Physiological Regulation of an Alkaline-Resistant Laccase Produced by *Perenniporia tephropora* and Efficiency in Biotreatment of Pulp Mill Effluent

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Abstract  Regulation of alkaline-resistant laccase from *Perenniporia tephropora* KU-Alk4 was proved to be controlled by several factors. One important factor was the initial pH, which drove the fungus to produce different kinds of ligninolytic enzymes. *P. tephropora* KU-Alk4 could grow at pH 4.5, 7.0, and 8.0. The fungus produced laccase and MnP at pH 7.0, but only laccase at pH 8.0. The specific activity of laccase in the pH 8.0 culture was higher than that in the pH 7.0 culture. At pH 8.0, glucose was the best carbon source for laccase production but growth was better with lactose. Low concentrations of glucose at 0.1% to 1.0% enhanced laccase production, while concentrations over 1% gave contradictory results. Veratryl alcohol induced the production of laccase. A trace concentration of copper ions was required for laccase production. Biomass increased with an increasing rate of aeration of shaking flasks from 100 to 140 rpm; however, shaking at over 120 rpm decreased laccase quantity. Highest amount of laccase produced by KU-Alk4, 360 U/mL, was at pH 8.0 with 1% glucose and 0.2 mM copper sulfate, unshaken for the first 3 days, followed by addition of 0.85 mM veratryl alcohol and shaking at 120 rpm. The crude enzyme was significantly stable in alkaline pH 8.0~10.0 for 24 hr. After treating the pulp mill effluent with the KU-Alk4 system for 3 days, pH decreased from 9.6 to 6.8, with reduction of color and chemical oxygen demand at 83.2% and 81%, respectively. Laccase was detectable during the biotreatment process.

Keywords  Laccase, *Perenniporia tephropora*, Physiology, Pulp mill effluent, White-rot fungi

White-rot fungi are a heterogeneous group of ligninolytic basidiomycetes. They have received considerable attention for their bioremediation potential due to an extracellular nonspecific enzyme system composed of laccases, lignin peroxidases (LiP) and manganese peroxidases (MnP), which function together with H₂O₂-producing oxidases and secondary metabolites [1]. Biotechnological applications of white-rot fungi or their ligninolytic enzymes, especially laccase, include degradation of highly toxic environmental pollutants such as dye residues, polyaromatic hydrocarbons, chlorinated phenols, and lignin in pulp mill wastewater, as well as applications in biopulping and biobleaching of pulp and paper [2].

Lignin and chlorinated phenols are major pollutants discharged from the pulp bleaching process of the pulp mill industry causing toxic and intensely brown colored waste effluents. Conventional biological processes such as aerated lagoons and activated sludge plants are ineffective in removing the brown color and phenolic compounds from pulp mill wastewater. This effluent is discharged into rivers, causing problems for communities and the environment. Biological treatment by white-rot fungi and their ligninolytic enzymes removed the color and reduced the chemical oxygen demands (COD) in the pulp mill effluent [3]. However, some characteristics of white-rot fungi such as slow growth and glucose requirement, limit their ability for the practical treatment of pulp mill effluent under extreme environmental conditions, strong alkaline pH, and oxygen limitation [4]. A fungal strain is required which can tolerate alkaline pH to survive in extreme conditions, and still have the ability for extracellular ligninolytic enzyme production. Thus, the efficiency of a white-rot fungal system for effluent treatment depends on allowing the right strain to work, and control...
its ligninolytic enzyme production.

Research into the physiology of white-rot fungal growth and their extracellular ligninolytic enzyme production is important not only from the viewpoint of comparative biology, but also for the potential of designing environmental conditions or systems appropriate to each particular purpose and fungal strain. The physiological regulation of ligninolytic enzyme production by white-rot fungi has been extensively studied in Phanerochaete chrysosporium and Trametes versicolor [5]. Expression of ligninolytic enzyme by fungi is influenced by various physiological parameters including the type and concentration of the carbon source, pH of the fermentation broth, and the presence of an inducer such as copper [5, 6].

The strain of Polyporaceae designated as KU-Alk4 was isolated in Thailand by our group, and preliminary studies on its internal transcribed spacer (ITS) DNA sequence suggested that it was closely related to Ganoderma philippii, with 93% identity [7]. In this study, the ITS sequence of KU-Alk4 was re-analyzed for sequence homology, and it was found to be a strain of Perenniporia tephropora. The laccase from strain KU-Alk4 has been used to decolorize synthetic dye [8]. Statistical design was employed to optimize the culture medium composition for KU-Alk4, resulting in high laccase production [9]. One interesting finding was that glucose concentration regulated the expression of different laccase isozymes by KU-Alk4 cultivated at alkaline pH, and two isozymes were found to be novel laccases [7].

Although the laccase of Perenniporia has been reported, the physiological parameters affecting its ligninolytic enzyme production are little known, and to date no studies on the biotreatment of pulp mill effluent have been reported with Perenniporia. Hence, this research aimed to investigate the physiological factors affecting the growth of P. tephropora strain KU-Alk4 and its ligninolytic enzyme production. Additionally, the capability of strain KU-Alk4 to treat pulp mill effluent was investigated under non-sterile process. This study focused on the treatment efficiency of color reduction, COD, and the pH of the effluent.

**MATERIALS AND METHODS**

**Fungal strain.** *P. tephropora* KU-Alk4 was isolated from a living tree, *Terminalia bellirica* Roxb., at Kasetsart University, Thailand. The fungus was maintained on potato dextrose agar (PDA) slants and stored at 4°C. The ITS sequence of KU-Alk4 was re-analyzed for sequence homology using the BLASTN program (http://www.ncbi.nlm.nih.gov/BLAST) against the updated GenBank database [10]. Sequence alignment was performed using the ClustalW program in BioEdit ver. 7.0.9.0 [11]. The phylogenetic tree was constructed by the neighbor-joining method and bootstrap analysis with 1,000 replications using the MEGA4 program [12].

**Culture conditions for physiological studies.** The fungus was grown on PDA plates at 30°C for 4 days. Fifteen plugs, 5 mm in diameter from the growing edge of mycelia were used as inoculum in 250 mL Erlenmeyer flasks containing 50 mL Kirk’s liquid medium [13]. The original medium contained 1% glucose and 0.02 mM cupric sulfate. The initial pH of the media was adjusted to start in acidic, neutral, and alkaline conditions of 4.5, 7.0, or 8.0, respectively, and pH was not controlled during the cultivation. The culture was grown unshaken at 30°C for 3 days. Then an inducer as 0.85 mM of veratryl alcohol, guaiacol, or 2,6-dimethoxyphenol (DMP) was added to the medium. Incubation of the culture was continued for 6 days, shaken at 100, 120, or 140 rpm. Glucose, lactose, glycerol, and carboxymethylcellulose (CMC) at 1% were compared as carbon sources. The effects of glucose concentration of 0.1%, 0.5%, 1%, 2%, 3%, or 4% were examined, as was the effect of copper at different concentrations. Cell pellets from three replicates were dried at 60°C to a constant weight to obtain the cell dry weight, and the filtrate was treated with crude ligninolytic enzyme.

**Analytical determinations.** Ligninolytic enzyme activities of laccase, MnP, and LiP were assayed. Laccase activity was assayed using 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) as substrate, as previously described [9]. MnP and LiP activities were assayed following the method of Kondo et al. [14]. They were expressed as units per milliliter, where 1 U was defined as 1 μmol of substrate oxidized per min. Total activities of ligninolytic enzyme were defined as the total activities of laccase, MnP, and LiP. Specific activity (U/mg cell dry weight) was defined as enzyme units per mg dry weight of fungal cells. The stability of the crude enzyme to pH was examined after incubating the enzyme in buffers of different pH (pH 2.0–10.0) at 30°C for 24 hr.

**Biotreatment of pulp mill effluent.** Raw wastewater from the pulping process was obtained from the wastewater treatment facility of pulp and paper factory in Thailand. The samples were transported to the laboratory in iceboxes and immediately used after arrival. We established and optimized a suitable procedure for the growth of KU-Alk4 and biotreatment of the pulp mill effluent by shake flask method as follows. An inoculum was prepared as described in section 2.2. Fifteen plugs of inoculum were inoculated into 250 mL Erlenmeyer flasks containing 50 mL potato dextrose broth (PDB) medium, pH 8.0, under sterile condition. The culture was grown unshaken at room temperature (RT, 32 ± 2°C) for 1 day, and then further incubated at RT, with continuous shaking at 120 rpm on a rotary shaker for 3 days. The process of pulp mill effluent biotreatment was carried out under non-sterile condition. The mycelial mats of KU-Alk4 obtained from PDB were filtered through double-layers of nylon cloth, and then 6 g wet weight of the mycelial mats was transferred into 50 mL of freshly pulped mill effluent in 250 mL flasks. The biotreatment process was incubated at RT under shaking at 120 rpm on a rotary shaker. The experiment was performed in triplicate using three separate flasks. A control experiment was carried out...
in triplicate, using the autoclaved sterile fungal mycelium of a 4-day-old culture from PDB instead of the living culture. Samples were taken daily by filtration through double-layers of nylon cloth. The filtrates were analyzed for reduction of COD, pH, and color in ADMI color units (American Standard Methods 2120 E).

RESULTS AND DISCUSSION

Identification of strain KU-Alk4. In nature, the fruiting body of strain KU-Alk4 grows out laterally like a shelf on the bark of a tree. The dorsal surface shows numerous pores (Fig. 1A). A previous report compared the taxonomy of the ITS gene sequence of KU-Alk4 with the GenBank database using the BLASTN program from the NCBI website [7]. Based on ITS sequence analysis, KU-Alk4 was most similar to *G. philippii*, but only 93% homologous. Hence, the ITS sequence of KU-Alk4 (AY605709) was re-analyzed for sequence homology. The result showed 99% sequence similarity to *P. tephropora* Cui9029 ITS 1, partial sequence; 5.8S ribosomal RNA gene and ITS 2, complete sequence; and 28S ribosomal RNA gene, partial sequence (HQ876601) [15]. Phylogenetic analysis showed that the position of the strain Alk4 in the phylogenetic tree was close to *P. tephropora* Cui9029 and Cui6631 (Fig. 1B). From morphological characteristics and ITS gene sequences, KU-Alk4 was identified as *P. tephropora*.

A preliminary study found that KU-Alk4 secreted two kinds of ligninolytic enzyme, laccase and MnP, but not LiP, in Kirk’s liquid medium, pH 7.0. The fungus grew and formed pellets better under static conditions than when shaken during the first 3 days. No ligninolytic enzymes were produced during these first 3 days. Production of the enzymes started after the addition of 0.85 mM veratryl alcohol as an inducer on day 3, after which the cultures were shaken. Enzyme activities increased dramatically, reaching a maximum on day 9 under shaking conditions. Shaking the 3-day-grown cells after inducer addition gave 5 times higher production of the enzymes than continuing with static growth. Moreover, the cultures grown under the regime of 3 days static then 6 days shaken, with addition of the inducer at day 3, produced 2.5 times higher activity of enzyme production than cultures shaken throughout the entire 9-day period. Thus, the culture regime of 3 days static then 6 days shaken was chosen for further study on the physiological factors affecting the growth of *P. tephropora* strain KU-Alk4 and its ligninolytic enzyme production, using the one-factor-at-a-time method. These parameters were media pH, carbon source and inducer, copper ions, and aeration rate.

Regulation of ligninolytic enzyme production by pH. The initial pH of the medium not only affected fungal growth but also regulated the production of ligninolytic

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Fig. 1. A, Fruiting body of *Perenniporia tephropora* KU-Alk4 in nature; B, Phylogenetic tree of KU-Alk4 and related species in the genus *Perenniporia* constructed on the basis of internal transcribed spacer DNA sequences using the neighbor-joining method. Bar = 0.005 base substitutions per site. There were a total of 540 positions in the final dataset. Bootstrap values are shown next to the branches.
Alkaline-resistant Laccase from *Perenniporia tephropora* enzyme. Fig. 2 compares the growth of the fungus and production of individual enzymes, laccase and MnP, in media with initial pH of acid (4.5), neutral (7.0), and alkaline (8.0). The pH was not controlled during the incubation. The best growth of mycelial pelleted formation was achieved at pH 7.0, with the early lag phase in 2 days. The fungus also grew well but formed smaller mycelial pellets at pH 8.0, at the same growth rate by day 9, but with a longer lag phase of 4 days. At the relatively acidic pH of 4.5 poor growth was observed, and no ligninolytic enzymes were detected. The enzymes were only detected from the cultures grown at pH 7.0 and 8.0. At pH 7.0, the fungus produced equal activities of laccase and MnP. In the relatively alkaline condition of pH 8.0, only laccase activity was detected. The specific activity of laccase recovered after 9 days cultivation at alkaline pH was 4 times higher than that recovered at neutral pH. Total activities of the ligninolytic enzyme produced at pH 8.0 and 7.0 were 67 and 37 U/mg cell dry.

![Fig. 2. Effect of pH on growth and production of laccase and manganese peroxidase (MnP) by *Perenniporia tephropora* KU-Alk4 in Kirk's medium. The arrow indicates the addition of veratryl alcohol. Values are the average of three independent experiments. Error bars show the standard deviation of triplicates.](image)

![Fig. 3. Effects of various carbon sources (A) and glucose concentration (B) on laccase production by *Perenniporia tephropora* KU-Alk4 in Kirk’s medium, pH 8.0. The arrow indicates the addition of veratryl alcohol. Values are the average of three independent experiments. Error bars show the standard deviation of triplicates. CMC, carboxymethylcellulose.](image)
weight, respectively. The productivity and ability of the enzymes to retain their activities at high pH were noteworthy when compared to those of *Pleurotus ostreatus* and *Cyathus stercoreus*, and these produced ligninolytic enzyme only at pH 5.0–6.0 [16, 17]. The alkaline condition pH 8.0 regulated KU-Alk4 to produce only laccase. Thus, for the characteristics and potential application of laccase in the pulp and paper industry, pH 8.0 was chosen for further testing of KU-Alk4 laccase production.

**Effect of carbon sources.** At pH 8.0, KU-Alk4 preferred lactose for growth to glucose and CMC, each at 1% (Fig. 3A). When glycerol was used as a carbon source, KU-Alk4 did not grow in the medium. Glucose was determined as the best substrate for laccase production by KU-Alk4. The specific activities of laccase in media with 1% glucose, lactose or CMC were 126, 28, and 11 U/mg cell dry weight, respectively. The effects of lactose were similar to those observed for *Ganoderma lucidum* which produces α-galactosidase. This allows the fungus to use lactose for growth, and causes the repression of laccase production [18].

We previously reported that KU-Alk4 produced different laccase isozymes at low (1%) and high (4%) glucose concentration [7]. This study reported on the physiology of growth and laccase secretion of KU-Alk4 in alkaline medium, with different glucose concentrations ranging from 0.1 to 4.0%. In medium at pH 8.0, the higher the glucose concentration (from 0.1% to 4.0%), the higher the fungal biomass obtained (Fig. 3B). On the other hand, total laccase activity produced by KU-Alk4 increased only when glucose concentration increased from 0.1% to 1.0%, and then decreased. The specific activities of laccase produced by cultures grown for 9 days in medium with 1%, 2%, 3%, and 4% glucose were 126, 59, 40, and 33 U/mg cell dry weight, respectively. This primarily suggested that glucose repressed the enzyme synthesis of KU-Alk4, similar to a phenomenon found in several fungi, which is thought to be an energy-saving response [19, 20].

**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 inducible laccase is mainly affected by the chemical nature and time of inducer addition. KU-Alk4 grew in pH 8.0 medium without veratryl alcohol, but no laccase activity was detected (Fig. 4A). Addition of 0.85 mM veratryl alcohol induced KU-Alk4 to produce laccase. Veratryl alcohol at this concentration did not affect the fungus growth, only its laccase production. Addition of veratryl alcohol on day 3 of culture induced KU-Alk4 to produce 230 U/mL laccase. This was three times more than the culture with veratryl alcohol added at the beginning. Therefore, the production

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**Fig. 4.** Effects of induction control (A) and some phenolic compounds (B) as inducers of laccase production of *Perenniporia tephropora* KU-Alk4 in Kirk’s medium, pH 8.0. Values are the average of three independent experiments. Error bars show the standard deviation of triplicates.
of laccase by KU-Alk4 was induction controlled. Induction by 0.85 mM veratryl alcohol on day 3 of culture resulted in the production of the highest specific laccase activity of 126 U/mg cell dry weight, detected on day 9.

Low molecular weight phenolic compounds as veratryl alcohol, guaiacol, and DMP were tested for the production of laccase by KU-Alk4 (Fig. 4B). The fungus was first allowed to grow in a pH 8.0 medium without the phenolic compound for 3 days. Then, each of the above phenolic compounds was added at a final concentration of 0.85 mM. KU-Alk4 grew in the presence of these inducers, but guaiacol and DMP showed only slight effects on fungus growth. Although the fungus also grew in the presence of guaiacol or DMP, very low activities of laccase were detected. The specific activities of laccase produced in the cultures with veratryl alcohol, guaiacol, and DMP were 126, 12, and 4 U/mg cell dry weight, respectively. Results suggested that guaiacol and DMP were not good inducers for laccase production by KU-Alk4 under these growth conditions. Thus, 0.85 mM veratryl alcohol was chosen as the best inducer. The production of laccase by Trametes modesta, T. versicolor, Phlebia radiata, and Dichomitus squalens was also induced by veratryl alcohol [21]. However, other white-rot fungi responded differently toward the two low molecular weight aromatic compounds. Guaiacol induced laccase production by Ganoderma sp. WR-1, Trametes pubescens, and Daedalea flavida [21-23], while DMP was the best inducer of laccase production by Lentinus strigosus [24].

**Effect of copper ions.** Copper, essential heavy metal, is required for growth and ligninolytic enzyme production of white-rot fungi, but only in trace amounts; excess amounts are toxic. At high concentrations, the free forms of cupric ions are extremely toxic to microbial cells. Since laccase is a copper-containing protein, copper supplementation of the culture medium enhances both laccase transcription and activity [22, 25]. Positive effects of copper addition on the production of laccase were also observed in P. tephropora, Ceriporiopsis subvermispora, Marasmius quercophilus, T. versicolor, Pleurotus ostreatus, Ganoderma applanatum, and G. lucidum [6, 25-28]. Addition of copper to KU-Alk4 culture medium affected both fungal growth and laccase production. Addition of copper sulfate (CuSO₄) at low concentrations (0.02 and 0.2 mM) stimulated growth of KU-Alk4 and had a positive effect on laccase production (Fig. 5A). Copper at 0.2 mM stimulated higher production of laccase but caused slight inhibition of growth compared with 0.02 mM CuSO₄. Copper at 2 mM is toxic to KU-Alk4, which resulted in complete growth inhibition. Specific activities of laccase with 0.02 and 0.2 mM CuSO₄ were 126 and 186 U/mg cell dry weight, respectively. In contrast, Trametes pubescens produced the highest amount of laccase with 2 mM copper

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**Fig. 5.** Effects of copper ion (A) and shaking rate (B) on laccase production by *Perenniporia tephropora* KU-Alk4 cultivated in Kirk’s medium, pH 8.0. The arrow indicates the addition of veratryl alcohol. Values are the average of three independent experiments. Error bars show the standard deviation of triplicates.
Effect of aeration on laccase production. Aeration provided to KU-Alk4 from day 3 to day 9 of cultivation was important for the production of laccase as well as fungal growth (Fig. 5B). More laccase was produced in shaken than in unshaken cultures. Increasing rates of aeration by changing the shaking rate of 50 mL culture supplemented with 0.2 mM copper sulfate in a 250 mL flask from 100 to 120 and 140 rpm resulted in increased biomass, but shaking at over 120 rpm resulted in lower production of laccase. The specific activity of laccase produced in these conditions was 247 U/mg cell dry weight, while those at 100, 140, and 0 rpm were 216, 126, and 62 U/mg cell dry weight, respectively.

Results of physiological study by the one-factor-at-a-time method summarized that the optimum conditions were as follows: Kirk’s medium at initial pH 8.0, with pH not controlled throughout the experiment; 1% glucose as a carbon source; 0.2 mM copper sulfate; 0.85 mM veratryl alcohol as an inducer; and incubation without shaking for the first 3 days, then with shaking at 120 rpm. Under these conditions that regulated the fungus to produce only laccase, the secondary metabolic state of KU-Alk4 produced the highest laccase of 360 U/mL (Fig. 5B). Our strain produced significantly higher laccase activity in comparison with most fungi, which typically produce 4-100 U/mL [22, 30]. The optimum conditions for laccase production suggested by the one-factor-at-a-time method appeared to differ from those previously reported by our group, using Box-Behnken design for medium optimization [9]. The obvious difference was that the results of Box-Behnken design showed that the culture with pH controlled at 6.0 throughout the experiment and 4% glycerol was suitable for laccase production by KU-Alk4. However, KU-Alk4 could not grow in glycerol when the initial pH was 8.0, whether the pH of the culture was controlled or not throughout the experiment. The result obtained in this study showed that the initial pH of 8.0 did control the utilization of glycerol by KU-Alk4. Nevertheless, in pH 8.0 medium with glucose as a carbon source and when pH was not controlled throughout the experiment, laccase production by KU-Alk4 was 1.4 times higher than that produced in medium with 4% glycerol and with pH controlled at 6.0 throughout the designed experiment [9]. These results led us to consider controlling the pH of the medium, which had a significant effect on fungal growth and laccase production, and then selecting the carbon source, both type and concentration.

Crude enzyme from the optimum conditions, suggested by the one-factor-at-a-time method was composed of three dominant proteins which all exhibited laccase activity [7]. The crude enzyme was stable for 24 hr in alkaline regions up to pH 10.0 (Fig. 6). The relative activity of the crude enzyme was highly stable at alkaline pH 8.0–9.0. The activity of crude enzyme was more sensitive at lower pH 2.0–7.0 and decreased by 15% to 20% when the enzyme was kept at lower pH for 24 hr. KU-Alk4 showed extreme alkaline-resistance for laccase production. Thus, KU-Alk4 was considered to be an attractive fungus for further study in the biotreatment of pulp mill effluent which has a strongly alkaline pH and is an intense brown color.

Biotreatment of pulp mill effluent. The strain KU-Alk4 was grown to form mycelia mats, and induced into the secondary metabolic state in a pH 8.0 of PDB medium for 4 days. Mycelial mats of KU-Alk4 were then immersed in the pulp mill effluent under non-sterile condition. The biotreatment of effluent by KU-Alk4 was conducted for 3 days on pulp mill effluent containing 1,621 ADMI color unit with a COD of 3,024 mg/L at pH 9.6. After treating the effluent with KU-Alk4 for 3 days, results indicated an 83.2%, and 81% reduction of color and COD, respectively (Fig. 7A and 7B). The reduction of color using biological treatment correlated with the degradation of lignin and its related phenolic compounds which are major components of pulp mill effluent, and the cause of a higher COD and color as well as harmful effects on the environment [31, 32]. This result showed that KU-Alk4 was capable of a significant reduction in the pollutant load of the effluent. After 2 days of biotreatment, the pH of the effluent was neutralized (Fig. 7C), which caused by aromatic acids, lignin biodegradation products such as vanillic acid, syringic acid, benzoic acid, and protocatechuic acid [33]. The control experiment using autoclaved fungal mycelium showed no reduction in color, COD, or pH. Thus, the effective biotreatment of pulp mill effluent was due to the fungal action. Only one ligninolytic enzyme, laccase, was detectable during the biotreatment of effluent, and the activity of laccase gradually increased to 230 U/mL on day 3 (Fig. 7D). No enzyme activities were detected in the control experiments. Induction of LiP, MnP, and laccase in white-rot fungi during decolorization of paper mill effluent has previously been reported [34, 35]. Generally, the use of fungal systems
Alkaline-resistant Laccase from *Perenniporia tephropora* for effluent treatment has been constrained due to their requirement of narrow pH 4.0~6.0, nutrient supplementation, and longer retention time for their growth and enzyme production. However, the significant differences between this and previous studies concerning the biotreatment of pulp mill effluent by white-rot fungi were the utilization of a fungal system without any need for pH adjustment and nutrient supplementation, and therefore the reduction of additional process costs.

The ability of the crude enzyme with laccase activity of KU-Alk4 to decolorize the pulp mill effluent was tested under non-buffered condition. Treating the effluent with 100 U of the crude enzyme for 3 days resulted in a 42% reduction of color, indicating that this enzyme was directly involved in the decolorization process.

In conclusion, *P. tephropora* KU-Alk4 grew in a wide range of pH 4.5~8.0 and produced the ligninolytic enzyme laccase and MnP. Alkaline conditions drove the fungus to produce only laccase. At pH 8.0, KU-Alk4 produced higher specific activity of laccase than at pH 7.0. KU-Alk4 preferred 1% glucose to lactose for laccase production at pH 8.0, but glucose at higher concentrations repressed enzyme production. CMC and glycerol were not utilized by the fungus at pH 8.0. Veratryl alcohol was the best inducer of ligninolytic enzyme production, compared with guaiacol and DMP at the same concentration. Copper was required for growth and laccase production. Aeration of the 3-day culture was important for fungal growth and enzyme production, however, excess oxygen inhibited laccase generation. The results indicated that utilization of *P. tephropora* KU-Alk4 is suitable for the treatment of color, COD, and pH of pulp mill effluent. Future study is required to explore the potential of this selected fungal system, and upscale bioreactors to implement better industrial operational conditions for effluent treatment. Further investigation of these issues is in progress.

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