5.3.3 Optimum pH and pH stability

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 (Figure 52A) which was the same as laccase of *G. lucidum* (GaLcs) (Ko *et al*., 2001) and those of *Pleurotus eryngii* (Muñoz *et al*., 1997) and *Botrytis cinerea* (Slomozynski *et al*., 1995). Most fungal laccases have optimum pH, in general, in the acidic region, between 3.0 and 5.0. At 25ºC for 1 h, KULacs were stable over a wide range of pH (3.0-10.0).

It is very interesting that all KULacs were atable at pH 10.0 for 1 hour especially KULac 5 whose activity still retained 100%. The activities of KULac 1, 2 and 3 could retain 80% at such alkaline pH (Figure 52B).

5.3.4 Optimum temperature and temperature stability

All KULacs had high optimum temperature between 55-90ºC (Figure 53). They were relatively stable at elevated temperatures, 40-60ºC. KULac3 had an extremely high optimum temperature (90ºC), when the initial rate was measured using DMP for 1 min, though it was not stable at that high temperature for more than 2 min. However, KULac 3 was the most thermostable. It could maintain 100% activity at 55ºC for 1 h and 95 and 50% at 60 and 70ºC, respectively, and the enzyme was completely inactivated when it was at 80ºC for 1 h. Most laccases previous reported have optimum temperatures between 50ºC to 60ºC (Slomczynski *et al*., 1995; Xiao *et al*., 2003; Ryan *et al*., 2003). The highest optimum temperature of laccase was that of *Marasmius quercophilus*, a litter decomposing fungus that was 80ºC (Farnet *et al*., 2000). To the best of our knowledge, KULac 3 is a novel enzyme with extremely high temperature and stability.

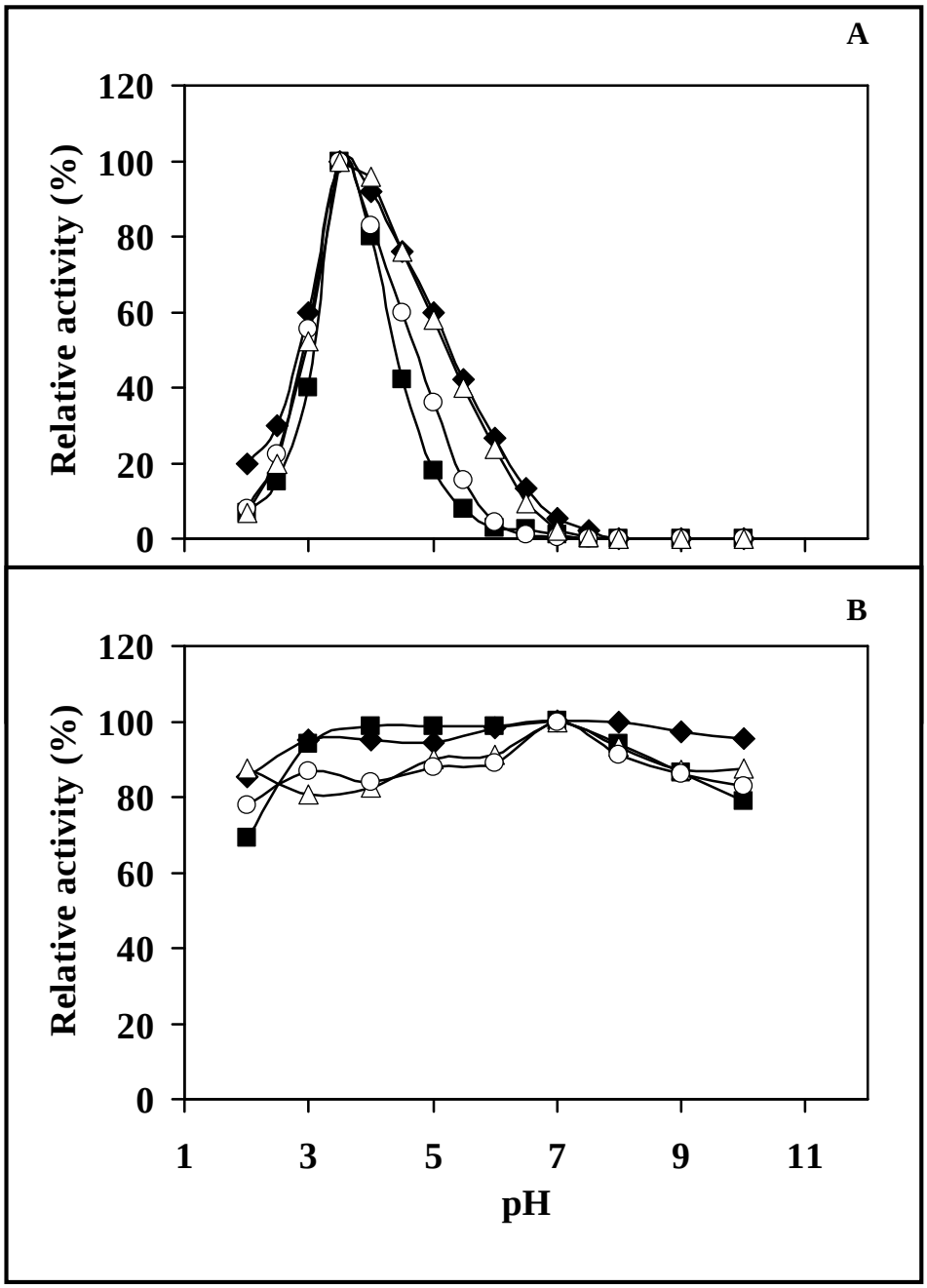

Figure 52 (A) Optimum pH and (B) pH stability for KULac 1; (○), KULac 2; (△), KULac 3; (■) and KULac 5; (◆).Values are the average of three independent experiments and the maximal mean deviation is ± 5% of values.

KULac 5, another isozyme that *Ganoderma* sp. KU-Alk4 produced in G4% medium had its optimum temperature at 70ºC, second to KULac 3, while those of KULac 1 and KULac 2 were 55 and 65ºC, respectively. It appeared that laccase isozymes that the fungus produced in 4% concentrated glucose had higher optimum temperature than those produced in 1% glucose.

On the other hand, temperature stability of KULac 1 was slightly better than that of KULac 5. Both isozymes retained 90% activities at 55ºC but half life of KULac 5 was at 60ºC while that of KULac 1 was nearly 65ºC.

KULac 2, the new laccase, proposed from its N-terminal amino acid sequence, was the most sensitive isozyme. Its 100% activity could be rbtained for 1 h at only 40ºC. At 45, 50, 55 and 60ºC, the activities were 90, 75, 50 and 35% remained, respectively, and no activity at 70ºC.

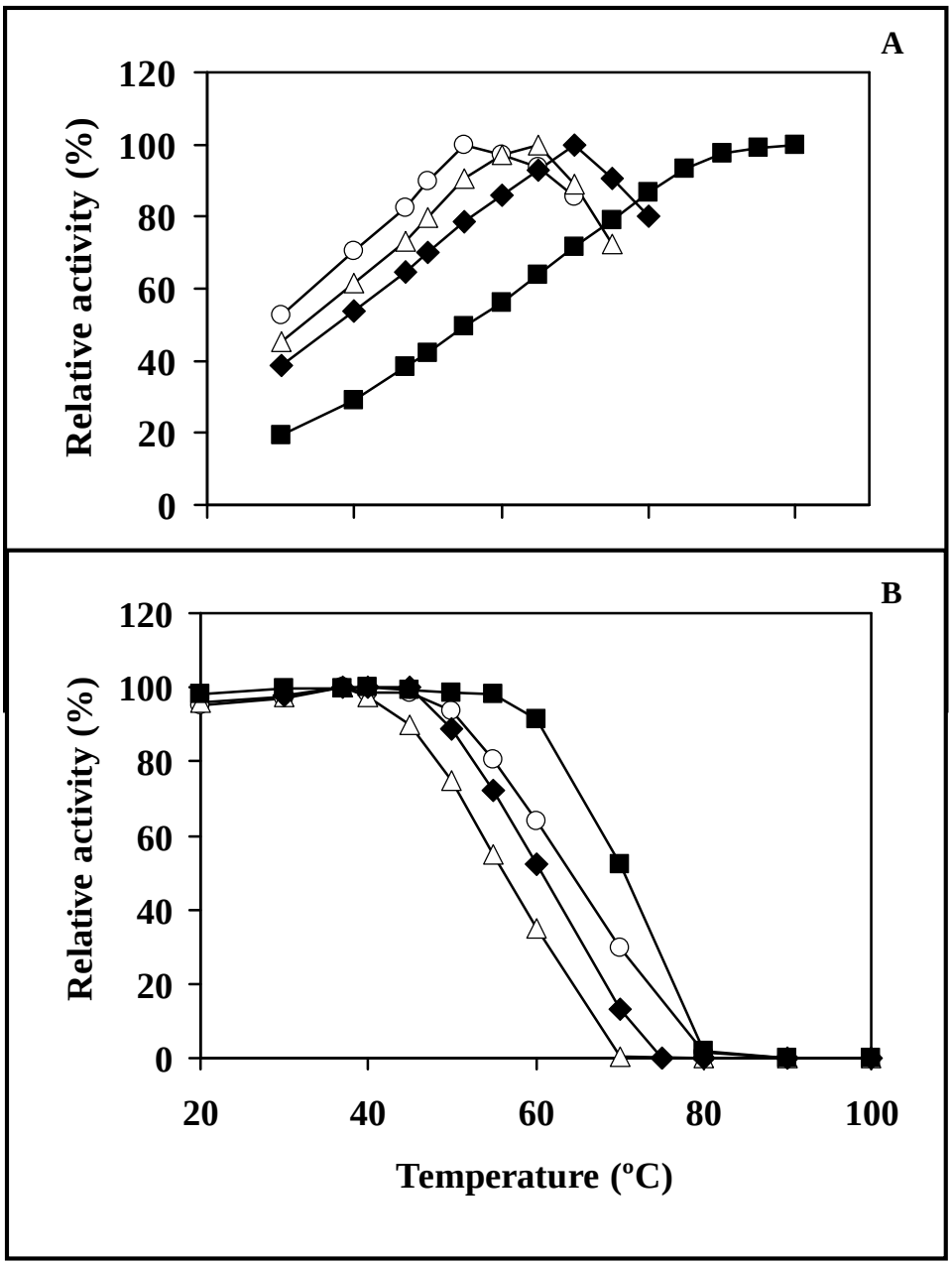

Figure 53 (A) Effect of temperature on the activity and (B) thermal stability of KULac 1; (○), KULac 2; (△), KULac 3; (■) and KULac 5; (◆). Values are the average of three independent experiments and the maximal mean deviation is ± 5% of values.

5.3.5 Substrate specificity

Similar to the other fungal laccases, ABTS was the best substrate of all KULacs (Table 29). 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 4 KULacs could oxidize DMP, hydroquinone and guaiacol, too. KULac 3 and 5 oxidized DMP approx. 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.

5.3.6 Oxidization of phenolic compounds

KULac 1 and 3 were able to oxidize a series of phenolic compounds with different degrees of substitution (Table 30). Both isozymes presented comparable rates of oxidation of these substrates. Like the other fungal laccases (Gianfreda, 1999; Leonowicz *et al*., 2001), the 2 isozymes obtained from *Ganoderma* sp. KU-Alk4 oxidized, non-specifically, a wide range of phenolic and non-phenolic aromatic substrates. The KULacs did not oxidize tyrosine as it is a characteristics of true laccase (Robles *et al*., 2002; Jung *et al*., 2002 Litthauer *et al*., 2006). The nature and substitution on the phenolic ring affected the oxidation rate of laccases. Both isozymes presented comparable rates of oxidation of these substrates. They could oxidize substituted phenols (DMP, hydroquinone, resorcinol, 4-chlorophenol and guaiacol), phenolic aldehyde (vanillin), alcohols (vanillyl and veratryl alcohol) and acids (syringic, vanillic, ferulic, caffeic, *ρ*-coumaric, and abietic acid). They could oxidize naphthols, both 1- and 2-naphthol, the former more rapidly than the latter. No activity was observed with catechol, veratraldehyde, benzaldehyde, benzoic acid, veratric acid or cinnamic acid including non-phenolic aromatic compounds.

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. As well, *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*., 2006) which had no activity with the *m*-diphenol substrates. Moreover, laccases in *Trametes trogii, Sclerotium rolfsii* and *Dichomitus squalens* were able to oxidize catechol while KULacs showed no activity to that *o*-diphenol (Zouari-Mechichi *et al*. 2006; Ryan *et al*., 2003; Périé *et al*., 1998).

Table 29 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 30 Rates of oxidation of various substrates catalyzed by KULac 1 and 3 from *Ganoderma* sp. KU-Alk4.

Substrates	Dissolved O_2 consumption rate[a] ($mgL^{-1}min^{-1}$)	
	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

Table 30 (Continued)

Substrates	Dissolved O_2 consumption rate[a] ($mgL^{-1}min^{-1}$)	
	KULac 1	KULac 3
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

[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)

NC: no changes in the reaction

Table 31 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*, *Trametes versicolor* and *T. hirsuta* (Abadulla *et al*., 2000, Ryan *et al*., 2003). Azide made 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 suggested 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.

Table 31 Effect of various reagents and organic solvents on laccase isozymes from *Ganoderma* sp. KU-Alk4.

Reagents	Final concentration (mM)	Relative activity[a] (%)	
		KULac 1	KULac 3
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[b]	1.3	100	100
EDTA	6.5	25	25
Solvents	Final concentration (%)	Relative activity[a] (%)	
		KULac 1	KULac 3
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

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

[b] EDTA: Ethylenediaminetetraacetic acid