Alk4 was prepared by culturing the fungus in pH 8.0-medium contained 0.2 mM $CuSO_4$. Glucose at of 1% (G1%) and 4% (G4%) was used as carbon source to compare their enzyme properties. The culture was incubated at 30ºC in static conditions for 3 days. On the third day, 0.85 mM final concentration of veratryl alcohol was added as inducer of laccase production, then culture was continued in shaking condition at 120 rpm.

3.1 Optimum pH and pH stability

3.1.1 DMP was used as substrate to determine optimum pH. Reaction mixture of the enzymes with 3 mM DMP was incubated in 50 mM buffers at various pH values. Buffers were hydrochloric acid-potassium chloride (pH 2.0-2.5), glycine-HCl (pH 2.5-3.5), malonate (pH 3.0-4.5), citrate phosphate (pH 3.0-7.0), phosphate (pH 6.0-8.0), Tris (hydroxymethyl) aminomethane (pH 7.5-9.0) and glycine-NaOH (pH 9.0-10.0).

3.1.2 pH stability was examined with the enzymes preincubated in 50 mM buffer of different pH, 2.0-10.0, at 25ºC for 1 h. After 1 h incubation, the laccase activity was assayed by using DMP as substrate at its optimum pH. Buffers were as described in 3.1.1.

3.2 Optimum temperature and temperature stability

3.2.1 DMP was used as substrate to determine optimum temperature. Reaction mixture of the enzymes with 3 mM DMP was incubated in 50 mM malonate buffer pH 4.5 at various temperatures. Optimum temperature was determined within the range of 20-95°C using spectrophotometer Libra S12 (Biochrom).

3.2.2 The enzyme stability was done by preincubating the enzyme solution at various temperatures, 20-95°C, for 1 h. Remaining activity of laccase was assayed at optimum temperature.

4. Biotechnological use of the laccase from *Ganoderma* sp. KU-Alk4

4.1 Dye decolorization by using crude enzymes

4.1.1 Dyes: Twelve reactive dyes were Congo Red, Direct Blue 15, Direct Red 23, Direct Yellow 12, Reactive Red 4, Reactive Yellow 2, Ponceau S, Bromophenol blue, Crystal Violet, Malachite Green, Indigo Carmine, Reactive Blue 15. Wavelengths resulting in maximum absorbance (λ_{max}) of each dye were shown in Table 14.

4.1.2 Decolorization procedure: Crude enzymes from G1% and G4% culture were used. Crude enzyme, 180 IU, 0.3 mL was added into 2.7 mL of 25 mg/L dye dissolved in 50 mM malonate buffer, pH 3.5. The reaction mixtures were incubated at 25ºC, shaken in reciprocal shaker at 200 rpm for 6 h. Decolorization of dye was determined as the decreasing of the dye maximum absorbancy. The reactions were done in three replicates.

Table 14 Wavelengths resulting maximum absorbance (λ_{max}) of various dyes.

Dye	λ_{max} (nm)
Azo dye	
Congo Red	570
Direct Blue 15	615
Direct Red 23	560
Direct Yellow 12	420
Reactive Red 4	540
Reactive Yellow 2	410
Azoic dye	
Ponceau S	540
Triphenylmethane dye	
Bromophenol blue	590
Crystal Violet	630
Malachite Green	650
Indigoid dye	
Indigo Carmine	610
Phthalocyanine dye	
Reactive Blue 15	620

4.2 Dye decolorization using immobilized laccase entrapped in copper alginate

4.2.1 Chemicals

Alginate is a co-polymer of D-mannuronate and L-guluronate. Di- or polyvalent cations cause gelation by cross-linkage of L-guluronate residues (Haug *et al*., 1967). Two types of alginate, type A and B, were used. Type A is a high guluronate alginate from *Laminaria digitata* (BDH Chemicals Ltd.) which contains around 70% L-guluronate. Type B is a high mannuronate alginate from *Macrocystis pyrifera* (low viscosity, Sigma Chemical Company) which contains less than 30% L-guluronate (Bucke, 1987). Other chemicals were of analytical grade and purchased from Sigma Chemical Company.

4.2.2 Fungal laccase

Lyophilized laccase of a *Ganoderma* sp. designated as KU-Alk4 was used. *Ganoderma* sp. KU-Alk4 secretes laccase as a dominant protein in a specific medium of Kirk at 1% glucose. No other lignin-degrading enzymes are produced at pH 8.0. Laccase activity was determined spectrophotometrically (Perkins Elmer Lambda 29) by the oxidation of the ABTS.

4.2.3 Enzyme immobilization

Standard immobilization procedure was used. The lyophilized laccase was dissolved in distilled water to a final activity of 0.1 IU/mL. Sodium alginate powder (type A), 3.0% w/v, was added to the enzyme solution and then the mixture was stirred thoroughly to ensure complete mixing. The mixture was forced through a needle (18 gauge) with a peristaltic pump at the flow rate of 8 mL/min into 0.15 M cross linking solutions, $CuSO_4$ dissolved in distilled water. Spherical beads of Cu-alginate that entrapped the enzyme inside were formed. After 30 min the spherical beads were separated and washed with distilled water. The immobilization yield was

determined as residual laccase activity found after dissolution of beads by incubating in 0.1 M sodium tartrate buffer pH 4.5 for 15 min at 4ºC and the activity in the bead was compared with that added to the alginate solution.

The same procedure was used to study effects of cross-linking agents which were 0.15 M $CuSO_4$, $CaCl_2$ or $AlCl_3$ and the combination of 2 ions, each of 0.075 M.

4.2.4 Experimental design and evaluation

Immobilization design was based on the standard procedure to produce Cu-alginate beads. Effects of alginate composition, concentrations of alginate and cross-linking agent ($CuSO_4$) were tested. A two 3×3 Latin Square with 3 factors at 3 levels each was used in the experimental design to obtain the most effective Cu-alginate beads for dye decolorization by the immobilized laccase of *Ganoderma* sp. KU-Alk4. Table 15 shows factors coded and natural values used in this experiment. The factors are prescribed into 3 levels, coded -1, 0, and +1 for low, middle and high concentration (or value). Alginates type A and B, which have low and high mannuronate contents, respectively, were used. The alginate compositions, defined as A:B ratios, were set at 100:0, 50:50, and 0:100 % w/w. Alginate solutions at 1.5, 3.0 and 4.5% w/v were used to form beads using 0.075, 0.15 and 0.225 M $CuSO_4$ as cross-linking agent. The design matrix of the 9 trial experiments is represented in Table 16. Each experiment was carried out with 3 replicates and the results were evaluated statistically.

Table 15 Experimental design combinations for factors and levels used for optimisation of the laccase entrapment in Cu-alginate bead based alginate composition and, concentration and copper sulphate as cross-linking agent.

Key	Factors	Levels		
		Low	Medium	High
		-1	0	+1
F_1	Alginate type, A:B[a] (w/w)	100:0	50:50	0:100
F_2	Alginate concentration (% w/v)	1.5	3.0	4.5
F_3	$CuSO_4$ concentration (M)	0.075	0.15	0.225

[a] Alginate type A from BDH Chemicals Company

[b] Alginate type B from Sigma Chemical Company

Table 16 The coded and Latin Square Experimental Design

Treatment	Factors		
	F_1	F_2	F_3
1	-1	-1	-1
2	-1	0	0
3	-1	+1	+1
4	0	-1	0
5	0	0	+1
6	0	+1	-1
7	1	-1	+1
8	+1	0	-1
9	+1	+1	0

4.2.5 Dye decolorization

Free and immobilized laccase, as designed, each of 45 IU, 0.3 mL were added into 2.7 mL of 25 mg/L Indigo Carmine dissolved in distilled water. The reaction mixtures were incubated at 25ºC, stirred at 200 rpm for 12 h. Decolorization of dye was determined as the decrease of the dye absorbancy at 610 nm.

With free laccase, the efficiency of dye decolorization was followed until 100% completed, then new dye was added to the liquid to give the same concentration. The efficiency of the second batch was followed up to 12 days.

With immobilized laccase, repeated batches were used to complete the dye decolorization. After the decolorization was completed, the liquid phase was drained and supplemented with fresh aliquots of the dye solution. The experiments were run for a total of 7 days. Efficiency in decolorization was followed. Controls with denatured laccase were run in parallel under identical conditions.

To confirm the optimal conditions forecasted for the decolorization of Indigo Carmine, a set of five replicates were done with the optimal compositions and concentrations of alginates and concentration of cross-linking agents.

4.2.6 Statistical analyses

Data of the activity of the immobilized laccase beads and of dye decolorization were subjected to analysis of variance in the experimental design module of Statistica (SoftStat, USA). Differences among treatments were determined using the least squares means procedure (PDIFF option) of SAS, 1996. Treatment effects were declared significant if $P<0.05$ and trends were discussed at $P<0.10$, unless otherwise noted.

Data of total amount of dye decolorized in repeated batches over 7 days were analysed using the experimental design procedure in the Statistica Software Version 7.0 (SoftStat, 2004).

4.3 Using airlift bioreactor for Indigo Carmine decolorization by copper alginate immobilized laccase of *Ganoderma* sp. KU-Alk4

A 5 L-airlift bioreactor (B. Braun BioLab, Germany) comprised a vessel of 1000 mm high, 80 mm diameter except the top part was 160 mm and 250 mm dia. Its working volume was 4 L (Figure 9). Diameter of air sparger was 60 mm. Initially air was supplied by an air pump at the fixed rate of 10 L/min. Optimization of the airflow rate was investigated.

The reactor was filled with 3.6 L of 25 mg/L Indigo Carmine dissolved in distilled water. The immobilized laccase with 3.6% w/v BDH alginate and 0.15 M $CuSO_4$, suggested by Latin Square Design, was used to decolorize Indigo Carmine in repeated batch. The reaction was initiated when immobilized laccase of 6×10^4 IU, 400 mL were added. The experiment was run with 2 replicates of bioreactor at 25ºC.

Decolorization of the dye was determined as the decreasing of the dye absorbancy at 610 nm. Four-milliliter samples were drawn from the airlift bioreactor to follow efficiency in decolorization. After the decolorization was completed, the liquid phase was drained and supplemented with fresh aliquots of the dye solution.

Determination of residual laccase activity was done at the end of the incubation. Cu-alginate beads were recovered from the reaction mixture, and subsequently washed with distilled water. Residual laccase activity was assayed from dissolution of beads by incubation in 0.1 M sodium tartrate buffer pH 4.5 for 15 min at 4ºC, then compared with the original laccase activity added to the alginate solution.

Figure 9 A 5 L-airlift bioreactor used in Indigo Carmine decolorization by copper alginate immobilized laccase of *Ganoderma* sp. KU-Alk4.

5. Purification and characterization of laccases of *Ganoderma* sp. KU-Alk4

5.1 Inoculum and culture conditions

The fungus was grown on PDA plates at 30ºC for 4 days. Fifteen plugs of 5-mm diameter from the growing edge of mycelia were used as inoculum in a 250-mL Erlenmeyer flask containing 50 mL of Kirk's liquid medium contained glucose and 0.2% $CuSO_4$. Initial pH of the culture was 8.0. Glucose at 1% (G1%) and 4% (G4%) was used as carbon source. Laccase isozymes from the different glucose concentration culture were compared. The culture was incubated at 30ºC in static conditions for 3 days. On the third day, 0.85 mM final concentration of veratryl alcohol was added as inducer, then culture was continued in shaking condition at 120 rpm. The contents of 3 flasks, as replicates, were randomly taken every day. Cell pellets were filtrated and dried at 60ºC to a constant weight to obtain the mycelial dry weight and the filtrate was treated as crude enzyme.

5.2 Analytical determinations

Lignin-degrading enzymes, laccase, MnP and LiP, were assayed using the DMP methodology previously described.

Protein concentration was measured followed the method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

Isozyme analysis was done using zymograms. Crude enzyme from the fungal culture grown for 9 days was separated using native polyacrylamide gel electrophoresis (native-PAGE) (Laemmli *et al*., 1970) performed under nondenaturing conditions as previously described.

5.3 Purification of laccase isozymes

All the procedures of purification were carried out at 4ºC. The crude enzymes of each 9-day culture were concentrated by Amicon ultrafiltration using a 10 kDa molecular weight cut off Millipore membrane. Two columns of DEAE-Toyopearl ion exchange chromatograph were used to purify all laccase isozymes.

The first DEAE-Toyopearl ion exchange column was 15 mm dia. and 60 mm high and was pre-equilibrated with 50 mM Tris-HCl, pH 7.5. The enzyme was eluted with a linear gradient of 0-0.8 M NaCl in the same buffer at a flow rate of 10 mL/h. The laccase fractions were pooled, dialyzed against 50 mM Tris-HCl, pH 7.5 and concentrated by Amicon ultrafiltration, then subsequently applied onto a second column of DEAE-Toyopearl (10 × 50 mm). A linear gradient of 0-0.6 M and 0-0.4 M NaCl was used for elution of laccase isozymes from G1% and G4% medium at a flow rate of 5 mL/h.

5.4 Characterization of the purified laccase isozymes (KULacs)

All main isozymes separated as purified proteins were designated as KULacs coded in different numbers.

5.4.1 Molecular weight and numbers of subunit determination

Molecular weight of each laccase isozyme was determined with SDS-PAGE and native PAGE (Laemmli *et al*., 1970). Molecular weight markers (DAIICHI Pure Chemicals) were myosin, 200.0 kDa; *β*-galactosidase, 116.3 kDa; albumin, 66.3 kDa and aldolase, 42.4 kDa. Protein was visualized by staining gel with Coomassie Brilliant Blue-R250. Molecular weights of native proteins of the isozymes were determined followed the Hedrick and Smith method (1968). Native PAGE was carried out on 8, 10 and 12% gels. Nondenatured protein markers (DAIICHI Pure Chemicals) were thyroglobulin, 669.0 kDa; ferritin, 443.0 kDa; lactate dehydrogenase, 139.9 kDa, albumin, 66.3 kDa and trypsin inhibitor, 20.1 kDa.

5.4.2 N-terminal amino acid sequence analyses

To determine the amino acid sequence at the N-terminal of each purified protein of laccases isozyme was electroblotted directly from the native-PAGE gel to a polyvinylidene difluoride membrane (PVDF, Bio-Rad). Amino acid sequence at the N-terminal was analyzed by using an Applied Biosystem Procise Amino Acid Sequencer.

5.4.3 Optimum pH and pH stability

Optimum pH was determined using various buffers at different pH as followed, hydrochloric acid-potassium chloride (pH 2.0-2.5), glycine-HCl (pH 2.5-3.5), malonate (pH 3.0-4.5), citrate phosphate (pH 3.0-7.0), phosphate (pH 6.0-8.0), Tris (hydroxymethyl) aminomethane (pH 7.5-9.0) and glycine-NaOH (pH 9.0-10.0). The pH stability was examined after the enzymes were incubated in the above described buffer of different pH (pH 2.0-10.0) at 25ºC for 1 h. The methodology was as described in part 3.2.

5.4.4 Optimum temperature and temperature stability

Optimum temperature was determined within the range of 20-95°C using spectrophotometer Libra S12 (Biochrom). Temperature stability was investigated by incubating the enzyme in 50 mM malonate buffer pH 4.5 at various temperatures for 1 h. The methodology was as described in part 3.2.

5.4.5 Substrate specificity

Substrate specificity of KULac 1, 2, 3 and 4 were compared by using the method of laccase activity assay as described in part 1.1.3. The buffer was 50 mM malonate, pH 3.5 and the enzymes concentration was 0.9 U/mL. The reaction was performed at 25ºC. The product formation occurring during the reactions with the various substrates was followed through the absorption change at the maximum absorbancy of each substrate. The substrates were ABTS, DMP, hydroquinone and guaiacol.

5.4.6 Oxidation of phenolic compounds

Capability of each purified laccase isozyme on oxidation of some phenolic compounds was determined by following the uptake of oxygen in the reaction mixture at 25ºC, using an oxygen electrode (Jenway 9300). Quantitative determination was defined as mg oxygen per litre of the reaction per minute. This method was used with some phenolic and non-phenolic substrates whose initial products of laccase activity underwent further non-enzymatic chemical changes that changed the spectra of the reaction mixtures. The reaction mixture composed of 2 mL of 3 mM substrates dissolved in 5% ethanol, 3 mL of 50 mM malonate buffer pH 3.5 and 1 mL of enzyme (90 U/mL).

5.4.7 Effect of chemical reagents and organic solvents on the purified isozymes activity

Chemical reagents and organic solvents were added to total 3 mL reaction mixture. The reaction mixture contained 1 mM DMP in 3 mL of 50 mM malonate buffer, pH 3.5 with 0.3 U of enzyme. Volume and concentrations of reagents and solvents were calculated to give the desired final concentration.

6. Molecular taxonomy of *Ganoderma* sp. KU-Alk4, macrofungal strain

6.1 Internal transcribed spacer (ITS) of 28S ribosomal DNA (rDNA) sequence determination

DNA extraction: Mycelium collected from Kirk's medium by filtration through filter paper (Whatman No.4) was grounded with liquid nitrogen in a mortar. DNA was extracted by using DNA Trap kit (DNA Tec, BIOTEC, Kasetsart University Kamphaengsaen).

PCR amplification of ITS of 28S rDNA was done. The fungal purified DNA was dissolved in TE buffer and used as template in amplification. PCR was performed using an automated thermal cycler (Perkin Elmer, Applied Biosystems Division) PCR was performed with initial denaturation at 95ºC for 1 min and followed by a total of 25 cycles with 15 sec denaturation at 95ºC and 30 sec annealing at 50ºC. Each annealing step was followed by 2 min extension at 72ºC. The primers used were ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC). The PCR product was generated with AmpliTaqGold^{TM} DNA polymerase (Perkin Elmer, Applied Biosystems Division) and further cloned using pGEM-T Easy cloning kit (Promega®) (Hongoh *et al*., 2003).

Sequencing of ITS4 was done. The sequencing reactions were performed with a Big Dye^{TM} Terminator Cycler Sequencing kit (Perkin Elmer, Applied Biosystems Division). Sequencing was carried out with an ABI Model 377 DNA Sequencer (Perkin Elmer, Applied Biosystems Division). The ITS4 sequences were analyzed and aligned using CLUSTAL X version 1.8 with a selection of 22 reference sequences obtained from the GenBank database by the Blast N program from NCBI web site.

6.2 18S rDNA sequence determination

DNA was extracted by using DNA Trap kit (DNA Tec, BIOTEC, Kasetsart University Kamphaengsaen). PCR amplification of 18S rDNA was done by using the primer GTAGTCATATGCTTGTCTC and TCCGCAGGTTCACCTACGGA.

Sequencing was carried out with an ABI Model 377 DNA Sequencer (Perkin Elmer, Applied Biosystems Division). The 18S rDNA sequences were analyzed and aligned using CLUSTAL X version 1.8 with a selection of 20 reference sequences obtained from the GenBank database by the Blast N program from NCBI web site.

6.3 Phylogenetic analyses

Phylogenetic comparison was done using both ITS4 of 28S rDNA and 18S rDNA genes. The phylogenetic trees were constructed from the evolutionary distance data by applying both gene sequences to the algorithm of the Neighbor-Joining (NJ), Unweighted Pair Group Method with Arithmic Mean (UPGMA), Minimum Evolution (ME) and Maximum Parsimony (MP) methods. To evaluate the robustness of branches in the inferred tree, the bootstrap resampling method of Felsenstein with 100 replicates was used. CLUSTAL X1.8, GENETYX 5.1 and MEGA2 software were used to construct the phylogenetic trees for comparison.