APPENDICES

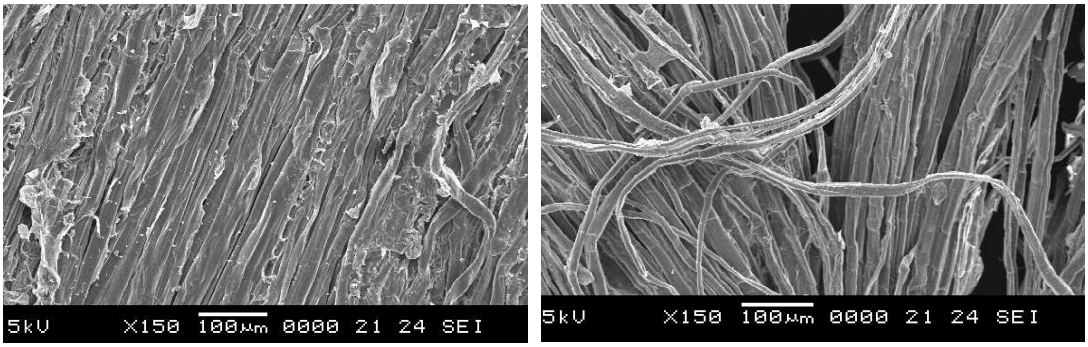
Appendix A

Using of lignin-degrading enzymes in biopulping process

Appendix Table A1 Some chemical properties of the enzyme-treated pulp of paper mulberry from the process of soaking the bark in the lignin-degrading enzymes from KU-Alk4 for 12, 24, 36 and 48 h compared with that soaked in water.

Samples	Extractives (%)		lignin		Holocellulose (%)
	Extract with Benzene: ethanol	water	Klason lignin (%)	Acid soluble lignin (%)	
1. control	5.52	10.13	7.04	2.13	71.00
2. 12 h	5.73	8.84	8.83	1.78	78.23
3. 24 h	7.18	9.45	8.43	1.91	78.44
4. 36 h	6.33	10.78	7.17	1.87	78.83
5. 48 h	6.96	11.60	6.51	1.52	78.05

(A) **(B)**



(C)

Appendix Figure A1 Electron microscopic picture of paper mulberry bark pulped with the lignin-degrading enzymes of KU-Alk4 for 48 h. (A) bark in water (B) bark with 7% NaOH (C) Bark with lignin-degrading enzymes of KU-Alk4.

Appendix B

Culture media

Kirk's medium

Glucose	10.0	g/L
Ammonium tartrate	0.2	g/L
Sodium acetate	1.6	g/L
Kirk's trace element stock solution	60.0	mL/L
Kirk's salts stock solution	100.0	mL/L
Tween 80	1.0	g/L

Kirk's trace element stock solution

Nitrilo triacetate	9.0	g/L
$MgSO_4 \cdot 7H_2O$	3.0	g/L
$MnSO_4$	2.7	g/L
NaCl	6.0	g/L
$FeSO_4 \cdot 7H_2O$	0.6	g/L
$CoSO_4 \cdot 7H_2O$	1.1	g/L
$CaCl_2 \cdot 2H_2O$	0.6	g/L
$ZnSO_4 \cdot 7H_2O$	1.1	g/L
$CuSO_4 \cdot 5H_2O$	60.0	mg/L
$AlK(SO_4)_2 \cdot 12H_2O$	110.0	mg/L
H_3BO_3	60.0	mg/L
$Na_2MoO_4 \cdot 2H_2O$	70.0	mg/L

Kirk's salts stock solution

KH_2PO_4	20.0	g/L
$MgSO_4 \cdot 7H_2O$	5.0	g/L
$CaCl_2 \cdot 2H_2O$	1.3	g/L
Kirk's trace element stock solution	16.7	ml/L
Thiamine-HCl	10.0	mg/L

Wood meal medium

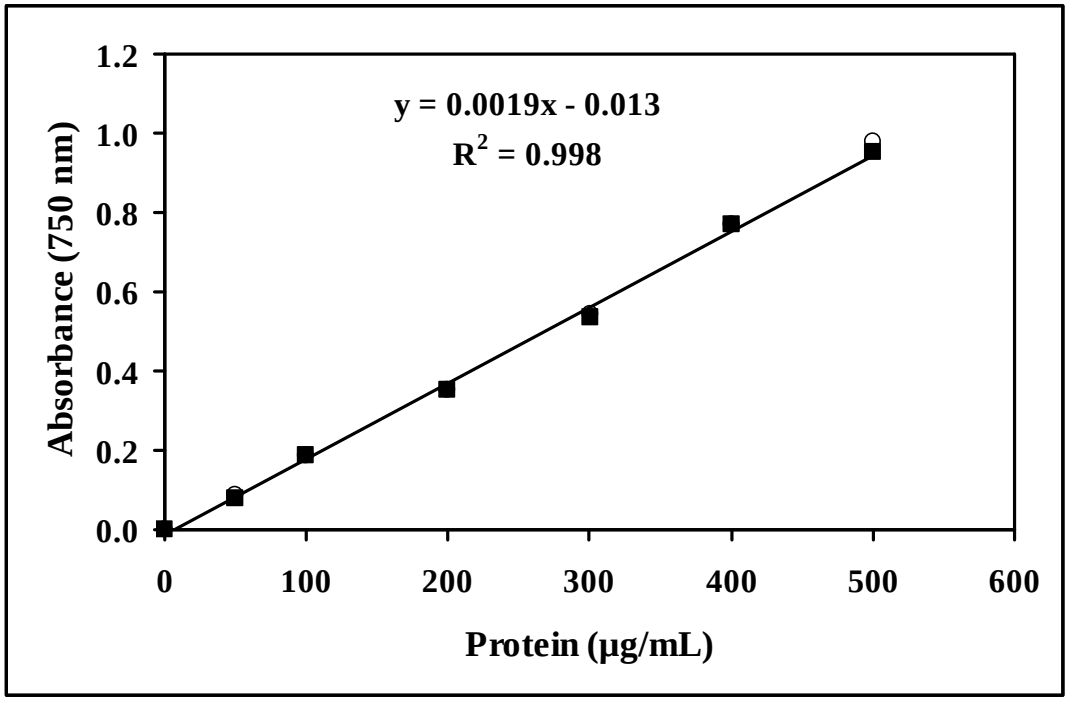
Beech wood meal	20	g/L
Guaiacol	0.2	mL/L
Benomyl	0.01	g/L
Agar	16	g/L

Appendix C

Reagents

Lowry-Folin protein assay reagents;

Solution A	:	1% (w/v) $CuSO_4.5H_2O$
Solution B	:	2% (w/v) Sodium potassium tartrate
Solution C	:	0.2 M Sodium hydroxide
Solution D	:	4% (w/v) Sodium carbonate
Solution E	:	Mixed 49 mL of Solution C with 49 mL of Solution D. Added 1 mL of Solution A and 1 mL of Solution B. (Freshly prepare before use)
Solution F	:	Diluted 1:1 (v/v) Folin-Ciocalteau reagent (Merck®) with distilled water



Appendix Figure C1 Standard curve of protein (bovine serum albumin) assay by Lowry-Folin method.

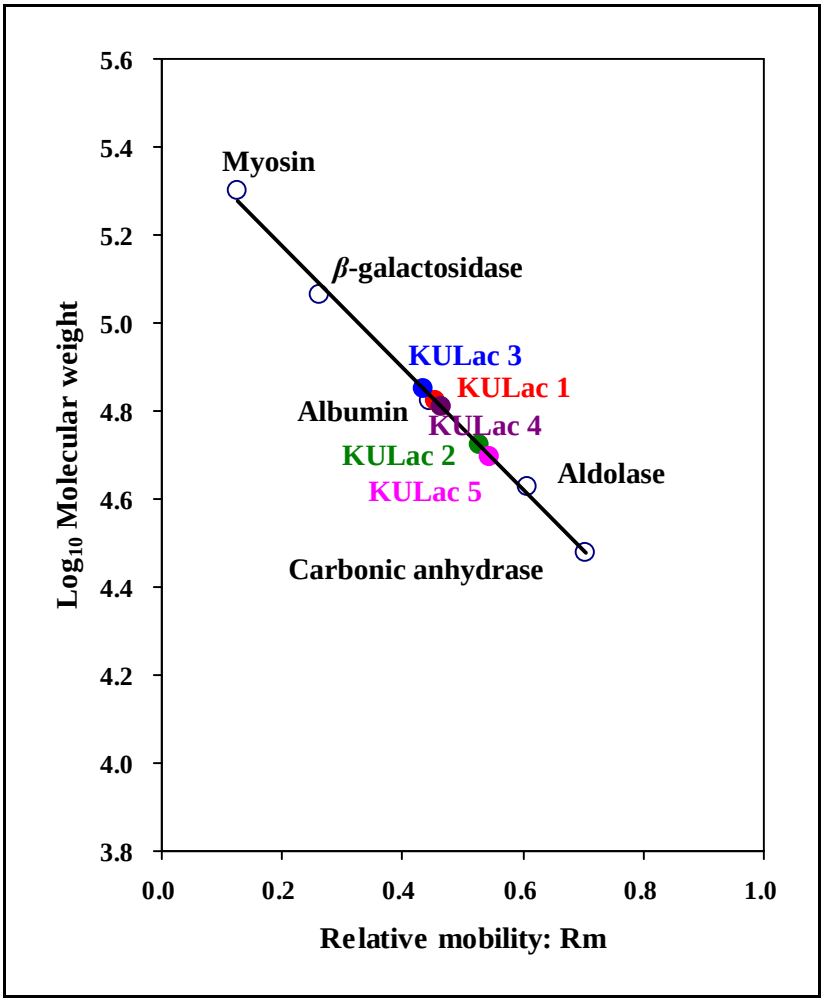
Composition of reagents of SDS-PAGE were as follows:

1. Acrylamide-bis Stock, 100 mL:	30% acrylamide
	0.8% N,N'-methylene bis acrylamide
2. 2x SDS-Running Buffer, 100 mL:	0.75 M Tris-HCl, pH 8.8
	0.2% SDS
3. 2x SDS-Stacking Buffer, 100 mL:	0.25 M Tris-HCl, pH 6.8
	0.2% SDS
4. 5x SDS-Electrode Buffer, 100 mL:	0.125 M Tris-HCl, pH 8.3
	0.96 M glycine
	0.5% SDS
5. TEMED	full strength
6. 2x SDS-SAB	0.125 M Tris-HCl, pH 6.8
	4% SDS
	20% glycerol
	0.002% Bromphenol blue
	10% mercaptoethanol
	10% mercaptoethanol
	(add just before use)

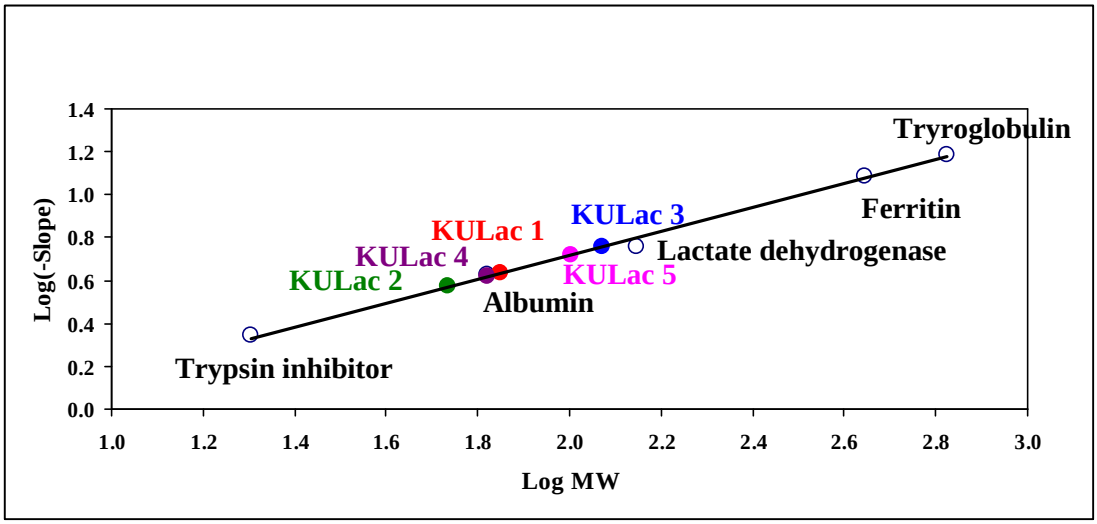
Comassie brilliant blue staining for protein from PAGE

Compositions of Coomassie Brilliant Blue stain and destain solution were as follows:

1. Stain:	450 mL water
	500 mL methanol
	75 mL acetic acid
	5 g Coomassie Brilliant Blue
2. Destain I:	1.0 litre water
	1.0 litre methanol
	200 mL acetic acid
3. Destain II:	150 mL methanol
	225 mL acetic acid
	Bring to 1.0 litre with water



Appendix Figure C2 Standard curve of molecular weight standard protein from SDS-PAGE.



Appendix Figure C3 Standard curve of molecular weight standard protein from native-PAGE by Hedrick and Smith method.

Reagent for analysis of ITS4 and 18S rDNA

TE buffer

Tris-HCl	10.8	g
EDTA-2Na	0.83	g
pH was adjusted to 8.0 with HCl		
Add MilliQ water to	1,000	mL

Appendix D

Published papers

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ORIGINAL PAPER

Novel laccases of *Ganoderma* sp. KU-Alk4, regulated by different glucose concentration in alkaline media

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Abstract Physiological regulation of laccase production from *Ganoderma* sp. KU-Alk4, isolated in Thailand, was controlled by the initial glucose concentration in liquid culture. Different laccase isozymes were produced using different starting concentrations of glucose. With 1% glucose, two isozymes, KULac 1 and 2 were produced, while with 4% glucose, three different isozymes, KULac 3, 4 and 5, were produced. The KULacs differed in their molecular mass, ranging from 53 to 112 kDa. KULac 2 was a new laccase that had a different N-terminal amino acid sequence from other laccases previously reported. All the isozymes had optimum pH at 3.5 and were stable over the wide range of pH, 3.0–10.0, especially in alkaline pH. It is noteworthy that the activities of the four KULacs with 2,6-dimethoxyphenol were extremely high up to 90°C. They retained 100% of their activities at 60°C for 1 h.

Keywords *Ganoderma* · Glucose effect · Isozyme · Laccase · Purification

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Introduction

Laccases (benzenediol: oxygen oxidoreductase, E.C. 1.10.3.2) are oxidoreductases belonging to the family of multi copper-containing oxidases that reduce bimolecular oxygen to water and simultaneously perform monoelectronic oxidation of a wide range substrates, including mono-, di- and polyphenols, aminophenols, methoxyphenols, polyamines, aryl diamines and lignins (Yaropolov et al. 1994; Solomon et al. 1996; Xu 1999). Due to their broad substrate specificity, laccases are very useful in several biotechnological processes, such as the detoxification of industrial effluents, for instance from the paper and pulp, textile and petrochemical industries (Rodríguez and Luis 2006; Poonpairoj et al. 2001; Wesenberg et al. 2003; Grönqvist et al. 2003) including pesticides or insecticides and explosives in soil (Durán and Esposito 2000). Laccases have also been supplemented in food and cosmetics as oxidizing agents, replacing chemical agents that were previously used (Minussi et al. 2002; Golz-Berner et al. 2004). Moreover, they have been used in organic synthesis, where their typical substrates are phenols and amines (Riva 2006).

White-rot fungi typically produced multiple isozymes of laccase depending on the fungal species and environmental conditions. Although molecular properties of isozymes and purification procedures were described for laccases from several fungi (Yaver et al. 1996; Périé et al. 1998; Farnet et al. 2000; Ko et al. 2001; Xiao et al. 2003; Zhang et al. 2006), there is still a lack of information on physiology of the formation of different isozymes. *Ganoderma*, a genus in white-rot fungi, possess important medicinal properties (Jong and Birmingham 1992). One of the important aspects of *Ganoderma* sp. is the use of its ligninolytic potential, Manganese peroxidase (MnP), Lignin peroxidase (LiP) and

laccase (Silva et al. 2005; D'Souza et al. 1999). Little is known about the ligninolytic enzymes of *Ganoderma* species but Ko et al. (2001) described purification and characterization of laccases from *G. lucidum.*

Ganoderma sp. strain KU-Alk4 was isolated in Thailand by our group. The identification and phylogenetic comparison will be reported elsewhere. Preliminary studies suggested that it is a strain of *G. philippii.* Ligninolytic enzymes from this strain have been used for removing lignin during the pulping process to produce paper from mulberry bark (Poonpairoj et al. 2001). Production of laccases by *Ganoderma* sp. strain KU-Alk4 was affected by the initial glucose concentration in batch cultures (Teerapatsakul and Chitradon 2003). The crude enzyme from 1 to 4% glucose cultures showed differences in the laccase isozymes produced. To date, no studies on the effect of glucose concentration to the profiles of laccase isozymes have been reported with white-rot fungi. Thus, the aim of this work was to compare physical and catalytic characteristics of the laccase isozymes produced by *Ganoderma* sp. in batch culture using 1 and 4% initial glucose concentrations.

Materials and methods

Fungal strain

Ganoderma sp. designated as KU-Alk4 isolated from a living tree, *Terminalia bellerica* Roxb., at Kasetsart University, Bangkok, Thailand. The fungus was maintained on a potato dextrose agar (PDA) slants and stored at 4°C and subcultured monthly. For longer storage the culture was maintained on rice grain solid state at room temperature.

Inoculum and culture conditions

The fungus was grown on PDA plates at 30°C 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 liquid medium (Tien and Kirk 1988). The initial pH of the culture was 8.0. To study the effects of glucose concentration on laccase production, glucose at of 1% (G1%) and 4% (G4%) was used as carbon source. The culture was incubated at 30°C 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 140 rpm. The contents of three flasks, as replicates, were randomly taken every day and filtered. Cell pellets were dried at 60°C to a constant weight to obtain the mycelial dry weight and the filtrate was treated as crude enzyme.

Analytical determinations

Ligninolytic enzymes were assayed using the methodology described by Kondo et al. (1994). Laccase and MnP activity were measured by monitoring the oxidation of 2,6-dimethoxyphenol (DMP) at 469 nm. LiP activity was evaluated by monitoring the oxidation of veratryl alcohol at 310 nm. All enzyme assays were performed with 3-mL reaction mixtures at 25°C. Enzyme activity was expressed as the amount of spectral change (change in absorbance per minute) within 3 min. Rate of the reaction was converted into a quantitative amount of enzyme. Unit (U) of laccase was defined as the amount of enzyme causing 1.0 increase in absorbance per minute at the appropriate wavelength.

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 1970) performed under nondenaturing conditions. Laccase activity in gels was visualized by active staining using 3 mM DMP as a substrate in 50 mM malonate buffer pH 4.5, during 30 min.

Purification of laccase isozymes

All the procedures of purification were carried out at 4°C. The crude enzymes of each 9-day culture produced 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.

Characterization of the purified laccases

The molecular weight of each laccase isozyme was determined by SDS-PAGE and native PAGE (Laemmli 1970). Molecular weight markers (DAIICHI Pure Chemicals) were myosin 200.0 kDa, β-galactosidase 116.3 kDa, albumin 66.3 kDa aldolase 42.4 kDa and carbonic anhydrase 30.0 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 (1968) method. 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.

To determine the amino acid sequence at the N-terminal of the purified laccases, each isozyme was electroblotted directly from the native-PAGE gel to a polyvinylidene

difluoride membrane (PVDF, Bio-Rad) and N-terminal amino acid sequence was analyzed by using an Applied Biosystem Procise Sequencer.

Optimum pH was determined using 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 buffer of different pH (pH 2.0–10.0) at 25°C for 1 h. 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.

Substrate specificity of the enzyme was done using two methods. Firstly, product formation occurring during the reactions with the various substrates was followed through the absorption change at the maximum absorbance. The second method was to follow the uptake of oxygen in the reaction mixture at 25°C, using an oxygen electrode (Jenway 9300), resulting in the qualitative determination in mg oxygen per liter of the reaction per minute. This method was used with those 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.

Chemical reagents and organic solvents were added to the reaction mixture containing 1 mM DMP in 3 mL of 50 mM malonate buffer, pH 3.5 to give the desired final concentration.

Oxidation of polycyclic aromatic hydrocarbons (PAHs) by laccase-mediator system was determined by incubating a mixture of the individual PAHs (20 μM), and ABTS (1 mM), in 15% acetonitrile in 3 mL of 50 mM malonate buffer pH 3.5 with 1 U of laccase. The reactions were incubated at 30°C for 3 h and stopped by the addition of acetonitrile to a final concentration of 65%. After centrifugation at 5,000×*g* for 10 min, the supernatant was measured by UV-visible spectrophotometer at wavelength 225, 300 and 326 nm for determination of anthracene, fluorene and pyrene oxidation, respectively. Control was identically prepared, except that the 10 min boiled-denatured enzyme was used.

Results and discussion

Effect of glucose concentration on laccase production

Laccase could be regulated so that it was the only lignin-olytic enzyme that was produced by *Ganoderma* sp. KU-Alk4 in Kirk medium by shifting the medium pH to 8.0, in which conditions no activities of LiP or MnP were detected. Laccase production started on day 3 on the addition of 0.85 mM veratryl alcohol as inducer (Fig. 1). Higher activity of laccase was detected in G1% than in G4% culture. The fungus produced overall laccase activity (U/mL) about twofold higher in G1% medium than in G4%. However, the mycelial dry weight of fungus in G1% medium was 1.6-fold lower than that in G4% at day 9, the time at which the maximal laccase activities were observed. The specific activities of laccase at day 9 in G1 and G4% media were 126.8 U/mg cell dry weight and 33.3 U/mg cell dry weight, respectively.

Zymograms of laccase isozymes in the crude enzymes collected at day 9 from G1% to G4% cultures (Fig. 2) showed two major active bands reacting with DMP (KU-Lac 1 and 2) in G1% culture (Fig. 2a). Three major active bands were observed from G4% cultures (KULac 3, 4 and 5; Fig. 2b). The five isozymes showed different molecular weights. From the results, ligninolytic enzyme production by this new isolate, *Ganoderma* sp. KU-Alk4, was controlled by the initial glucose concentration which affected not only to the total amount of laccase activity but also regulated the production of different isozymes.

Purification of laccase isozymes

KULac 1 from G1% culture was purified fourfold with giving specific activity 6,133 U/mg protein. Purified KU-Lac 2 obtained from G1% culture had specific activity of 4,205 U/mg protein (data not shown). After purification KULac 1 showed 70% of the initial activity and KULac 2 2%. Chromatography of the protein from G1% culture on a second column of DEAE-Toyopearl (Fig. 3) showed that

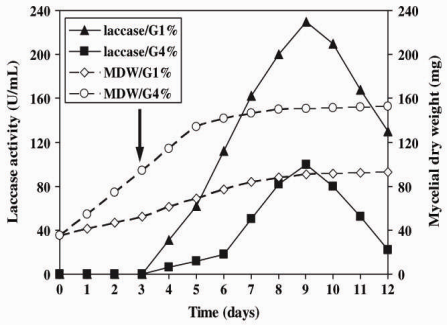

Fig. 1 Effect of glucose concentrations on laccase production and mycelial dry weight of *Ganoderma* sp. KU-Alk4. The cultures grown in G1% and G4% Kirk media supplemented with veratryl alcohol as inducer. *Arrow* indicates the time of the inducer addition. Values are the average of three independent experiments and the maximal mean deviation is ±8% of values

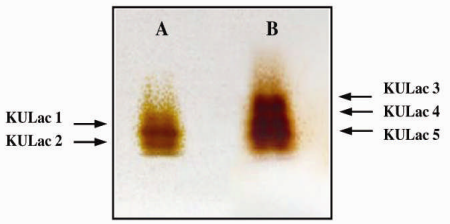

Fig. 2 Zymograms of laccase isozymes monitored in the crude enzyme of *Ganoderma* sp. KU-Alk4 grown in G1% (**A**), and G4% (**B**), Kirk media. Samples containing 1.5 U of laccase activity collected at 9 days were used

KULac 1 to be the main isozyme, eluted using 0.23 M NaCl, while KULac 2 was eluted by 0.37 M NaCl.

From G4% culture, purified KULac 3 that was eluted from the second DEAE-Toyopearl column (Fig. 4) at 0.14 M NaCl comprised 53% of the total activity and had a specific activity of 3,796 U/mg protein. Very small amounts of KULac 4 were obtained (less than 1%) and its specific activity was 2,652 U/mg protein. KULac 5 was eluted by 0.18 M NaCl, showed 24% of the total activity and had the highest specific activity 4,918 U/mg protein. The overall extent of purification was only 2.5–8.7 which suggests that *Ganoderma* sp. KUAlk4 secreted laccases as a dominant protein in the cultivated conditions.

Characterization of the laccase isozymes

The molecular weights of KULac 1, 2, 3, 4 and 5 were 67, 50, 71, 47 and 65 kDa, respectively (Fig. 5) The native protein of each isozyme on 10% non-denaturing native-PAGE showed that KULac 1, 2, 3, 4 and 5 had molecular masses of 90, 53, 112, 100 and 74 kDa, respectively (Fig. 6). Therefore, KULac 4 might be composed of two

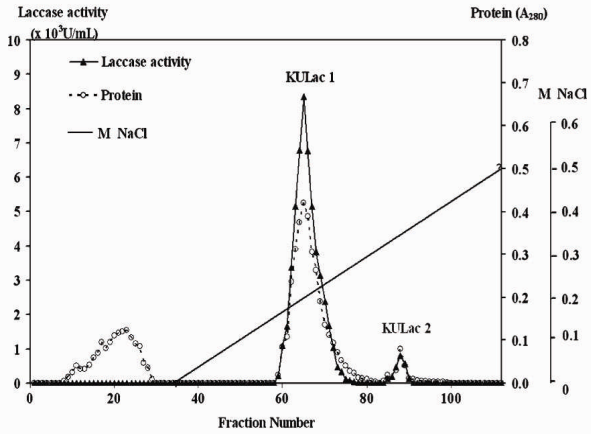

Fig. 3 Elution profile of laccase isozymes from 1% glucose concentration culture of *Ganoderma* sp. KU-Alk4 on DEAE-Toyopearl column (10 mm × 50 mm), 50 mM Tris–HCl buffer, pH 7.5 with 0–0.6 M NaCl as an elution buffer, flow rate 5 mL/h. 1.5 mL fractions were collected

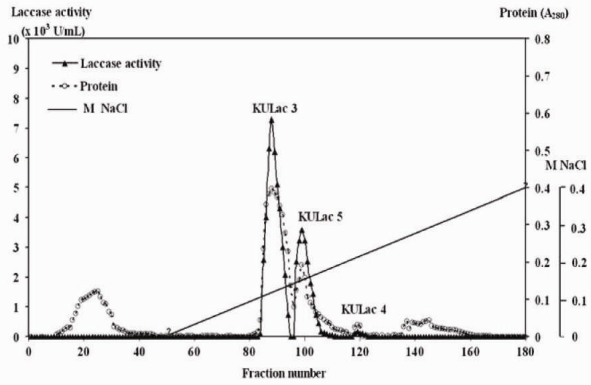

Fig. 4 Elution profile of KULac 3, KULac 4 and KULac 5 from G4% culture of *Ganoderma* sp. KU-Alk4 on DEAE-Toyopearl column (10 mm × 50 mm), 50 mM Tris–HCl buffer, pH 7.5 with 0–0.4 M NaCl as an elution buffer, flow rate 5 mL/h. 1.5 mL fractions were collected

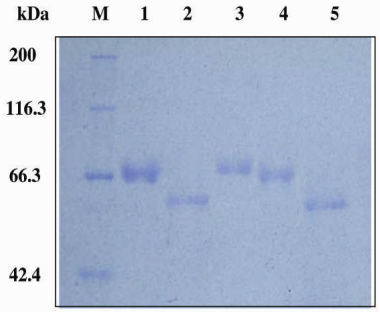

Fig. 5 SDS-PAGE of the purified laccase isozymes from *Ganoderma* sp. KU-Alk4. *Lanes M* nolecular weight marker, *lane 1* KULac 1, *lane 2* KULac 2, *lane 3* KULac 3, *lane 4* KULac 5, *lane 5* KULac 4. Samples containing 2 μg of protein

subunits of the same molecular weight, whereas KULac 1, 2, 3 and 5 were homogeneous proteins. This confirms that laccases from basidiomycetes, including *G. lucidum*, are generally monomeric proteins (Yaropolov et al. 1994; Ko et al. 2001). Most fungal laccases have molecular weights ranging from 59 to 110 kDa, mostly 66 kDa (Luisa et al. 1996; Wesenberg et al. 2003). Ko et al. (2001) reported that *G. lucidum*, a Korean isolate, produces three laccase isozymes in a complete medium without any specific induction, whose molecular weights were between 65 and 68 kDa. D'Souza et al. (1999) also reported five major laccase isozymes produced by *G. lucidum* in high nitrogen medium, two of which were 40 and 66 kDa. Our *Ganoderma* sp. KU-Alk4 produced different types and numbers of laccase isozymes. Two isozymes (KULac 1 and 2) and three different isozymes (KULac 3, 4, 5) were produced in G1% and G4% medium, respectively. Molecular weights of the isozymes that were produced in G1% medium were lower than that in G4% medium. It is typical of white-rot fungi to produce multiple isozymes of laccase. A maximum number of ten isozymes was reported in *Flavodon flavus* (Raghukumar et al. 1999) but there was no evidence showing that concentration of glucose could regulate the production of different types of laccases.

Fifteen amino acid sequences at the N-terminal of the five isozymes were identified and aligned to compare with the other laccases (Table 1). KULac 2 had the N-terminal sequence GIGPVADLTVRGGDI but KULac 1, 3, 4 and 5 the identical N-terminal, GIGPVTDLTISNADI. The N-terminal sequence of five amino acid residues of laccase from *G. lucidum*, reported briefly (Ko et al. 2001), was different from all KULacs by one amino acid. When all 15 amino acid residues of KULac 2 were compared with the others previously reported, the sequence of KULac 2 showed the closest similarity to that of *Dichomitus squalens* but by only 67% (Périé et al. 1998). The N-terminal sequences of KULac 1, 3, 4, and 5 showed the closest similarity, 93%, to the laccase of *D. squalens* (Périé et al. 1998). When compared with laccase II and III of *T. versicolor* and that of *Panus rudis* and *Coriolus hirsutus*, they were 67% similar (Bourbonnais et al. 1995; Zhang et al. 2006; Kojima et al. 1990). *Ganoderma* sp. KU-Alk4 was closely related to *G. philippii* when its phylogenic comparison was done with ITS four (report elsewhere). Therefore, KULac 2 was a new laccase isozyme that could be produced by *Ganoderma*, KU-Alk4, in a certain stress of glucose.

The chemical properties of KULacs were investigated and compared with those of other fungal laccases. The effect of pH and temperature on all KULacs was examined except KULac 4, because of the small amounts available of this isozyme. The optimum pH of all of the KULacs against DMP was 3.5 (Fig. 7a). Most fungal laccases have optimum pH, in general, between 3.0 and 5.0. At 25°C for 1 h, KULacs were stable over a wide range of pH (3.0–10.0), especially in alkaline range (Fig. 7b). These laccases were relatively stable at elevated temperatures up to 55°C (Fig. 8b). KULac 3 was the most thermostable, maintaining its activity up to 60°C. It was more stable than the KULacs of G1% culture. KULac 2, the new laccase, was

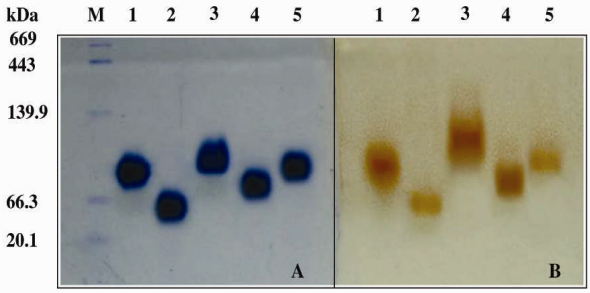

Fig. 6 Non-denaturing native PAGE (10%) of laccase isozymes from *Ganoderma* sp. KU-Alk4. **a** Protein staining by Coomassie blue R-250 staining. **b** Activity staining with DMP. Samples contained 6 μg (**A**) and 2 μg (**B**) protein. *Lanes M* molecular weight markers, *lanes 1* KULac 1, *lanes 2* KULac 2, *lanes 3* KULac 3, *lane 4* KULac 5 and *lane 5* KULac 4

Table 1 Comparison of N-terminal amino acid sequences of *Ganoderma* sp. KU-Alk4 laccases and other fungal laccases

Microorganisms	N-terminal amino acid sequences																				Reference
Ganoderma sp. KU-Alk4																					
KULac 2	G	I	G	P	V	A	D	L	T	V	R	G	G	D	I						
KULac 1; 3; 4; 5	G	I	G	P	V	T	D	L	T	I	S	N	A	D	I						
Ganoderma lucidum	G	I	G	P	T																Ko et al. 2001
Dichomitus squalens	G	I	G	P	V	T	D	L	T	I	T	N	A	D	I	A	P				Périé et al. 1998
Trametes versicolor																					
I	A	I	G	P	V	A	S	L	V	V	A	N	A	P	V	S	P	P	P		Bourbonnais et al. 1995
II	G	I	G	P	V	A	D	L	T	I	T	D	A	A	V	S					
III	G	I	G	P	V	A	D	L	T	I	T	D	A	E	V	S					
Panus rudis	A	I	G	P	V	T	D	L	H	I	V	N	D	N	I	A	P	D	G	F	Zhang et al. 2006
Coriolus hirsutus	A	I	G	P	T	A	D	L	T	I	S	N	A	E	V	S	P				Kojima et al. 1990
Ceriporiopsis subvermispora	A	I	G	P	V	T	D	L	E	I	T	D	A	F	V	A	P				Fukushima and Kirk 1995
Pycnoporus cinnabarinus	A	I	G	P	V	A	D	L	T	L	T	N	A	A	V	S	P				Eggert et al. 1996
Trametes villosa I	A	I	G	P	V	A	D	L	V	V	A	N	A	P	V	S	P				Yaver et al. 1996
Trichophyton rubrum	A	I	G	P	V	A	D	L	H	I	T	D	D	T	I	A	P				Jung et al. 2002
Pleurotus ostreatus	A	I	G	P	T	G	D	M	Y	I	V	N	E	D	Y						Palmieri et al. 1997
Phlebia radiata	S	I	G	P	V	T	D	F	H	I	V	N	A	A	V	S	P				Saloheimo et al. 1991
Phellinus ribis	A	I	V	S	T	P	L	L	I	P	N	A	N	C	L						Min et al. 2001
Agaricus bisporus	D	T	X	K	T	F	N	F	D	L	V	N	T	R	L	A	P				Perry et al. 1993
Neurospora crassa	G	G	G	G	G	C	N	S	P	T	N	R	Q	C	W	S	P				Germann et al. 1988

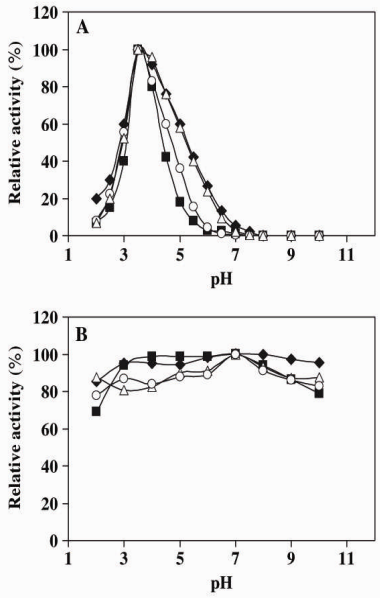

Fig. 7 **A** Optimum pH and **B** pH stability for KULac 1 (○), KULac 2 (△), KULac 3 (■) and KULac 5 (◆).Values are the average of three independent experiments and the maximal mean deviation is ±5% of values

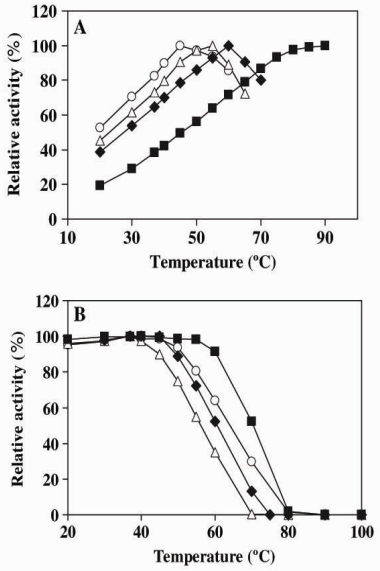

Fig. 8 **A** Effect of temperature on the activity and **B** thermal stability of KULac 1 (○), KULac 2 (△), KULac 3 (■) and KULac 5 (◆). Values are the average of three independent experiments and the maximal mean deviation is ±5% of values

the most sensitive isozyme to high temperature. KULac 3 had an extremely high optimum temperature (90°C), when the initial rate was measured using DMP for 1 min though it was not stable at that temperature for more than 2 min (Fig. 8a). To the best of our knowledge, KULac 3 is a novel enzyme with extremely high optimum temperature and stability. Other laccases have optimum temperatures between 50 and 60°C (Slomczynski et al. 1995; Xiao et al. 2003; Ryan et al. 2003). The highest temperature previously reported for laccase activity was 80°C in *Marasmius quercophilus*, a litter decomposing fungus (Farnet et al. 2000).

Similar to the other fungal laccases, ABTS was the best substrate of all KULacs (Table 2). Specific activities of KULac 1, 2, 3 and 5 to ABTS was 1.70, 1.11, 1.05 and 1.38 kIU/mg protein, respectively. The four KULacs could oxidize DMP, hydroquinone and guaiacol, also. KULac 3 and 5 oxidized DMP twice as rapidly as KULac 1 and 2. KULac 1 and 2 reacted with DMP and hydroquinone at similar rates. Oxidation of guaiacol was very slow. KULac 1 and 3 were able to oxidize a series of phenolic compounds with different degrees of substitution (Table 3). Both isozymes presented comparable rates of oxidation of these substrates. Like the other fungal laccases (Gianfreda 1999; Leonowicz et al. 2001), those obtained from *Ganoderma* sp. KU-Alk4 oxidized, non-specifically, a wide range of phenolic and non-phenolic aromatic substrates. In common with true laccases (Robles et al. 2002; Jung et al. 2002; Litthauer et al. 2007) the KULacs did not oxidize tyrosine. The nature and substitution on the phenolic ring affected the oxidation rate of laccases. KULacs showed greater selectivity for dimethoxy substituted phenols such as DMP and syringic acid than for those phenols with only one methoxy substitution, such as vanillyl alcohol, vanillin, vanillic acids and ferulic acid. *p*-Diphenols (hydroquinone) were better substrates than *m*-diphenols (resorcinol). The capability of KULacs to oxidize *m*-diphenols contrasted with that of *Pycnoporous sanguineus* (Litthauer et al. 2007) which had no activity with the *m*-diphenol substrates. Moreover, laccases in *T. trogii*, *Sclerotium rolfsii* and *D. squalens* are able to oxidize catechol while KULacs show no activity to that *o*-diphenol (Zouari-Mechichi et al. 2006; Ryan et al. 2003; Périé et al. 1998). The three PAHs anthracene, fluorine and pyrene were not oxidized by KULacs. In the presence of the mediator 1 mM ABTS, KULacs 1 and 3 oxidized the three PAHs at similar rates. The three-ring PAHs anthracene and fluorine were oxidized

Table 2 Substrate specificity of laccase isozymes from *Ganoderma* sp. KU-Alk4

Substrate	λ_{max}	Relative activity[a] (%)			
		KULac 1	KULac 2	KULac 3	KULac 5
ABTS	420	100	100	100	100
2,6-Dimethoxyphenol	469	31	16.7	40	50
Hydroquinone	247	31	16.7	10	20
Guaiacol	465	6.3	1.7	5	10

[a] Values are the average of three independent experiments and the maximal mean deviation is ±5% of values

Table 3 Rates of oxidation of various substrates catalyzed by KULac 1 and 3 from *Ganoderma* sp. KU-Alk4

Substrates	Dissolved O_2 consumption rate[a] (mg $L^{-1}min^{-1}$)	
	KULac 1	KULac 3
ABTS[b]	23.085	23.085
2,6-Dimethoxyphenol	3.368	5.389
1,4-Benzenediol (hydroquinone)	1.619	2.105
2-Methoxyphenol (guaiacol)	0.088	0.094
1,2-Benzenediol (catechol)	NC	NC
1,3-Benzenediol (resorcinol)	0.438	0.482
4-Chlorophenol	0.273	0.328
Benzaldehyde	NC	NC
4-Hydroxy-3-methoxybenzaldehyde (Vanillin)	0.254	0.305
3,4-Dimethoxybenzaldehyde (Veratraldehyde)	NC	NC
Benzoic acid	NC	NC
3,5-Dimethoxy-4-hydroxybenzoic acid (Syringic acid)	1.396	2.234
4-Hydroxy-3-methoxybenzoic acid (Vanillic acid)	0.147	0.221
3,4-Dimethoxybenzoic acid (veratric acid)	NC	NC
4-Hydroxy-3-methoxybenzyl alcohol (vanillyl alcohol)	0.506	0.810
3,4-Dimethoxybenzyl alcohol (veratryl alcohol)	0.255	0.383
Cinnamic acid	NC	NC
4-Hydroxy-3-methoxycinnamic acid (ferulic acid)	0.838	1.089
3,4-Dihydroxycinnamic acid (caffeic acid)	0.746	0.895
4-Hydroxycinnamic acid (*p*-coumaric acid)	0.052	0.078
1-Naphthol	1.896	2.654
2-Naphthol	0.376	0.564
Cyclohexene	NC	NC
Abietic acid	0.277	0.285
Tyrosine	NC	NC
Anthracene	NC	NC
Fluorene	NC	NC
Pyrene	NC	NC

NC no changes in the reaction

[a] Values are the average of three independent experiments and the maximal mean deviation is ±5% of values

[b] ABTS: 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonate)

by 60 and 48%, respectively during the 3 h of incubation. Pyrene, the four-ring PAH was oxidized by 22% in similar circumstances. Pickard et al. (1999) found no oxidation of pyrene by a purified laccase from *Coriolopsis gallica* UAMH 8260 in the presence of ABTS.

Table 4 shows the effects of various reagents and organic solvents on KULac 1 and 3. Both isozymes were completely inhibited by 1.3 mM sodium azide like laccases of *Pleurotus ostreatus*, *T. versicolor* and *T. hirsuta* (Abadulla et al. 2000, Ryan et al. 2003). Azide makes a bridge between Cu, both type II and III, within the protein structure (Périé et al. 1998). Both KULac 1 and KULac 3 were inhibited by the reducing agents, 2-mercaptoethanol and dithiothreitol. This suggests that cysteine occurs at the active site of the laccases (Thurston 1994). The chelating agent, EDTA, inhibited at 6.5 mM final concentration.

Organic solvents had variable effects on laccase activity depending on the degree of polarity. The KULacs retained 25% of their maximum activity in the presence of 50% methanol or 50% ethanol, suggesting that they may be suitable for use in the oxidation of water-insoluble substrates.

Conclusions

The newly isolated *Ganoderma* sp. KU-Alk4 produces five isozymes of laccase. When the initial glucose concentration used in liquid culture is low (1%) different isozymes are produced than when a higher initial glucose concentration (4%) is employed. Two isozymes, KULac 1 and KULac 2 were produced in the former conditions, three, KULac 3, 4 and 5 in the latter. KULac 2 and 4 were relatively minor components of the total laccase.

Table 4 Effect of various reagents and organic solvents on laccase isozymes from *Ganoderma* sp. KU-Alk4

	Final concentration (mM)	Relative activity[a] (%)	
		KULac 1	KULac 3
Reagents			
Control	–	100	100
$CuSO_4$	1.3	100	100
NaN_3	1.3	0	0
2-Mercaptoethanol	1.3	28.6	66
Dithiothreitol	1.3	28.6	44.5
EDTA	1.3	100	100
	6.5	25	25
Solvents			
Control	–	100	100
Methanol	50	25	37.5
Ethanol	50	25	25
Acetonitrile	50	16.7	13.1
Acetone	50	16.7	12.5
Isopropanol	50	16.7	14.2
1,4-Dioxane	50	8.3	3.8

EDTA ethylenediaminetetraacetic acid

[a] Values are the average of three independent experiments and the maximal mean deviation is ±5% of values

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ORIGINAL PAPER

Improvement of laccase production from *Ganoderma* sp. KU-Alk4 by medium engineering

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Abstract To engineer the production of laccase by *Ganoderma* sp. KU-Alk4, a newly isolated white-rot fungus, a seven-level Box–Behnken factorial design was employed to optimize the culture medium composition. A mathematical model was developed to show the effect of each medium component and their interactions on the production of laccase activity in submerged fermentation. The model estimated the optimal concentrations of glycerol, yeast extract and veratryl alcohol as 40, 0.22 g/l and 0.85 mM, respectively, with the medium pH of 6.0. These predicted conditions were verified by validation experiments. The optimized medium gave laccase activity of 240 U/ml, which is 12 times higher than that produced in non-optimized medium. Thus, this statistical approach enabled rapid identification and integration of key medium parameters for *Ganoderma* sp. KU-Alk4, resulted the high laccase production.

Keywords Box–Behnken design · *Ganoderma* · Glycerol · Laccase · Media optimization

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Introduction

White-rot fungi, a heterogeneous group of lignin-degrading basidiomycetes, have received considerable attention for their bioremediation potential (Smith and Thurston 1997) which is due to their extracellular nonspecific and nonstereoselective enzyme system composed of laccases (EC 1.10.3.2), lignin peroxidases (LiP, EC 1.11.1.14) and manganese peroxidases (MnP, EC 1.11.1.13), which function together with H_2O_2-producing oxidases and secondary metabolites (Field et al. 1993; Barr and Aust 1994; Kuhad et al. 1997). They degrade a wide range of pollutants, including polycyclic aromatic hydrocarbons, chlorinated phenols, polychlorinated biphenyls, dioxins, pesticides, explosives and dyes (Kirk et al. 1978; Higson 1991). Studying the lignin-modifying enzymes of white-rot fungi is valuable not only from the standpoint of comparative biology but also with the expectation of finding better lignin-degrading systems for use in various biotechnological applications. The biochemistry of the genus *Ganoderma* has been extensively studied because most of its species possess medicinal properties (Jong and Birmingham 1992). Additionally one of the important properties of *Ganoderma* spp. is their ligninolytic potential. The ligninolytic enzymes from *Ganoderma* sp. KU-Alk4 have been used to remove lignin from paper mulberry bark in a more environmentally friendly pulping process than the alkaline extraction previously used (Poonpairoj et al. 2001b).

The expression of laccase isozymes of *Ganoderma* sp. KU-Alk4 was found to be influenced by culture conditions such as the nature and concentration of carbon source, initial pH and the presence of inducers (report elsewhere). Optimization of the culture conditions and medium composition is necessary for laccase production, especially in large scale production. Application of statistical

methodologies is helpful in finding the effects and interactions of the physiological factors that play roles in biotechnological processes. The use of different statistical designs has been recently employed for medium optimization for the production of enzymes such as lysozyme, xylanase and amylase by fungal cultures (Ghanem et al. 2000; Dey et al. 2001; Francis et al. 2003; Parra et al. 2005).

To date no studies on medium engineering for laccase production of *Ganoderma* have been reported. Thus, the aim of this work was to optimize the medium for the laccase produced by the newly isolated *Ganoderma* sp. KU-Alk4 using a Box–Behnken experimental design. The type and concentration of carbon and nitrogen sources and inducers at different pH values were investigated.

Materials and methods

Fungal strain

A *Ganoderma* sp. designated as KU-Alk4 was isolated from a living tree, *Terminalia bellerica* Roxb., at Kasetsart University, Thailand. It was selected for laccase production after an extensive screening of strains of basidiomycetes and filamentous fungi based on its high activity in the removal of lignin from paper mulberry bark (Poonpairoj et al. 2001a, b). Identification and phylogenetic study of this strain will be reported elsewhere. Preliminary studies based on ITS4 sequence analysis suggested that it is 93% identity to *G. philippii*. In addition, crude enzyme of KU-Alk4 proved to be able to decolorize dyes effectively. The fungus was maintained on a potato dextrose agar (PDA) slants and stored at 4°C and subcultured monthly.

Chemicals

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

Media design and composition

The medium design was based on that of Tien and Kirk (1988). A standard Kirk's medium was the following composition (per l): 10 g glucose, 0.22 g ammonium tartrate, 1.64 g sodium acetate, 1 g Tween80, 60 ml trace element stock solution and 100 ml salts stock solution. The trace element stock solution consisted of (per l): 9 g nitrilotriacetate, 3 g $MgSO_4 \cdot 7H_2O$, 2.73 g $MnSO_4$, 6 g NaCl, 0.6 g $FeSO_4 \cdot 7H_2O$, 1.1 g $CoSO_4 \cdot 7H_2O$, 0.6 g $CaCl_2 \cdot 2H_2O$, 1.1 g $ZnSO_4 \cdot 7H_2O$, 60 mg $CuSO_4 \cdot 5H_2O$, 110 mg $AlK(SO_4)_2 \cdot 12H_2O$, 60 mg H_3BO_3 and 70 mg $Na_2MoO_4 \cdot 2H_2O$. The salts stock solution composed of (per l): 20 g KH_2PO_4, 5 g $MgSO_4 \cdot 7H_2O$, 1.3 g $CaCl_2 \cdot 2H_2O$, 10 mg thiamine–HCl and 16.7 ml trace element stock solution. Non-optimized control medium contained 10 g glucose/l and 0.22 g ammonium tartrate/l as C- and N-sources with 0.85 mM veratryl alcohol as an inducer. The pH was not controlled through the culture period. Medium composition was modified by changing the nature and concentration of carbon and nitrogen sources, the pH and by the addition of potential inducers of laccase activity. The pH of modified media was controlled throughout the culture period with 0.1 M citrate-phosphate buffer.

Fifteen plugs of 5-mm diameter grown on PDA at 30°C for 4 days, were used as inoculum in 50 ml medium. The cultures were incubated at 30°C in static condition for 3 days. On the third day, an inducer was added and the culture continued, now with shaking at 30°C. Samples were taken from the flask everyday and centrifuged at 10,000 rev/min, 4°C, for 15 min. The supernatant was used for the determination of laccase activity. To obtain the mycelial dry weight, the contents of three flasks were taken and centrifuged. Then, the cell pellet was dried at 60°C to a constant weight.

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 ($\varepsilon = 3.6 \times 10^4\ M^{-1}\ cm^{-1}$). 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 is defined as the amount of enzyme required to oxidize 1 µmol of ABTS per min at 25°C.

Experimental design and evaluation

Effects of seven factors such as pH, type of C-source, concentration of C-source, type of N-source, concentration of N-source, inducer type and inducer concentration on laccase production were statistically tested for the best combination of those factors on laccase produced by *Ganoderma* sp. KU-Alk4. The optimization was based on a Box–Behnken design with 66 combinations and ten replications of the centre point. (Box 1965). Table 1 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 three different levels were selected based on results of previous study in 50 ml medium

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

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

without any pH control. 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 2 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.

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:

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

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 X_i, X_j (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 applicability 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).

Results

Laccase from Ganoderma sp. KU-Alk4

In the basic Kirk's medium, production of laccase by the new isolate, KU-Alk4, started when the inducer veratryl alcohol was added to the culture in day 3 (Fig. 1). The control treatment showed a typical curve of microbial growth. The onset of the secondary growth phase was on day 5 and laccase production could be observed in the secondary phase. The maximal laccase production, 20 U/ml, was in day 9. This titre was low compared to typical

Table 2 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	Laccase[a] (U/ml)	Runs	X_1	X_2	X_3	X_4	X_5	X_6	X_7	Laccase[a] (U/ml)
1	0	0	0	−1	−1	−1	0	25.8	30	+1	−1	0	+1	0	0	0	0
2	0	0	0	+1	−1	−1	0	19.8	31	−1	+1	0	+1	0	0	0	0
3	0	0	0	−1	+1	−1	0	8.4	32	+1	+1	0	+1	0	0	0	0
4	0	0	0	+1	+1	−1	0	29.8	33	0	0	−1	−1	0	0	−1	14.4
5	0	0	0	−1	−1	+1	0	0	34	0	0	+1	−1	0	0	−1	12.2
6	0	0	0	+1	−1	+1	0	0	35	0	0	−1	+1	0	0	−1	4.9
7	0	0	0	−1	+1	+1	0	21.3	36	0	0	+1	+1	0	0	−1	3.3
8	0	0	0	+1	+1	+1	0	5	37	0	0	−1	−1	0	0	+1	0
9	−1	0	0	0	0	−1	−1	0	38	0	0	+1	−1	0	0	+1	0
10	+1	0	0	0	0	−1	−1	0	39	0	0	−1	+1	0	0	+1	0
11	−1	0	0	0	0	+1	−1	0	40	0	0	+1	+1	0	0	+1	0
12	+1	0	0	0	0	+1	−1	0	41	−1	0	−1	0	−1	0	0	0
13	−1	0	0	0	0	−1	+1	0	42	+1	0	−1	0	−1	0	0	0
14	+1	0	0	0	0	−1	+1	0	43	−1	0	+1	0	−1	0	0	0
15	−1	0	0	0	0	+1	+1	0	44	+1	0	+1	0	−1	0	0	0
16	+1	0	0	0	0	+1	+1	0	45	−1	0	−1	0	+1	0	0	0
17	0	−1	0	0	−1	0	−1	4	46	+1	0	−1	0	+1	0	0	0
18	0	+1	0	0	−1	0	−1	8.5	47	−1	0	+1	0	+1	0	0	0
19	0	−1	0	0	+1	0	−1	18.5	48	+1	0	+1	0	+1	0	0	0
20	0	+1	0	0	+1	0	−1	27.5	49	0	−1	−1	0	0	−1	0	65.1
21	0	−1	0	0	−1	0	+1	0	50	0	+1	−1	0	0	−1	0	95.2
22	0	+1	0	0	−1	0	+1	0	51	0	−1	+1	0	0	−1	0	70.7
23	0	−1	0	0	+1	0	+1	0	52	0	+1	+1	0	0	−1	0	149
24	0	+1	0	0	+1	0	+1	0	53	0	−1	−1	0	0	+1	0	15.2
25	−1	−1	0	−1	0	0	0	0	54	0	+1	−1	0	0	+1	0	15.2
26	+1	−1	0	−1	0	0	0	0	55	0	−1	+1	0	0	+1	0	19.1
27	−1	+1	0	−1	0	0	0	0	56	0	+1	+1	0	0	+1	0	13
28	+1	+1	0	−1	0	0	0	0	57–66	0	0	0	0	0	0	0	0
29	−1	−1	0	+1	0	0	0	0									

X_1 pH, X_2 carbon source, X_3 concentration of carbon source, X_4 nitrogen source, X_5 concentration of nitrogen source, X_6 inducer type, X_7 = inducer concentration

[a] Laccase activity from day 13, when laccase reached its highest peak

reported strains, 4–100 U/ml (Revankar and Lele 2006). Hence, in order to improve laccase production by KU-Alk4, a Box–Behnken experimental design was applied for investigation of the relationship between substrate medium components, their concentration and the pH of the medium to optimize the production of laccase. To the best of our knowledge the optimization of the medium ingredients for laccase production by using this design has not been reported.

Box–Behnken experimental design

By the design of Box–Behnken (Table 1) followed with 66 trial experiments (Table 2), laccase production varied from 0 to 149 U/ml in the 66 different media tested. A constant increase of laccase production was observed from the day 5 to day 13 when laccase reaches its peak of production. The five best conditions were run no. 4 (25 g of lactose/l; 0.44 g of malt extract/l; 0.85 mM veratryl alcohol), run no. 20 (25 g of glycerol/l; 0.44 g of yeast extract/l; no inducer), run no. 49 (10 g of glucose/l; 0.22 g of yeast extract/l; 0.85 mM veratryl alcohol), run no. 51 (40 g of glucose/l; 0.22 g of yeast extract/l; 0.85 mM veratryl alcohol) and run no. 52 (40 g of glycerol/l; 0.22 g of yeast extract/l; 0.85 mM veratryl alcohol). The time course of laccase production of the five best runs are compared in Fig. 2. The medium pH was controlled at pH 6.0 throughout the experiment. The best conditions were the medium with 40 g glycerol/l, 0.22 g yeast extract/l and 0.85 mM veratryl alcohol with the culture pH controlled at

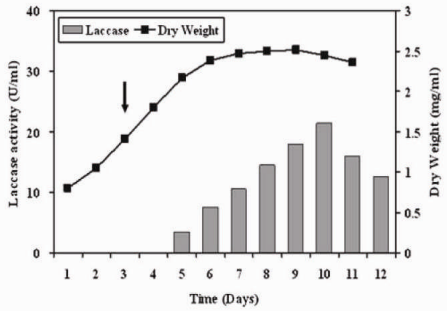

Fig. 1 Laccase activity and growth curve of *Ganoderma* sp. KU-Alk4 in non-optimized Kirk's liquid medium, initial pH 7.0. No pH controlled throughout the experiment. Culture conditions: first 3 days as static culture; after 3 days, cultures were shaken at 140 rev/min 30°C. *Arrow* indicates the addition of 0.85 mM veratryl alcohol and the starting time of shaking

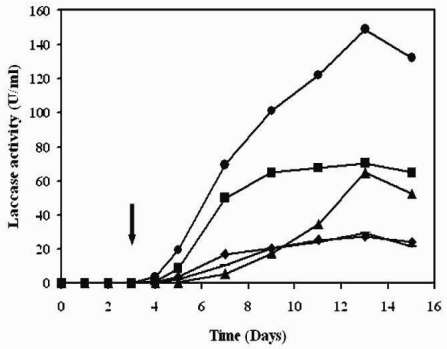

Fig. 2 Laccase production by *Ganoderma* sp. KU-Alk4 at time course of fermentation in the selected optimized media by the Box–Behnken factorial designed, run nos., 4 (—); 20 (◆); 49 (▲); 51 (■); 52 (●). Conditions of growth: static condition for 3 days and then shaken at 140 rev/min at 30°C. The medium pH was controlled throughout the experiment. *Arrow* indicates the addition of inducer and the starting time of shaking

pH 6.0 and the maximum activity obtained in day 13 was 149 U/ml.

Table 3 shows the ANOVA of the results on the peak of laccase production over the time course of the fermentation. The pH, nature and concentration of carbon sources and the nature and concentration of inducers, were highly significant ($P < 0.0005$). Furthermore, the interaction between the sources of carbon and the type of inducer was significant ($P < 0.005$). The statistical analysis shows that the carbon sources and inducer type are the most important factors for laccase production. On contrary, sources and concentrations of nitrogen were not significant for laccase production.

In order to find the optimum and statistically significant interactions between factors, a second order (quadratic) polynomial equation fitted the experimental data for laccase produced by KU-Alk4 was constructed with a multiple correlation coefficient (R^2) of 0.98 (residual 0.045, variance explained 93%):

$$\begin{aligned}&\text{Laccase production (U/ml)}\\&= 179.4 + 67.3X_1^2 + 79.6X_2 - 89.3X_2^2 + 52.4\\&X_3 - 81.9X_3^2 - 15.0X_4 + 22.0X_4^2 + 20.1X_5 + 14.6X_5^2\\&- 240.6X_6 - 105.4X_6^2 - 34.3\\&X_7 + 38.1X_7^2 + 0.0X_1X_2 + 0.0X_1X_2^2 + 8.5X_1^2X2\\&- 2.4X_1^2X_3 - 0.5X_1^2X_4 + 11.9X_1^2\\&X_4 - 36.0X_1^2X_6 - 21.8X_1^2X_7 + 52.6X_2X_3 - 55.5X_1X_3^2\\&- 40.5X_2^2X_3 + 5.6X_2\\&X_5 - 9.1X_2^2X_5 - 143.2X_2X_6 + 162.5X_2^2X_6 - 16.9X_2X_7\\&+ 14.8X_2^2X_7 + 0.7X_3X_4 + 11.0X_3^2X_4 - 72.2X_3X_6 + 4.8X_3X_7\\&+ 14.0X_4X_5 - 39.6X_4X_6 + 23.1X_4X_7\\&+ 41.9X_5X_6 - 42.0X_5X_7\end{aligned} \quad (2)$$

where X is the coded value (between -1 and +1) for the factor indicated by the attached subscript. The coefficients of pH (quadratic), carbon sources (linear and quadratic), carbon source concentrations (linear and quadratic) sources of carbon (quadratic), inducer (linear and quadratic) and the interactions between carbon sources and carbon source concentrations (linear and quadratic), carbon sources (quadratic) and carbon source concentrations (linear), carbon sources (linear and quadratic) and inducer levels (linear) and the levels of sources of carbon and inducer (linear) were all statistically significant at a level of $P < 0.005$. The least significant terms were included in the equation to maintain the hierarchy in the model.

The response contour plots described by the regression model were drawn to illustrate relationships between factors on laccase activity produced under the sets of conditions and treatment levels tested. Results of using different carbon sources at three concentration levels of 10, 25 and 40 g/l, at which the pH was controlled at 4.0, 6.0 or 8.0, showed that glucose and glycerol were efficient carbon sources compared to lactose. The best carbon source was 40 g glycerol/l at pH 6.0 that gave the highest activity of 80 U/ml which was 78% higher than that obtained with 40 g glucose/l. With different nitrogen sources at three different levels when the pH was controlled at 4.0, 6.0 or 8.0, the result showed that the fungus produced the highest laccase activity with 0.22 g yeast

Table 3 The analysis of variance of the Box–Behnken experimental design for the laccase production by Ganoderma sp. KU-Alk4

	F	*P*
(1) pH L + Q	35.75	0.00000***
(2) Carbon sources L + Q	69.61	0.00000***
(3) Levels SC L + Q	55.83	0.00000***
(4) Nitrogen sources L + Q	4.06	0.03754^{NS}
(5) Levels SN L + Q	2.11	0.15404^{NS}
(6) Inducer L + Q	148.61	0.00000***
(7) Concentration L + Q	12.69	0.00050**
2 × 3	8.56	0.00128*
2 × 6	77.15	0.00000***
3 × 6	10.96	0.00442

Levels of statistical significance ***$P < 0.00001$, **$P < 0.0005$ and *$P < 0.005$; ^{NS}not significant

extract/l 50 U/ml at pH 6.0 which was four times higher than that produced with ammonium tartrate and malt extract at the same pH 6.0. Inducer was important factor affected to the ability of the fungus on laccase production. Veratryl alcohol at 0.85 mM in the medium that controlled pH at 6.0 statistically proved the most effective on laccase production of KU-Alk4. The laccase activity obtained was 58 U/ml.

Comparison of the observed versus predicted yields is shown in Fig. 3. The points above or below the diagonal line represent areas of over- or under-prediction of the model. This showed that no significant violations of the model were found in the analysis, with 98% correlation of the model with the experimental data obtained.

Optimal condition for laccase production by KU-Alk4 suggested by the Box–Behnken design was glycerol (40 g/l) as carbon source and yeast extract (0.22 g/l) as nitrogen source with veratryl alcohol (0.85 mM) as inducer and the medium pH was controlled at pH 6.0 through out the experiment. To confirm the optimal condition predicted for the production of laccase, a set of five replicates using the optimal combination of substrates and concentrations were used. The highest activity of laccase obtained from KU-Alk4 in confirmed experiments was as high as 240 U/ml (Fig. 4) which was 12 times higher than the non-optimized medium. It was found that the more active culture also resulted in higher enzyme activity. This experiment was robust with high reproducibility, shown by the small error bars.

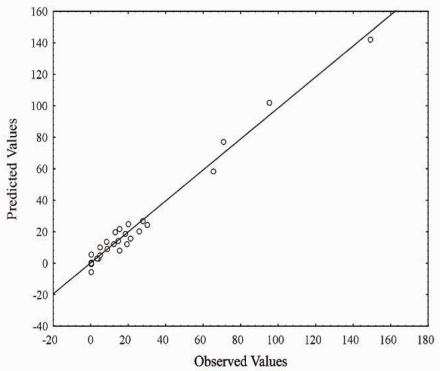

Fig 3 Observation and prediction of laccase activity of *Ganoderma* sp. KU-Alk4 calculated with the model

Discussion

This study considered seven factors with three levels on laccase production of KU-Alk4. To achieve the results obtained in this study using a full factorial design would

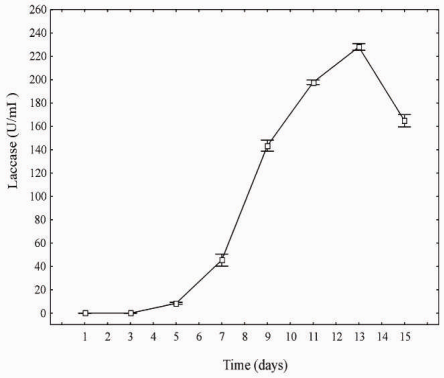

Fig. 4 Confirmatory run using the best medium: glycerol (40 g/l), yeast extracts (0.22 g/l) and veratryl alcohol (0.85 mM) at pH 6.0. *Bars* represent the mean and ± standard error of the five confirmed experiments

have required $3^7 \times 3$ replicates experiments taking into account all the variables involved. By using Box–Behnken design, a significantly smaller combination of factors and levels could be used for effectively examining the effect of interacting factors on laccase production. Thus, only a limited number of experiments (66) were suggested. Optimal medium composition and condition was found that represented a 12 times increase in titre compared to the non-optimized medium. The laccase activity of KU-Alk4 achieved in this work of 240 U/ml represented a significant improvement and demonstrating success in medium engineering using statistical design of Box–Behnken.

In this study, laccase was produced under a variety of selected culture conditions to investigate their effects on the amount of laccase. Results led us to consider on the medium pH that the culture pH were controlled throughout the experiment had significant effect on the fungal growth and laccase production, that resulted to the selection of carbon source in both types and concentration. We also realize that the other factors such as dissolved oxygen which were not controlled in shake flasks, ethanol and copper, would influence laccase production by the fungus, and may increase the titre values beyond those predicted by the model. However, to study on the effect of dissolved oxygen, a controllable fermenter would need to be used. For example, Eggert et al. (1996) using a 100-l fermenter to culture *Pycnoporus cinnabarinus* succeeded in doubling the laccase titres over those obtained in shake flasks. Laccase production from *Trametes versicolor* was increased 20-fold by ethanol, which however was comparable to that with veratryl alcohol (Lee et al. 1999). Addition of copper, a micronutrient that has key role as metal activator in fungal laccase, enhanced laccase production 30-fold with *T. pubescens* (Galhaup and Haltrich 2001) and twofold with *Ganoderma* sp. *WR-1* (Revankar and Lele 2006).

Optimum conditions for laccase production appeared to be different to those previously reported for the other fungi. Clearly, the most obvious difference is that the results of ANOVA which showed carbon sources and inducer types were the two most important factors for laccase production in KU-Alk4. Sources and concentrations of nitrogen were the least important nutrient factor. In most of the lignino-lytic fungi, the C:N ratio is a factor that influences laccase production. Nitrogen limitation usually stimulates the production of laccase in *P. cinnabarinus* (Eggert et al. 1996) and *Botryosphaeria* sp. (Vasconcelos et al. 2000). In contrast, high levels of laccase were observed when *Ganoderma lucidum* (D'Souza et al. 1999), *Cyathus stercoreus* (Sethuraman et al. 1999) and *Ceriporiopsis subvermispora* (Lobos et al. 1994) were grown in media with high nitrogen. Our results demonstrate that the addition of sufficient organic nitrogen in the form of yeast extract is suitable for laccase production by KU-Alk4.

In general in fungi, substrates such as glucose that are efficiently and rapidly utilized by the organism result in high level of laccase activity (Galhaup et al. 2002; Nyanhongo et al. 2002) but laccase production by KU-Alk4 was found to be optimal with glycerol as carbon source, though it was consumed more slowly than glucose.

Various aromatic compounds such as veratryl alcohol are able to induce laccase production (Arora and Gill 2001; Dekker and Barbosa 2001). The most widely reported inducer of laccase production is 2,5-xylidine (Galhaup and Haltrich 2001; Galhaup et al. 2002; Rancano et al. 2003; Revankar and Lele 2006). However, Lee et al. (1999) re-

Table 4 Comparison of laccase production by *Ganoderma* sp. KU-Alk4 with some reference fungi

Strain	Inducer	Concentration (mM)	Activity (U/ml)	Reference
Ganoderma sp. KU-Alk4	Veratryl alcohol	0.85	240	Present work
Trametes pubescens	Gallic acid	1	350	Galhaup et al. (2002)
	2,5-Xylidine	1	275	
	$CuSO_4$	2	325	
Trametes versicolor	Veratryl alcohol	1	80	Lee et al. (1999)
	2,5-Xylidine	1	30	
Trametes versicolor	2,5-Xylidine	1	10.9	Bollag and Leonowicz (1984)
Coriolus hirsutus	Syringaldazine	0.1	50	Koroljova-Skorobogat'ko et al. 1998
Trametes pubscens	2,5-Xylidine	1	8	Galhaup and Haltrich (2001)
Trametes versicolor	2,5-Xylidine	1	1.5	Rancano et al. (2003)
Trametes multicolor	$CuSO_4$	1	18	Hess et al. (2002)
WR-1	2,5-Xylidine	0.8	692	Revankar and Lele (2006)
	$CuSO_4$	1	410	

Modified from Revankar and Lele (2006). Note: In all the above cases one unit of laccase activity was defined as the amount of enzyme required to oxidize 1 µmol of ABTS per min at 25°C

ported a doubling of laccase production by *T. versicolor* when veratryl alcohol was used instead of 2,5-xylidine. This is consistent with our observation that in KU-Alk4, only veratryl alcohol, among a number of compounds tested, showed effective stimulation of laccase production. Ferulic acid and guaiacol enhance laccase production in *P. cinnabarinus* (Herpoël et al. 2000), *Phlebia radiata* and *Daedalea flavida* (Arora and Gill 2001). These compounds slowed growth of KU-Alk4 and did not enhance laccase production, suggesting that they are toxic to the fungus.

Conclusions

By medium engineering we have increased laccase production of new isolated mushroom, KU-Alk4, by 12-fold. From an economic point of view, the most important parameters in screening and optimization of media are time and cost. The strategy used here demonstrates advantages in comparison with traditional methods and allows the development of a mathematical model that predicts where the optimum is likely to be located. This is the first report on optimization of the medium ingredients for laccase production of *Ganoderma* sp. by using Box–Behnken design. Table 4 The laccase activities produced by *Ganoderma* sp. KU-Alk4 in optimum conditions as designed were significantly higher than produced by most fungi using similar conditions.

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