RESULTS AND DISCUSSION

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

1.1 Production of the enzymes in liquid medium

Ganoderma sp. KU-Alk4 secreted 2 kinds of lignin-degrading enzymes, laccase and MnP, in Kirk liquid medium, pH 7.0 with 1% glucose, 0.02 mM $CuSO_4$ but not LiP. Preliminary study found that the fungus grew and formed pellet better under static condition than shaking at 140 rpm from the first 3 days. No lignin-degrading enzymes were produced during these 3 days. Production of the enzymes started after the addition of 0.85 mM veratryl alcohol as inducer in day 3 (Figure 10). The enzymes activity increased dramatically to reach the maximum in day 9 in the shaking condition. Cells grew unshaking for 3 days, followed by shaking after the inducer addition for further 6 days, gave 5 times higher production of lignin-degrading enzymes than those grew under continuing static growth condition. Moreover, the culture grew under such conditions of 3-day static and 6 day shaking with the addition of the inducer at day 3 also gave 2.5 times higher activity of the lignin-degrading enzymes than that grew shaking throughout 9 days. Therefore, the best conditions suggested for study on the regulation of the lignin-degrading enzymes production by *Ganoderma* sp. KU-Alk4 was the cultivation in Kirk's liquid medium with the growth condition of 3-day static, followed by shaking for total 9 days and 0.85 mM veratryl alcohol added on day 3.

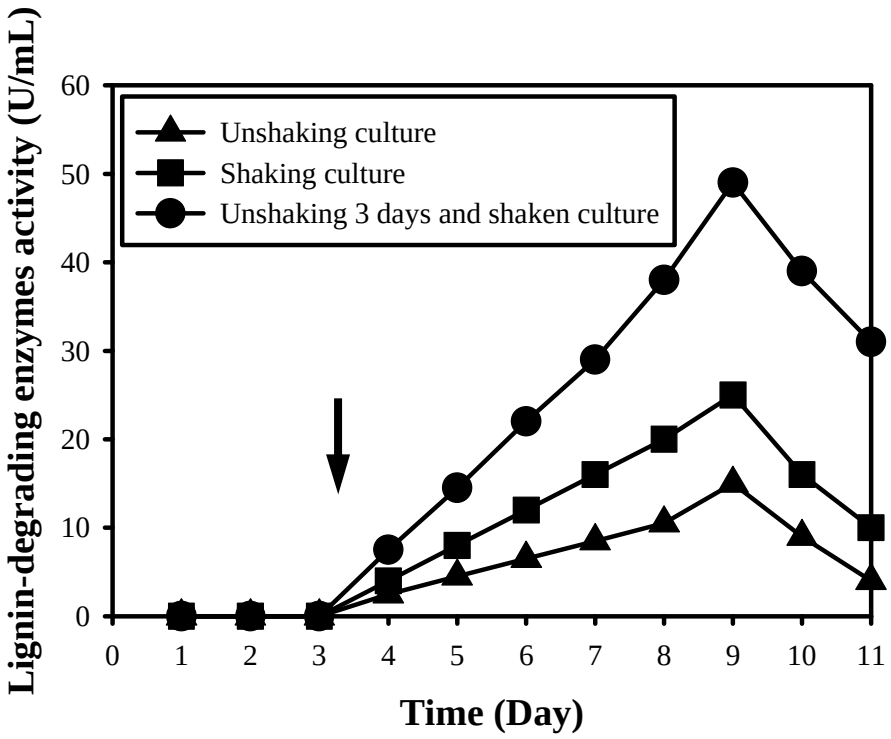


Figure 10 Time course of the lignin-degrading enzymes production by *Ganoderma* sp. KU-Alk4 in Kirk's medium, pH 7.0 and incubated in the different conditions. Arrow indicates the time of 0.85 mM veratryl alcohol addition. Shaking rate is 140 rpm. The activities were against 2,6-dimethoxyphenol.

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

1.2.1 Regulation of lignin-degrading enzymes production by pH

When *Ganoderma* sp. KU-Alk4 was screened for the process of paper mulberry biopulping, it was found that KU-Alk4 produced different ratios of laccase and MnP in the media with the different initial pH (Poonpairoj *et al*., 2001a, b). Initial pH of the medium affected both the fungal growth and the lignin-degrading enzymes production by the fungus.

Figure 11 compared growth of the fungus and individual enzymes, laccase and MnP, produced in the media with initial pH of 4.5, 7.0 and 8.0. No pH control was regulated during the incubation. The culture condition of 3 days static followed by 6 days shaking was employed. Veratryl alcohol, 0.85 mM, was added on the 3rd day. Glucose of 1%, 0.02 mM $CuSO_4$ and 140 rpm shaking rate were used.

Growth was the best at pH 7.0 with the early log phase started in 2 days (Figure 11A). The fungus also grew well at pH 8.0 at the same growth rate but with longer lag phase of 4 days. At acidic pH of 4.5, poor growth was observed and little lignin-degrading enzyme activity was detected (Figure 11A, B, C).

The enzymes could be detected only from the culture grown at pH 7.0 and 8.0. At pH 7.0, the fungus produced an almost equal amount of laccase and MnP (approx. 46 U/mL). On contrary, in alkaline condition of pH 8.0, only laccase activity could be detected with total activity of 153 U/mL. Cell dry weight on day 9 was 2.3 mg/mL. Specific activity of laccase recovered after 9 days cultivation in alkaline condition was 4 times higher than that recovered at neutral pH which correlated to the dense of active bands appeared from the same amount of protein (16 µg protein) on zymogram (Figure 11D). Specific activities of the laccase that the fungus produced at pH 8.0 and 7.0 were 68 and 17 U/mg cell dry weight, respectively (Figure 11C).

Interesting observation was that at pH 7.0 and 8.0, the fungus produced different numbers and molecular sizes of the isoforms (Figure 11D). Only an active band of the smallest molecular size appeared on the zymogram of pH 7.0-culture but not on that pH 8.0-culture. No active band was detected from the pH 4.5-culture.

To our knowledge, there was no report that showed that the difference in initial pH of the medium culture regulated in the production of the different isozymes of laccase. The productivity and capability of the enzymes to produce the enzyme at high initial pH (8.0) were of interested when compared to those of *Pleurotus ostreatus*, *Cyathus stercoreus* and *Botryosphaeria* sp. that produced the lignin-degrading enzymes only at lower pH of 5.0-6.0 (Ardon *et al*., 1996; Sethuraman *et al*., 1999; Dekker and Barbosa, 2001).

Alkaline condition, pH 8.0, regulates KU-Alk4 to produce only laccase. According to characteristics and potential applications of laccase, therefore, pH 8.0 was introduced to drive *Ganoderma* sp. KU-Alk4 produce laccase.

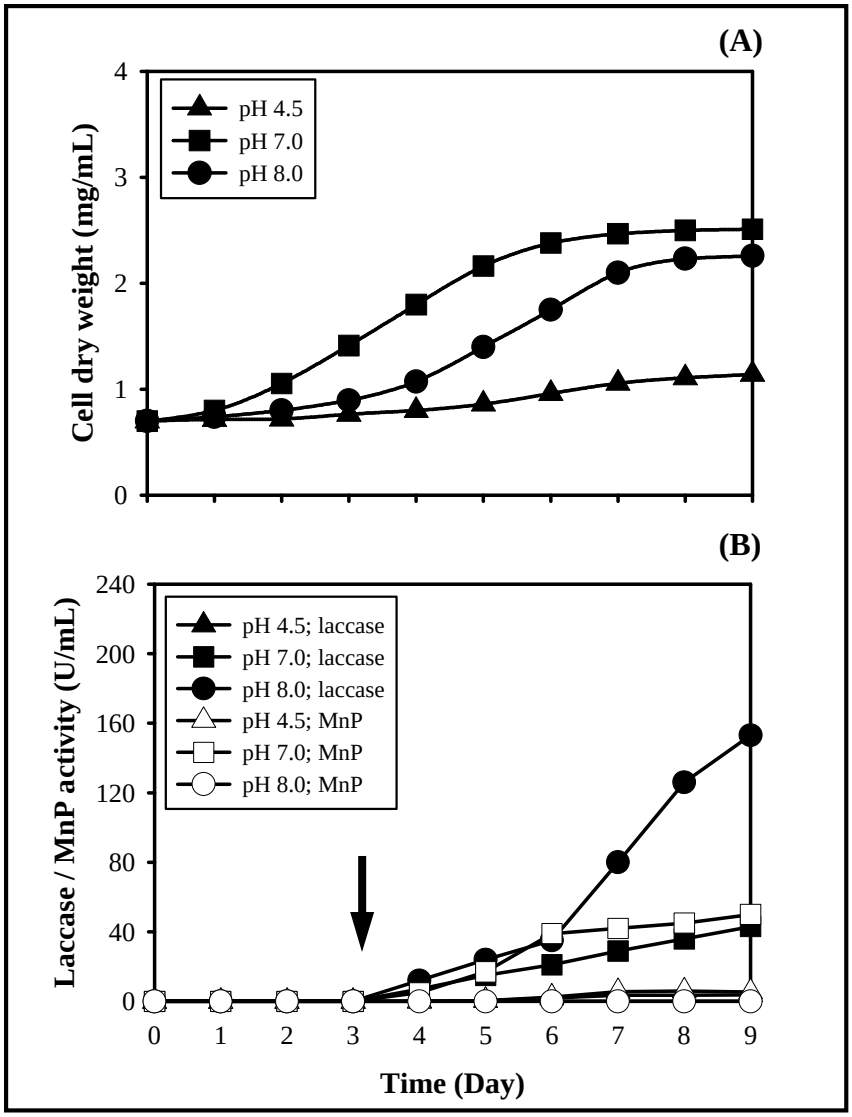
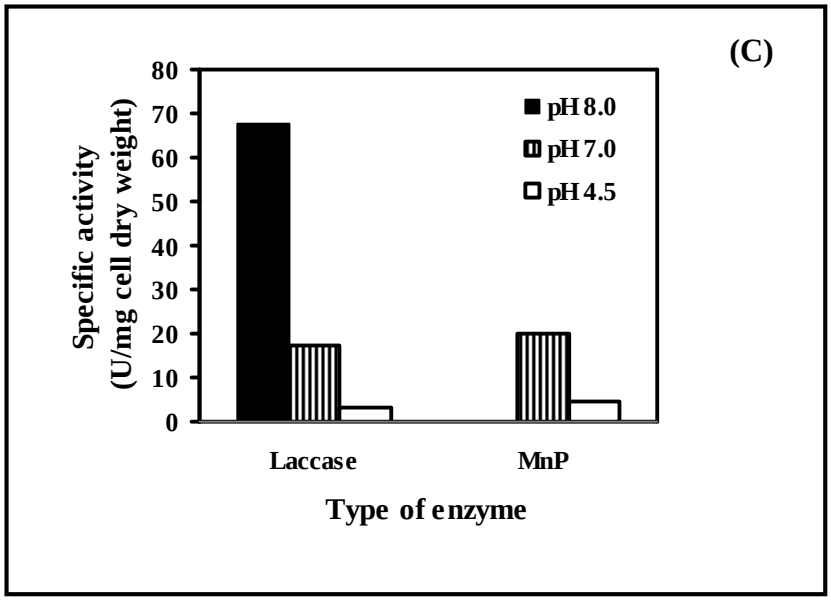


Figure 11 Effect of pH on production of laccase and MnP by *Ganoderma* sp. KU-Alk4 in Kirk's medium. Culture conditions are in 2 steps. First 3 days as unshaking culture, after 3 days, cultures were shaken at 140 rpm at room temperature. Arrow indicates the addition of veratryl alcohol, 0.85 mM. (A) mg cell dry weight, (B) activities of laccase and MnP against 2,6-dimethoxyphenol, (C) Effect of pH on specific activity of lignin-degrading enzymes of *Ganoderma* sp. KU-Alk4 and (D) Active bands of laccase (16 µg protein) on native PAGE, staining with 2,6-dimethoxyphenol.



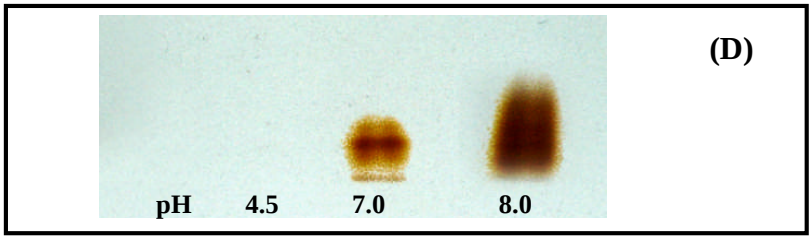


Figure 11 (Continued)

1.2.2 Regulation of laccase production by induction control

The production of laccase depends not only on the fungal strain but also on the presence of an inducer. Production of the inducible laccase is affected the most by the chemical nature and time of inducer added (Gianfreda, 1999). Figure 12A, B showed growth and laccase production of *Ganoderma* sp. KU-Alk4 in the pH 8.0-medium with and without 0.85 mM veratryl alcohol, a phenolic compound and addition of the alcohol was either at the beginning of the cultivation or on the 3rd day after the fungal pellets were formed under static condition. The medium contained 1% glucose, 0.02 mM $CuSO_4$ and the shaking rate used during day 3-9 was 140 rpm.

Results showed that veratryl alcohol at such concentration had no effect on the fungal growth, but only on its laccase production. *Ganoderma* sp. KU-Alk4 showed a typical growth rate, in the pH 8.0-medium with or without veratryl alcohol. Without veratryl alcohol, no laccase was produced. Addition of veratryl alcohol on the 3rd day after cell grew induced *Ganoderma* sp. KU-Alk4 produce laccase 230 U/mL which was 3 times more than that in the culture with veratryl alcohol added from the beginning. These proved that the production of laccase of *Ganoderma* sp. KU-Alk4 was induction controlled. This is contrast with *Ganoderma* sp. *WR-1* that produced high activity of laccase without any inducers (Revankar and Lele, 2006).

It was shown that veratryl alcohol at the concentration of 0.85 mM could be used as an inducer and the addition on the 3rd day after growth allowed the fungus to produce laccase with the highest specific activity detected on day 9 equal to 126 U/mg cell dry weight (Figure 12C).

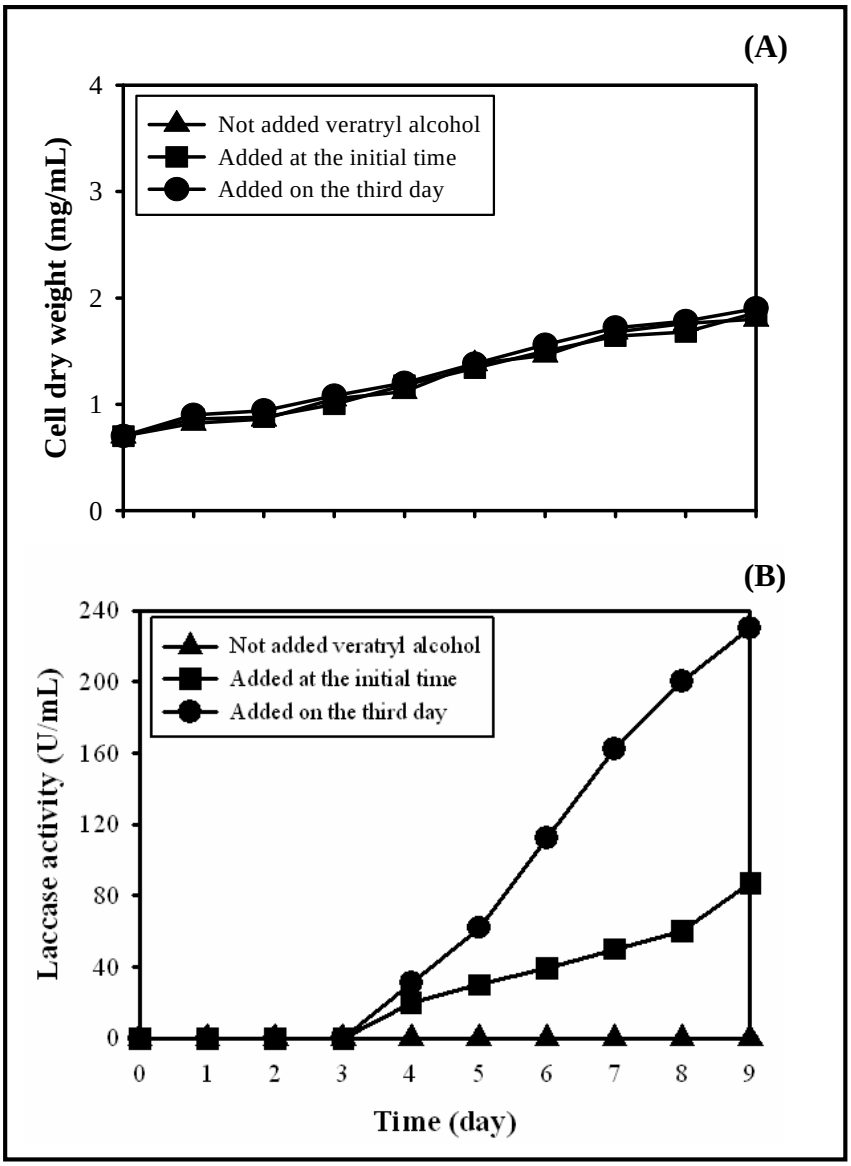


Figure 12 Induction control of laccase production of *Ganoderma* sp. KU-Alk4 in Kirk's medium pH 8.0 by 0.85 mM veratryl alcohol. Culture conditions in 2 steps. First 3 days as unshaking culture, after 3 days, cultures were shaken at 140 rpm at room temperature. (A) mg cell dry weight, (B) laccase activity against 2,6-dimethoxyphenol. (C) Effect of induction control on specific laccase activity of *Ganoderma* sp. KU-Alk4 in Kirk's medium pH 8.0 by 0.85 mM veratryl alcohol.

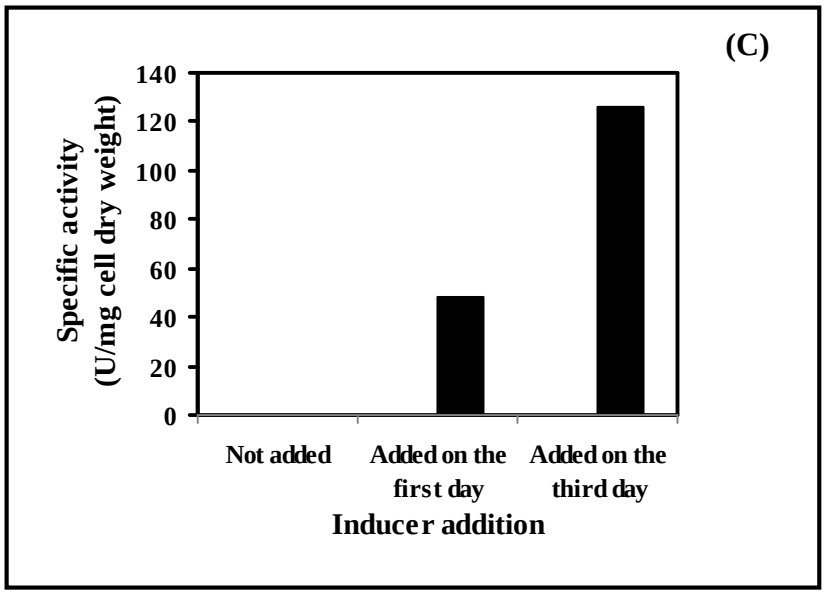


Figure 12 (Continued)

1.2.3 Induction of laccase production by different phenolic compounds

Low molecular weight phenolic compounds, veratryl alcohol, guaiacol and 2,6-dimethoxyphenol (DMP) were tested for their abilities to be an inducer for the laccase production of *Ganoderma* sp. KU-Alk4. The fungus was allowed to grow statically in a pH 8.0-medium without any phenolic compound for 3 days, then each of the selected phenolic compounds was added to the final concentration of 0.85 mM. The culture was then allowed to grow under shaking condition at 140 rpm. The media contained 1% glucose and 0.02 mM $CuSO_4$. *Ganoderma* sp. KU-Alk4 could grow in the presence of those inducers, however, guaiacol and DMP showed the growth of the fungus (Figure 13A). Veratryl alcohol was the best inducer for laccase production of *Ganoderma* sp. KU-Alk4 with total activity of 230 U/mL (Figure 13B). Cell dry weight on day 9 was 1.8 mg/mL. The fungus grew in the presence of guaiacol or DMP but little of laccase activity was detected in the cultures. Specific activity of laccase produced in the culture with veratryl alcohol, guaiacol and DMP were 126, 12 and 4 U/mg cell dry weight, respectively (Figure 13C). This suggested that 0.85 mM guaiacol and DMP were not the good inducers for laccase production of *Ganoderma* sp. KU-Alk4 at such growth conditions. Figure 13D showed that guaiacol and DMP did not induce the synthesis of the clump of isozymes that appeared with veratryl alcohol. The production of laccases of *Trametes modesta*, *Trametes versicolor*, *Botryosphaeria* sp., *Phlebia radiata*, and *Dichomitus squalens* were also induced by veratryl alcohol (Nyanhongo *et al*., 2002; Dekker and Barbosa, 2001; Arora and Gill, 2000; Schlosser *et al*., 1997). However, towards the other 2 low molecular weight aromatic compounds, different white-rot fungi responded differently. While guaiacol had less inductive to laccase production of *Ganoderma* sp. KU-Alk4, it was affected on that of *Ganoderma* sp. *WR-1*, *Trametes pubescens*, *Daedalea flavida*, and *Pleurotus cinnabarinus* (Revankar and Lele, 2006; Galhaup and Haltrich, 2001; Arora and Gill, 2001; Eggert *et al*., 1996).

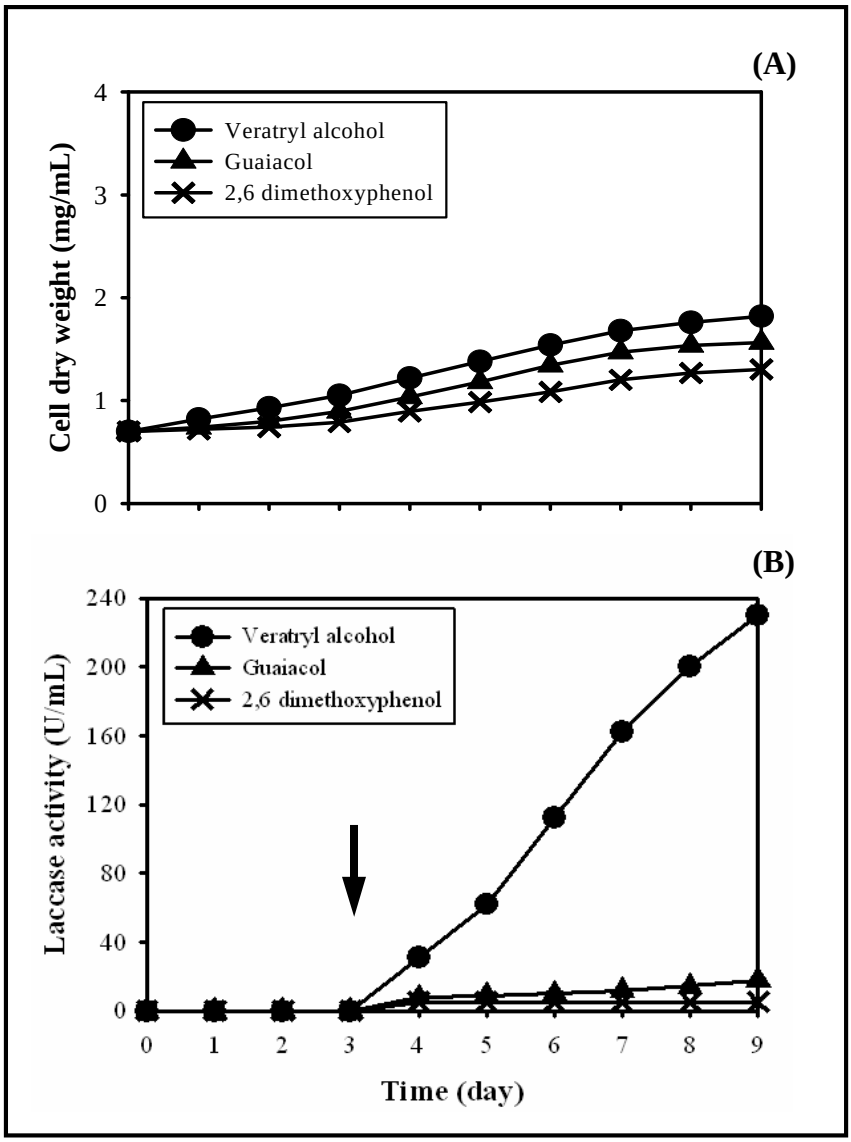


Figure 13 Effect of some phenolic compounds as inducer to the laccase production of *Ganoderma* sp. KU-Alk4 cultivated in Kirk's medium pH 8.0. Culture conditions in 2 steps. First 3 days as unshaking culture, after 3 days, cultures were shaken at 140 rpm at room temperature. Arrow indicates the addition of inducer, 0.85 mM. (A) mg cell dry weight, (B) laccase activity against 2,6-dimethoxyphenol. (C) Effect of some phenolic compounds as inducer to the specific laccase activity of *Ganoderma* sp. KU-Alk4 and (D) active bands of laccase (16 µg protein) on native PAGE, staining with 2,6-dimethoxyphenol. VA = veratryl alcohol, GA = guaiacol, DMP = 2,6-dimethoxyphenol.

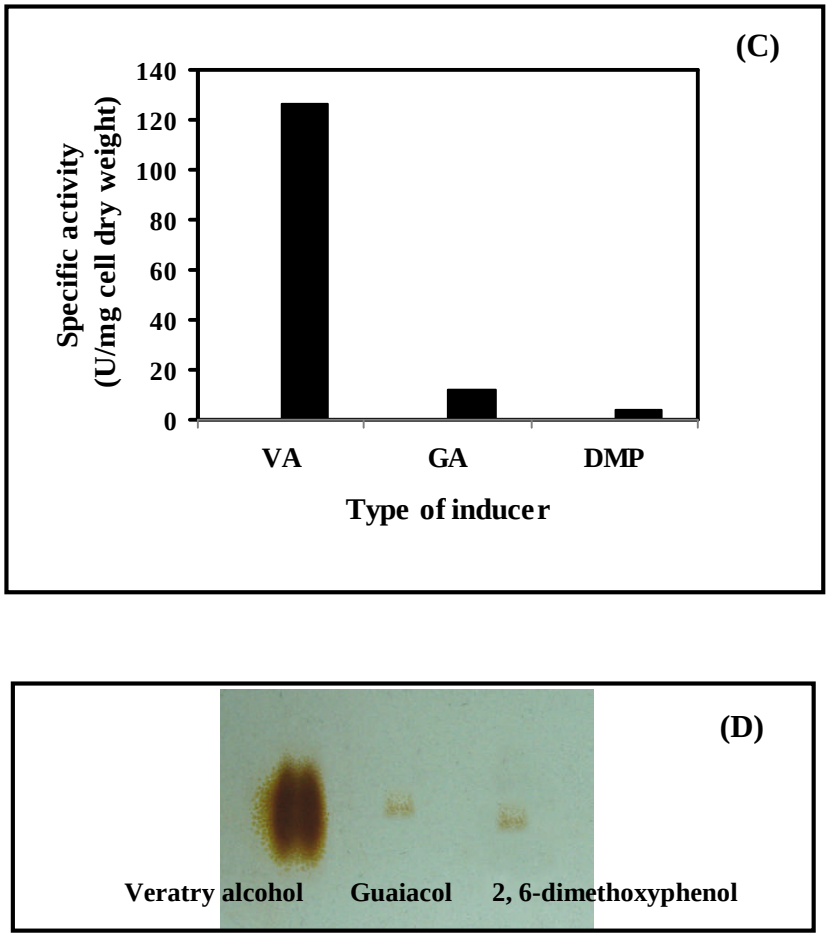


Figure 13 (Continued)

1.2.4 Effect of carbon sources

Three kinds of carbon sources, glucose, lactose and CMC, each of 1%, were used to grow *Ganoderma* sp. KU-Alk4 in the liquid Kirk's medium, pH 8.0 with 0.02 mM $CuSO_4$, 0.85 mM veratryl alcohol and the same culture condition with 140 rpm shaking rate.

Ganoderma sp. KU-Alk4 preferred lactose for growth to glucose and CMC (Figure 14A). On contrary, glucose was the best substrate for laccase production by KU-Alk4 with total activity of 230 U/mL (Figure 14B). Cell dry weight on day 9 was 1.8 mg/mL. Specific activity of laccase in the media with 1% glucose, lactose and CMC were 126, 28 and 11 U/mg cell dry weight, respectively.

Similar isozymes were produced by *Ganoderma* sp. KU-Alk4 cultivated in the pH 8.0-medium with glucose and lactose (Figure 14C). The observation within lactose agreed with a report on *Ganoderma lucidum* that the fungus produces *ß*-galactosidase and allowed the fungus to use lactose for growth and caused the repression of laccase production (Sripuan *et al*., 2003).

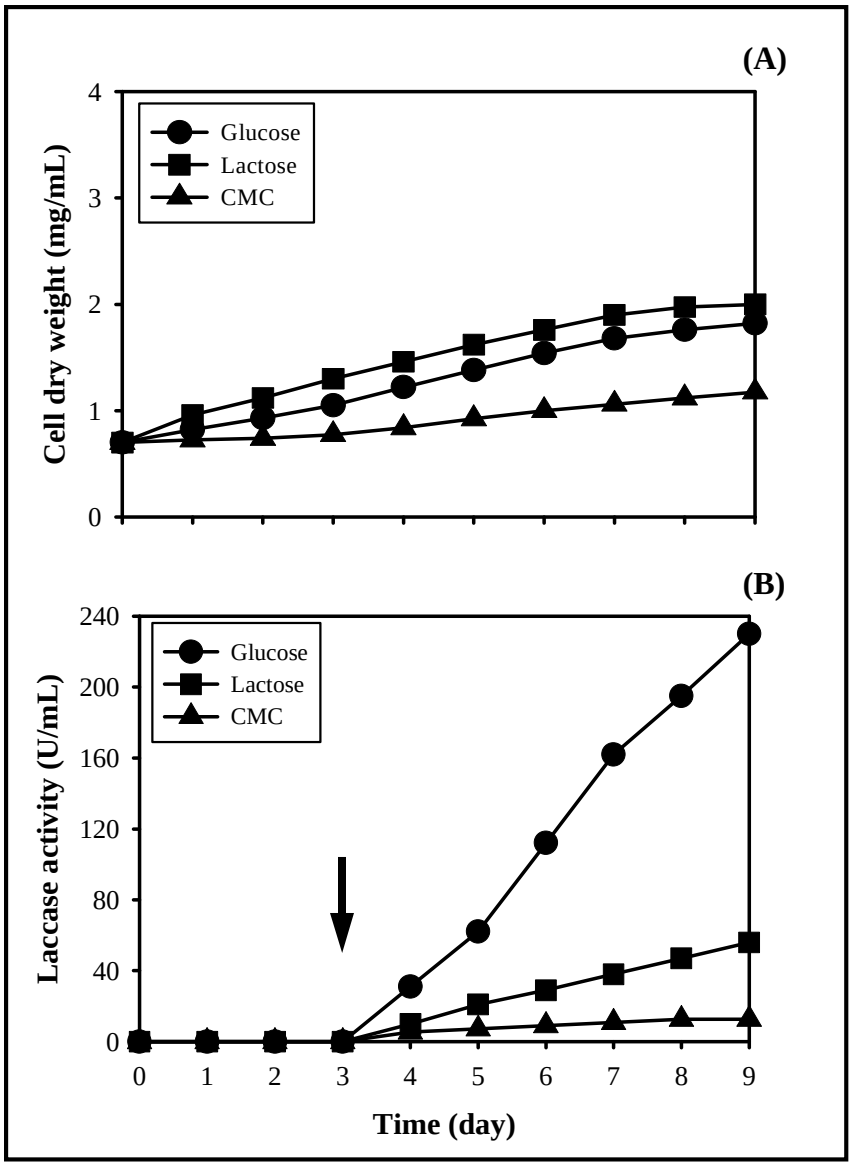
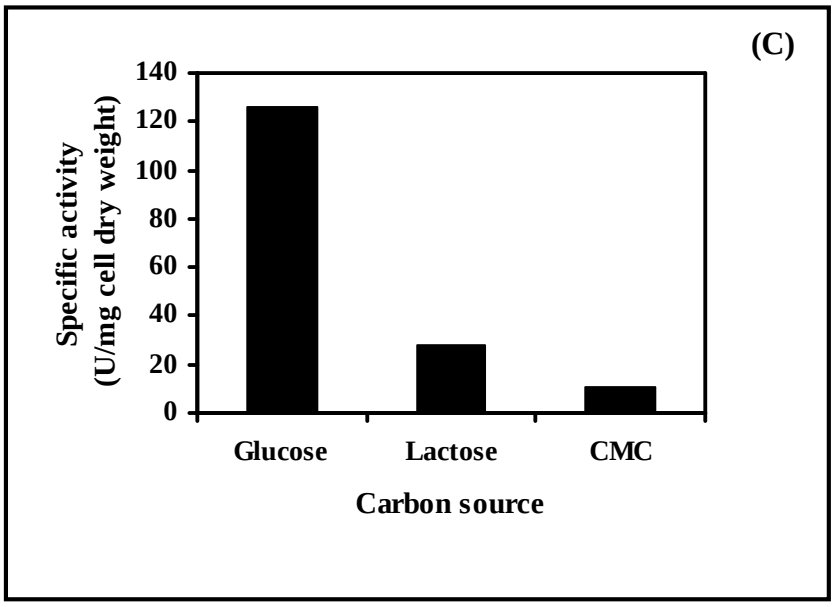


Figure 14 Effect of various carbon sources, each 1%, on laccase production of *Ganoderma* sp. KU-Alk4 cultivated in Kirk's medium pH 8.0. Culture conditions in 2 steps. First 3 days as unshaking culture, after 3 days, cultures were shaken at 140 rpm at room temperature. Arrow indicates the addition of veratryl alcohol, 0.85 mM. (A) mg cell dry weight, (B) laccase activity against 2,6-dimethoxyphenol. (C) Effect of various carbon sources, each 1%, 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



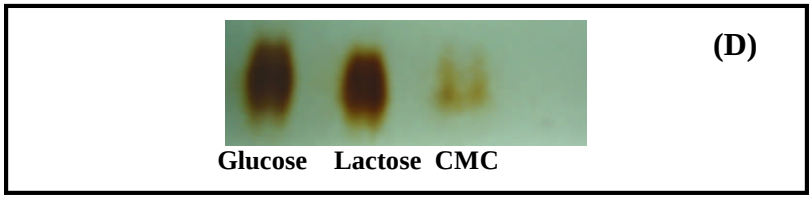


Figure 14 (Continued)