CONCLUSION

Physiological aspects of a new isolated Polyporaceae designated as KU-Alk4 on regulation of lignin-degrading enzymes synthesis were investigated. The fungus grew and produced the lignin-degrading enzymes in the media with wide range of initial pHs from 4.5-8.0. At initial pH 4.5 and 7.0, the fungus produced 2 kinds of lignin-degrading enzymes, laccase and MnP. On the other hand, in alkaline conditions, pH 8.0, the fungus produced only laccase. However, no pH was controlled during the cultivation. At pH 8.0, KU-Alk4 produced laccase with higher specific activity than that produced at pH 7.0. Amount of laccase isozymes that the fungus produced at pH 8.0 was more and bigger in sizes that those at pH 7.0. Regulation of the enzyme production of KU-Alk4 was proved to be negative induction control. Veratryl alcohol was the best inducer of lignin-degrading enzymes production compared with guaiacol and 2,6-dimethoxyphenol at the same concentration of 0.85 mM. The production of laccase of KU-Alk4 was also regulated by catabolite repression. KU-Alk4 preferred glucose to lactose, each of 1%, for laccase production at pH 8.0 but glucose at higher concentrations repressed the enzyme production. CMC was not utilized by the fungus at pH 8.0. Copper, 0.02-0.2 mM, was required for laccase production of KU-Alk4 but in trace. High concentration of $CuSO_4$ at 2 mM inhibited growth of the fungus. Aeration rate supplied to the 3 day-grown culture was important for fungal growth and the enzyme production. Excess oxygen inhibited laccase production. The cultivation conditions that gave the highest laccase activity was in 50 mL modified Kirk's medium at pH 8.0 with 1% glucose, 0.2 mM $CuSO_4$ and 0.85 mM veratryl alcohol as an inducer added on day 3 to the cell pellet that grew unshaken, then the culture was continued shaking at 120 rpm. The highest laccase recovered on day 9 was 327.5 IU/mL or 218 IU/mg cell dry weight which was 16 times improved.

Not only the synthesis of laccase was controlled by catabolite repression but also the different concentrations of glucose regulated the fungus to produce different laccase isozymes. In the medium pH 8.0, KU-Alk4 produced 2 main isozymes,

KULac 1 and KULac 2 with 1% glucose. In the pH 8.0 medium with 4% glucose, it produced 3 main isozymes, KULac 3, 4 and 5. Purification and characterization of the 5 isozymes showed that all isozymes had different molecular weights between 53-112 kDa. KULac 2 and 4 were relatively minor components of the total laccase. KULac 3 had the highest optimum temperature of 90ºC and stable to high temperature of 60°C compared to the others. Optimum temperature of the other KULacs were 55-70°C and stable at 40-45°C. Optimum pH of all KULacs were 3.5 while they were 80-100% stable to pH 3.0-10.0 in 1 h. KULac 2 was a new laccase according to its novel N-terminal amino acid sequence that is different from those previously reported.

Medium engineering using a Box-Behnken experiment design was employed for an improvement of laccase production of KU-Alk4. Five of nine selected factors, pH, kinds and concentration of carbon, kinds of inducer and combination of carbon source and inducer were highly significant factors that effected to the enzyme production predicted by the mathematical model. Concentration of inducer was also significant, too. With 5 reproducible experiments confirmed that the optimized condition predicted by the Box-Behnken design gave the maximal production of laccase from KU-Alk4 in the medium that pH was controlled throughout the experiment. The best medium predicted by the statistical design was that with 40g/L glycerol, 0.2 g/L yeast extracts, 0.02 mM $CuSO_4$ and 0.85 mM veratryl alcohol and pH of the medium was controlled at 6.0. The engineered medium was 12 times improved. Using the Box-Behnken design is an efficient method that the correlation of the experiments to the statistical design was 98%. This is the first report on optimization of the medium for laccase production by using the Box-Behnken design and it is worth noted that laccase activities achieved from KU-Alk4 in the experiment with optimum conditions as designed was 240 IU/mL that is significant higher than most of the previous reported fungi under the similar conditions.

Laccase of KU-Alk4 could decolorize several dyes, the best was Indigo Carmine. For practical use of the enzyme in industry and environmental prospect, immobilization of the KU-Alk4 laccase was done. Immobilization by entrapment in the alginate bead with copper and/or aluminum demonstrated that the activity was increased 1.15 times by the addition of 0.15 M $CuSO_4$ and 1.25 times with the combination of $CuSO_4$ and $AlCl_3$, each at 0.075 M. Though, the activity of laccase of KU-Alk4 was the best when entrapped in Cu-Al alginate according to that the trivalent cation, Al^{3+}, could improve gel strength and reduce pore size of beads, but the activity was not stable. We demonstrated, the first time to our knowledge, that aluminium ion inhibited laccase activity.

Immobilization of laccase of KU-Alk4 in Cu-alginate bead was selected. Optimization of laccase entrapment in copper alginate beads has not been fully examined elsewhere. In this study, we demonstrated the optimization of the enzyme immobilization. Latin Square Design was used to optimize the factors affecting to the residual activity as well as the efficiency in the decolorization of Indigo Carmine. To the best of our knowledge, this is the first description of the development of an effective method for the immobilization of laccase for use in dye decolorization. The dye decolorization system provides a reasonable and practical method for large-scale dye decolorization because it was a non-bufferized system and very little or no degradation of alginate gel that resulted no leakage of Cu ion. In a large-scale dye decolorization of 5 L-airlift bioreactor with the best performance that airflow rate was at 4 L/min, the decolorization efficiency was 100% completed over 14 cycles within 20 days with $6x10^4$ IU of the immobilized laccase. Each batch contained 125 mg Indigo Carmine that dissolved in water. Total amount of removed dye at the end of 20 days incubation was as high as 1.8 g.

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From morphological characteristics and sequences of ITS4 gene. KU-Alk4 was identified as *Ganoderma* sp. Comparative taxonomy of the ITS4 and 18S rDNA gene sequences of *Ganoderma* sp. KU-Alk4 with the sequences in the GenBank database using BLAST N program from NCBI website was done. From its ITS4 gene sequence, *Ganoderma* sp. KU-Alk4 was only 93% homologous to that of *Ganoderma philippii*. From 18S rDNA gene sequence, *Ganoderma* sp. KU-Alk4 was 99% similarity to that of *Ceriolopsis* sp. Phylogenetic relation using ITS4 and 18S rDNA genes of *Ganoderma* sp. KU-Alk4 and other fungi constructed by neighbor-joining method from the MEGA 2 program. From morphological characteristics and phylogenetic comparison, *Ganoderma* sp. KU-Alk4 would be a new *Ganoderma* species.

The work presentd in this thesis has been used in the following publications.

1. Teerapatsakul, C.; N. Abe. C. Bucke, N. Kongkathip, S. Jareonkitmongkol and L. Chitradon. 2007. Novel laccases of *Ganoderma* sp. KU-Alk4, regulated by different glucose concentration in alkaline media. World Journal of Microbiology and Biotechnology (in accepted and impress).

2. Teerapatsakul, C.; R. Parra; C. Bucke and L. Chitradon. 2007. Improvement of laccase production from *Ganoderma* sp. KU-Alk4 by medium engineering. World Journal of Microbiology and Biotechnology (In accepted and impress).

3. Teerapatsakul, C.; C. Bucke; R. Parra; T. Keshavarz and L. Chitradon. Dye decolorisation by laccase entrapped in copper alginate (Submitted for World Journal of Microbiology and Biotechnology).

4. Teerapatsakul, C.; C. Bucke and L. Chitradon. Physiology of ligninolytic enzyme production by a newly isolated *Ganoderma* sp. KU-Alk4 (In preparation for Journal of Molecular Catalysis B : Enzymatic).

5. Teerapatsakul, C.; C. Bucke and L. Chitradon. Dye decolorization by immobilized laccase of *Ganoderma* sp. KU-Alk4 in an airlift bioreactor (In preparation for World Journal of Microbiology and Biotechnology).

6. Teerapatsakul, C. and L. Chitradon. 2003. Some physiological aspects of a white rot fungus, KU-Alk4 on production of ligninolytic enzymes, used in biopulping of paper mulberry. *In* Abstract in Microbial Utilization for Recycling of Agricultural Waste. NRCT-JSPS-DOST-LIPI-VCC, Mahidol University, 7-8 March.

7. Chitradon, L.*; C. Sittidilokrata; P. Poonpairoj; C. Teerapatsakul; S. Suthirawut; P. Siriacha and V. Punsuvon. 2003. Comparison of Using Fungal Polygalacturonase and bacterial pectate lyase in biopulping of paper mulberry. *In* Abstract in Microbial Utilization for Recycling of Agricultural Waste. NRCT-JSPS-DOST-LIPI-VCC, Mahidol University, March 7-8.

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