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Ligninolytic Enzyme Production by *Phanerochaete chrysosporium* in Plastic Composite Support Biofilm Stirred Tank Bioreactors

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Phanerochaete chrysosporium (ATCC 24725) produced lignin peroxidase (LiP) and manganese peroxidase (MnP) in defined medium in plastic composite support (PCS) biofilm stirred tank reactors. Laccase was not detected. The formation of the *Ph. chrysosporium* biofilm on the PCS was essential for the production of MnP and LiP. The bioreactor was operated as a repeat batch, and no reinoculation was required between batches. Peroxidase production was influenced by 5 min purging of the bioreactor with pure oxygen or continuous aerating with a mixture of air and oxygen at a flow rate of 0.005 vvm. Continuous aeration and 300 rpm agitation with 3 mM veratryl alcohol addition on days 0 and 3 demonstrated the highest lignin peroxidase production on day 6 with means of 50.0 and 47.0 U/L. Addition of veratryl alcohol and $MnSO_4$ on day 0 with 300 rpm agitation and continuous aeration at 0.005 vvm (air flow rate in L/min divided by the reactor working volume in liters) hastens the production of MnP with final yield of 63.0 U/L after 3 days. Fourteen repeated batches fermentation were performed without contamination due to low pH (4.5) and aseptic techniques employed.

KEYWORDS: Ligninolytic enzymes; *Phanerochaete chrysosporium*; PCS biofilm; bioreactor

INTRODUCTION

The white-rot wood-decaying basidiomycetes are capable of producing the lignin-degrading enzymes laccase, lignin peroxidase, and manganese peroxidase, or at least one of these enzymes (*1*, *2*). These enzymes have been intensively studied in *Phanerochaete chrysosporium* (*3*). The lignolytic system in *Ph. chrysosporium* is complex. It includes numerous enzymes or associated activities such as peroxidases, H_2O_2-generating oxidases (e.g, glucose oxidase and methanol oxidase), laccase, cellobiose dehydrogenase, glyoxal oxidase, aryl alcohol oxidase, transmembrane methyl transferase, transmembrane redox potential, and various low molecular weight compounds (*3*, *4*). The lignin peroxidase (LiP) and mangenase peroxidase (MnP) are families of extracellular isoenzymes (*5*). The ratio between the isoenzymes changes with the culture age and culture conditions (*6*−*8*).

Oxygen, temperatures, nutrients, and inducer compounds and other factors can affect the peroxidase production in *Ph. chrysosporium* (*9*, *10*). Veratryl alcohol (3,4-dimethoxybenzyl alcohol; VA) is synthesized de novo from glucose early in the secondary growth phase of *Ph. chrysosporium* cultures (*4*, *11*), but the addition of the VA to culture broth leads to increased LiP production (*12*−*14*). Addition of manganese (Mn^{2+}) to the white-rot fungi culture broth will induce the production of MnP to higher levels than the noninduced cultures (*15*, *16*). Also, ligninase production is promoted by other factors such as temperature and nutrients (*10*). In *Ph. chrysosporium* shake flask cultures, the production of ligninase was found to be suppressed by excess nutrients (*13*). Purging with pure oxygen markedly enhances the production of H_2O_2 in white-rot cultures (*9*); thus supplying oxygen to the *Ph. chrysosporium* culture broth instead of air will increase lignin degradation and LiP activity (*13*). However, a continuous oxygen supply will promote LiP and MnP production and decay in submerged cultures of *Ph. chrysosporium* (*17*).

Scale-up peroxidase production continues to be an important research interest. The shallow stationary cultures, agitated cultures, and cell immobilization bioreactors have been evaluated (*16*, *18*−*23*). Under both stationary and agitated incubation, the activity of ligninase reaches a maximum on day 5−6 in benchtop reactors, but the attempts to scale-up production result in lower activity (*23*). Immobilization of the *Ph. chrysosporium* mycelia was more effective in promoting cell growth, and LiP production improved up to 8100 U/L for a hypersecreting strain (*24*) compared to 75 U/L in conventional stationary liquid culture with wild type (*18*, *23*, *25*).

Biofilms are the natural form of cell immobilization (*26*). The types of reactors that rely on immobilized cell or biofilm include trickling filters, fixed-film or fixed-bed bioreactors, fluidized-bed reactors, and rotating bioreactors (*10*, *17*, *19*, *25*, *27*). However, work is still being carried out on the development

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Table 1. Repeated Batch Abbreviations and Fermentation Condition Evaluated

batch abbreviation	agitation speed (rpm)	Time of addition (3 mM final concn)[a]		aeration conditions
		on day 3	on day 0	
P_1V_3 1	120	veratryl alcohol	*b*	purging[c]
P_1V_3 2[d]	120	veratryl alcohol		purging
P_1VA_3 3	120	veratryl aldehyde		purging
P_1M_3 4	120	$MnSO_4$		purging
P_3V_3 5	300	veratryl alcohol		purging
P_3VA_3 6	300	veratryl aldehyde		purging
P_3M_3 7	300	$MnSO_4$		purging
C_3V_3 8	300	veratryl alcohol		continuous[e]
C_3VA_3 9	300	veratryl aldehyde		continuous
C_3M_3 10	300	$MnSO_4$		continuous
C_3V_0 11	300		veratryl alcohol	continuous
C_3VA_0 12	300		veratryl aldehyde	continuous
C_3M_0 13	300		$MnSO_4$	continuous
C_3VM_0 14	300		veratryal alcohol and $MnSO_4$	continuous

[a] A 3 mM final concentration for each inducer in the bioreactor was aseptically added on day 0 or 3. [b] No addition of inducer. [c] Bioreactor was purged for five minutes with pure oxygen at 0.005 vvm on day 0, then daily on day 3. [d] This batch only received a limited carbon source (10 g/L glucose). [e] Continuous aeration with mixture of air and oxygen at 0.005 vvm.

of an optimal bioreactor configuration and conditions to increase *Ph. chrysosporium* ligninolytic enzyme production. Several studies have demonstrated the effectiveness of the plastic composite support (PCS) biofilm reactor for increased end-product production rate, minimal lag phase, tolerance to high concentration of nutrient, reduced requirement of micronutrients, and increased cell density for ethanol, lactic acid, and succinic acid production (*28*−*33*).

In this study, the bioreactor design of Cotton et al. (*28*) was employed. The PCS tubes were fixed to the agitator shaft in the bioreactor to develop the *Ph. chrysosporium* PCS biofilm. *Ph. chrysosporium* attached and covered the whole surface of the PCS tubes. Biofilm thickness was controlled by agitation speed. The PCS biofilm increases the cell interfacial contact with culture fluid, decreases the shear force, and permits medium to circulate through the PCS tubes. Also, in this study, the effects of agitation, oxygen flow, and addition of veratryl aldehyde, veratryl alcohol, manganese (Mn^{2+}), and combinations of them to increase the ligninolytic enzymes of *Ph. chrysosporium* in PCS stirred tank biofilm reactor were investigated.

MATERIALS AND METHODS

Microorganism and Inoculum Preparation. *Phanerochaete chrysosporium* (ATCC 24725) was obtained from American Type Culture Collection (Manassas, VA). The *Ph. chrysosporium* was maintained on potato dextrose agar slants at 4 °C. The active *Ph. chrysosporium* was cultured on media containing 10.0 g/L malt extract, 2.0 g/L peptone, and 2.0 g/L yeast extract (Difco lab, Deroit, MI), 10.0 g/L glucose, 1.0 g/L asparagine, 2.0 g/L KH_2PO_4, 1.0 g/L $MgSO_4 \cdot 7H_2O$, 0.001 g/L thiamin, and 20 g/L agar (Sigma Chemical Co., St. Louis, MO) (*23*). The slants were incubated for 6 days at 39 °C. Spore suspensions were prepared in sterile water followed by passage through sterile glass wool to remove mycelia. The spore suspension concentration was determined by measuring absorbance at 650 nm.

Medium Preparation. Peroxidase production medium contained 20.0 g/L glucose, 0.22 g/L ammonium tartrate, 2.0 g/L KH_2PO_4, 5.0 g/L $MgSO_4 \cdot 7H_2O$, 0.1 g/L $CaCl_2 \cdot H_2O$, 1.2 g/L acetic acid, 0.4 g/L NaOH (*34*), 11.7 mL of trace elements (*23*), 1.0 g/L Tween 80, and 2.5% (v/v) spore suspension. The inoculum size of spore suspension was adjusted to reflect an optical density reading of 0.83 at 650 nm using a proportion for all batch experiments unless indicated in the text. To feed the fermentor, 90 L of sterile culture medium was prepared in a B- Braun 100-D fermentor (Allentown, PA) with continuous agitation for 25 min at 121 °C. The trace elements solution was added after it was filter sterilized. The sterilized media was aseptically transferred into two sterilized 50 L carboys equipped with a carboy filling port, a medium delivery line with a liquid break, and an air vent capped with a 0.45 μm air filter for storage.

Plastic Composite Supports (PCSs). PCS tubes composed of 50% (w/w) polypropylene, 40% (w/w) ground dried soybean hull (Cargill Soy Processing Plant, Iowa Falls, IA), 5% (w/w) dried bovine albumin (Proliant Corp., Ames, IA), 5% (w/w) yeast extract (Ardamine Z from Sensient Flavor, Juneau, WI), and mineral salts were produced according to Ho et al. (*35*). These dry ingredients were mixed in a separate container prior to being poured into an extruder hopper. The twin screw corotating Brabender PL2000 extruder (model CTSE-V; C. W. Bra bender Instruments, Inc; South Hackensack, NJ) was operated at a rate of 11 rpm, barrel temperatures of 200, 220, and 200 °C, and a die temperature of 167 °C to a continuous tube. The PCS tubes had a wall thickness of 3.5 mm and an outer diameter of 10.5 mm.

PCS Biofilm Stirred Tank Bioreactor. The bioreactor design of Cotton et al. (*28*) was employed. Six PCS tubes, each 10 cm in length, were stacked in three rows of two parallel tubes, then bound to the agitator shaft in a grid fashion. The bioreactor was a computer controlled New Brunswick Bioflo 3000 (Edison, NJ) equipped with controllers of the pH, temperature, agitation, and dissolved oxygen. The 1.2 L vessel (inside diameter of 12 cm) was equipped with filtered sterilized air inlet and outlet. The broth removal port was connected with two branched lines to withdraw a sample and to remove all culture media from the vessel. The reactor with PCS attached to the agitator shaft was sterilized with 800 mL of water for 1.25 h at 121 °C. After sterilization and cool-down, the bioreactor was drained and sterilized culture medium was pumped into the bioreactor and kept overnight before inoculation to check sterility. Clear culture broth the next day confirmed sterility.

Batch Culture Protocols. The peroxidase production was evaluated in 14 different repeated batch fermentations at 39 °C with 1-L working volume and 6 day incubations per batch. Three samples were collected from each batch on days 0 (control), 3, and 6.

Table 1 describes the treatments evaluated. Four batch fermentations (P_1V_3 1, P_1V_3 2, P_1VA_3 3, and P_1M_3 4) investigated the effect of adding on day 3 3 mM veratryl alcohol, veratryl alcohol with limited carbon source (10 g/L glucose instead of 20 g/L), veratryl aldehyde, and $MnSO_4$, respectively. In P_1V_3 1, the bioreactor was inoculated with 2.5% (v/v) spore suspension with optical density of 0.83 at 650 nm (*36*). This was the only inoculation used for the study. After each batch, the bioreactor was drained and filled with sterile water. The agitation was increased to 500 rpm for 20 min to remove excess biofilm formed on PCS tubes, and then the bioreactor was drained again and refilled with fresh sterile media. No reinoculation was necessary, because the mycelia of the *Ph. chrysosporium* remain on and in the PCS tubes attached to the agitator shaft.

The repeated batch fermentations also investigated the effect of increasing the agitation to 300 rpm with spiking on day 3 with 3 mM veratryl alcohol for P_3V_3 5, veratryl aldehyde for P_3VA_3 6, or $MnSO_4$ for P_3M_3 7. All other fermentations were performed as described with P_1V_3 1.

The effect of continuous oxygen addition was also investigated at 300 rpm with spiking on day 3 with 3 mM veratryl alcohol for C_3V_3 8, veratryl aldehyde for C_3VA_3 9, or $MnSO_4$ for C_3M_3 10. Finally, the addition of enzyme inducers at time 0 was evaluated with continuous oxygen addition, 300 rpm agitation, and spiking with 3 mM veratryl alcohol for C_3V_0 11, veratryl aldehyde for C_3VA_0 12, $MnSO_4$ for C_3M_0 13, or a mixture of veratryl alcohol and $MnSO_4$ for C_3VM_0 14.

Sample Analysis. To determine enzyme activity, each batch was aseptically sampled (5 mL) on days 0, 3, and 6. Each sample was centrifuged at 3000 × *g* for 10 min at 4 °C, and the supernatant was evaluated for extracellular protein and enzyme activity. Oxygen consumption, CO_2 production, and change in pH were monitored via probes in the bioreactor connected to a computer.

Lignin Peroxidase (LiP). The level of LiP activity was determined using veratryl alcohol as substrate (*23*). One milliliter of the final assay mixture contained 2 mM veratryl alcohol, 0.4 mM H_2O_2 in 50 mM sodium tartrate buffer (pH 2.5), and 200 μL of culture supernatant. Oxidation of veratryl alcohol was measured as the increase in absorbance at 310 nm ($\epsilon_{310} = 9300\ M^{-1}\ cm^{-1}$). The increase in A_{310} was monitored during 300 s at 37 °C using a DU 640 UV/vis Backman spectrophotometer equipped with kinetics Soft-Pac no. 517033 for measuring enzyme activity (Beckman Instruments, Inc., Fullerton, CA). One unit (U) of LiP activity was defined as 1 μmol of veratryl alcohol oxidized in 1 min under defined conditions, and activities were reported as U/L.

Manganese Peroxidase (MnP). The level of MnP activity was determined by monitoring the enzyme oxidation of Mn^{2+} to Mn^{3+} (*37*). One milliliter of the final assay mixture contained 200 μL of culture supernatant, 0.1 M sodium tartrate, pH 5.0, 0.1 mM H_2O_2, and 0.1 mM $MnSO_4$. The product, Mn^{3+}, forms a transiently stable complex with tartaric acid, showing a characteristic absorbance at 238 nm ($\epsilon = 6500\ M^{-1}\ cm^{-1}$). Reactions were initiated by addition of H_2O_2. The increase in A_{238} was monitored during 300 s at 37 °C using a DU 640 UV/vis Backman spectrophotometer equipped with kinetics Soft-Pac no. 517033 for measuring enzyme activity. One unit (U) of MnP activity was defined as 1 μmol of Mn^{2+} oxidized in 1 min under defined conditions, and activities were reported as U/L.

Laccase Activity. Laccase activity was determined by using 2,2′-azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) as substrate (*38*). One milliliter of the final assay mixture contained 200 μL of culture supernatant and 2 mM ABTS in 0.1 M sodium citrate buffer (pH 3.0). Oxidation of ABTS was monitored spectrophotometrically as an increase at 420 nm (ϵ=36 000 $M^{-1}\ cm^{-1}$) by using a DU 640 UV/vis Backman spectrophotometer equipped with kinetics Soft-Pac no. 517033 for measuring enzyme activity. One unit (U) of laccase activity was defined as 1 μmol of ABTS oxidized in 1 min under defined conditions, and activities were reported as U/L.

Extracellular Protein Determination. The concentrations of extracellular protein were determined according to the Lowery reagent modified method (*39*) after 0, 3, and 6 days for each batch culture. Protein concentrations were determined spectrophotometrically at 750 nm by using a Spectrasonic 20 (Milton Roy, Rochester, NY). Bovine serum albumin was used to establish a standard curve for extracellular protein concentration (50−400 μg/mL; $r = 0.999$).

Statistical Analysis. Analysis for the enzyme activity was performed by using analysis of variance for a completely randomized design using JMP package. Comparison of pair of mean was done using the Tukey−Kramer LSD method with 5% level of significance. Determinations were made in replicates of three fermentation batches.

RESULTS AND DISCUSSION

To our knowledge, this is the first report aimed to employ wild type *Ph. chrysosporium* in a repeated batch with stirred tank bioreactor to produce ligninolytic enzymes. The results demonstrate that *Ph. chrysosporium* is able to develop a biofilm and produce ligninase in PCS biofilm stirred tank reactors. PSC bioreactors have been shown to stimulate microbial attachment (*29*) and help prevent wash-out of biomass from the reactor (*40*). The PCSs have also been shown to stimulate biofilm formation and enhance the production of end products (*28*−*30*). In this study, the PCSs stimulate the formation of a *Ph. chrysosporium* biofilm. In each batch, the PCS biofilm becomes significantly noticeable on day 2 and thick on day 6. The production of ligninase in 14 repeated batches was stimulated with the addition of inducers.

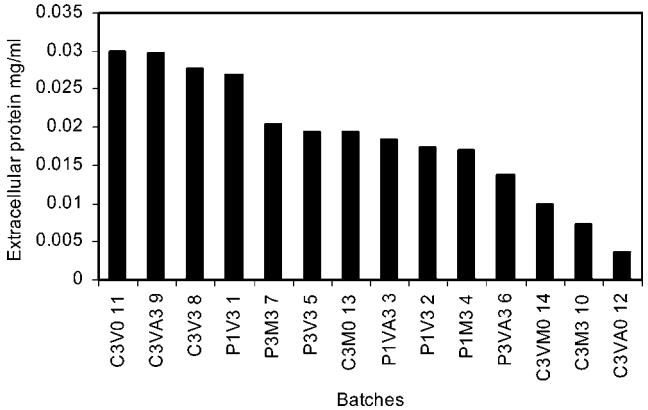

Figure 1. The concentrations of extracellular protein produced on day 6 for repeated batch fermentations arranged in descending order. Each value is the average of three replicates.

The enzyme production and extracellular proteins were determined during the fermentation days 0, 3, and 6 in each batch, and then the enzyme production and extracellular protein produced among all batches were compared for maximum production.

Analysis of variance for the 14 repeated batches of extracellular protein was statistically significant $F(13,41) = 51.2412$, $p < 0.0001$ (**Figure 1**). Tukey−Kramer pairwise comparison tests were conducted to define the significant pairwise batch. The mean concentrations of extracellular protein released were reported in descending order (**Figure 1**) for the 6 days of fermentation with each batch. The mean protein concentration demonstrated that C_3V_0 11, C_3VA_3 9, C_3V_3 8, and P_1V_3 1 with means of 0.03, 0.02967, 0.0276, and 0.0270 mg/mL, respectively, were not significantly different (LSD of 0.0059 mg/mL). Therefore, the extracellular protein production within the batches maybe grouped as C_3V_0 11, C_3VA_3 9, C_3V_3 8, P_1V_3 1 > P_3M_3 7, P_3V_3 5, C_3M_0 13, P_1VA_3 3, P_1V_3 2, P_1M_3 4 > P_3VA_3 6, C_3VM_0 14, C_3M_3 10, C_3VA_0 12 according to the mean values of extracellular protein production.

In MnP and LiP production among the 14 repeated batches, analysis of variance was statistically significant, $F(13,41) = 9.8134$, $p < 0.0001$ and $F(12,38) = 13.7699$, $p < 0.0001$, respectively. Tukey−Kramer pairwise comparison tests were conducted to define the significant pairwise batch fermentation. For day 6 samples, P_3VA_3 6 demonstrated the highest MnP production with mean of 56.7 U/L (**Figure 2**). For day 3 samples, C_3VM_0 14 demonstrated the highest MnP production with mean of 63.2 U/L. Also, comparison of the mean MnP production between days 3 and 6 demonstrates that the mean MnP production from all batches was higher on day 6 except for C_3VM_0 14.

For the 6 day samples, the C_3V_0 11 and C_3V_3 8 demonstrated the highest LiP production with means 50.0 and 47.3 U/L (**Figure 2**). For the 3 day samples, $C_3VM_0$14 demonstrated the highest LiP production with means of 28.7 U/L, which decreased

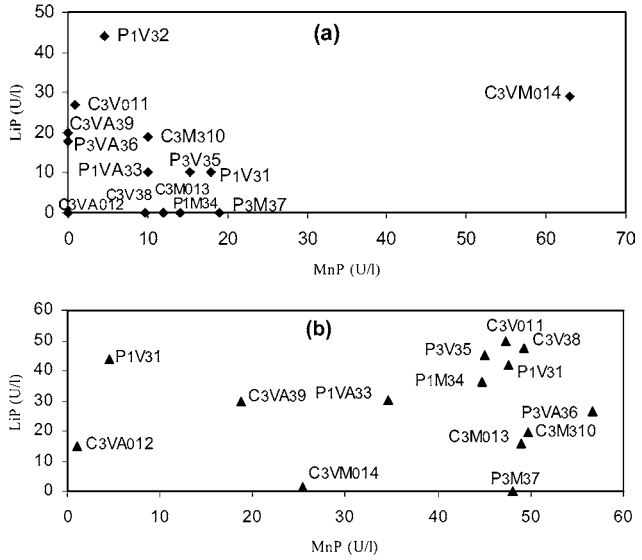

Figure 2. The pattern between the LiP and MnP production within the 14 repeated batch fermentations (a) on day 3 and (b) on day 6.

to 4.0 U/L on day 6. The LiP production results were also higher on day 6 in all batches except C_3VM_0 14, which was higher on day 3 (**Figure 2**).

Although the production of both LiP and MnP enzymes depends on nutrient limitations (*6*, *41*, *42*), this study indicates that also different conditions between the batches affect the production. However, no patterns were found between the production of LiP and MnP among the different treatments as day 3 samples illustrated (**Figure 2a**).

Day 6 samples demonstrated that 3 mM veratryl aldehyde alone was sufficient to induce both MnP and LiP (**Figure 2b**, P_1VA_3 3 and P_3VA_3 6). Furthermore, for day 6 samples, 3 mM $MnSO_4$ consistently induced MnP with little or no LiP production. There seems to be no direct relation between continuous and purged oxygen on enzyme production. Looking at the batches that differ in aeration, there is no difference between MnP and LiP productions, except on day 3 there was no LiP production for C_3V_3 8 compared to P_1V_3 1 and for P_1M_3 4 compared to C_3M_3 10 and also no MnP production for C_3VA_3 9 compared to P_1VA_3 3. Overall, continuous vs purged oxygen addition demonstrated no correlation to enzyme production among all batches with both procedures demonstrating excellent enzyme production (C_3V_0 11, P_1V_3 1, and C_3VA_3 9). As for the effect of increasing agitation rates (120 vs 300 rpm) on enzyme production, it seems that there is no pattern for the production between the compared batches. For example, while LiP production was the same comparing P_1V_3 1 to P_3V_3 5 and P_1VA_3 3 to P_3VA_3 6, a higher production was found in P_1M_3 4 compared to P_3M_3 7. On the other hand, MnP production only showed difference between P_1VA_3 3 compared to P_3VA_3 6. Veratryl aldehyde induction of LiP and MnP was affected by oxygen addition with continuous aeration significantly reducing enzyme production (C_3VA_0 12 and C_3VA_3 9) compared to purged aeration (P_3VA_3 6) under the same agitation (300 rpm). This may be due to the oxidation of veratryl aldehydes to veratric acid in the presence of continuous oxygen addition.

The *Ph. chrysosporium* PCS biofilm produced extracellular proteins with MnP and LiP enzymes in all batches. The *Ph. chrysosporium* produces a very low level of laccase when cultured with glucose (*3*). Laccase activity was demonstrated at slightly higher levels in the concentrated culture fluids when the *Ph.* chrysosporium was grown on cellulose and in plate assays, 3.1 U/L (*43*). However, no laccase activity was detected in our work.

The ligninolytic system in white-rot fungi, *Ph. chrysosporium*, has been considered noninducible by lignin and growth in the presence of low concentration of a synthetic lignin dose not significantly increase the rate of CO_2 evolution from lignin (*21*). Also, a limited nutrients condition for fungus production of ligninase is well documented (*4*). In this study, all batches were performed under defined limited nitrogen media and different inducers, agitation, and oxygen addition, which caused variations in enzyme production. The addition of veratryl alcohol to the culture medium of various white-rot fungi has repeatedly been found to increase LiP enzyme titers, which is explained as protecting LiP from H_2O_2-dependent reactions by offering a good reducing substrate (*17*, *44*), whereas $MnSO_4$ addition into white-rot fungi culture medium is considered a regulatory affecter for the production of LiP and MnP (*4*, *37*). Also, it has been reported that the manganese inhibited the endogenous production of veratryl alcohol by the white-rot fungi, which protects the LiP from H_2O_2-dependent reactions. In the absence of veratryl alcohol, H_2O_2-dependent reactions degrade the LiP, while MnP production continues (*4*). This study showed that the addition of $MnSO_4$ lowers the production of LiP but gives similar levels of MnP as in P_1V_3 1 compared to P_1M_3 4, P_3V_3 5 compared to P_3M_3 7, and C_3V_3 8 compared to C_3M_3 10. Thus, $MnSO_4$ suppressed the production of LiP but did not stimulate the production of MnP in the PCS biofilm reactor.

Various roles were suggested for veratryl alcohol in *Ph. chrysosporium* culture. Some studies considered veratryl alcohol as the real inducer of lignin peroxidase, which is needed to oxidize veratryl alcohol to veratryl aldehyde. The addition of veratryl aldehyde instead of veratryl alcohol to *Ph. chrysosporium* culture delays enzyme production as indicated in P_1VA_3 3, P_3VA_3 6, C_3VA_3 9, and C_3VA_0 12. Such results support the claim that veratryl alcohol will be formed de novo to induce and protect lignin peroxidase from the H_2O_2-dependent reactions (*11*, *44*).

The biofilm thickness, aeration, and shear stress have been shown to be the key parameters for controlling lignin peroxidase production. The biofilm thickness of *Ph. chrysosporium* was identified as the main scale-up parameter in a fixed trickle bed reactor that produced 110.0 U/L LiP activity after 200 h (*45*). Moreover, when the *Ph. chrysosporium* I-1512 (hypersecretory strain) was immobilized in an airlift bioreactor and supplied with continuous aeration (mixture of air and oxygen) with flow rate of 5 L/h, the MnP production reached 6600 U/L (*10*). Although the mechanism for the effect of oxygen is not clear, it is possible that the oxygen positively affects MnP induction of *mnp* gene transcription (*15*). Earlier reports showed that supplying *Ph. chrysosporium* culture with pure oxygen and agitation enhanced lignin degradation 3-fold (*46*, *47*).

Agitation conditions have been thought to cause different effects on the ligninolytic activity in *Ph. chrysosporium* culture flasks and bioreactor (*7*, *24*, *48*, *49*). In a pneumatically agitated bioreactor, high activity of MnP and LiP were obtained, 1812 and 4500 U/L, respectively (*24*). However, a negative effect of agitation on enzyme production (175 U/L) was found in a stirred tank reactor, which might result due to the shear stress effect (*48*). The effect of the low (120 rpm) and high (300 rpm) agitation with purging or continuous aeration on the *Ph. chrysosporium* PCS biofilm culture showed no significant difference in MnP and LiP enzymes production. For example, the P_1V_3 1, P_3V_3 5, and C_3V_0 11 demonstrated MnP activities of 45.0, 47.0, and 47.0 U/L, respectively, and LiP activities of

19.0, 27.0, and 30.0 U/L, respectively. *Ph. chrysosporium* ligninase productions in stationary or agitated culture with limited nitrogen medium were enhanced to reach a maximum on day 6 (*23*, *44*, *48*, *50*). However, 300 rpm agitation with addition of veratryl alcohol and manganese (Mn^{2+}) on day 0 and with continuous aeration (C_3VM_0 14) stimulated the best production of MnP and LiP on day 3, which reached 63.0 and 25.0 U/L, respectively (**Figure 2a**), whereas both enzymes decreased significantly on day 6 to 25.0 and 1.0 U/L, respectively (**Figure 2b**). Thus, these results suggest that these conditions stimulated some apparent extracelluar protease activity.

In conclusion, all different batch conditions in this study enhanced *Ph. chrysosporium* MnP and LiP enzymes synthesis. Through the present study, C_3V_0 11 and C_3V_3 8 are considered as good choices for the production of both enzymes, MnP activity of 47.3 and 49.3 U/L and LiP activity of 30.0 and 27.7 U/L, respectively. Furthermore, the conditions of C_3VM_0 14 (developing PCS biofilm, continuous aeration, 300 rpm agitation, and addition of 3 mM veratryl alcohol and manganese (Mn^{2+}) to the medium on day 0) were shown to be effective for the early production of MnP. These culture conditions are thought to be effective for the scale-up of this culturing technique. Decreasing the fermentation time can result in significant cost savings to the industry; therefore, the conditions of C_3VM_0 14 (**Figure 2**) could be considered the best choice to reduce fermentation time and to enhance MnP production on day 3. Moreover, the result of C_3VM_0 14 suggests that these conditions will be effective for scale-up production by the continuous culturing technique. Thus, the decline of LiP activity observed after day 6 in *Ph. chrysosporium* culture was found to be correlated with the appearance of idiophasic extracellular protease activity, but the daily addition of glucose started on day 6 resulted in low protease levels causing stable LiP activity (*51*).

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