1.2.5 Regulation of laccase production by catabolite repression control

As glucose was the best C-source, effect of glucose concentrations on *Ganoderma* sp. KU-Alk4 was investigated. The culture were grown in pH 8.0-media with various glucose concentration from 0.1-4.0%. $CuSO_4$ of 0.02 mM, 0.85 mM veratryl alcohol and 140 rpm shaking rate were employed in this experiment.

The higher the glucose concentration (from 0.1-4.0%), the higher the fungal biomass was obtained (Figure 16A). On the otherhand, total laccase activity produced by *Ganoderma* sp. KU-Alk4 increased only when glucose concentration increased from 0.1-1.0% but decreased when glucose is higher than 1.0% (Figure 16B). Specific activity of laccase produced by the culture grown for 9 days in the medium with 0.1, 0.5, 1, 2, 3 and 4% glucose were 2, 114, 126, 59, 40 and 33 U/mg cell dry weight, respectively (Figure 16C). This primarily suggested that glucose repressed the enzyme synthesis of *Ganoderma* sp. KU-Alk4, similar to that found in several fungi and yeast, and it was thought to be an energy-saving response (Ronne, 1995; Dekker and Barbosa, 2001; Galhaup *et al*., 2002). From the results, it was apparent that the physiology of this new isolate, *Ganoderma* sp. KU-Alk4, in lignin-degrading enzymes production was controlled by glucose concentration.

More interesting was that, though the total activity was decreased but the concentration of glucose influenced the fungus to produce different sizes and numbers of isozymes (Figure 16D). This is the first time, to our knowledge, that isozyme profile controlled by concentration of glucose has been revealed. With the higher concentration of glucose, the higher molecular weight of laccase isozymes were synthesized. This would occur from having high % glycosylation in the media with high glucose concentration resulted the increasing of high molecular weight isozymes (Eggert *et al*., 1996).

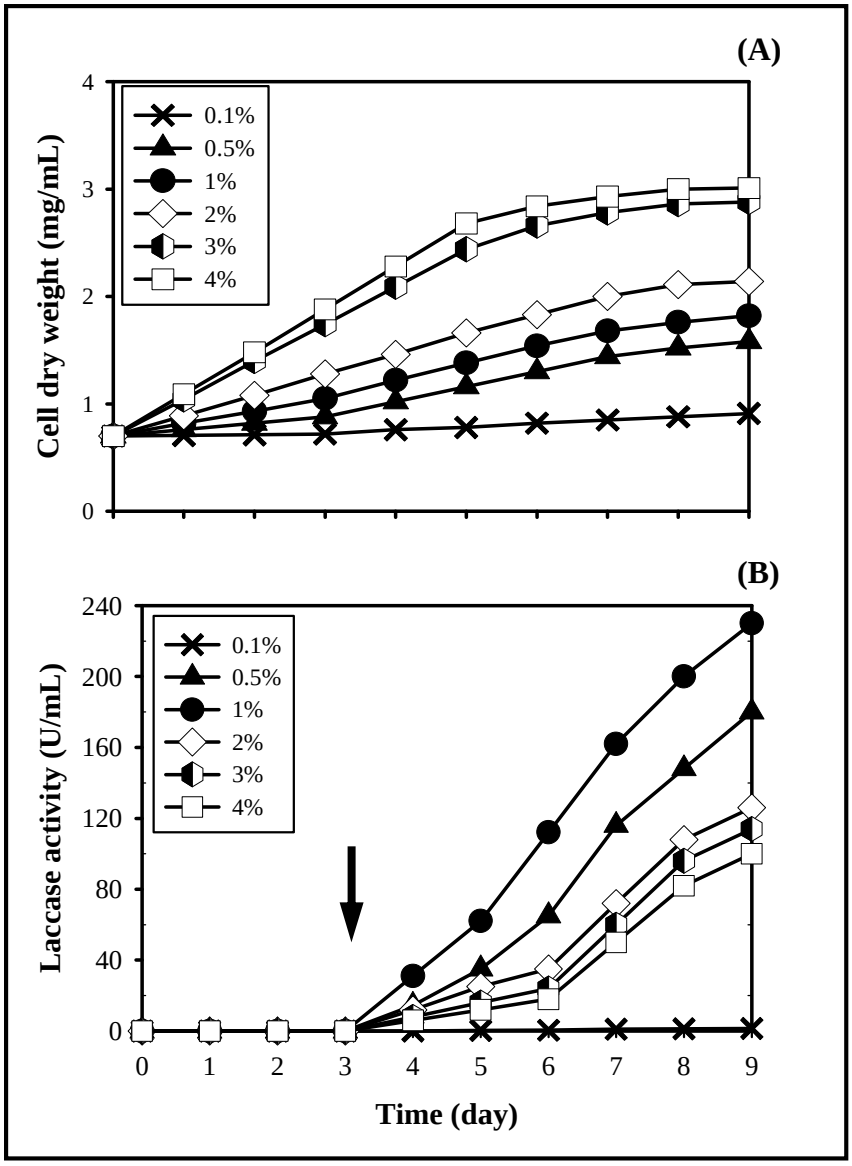
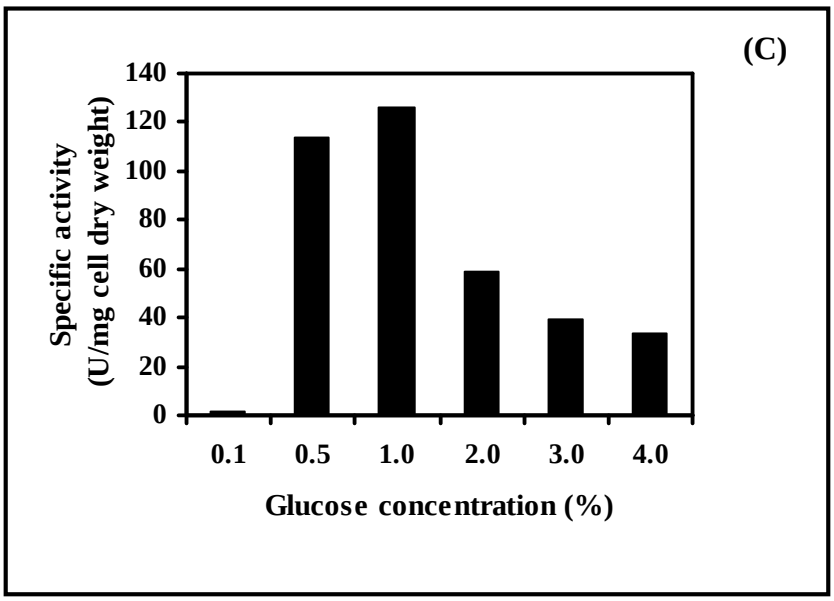


Figure 16 Effect of glucose concentration on laccase production of *Ganoderma* sp. KU-Alk4 in Kirk's medium pH 8.0. Culture conditions in 2 steps. First 3 days as unshaking culture, after 3 days, cultures were shaken at 140 rpm at room temperature. Arrow indicates the addition of veratryl alcohol, 0.85 mM. (A) mg cell dry weight, (B) laccase activity against 2,6-dimethoxyphenol, (C) Effect of glucose concentration on specific laccase activity of *Ganoderma* sp. KU-Alk4 in Kirk's medium pH 8.0 and (D) active bands of laccase (16 µg protein) on native PAGE, staining with 2,6-dimethoxyphenol.



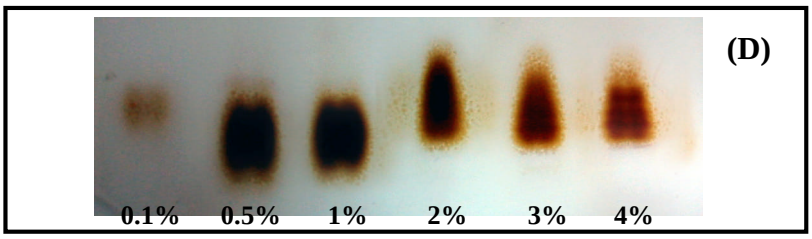


Figure 16 (Continued)

To prove that laccase production from *Ganoderma* sp. KU-Alk4 is controlled by catabolite repression, further experiment was performed. The fungus was cultivated in Kirk's medium pH 8.0 without the phenolic compound for 3 days, then veratryl alcohol was added to the final concentration of 0.85 mM. The media contained 1% glucose and 0.02 mM $CuSO_4$. The culture was then allowed to grow under shaking condition at 140 rpm for further 4 days to produce laccase. Then, mycelia were harvested. The mycelia was washed twice with saline and inoculated to the fresh Kirk's medium. The new media was pH 8.0 with various glucose concentrations from 1.0-4.0%. $CuSO_4$ (0.02 mM), veratryl alcohol (0.85 mM) and 140 rpm shaking rate were employed.

The result showed that pattern of the fungal growth and its laccase production was the same as the previous experiment. The higher the glucose concentration (1-4%), the higher the fungal biomass was obtained (Figure 17A). On the other hand, laccase activity produced by *Ganoderma* sp. KU-Alk4 decreased when glucose in the fresh medium was higher than 1% (Figure 17B). Therefore, the synthesis of laccase from *Ganoderma* sp. KU-Alk4 was controlled by catabolite repression.

1.2.6 Effect of metal ions

Essential heavy metals such as copper or manganese are required for growth and lignin-degrading enzymes production of white rot fungi but in trace, the excess amount is toxic. Copper in the high concentrations give the free form of copper (II) that is extremely toxic to microbial cells (Labbé and Thiele, 1999). Since laccase is a copper-containing protein, copper supplemented in the culture medium induces both laccase transcription and activity (Palmieri *et al*., 2000; Galhaup and Haltrich, 2001). Positive effect of copper addition on the production of laccase was reported in *Ceriporiopsis subvermispora*, *Marasmius quercophilus*, *Trametes versicolor*, *Pleurotus ostreatus*, and *Ganoderma applanatum* (Collins *et al*., 1997; Palmieri *et al*., 2000; Baldrian, 2003).

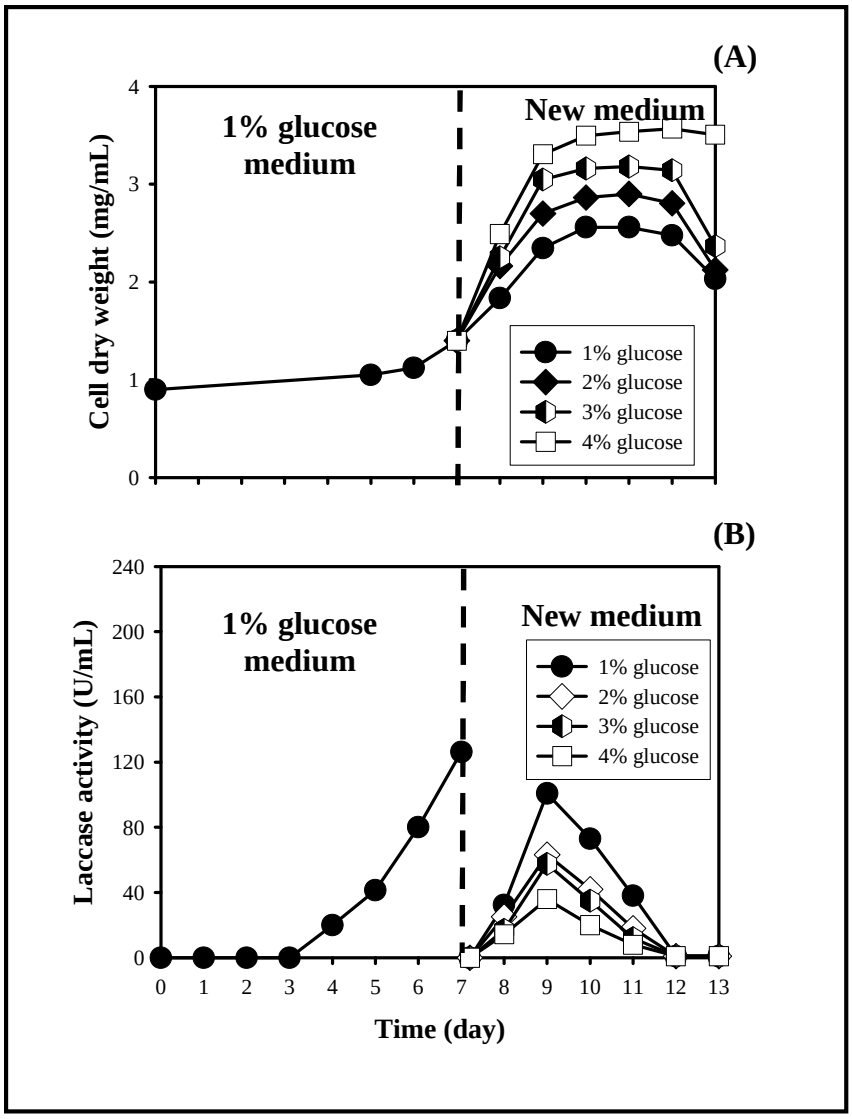


Figure 17 Catabolite repression control of laccase production by *Ganoderma* sp. KU-Alk4 in Kirk's medium pH 8.0. Culture conditions: first 3 days as static culture; After 3 days, cultures were shaken at 140 rpm at room temperature. Veratryl alcohol (0.85 mM) was added to the media in day 3. Harvested cells on day 7. Inoculated of twice saline washed cells to the same media contained 1-4% glucose with 0.85 mM veratryl alcohol. Culture were continue shaken. (A) biomass (mg cell dry weight) and (B) laccase activity against 2,6-dimethoxyphenol.

.

Effect of copper and manganese ions on growth and laccase production ability of *Ganoderma* sp. KU-Alk4 was investigated in the pH 8.0 medium with 1% glucose, and 0.85 mM veratryl alcohol was added after 3 days-pellet from under static condition followed by shaking condition at 140 rpm. The results showed that copper addition into the medium cultivated *Ganoderma* sp. KU-Alk4 affected the fungal growth and its laccase production (Figure 18A, B). Copper sulphate at low concentration of 0.02 and 0.2 mM stimulated growth of *Ganoderma* sp. KU-Alk4 and had positive effect on laccase production (Figure 18A, B). Copper at 0.2 mM stimulated higher production of *Ganoderma* sp. KU-Alk4 laccase, though it caused slight inhibition to the fungal growth compared to 0.02 mM $CuSO_4$, giving a higher specific activity of the enzyme. Specific activities of laccase with 0.2 and 0.02 mM $CuSO_4$ were 186 and 126 U/mg cell dry weight, respectively (Figure 18C). The higher concentration of copper at 2 mM, was so toxic to *Ganoderma* sp. KU-Alk4 that its growth was completely inhibited, and no laccase was detected. In contrast, *Trametes pubescens* produced the highest amount of laccase with 2 mM copper sulphate (Galhaup and Haltrich, 2001).

Bands of laccase isozymes could be observed on native-PAGE when copper sulphate concentrations were 0.02 and 0.2 mM (Figure 18D). The later one showed stronger active bands that related to the quantitative amount of laccase that was detected (Figure 18B, D).

Manganese at the concentration of 133 µM was reported to enhance laccase production in *Clitocybula dusenii* and *Nematoloma frowadii* (Scheel *et al*., 2000). However, manganese sulphate at 0-2.0 mM had relatively no effect on *Ganoderma* sp. KU-Alk4 laccase production, though fungal growth was slightly increased with higher Mn^{2+} ion (Figure 19A, B).

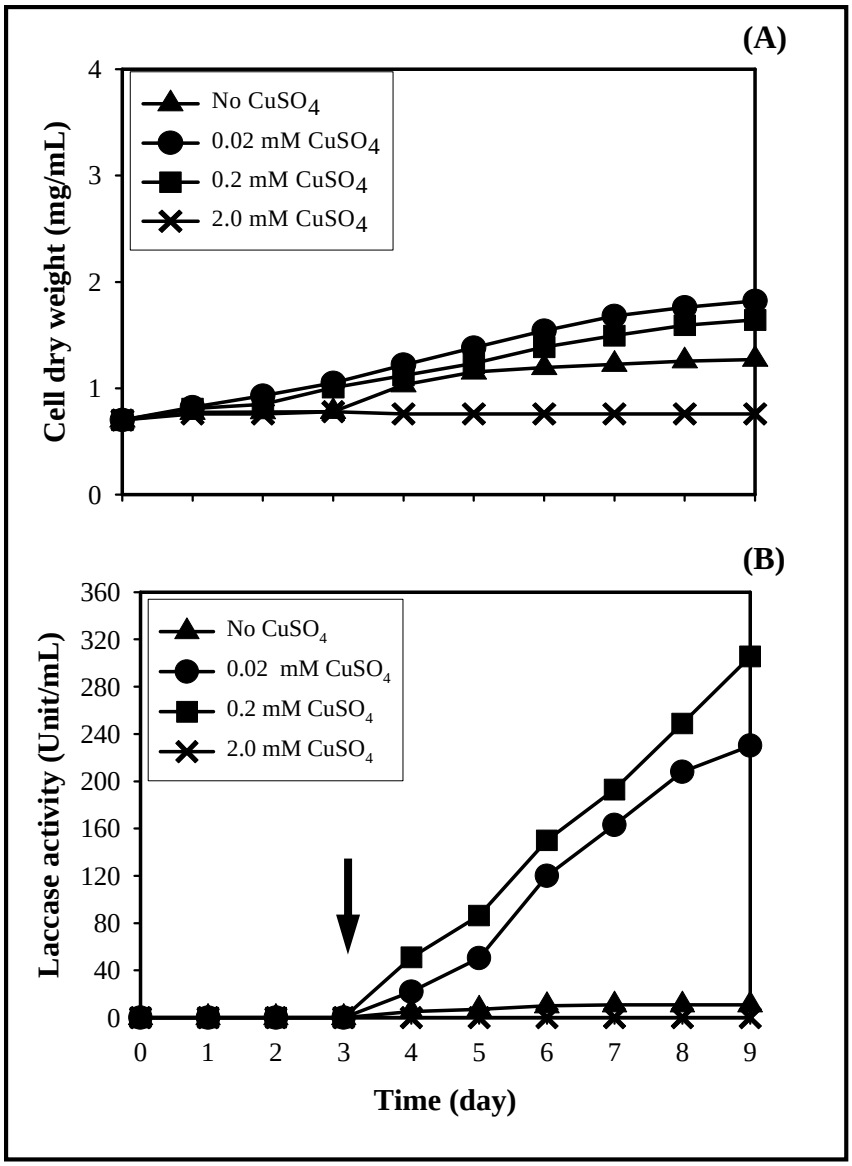
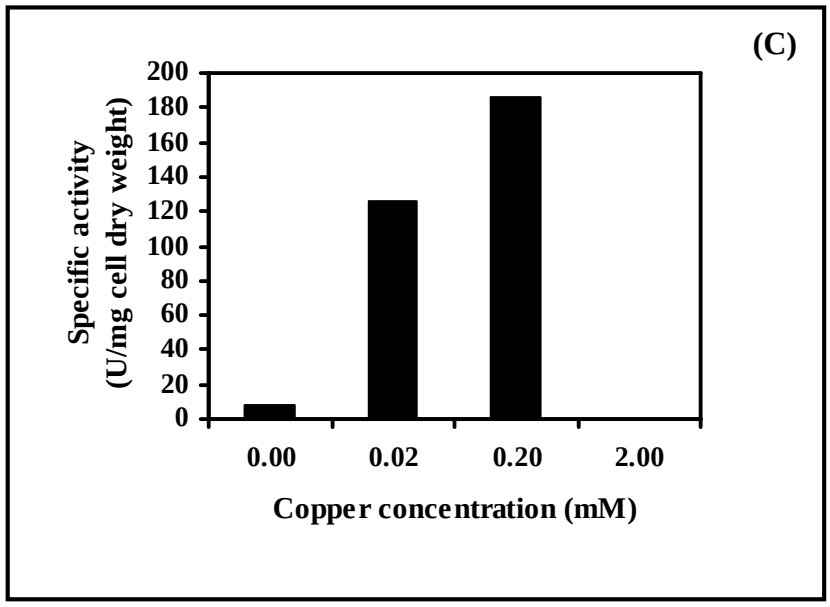


Figure 18 Effect of copper (II) ion on laccase production of *Ganoderma* sp. KU-Alk4 cultivated in Kirk's medium pH 8.0. Culture conditions in 2 steps. First 3 days as unshaking culture, after 3 days, cultures were shaken at 140 rpm at room temperature. Arrow indicates the addition of veratryl alcohol, 0.85 mM. (A) mg cell dry weight, (B) laccase activity against 2,6-dimethoxyphenol, (C) Effect of copper (II) ion on specific laccase activity of *Ganoderma* sp. KU-Alk4 cultivated in Kirk's medium pH 8.0 and (D) active bands of laccase (16 µg protein) on native PAGE, staining with 2,6-dimethoxyphenol.



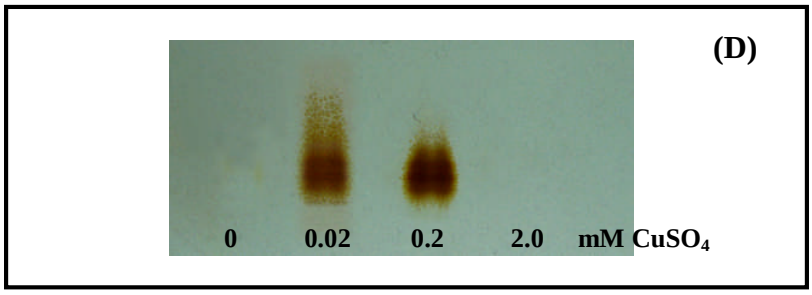


Figure 18 (Continued)

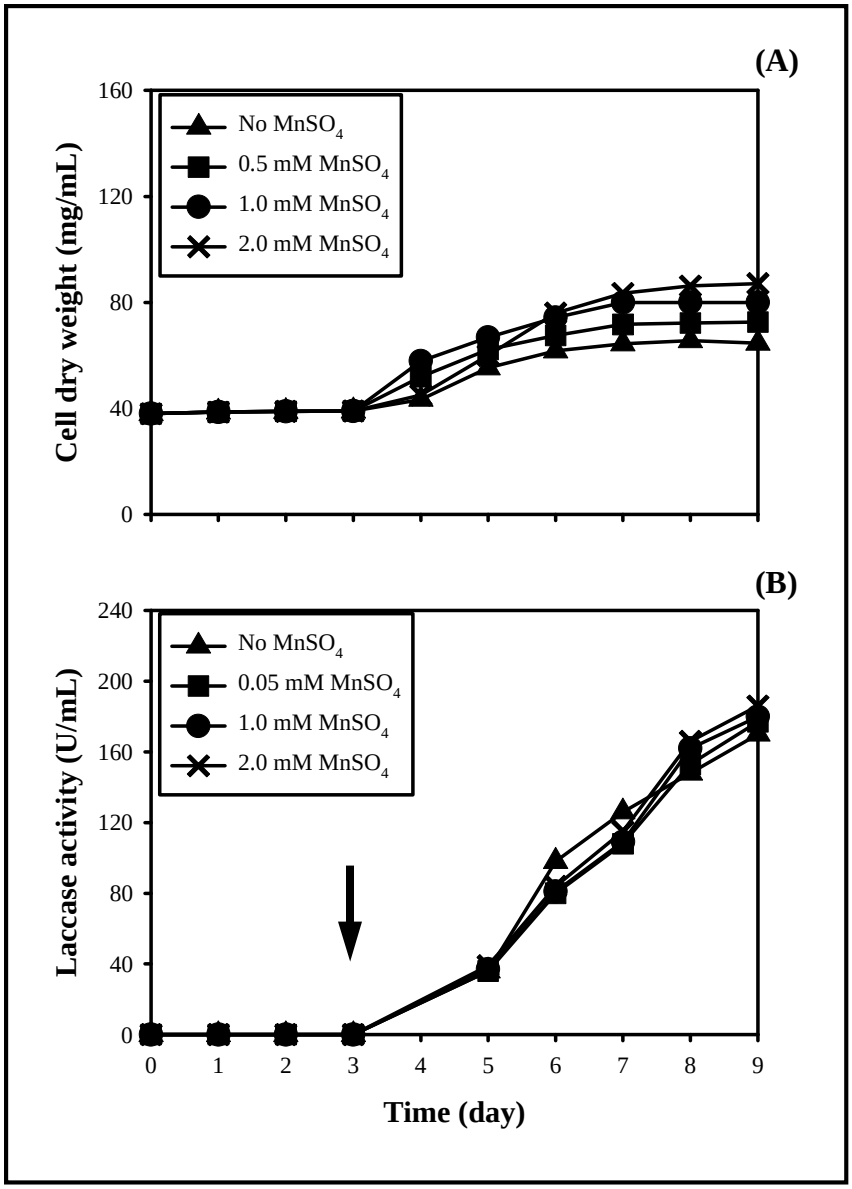


Figure 19 Effect of manganese (II) ion on laccase production of *Ganoderma* sp. KU-Alk4 cultivated in Kirk's medium pH 8.0. Culture conditions in 2 steps. First 3 days as unshaking culture, after 3 days, cultures were shaken at 140 rpm at room temperature. Arrow indicates the addition of veratryl alcohol, 0.85 mM. (A) mg cell dry weight, (B) laccase activity against 2,6-dimethoxyphenol.

1.2.7 Effect of aeration on laccase production

Aeration given to *Ganoderma* sp. KU-Alk4 during 3-9 days-cultivation was important to the production of laccase as well as the fungal growth (Figure 20A, B). The fungus was grown in pH 8.0 medium with 1% glucose, 0.2 mM $CuSO_4$ and 0.85 mM veratryl alcohol added on the 3rd day after growth appeared under static condition. Unshaking and shaking conditions at 100, 120 and 140 rpm were used to investigate the effect of oxygen amount to the fungal growth and laccase production.

Shaking caused the fungus to grow better and produce laccase than in unshaken condition. The amounts of oxygen needed for both physiologies were different. Increasing rates of aeration by changing the shaking rate of 50 mL culture in 250 mL flask from 100, 120, and 140 rpm caused increasing biomass (Figure 20A). On contrary, shaking at over 120 rpm resulted in lower production of laccase (Figure 20B). Specific activity of laccase that *Ganoderma* sp. KU-Alk4 produced under the shaking rate of 120 rpm was 247 U/mg cell dry weight. Shaking the culture at 100 rpm also gave higher laccase production higher than at 140 rpm, that was used previously. The unshaken cultures produced the least activity of the enzyme. Specific activities of laccase from the culture grown shaking at 100, 140 and 0 rpm were 216, 125 and 62 U/mg cell dry weight, respectively. Moreover, cell-free supernatant did not lose laccase activity at these levels of aeration since in the process of biopulping of paper mulberry that used *Ganoderma* sp. KU-Alk4 enzymes, oxygen showed its role as co-substrate and caused increasing of the enzyme activity (Poonpairoj *et al*., 2001b). Result of zymogram showed different electrophoretic movement of isozymes obtained in the culture grown with the different rates of shaking. At higher rate of shaking, large molecular mass isozymes were eliminated (Figure 20D).

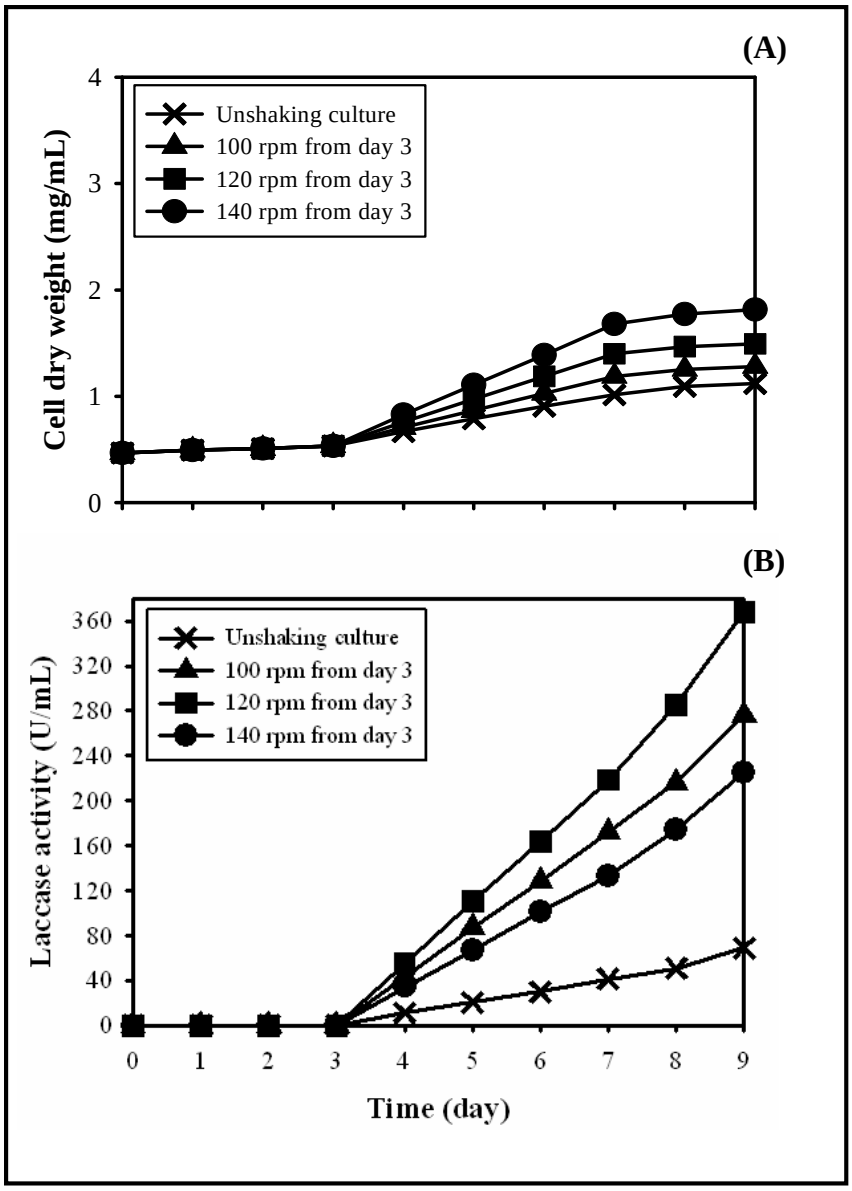
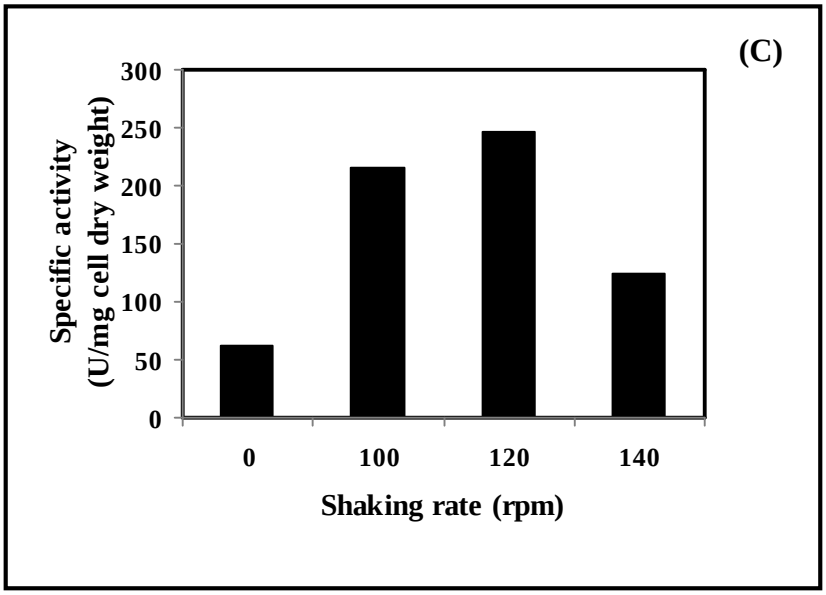


Figure 20 Effect of shaking rate on laccase production *Ganoderma* sp. KU-Alk 4 in Kirk's medium pH 8.0. Culture conditions in 2 steps. First 3 days as unshaking culture, after 3 days, cultures were shaken at different rpm at room temperature. Arrow indicates the addition of veratryl alcohol, 0.85 mM. (A) mg cell dry weight, (B) laccase activity against 2,6-dimethoxyphenol, (C) Effect of shaking rate on specific laccase activity of *Ganoderma* sp. KU-Alk 4 in Kirk's medium pH 8.0 and (D) active bands of laccase (16 µg protein) on native PAGE, staining with 2,6 dimethoxyphenol.



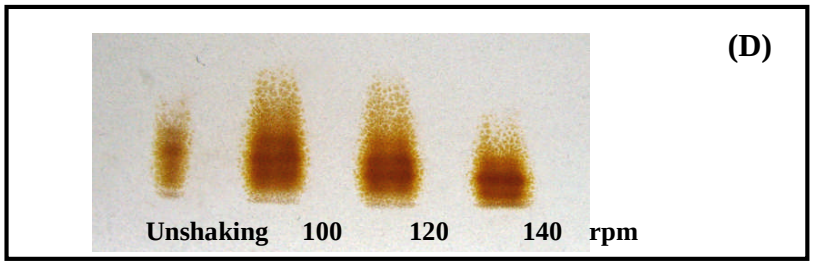


Figure 20 (Continued)

The best conditions for *Ganoderma* sp. KU-Alk4 to produce laccase could be performed in Kirk's medium at pH 8.0, with 1% glucose as C-source and 0.2 mM copper sulphate, 0.85 mM veratryl alcohol as inducer, incubated in unshaking condition for 3 days, then shaking at 120 rpm, *Ganoderma* sp. KU-Alk4 produced the highest laccase of 247 U/mg cell dry weight. The laccase activity to ABTS was 327.5 IU/mL or 218.3 IU/mg cell dry weight. The typical reported strains produce laccase activity in the range of 4-100 IU/mL (Lee *et al*., 1999, Galhaup and Haltrich, 2001, Hess *et al*., 2002). Therefore, our strain produced laccase significantly higher than that produced by most fungi. (IU = the amount of enzyme required to oxidize 1 µmol of ABTS per min at 25ºC)

1.3 Production of the enzymes on solid substrate

1.3.1 Various kinds of solid substrate

Chitradon *et al*. (2000) have studied on production lignin-degrading enzymes production of *Ganoderma* sp. KU-Alk4 on solid substrate for biopulping of paper mulberry. Cultivation of *Ganoderma* sp. KU-Alk4 on solid substrate composed of agricultural products such as rice bran, wheat bran and rice husk, was carried out in plastic bag (Figure 21). This method has been successful in production of glucoamylase and pectinase from filamentous fungi (Chitradon *et al*., 1996). However, *Ganoderma* sp. KU-Alk4 which is mushroom, took 1 month for growing on such substrates. In this research, production of lignin-degrading enzymes by *Ganoderma* sp. KU-Alk4 was carried out in glass bottle filled with only a single kind of grains. Different kinds of grain from corn, sweet sorghum and rice grains were used (Figure 22A). Initial pH and moisture content of each solid substrate were 7.0 and 45%, respectively. This is a commercial method for preparing inoculum of mushroom in Thailand.

Figure 21 Cultivation of *Ganoderma* sp. KU-Alk4 on solid substrates composed of agricultural products (Chitradon *et al*., 2000).

Growth of *Ganoderma* sp. KU-Alk4 was the best on sweet sorghum in 9 days followed by rice and corn grains, respectively (Figure 22B). Two kinds of lignin-degrading enzymes, MnP and laccase, could be detected from the solid substrates (Figure 23). No LiP was detected. *Ganoderma* sp. KU-Alk4 preferred rice grain for producing lignin-degrading enzymes to sweet sorghum and corn grains, respectively. Though, sweet sorghum was the best for growth. This could be because of more amount of lignin in rice grain that could induce *Ganoderma* sp. KU-Alk4 to produce more lignin-degrading enzymes. The fungus produced MnP in a higher ratio than laccase on all solid substrates in 9 days. On rice grain, the fungus produced MnP (3.5 U/g substrate), 4.4 times higher than laccase (0.5 U/g substrate). On sweet sorghum grain, amount of MnP (2 U/g substrate) was 4 times higher than that of laccase (0.5 U/g substrate). On corn grain, very small amount of lignin-degrading enzymes were detected, as well, trace amount of MnP was higher than laccase.

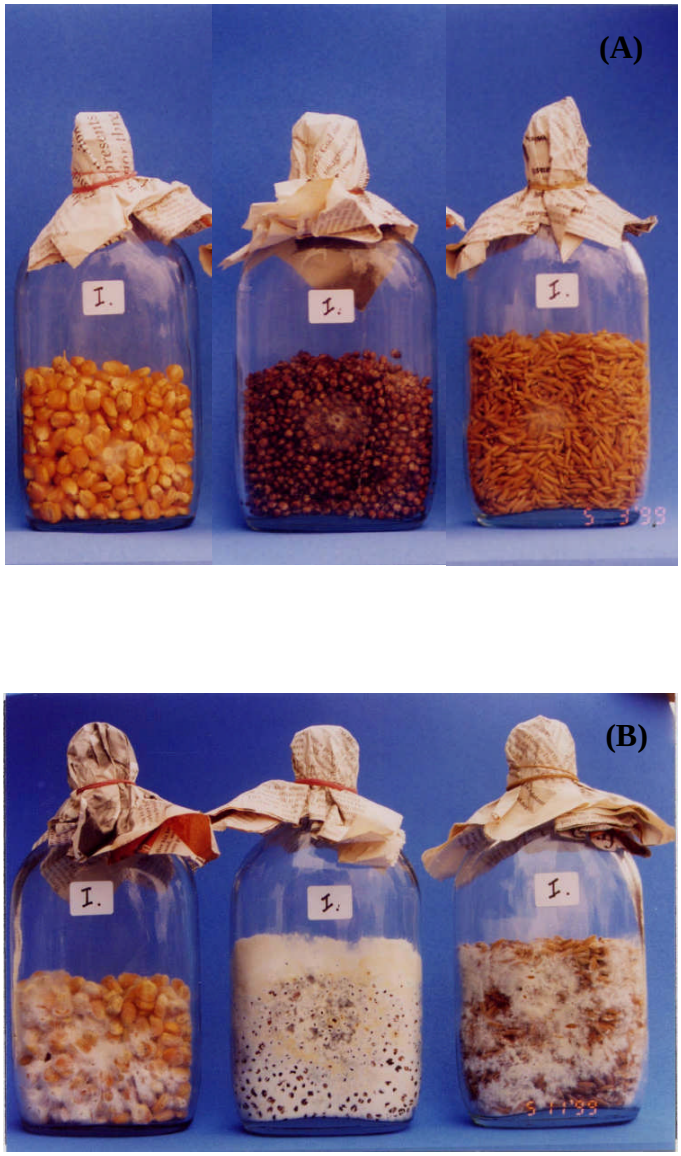


Figure 22 (A) Commercial method for preparing inoculum of mushroom in Thailand. (B) Cultivation of *Ganoderma* sp. KU-Alk4 on solid substrates of corn, sweet sorghum or rice grain, respectively, in 9 days at room temperature.

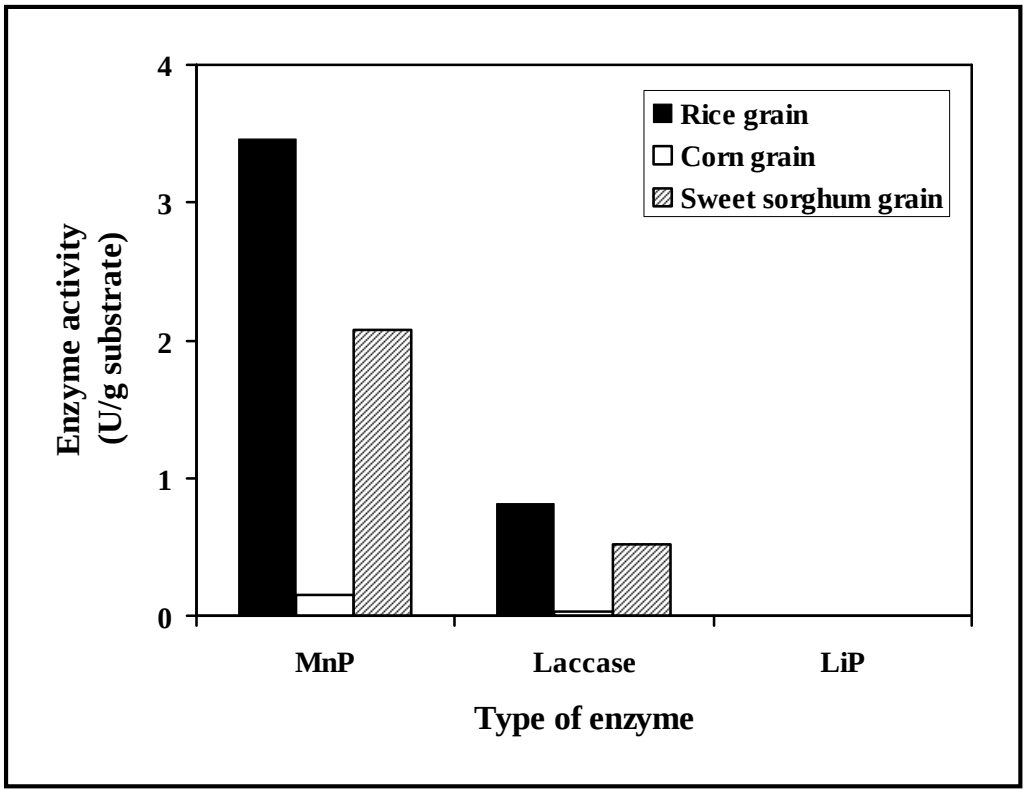


Figure 23 Lignin-degrading enzymes from *Ganoderma* sp. KU-Alk4 cultivated on agricultural solid substrates. Fifteen plugs of the fungal mycelia were inoculated and incubated at room temperature for 9 days. The enzyme was extracted from the solid substrates with 495 µL of toluene in 150 mL of 50 mM malonate buffer, pH 4.5.

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

Veratryl alcohol, 0.85 mM, induced the production of both MnP and laccase by *Ganoderma* sp. KU-Alk4 grown on solid substrates as well as liquid medium. The inducer could enhance the production of both MnP and laccase but not LiP when the fungus was grown on every solid substrates used (Figure 24, 25). Significant effect was on rice and sweet sorghum grains. Effect of veratryl alcohol on MnP production on sweet sorghum grains was similar when it was added either at the initial time of cultivation or on the 3rd day of cultivation (Figure 24). On sweet sorghum with 0.85 mM veratryl alcohol, the activity of MnP was 4 U/g substrate. On the other hand, veratryl alcohol enhancing MnP production on rice grain. The inducer enhanced the production of MnP on rice grain by two times when it was added at the 1st day of cultivation and three times when it was added to the 3 day-already grown cells. The latter gave the highest MnP activity of12 U/g substrate.

Veratryl alcohol, 0.85 mM, also enhanced the production of laccase on all kinds of solid substrates tested, especially those on sweet sorghum and rice grains (Figure 25). However, time of addition the inducer, either at the initial hour of incubation or on the 3rd day after incubation, did not make any difference. The effect was more on sweet sorghum grain than that on rice grain. Three times more of laccase production could be detected on sweet sorghum grain while only 2 times more of that could be detected on rice grain. Total activities of laccase obtained from both kinds of grains were the same at 1.3 U/g substrate.

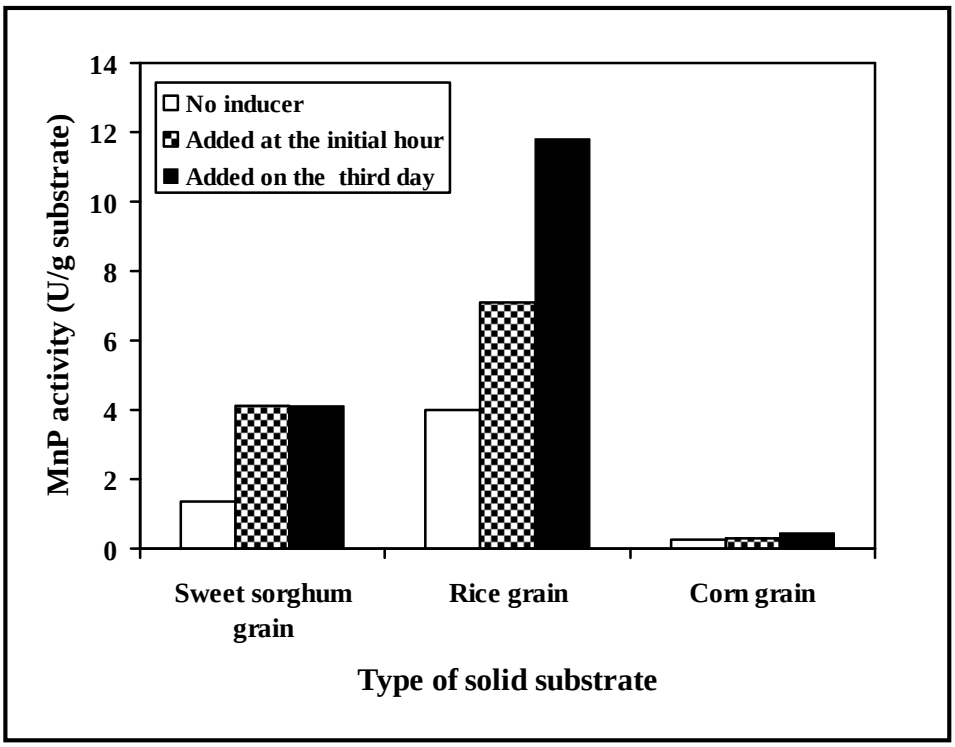


Figure 24 Induction control of production of MnP from *Ganoderma* sp. KU-Alk4 cultivated on agricultural solid substrates. Veratryl alcohol (1 mL, final conc. 0.85 mM) used as inducer. The culture was incubated at room temperature for 9 days.

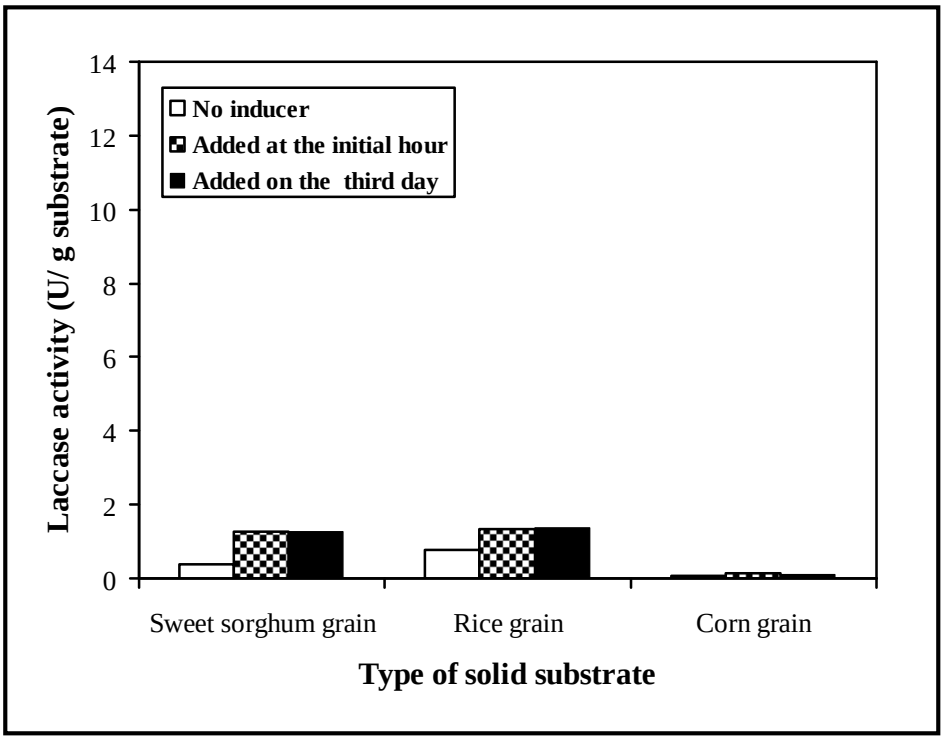


Figure 25 Induction control of production of laccase from *Ganoderma* sp. KU-Alk4 cultivated on agricultural solid substrates. Veratryl alcohol (1 mL, final conc. 0.85 mM) used as inducer. The culture was incubated at room temperature for 9 days.

Table 17 Comparison of liquid and solid state cultures of *Ganoderma* sp. KU-Alk4. Liquid medium composed of 1% glucose, 0.02 mM $CuSO_4$, shaking rate at 140 rpm. Solid state was rice grain with 0.85 mM veratryl alcohol with 45% moisture content.

Enzyme activities	Liquid pH 4.5 (U/mL)	Liquid pH 7.0 (U/mL)	Liquid pH 8.0 (U/mL)	Solid pH 7.0 (U/g substrate)	Solid pH 8.0 (U/g substrate)
Laccase	4	43	153	1.3	No growth
MnP	5	50	ND	12	No growth
LiP	ND	ND	ND	ND	No growth

ND = not detectable

When compared between the culture grown on solid substrates and liquid medium at pH 4.5, 7.0 and 8.0 (Table 17), the fungus grew only on solid substrates with neutral pH but not at alkaline pH. On solid substrate, the fungus produced MnP and laccase at the ratio of 4:1 while in the Kirk's liquid medium at pH 7.0 or 4.5, the fungus produced almost an equal amount of MnP and laccase and at pH 8.0, only laccase was produced. Though preparation of liquid medium was more complicated than preparation of solid medium, the advantage in the aspect of laccase production for further study was clear. Moreover, the enzymes production in liquid had less contaminating protein, easy to separate mycelium from enzyme solution and to purify laccase. However, cultivation of *Ganoderma* sp. KU-Alk4 on solid substrate was a good method for stock culture and could be storage at room temperature for more than 6 months.