Fungal Growth and Manganese Peroxidase Production in a Deep Tray Solid-State Bioreactor, and In Vitro Decolorization of Poly R-478 by MnP

Xinshan Zhao, Xianjun Huang, Juntao Yao, Yue Zhou, and Rong Jia*

College of Life Science, Anhui University, Hefei 230601, P.R. China

The growth of Irpex lacteus F17 and manganese peroxidase (MnP) production in a self-designed tray bioreactor, operating in solid-state conditions at a laboratory scale, were studied. The bioreactor was divided into three layers by three perforated trays. Agro-industrial residues were used both as the carrier of bound mycelia and as a nutrient medium for the growth of I. lacteus F17. The maximum biomass production in the bioreactor was detected at 60 h of fermentation, which was consistent with the CO₂ releasing rate by the fungus. During the stationary phase of fungal growth, the maximum MnP activity was observed, reaching 950 U/l at 84 h. Scanning electron microscopy images clearly showed the growth situation of mycelia on the support matrix. Furthermore, the MnP produced by I. lacteus F17 in the bioreactor was isolated and purified, and the internal peptide sequences were also identified with mass spectrometry. The optimal activity of the enzyme was detected at pH 7 and 25°C, with a long half-life time of 9 days. In addition, the MnP exhibited significant stability within a broad pH range of 4–7 and at temperature up to 55°C. Besides this, the MnP showed the ability to decolorize the polymeric model dye Poly R-478 in vitro.

Keywords: Irpex lacteus F17, tray bioreactor, solid-state fermentation, manganese peroxidase, polymeric dye Poly R-478, decolorization

Introduction

White-rot fungi produce extracellular ligninolytic enzymes, such as manganese peroxidase (MnP, E.C. 1.11.1.13), lignin peroxidase (LiP, E.C. 1.11.1.14), and laccase (Lac, E.C. 1.10.3.2) that can efficiently degrade lignin as well as many recalcitrant organic pollutants. Among these enzymes, MnP is one of the most frequently used for the transformation of environmental pollutants, such as synthetic dye decolorization as well as polycyclic aromatic hydrocarbons and tetracycline degradation [7, 11, 33]. MnP is a glycosylated heme-containing enzyme and can catalyze the oxidation of Mn²⁺ to Mn³⁺ in a H₂O₂-dependent reaction. Mn³⁺ is stabilized by organic acid, and the Mn³⁺-acid complex acting as the active redox mediator can attack phenolic lignin structures, resulting in the formation of unstable free radicals that tend to disintegrate spontaneously. A large number of white-rot fungi, including Phanerochaete chrysosporium, Trametes versicolor, Pleurotus ostreatus, Bjerkandera sp. BOS55, and others, have been reported to produce MnP [10].

Owing to the potential applications of MnP in treating environmental pollutants, there is general interest in producing the enzyme biotechnologically. However, large-scale enzyme production from white-rot fungi is limited mainly owing to the lack of suitable bioreactor configurations for fungal growth and their metabolism [27]. Although several studies on the development of innovative bioreactor systems have already been carried out [7, 25, 32], an ideal bioreactor for ligninolytic enzyme production from white-rot fungi has not yet been shown [6, 8]. Furthermore, the high production cost is another barrier to the commercialization of these enzymes.

Solid-state fermentation (SSF), which is carried out without the use of free-flowing water, is the most economic process
for ligninolytic enzyme production owing to the use of agro-industrial residues as the nutrient medium for white-rot fungi. This solid matrix can also be employed as a natural support for the development of filamentous fungi in SSF. In addition, these agro-industrial residues are rich in lignin or cellulose, which are inducers for ligninolytic enzyme production, thus stimulating much higher enzyme production in SSF processes. In fact, SSF has become an alternative large-scale production system for enzymes and secondary metabolites [9, 30].

*I. lacteus* F17 (CCTCC AF 2014020) — a local white-rot basidiomycete isolated by our laboratory — has been found to be a producer of MnP. The fungus *I. lacteus* has been reported to be very efficient in degrading a wide variety of recalcitrant organic pollutants, including various types of dyes and polycyclic aromatic hydrocarbons (PAH) [2, 13, 19, 26, 28]. MnP proved to be the main enzyme during the decolorization and PAH degradation by *I. lacteus* [2, 13, 29]. These features suggest this organism to be a good candidate for further applications at a larger scale and make it an interesting objective to scale up ligninolytic enzymes production.

In the present study, a laboratory-scale bioreactor was designed to examine the feasibility of utilizing agro-industrial residues as the nutrient and support for the growth of the white-rot fungus *I. lacteus* F17, as well as for scale-up of MnP production. Furthermore, the MnP produced by *I. lacteus* F17 in the bioreactor was purified and partially characterized to gain more information on its stability for application. Finally, the ability of the MnP to degrade aromatic pollutants was also assessed by monitoring the decolorization of the polymeric model dye Poly R-478.

**Materials and Methods**

**Microorganism and Growth Medium**

*I. lacteus* F17 was isolated from a decayed wood chip pile in the vicinity of Hefei, China, and was stored in the China Center for Type Culture Collection (CCTCC AF 2014020). The fungus was cultured on potato dextrose agar (PDA) slants for 7 days at 28°C and then preserved at 4°C. The PDA medium contains 200 g/l of potato extract, 20 g/l of glucose, and 20 g/l of agar.

**Solid-State Fermentation**

SSF substrate preparation was based on results of our previous research. Dried, ground, and sifted pine sawdust, rice straw, and soybean powder mixed at the ratio of 0.52:0.15:0.33 in weight were used both as an attachment place and to supply nutrients to the *I. lacteus* F17.

Prior to use, these solid materials were mixed and autoclaved at 121°C for 20 min. The grown mycelial mat collected from several slants was washed with sterile water and homogenized by using a Waring blender. Then, 500 ml flasks containing 150 ml of liquid medium (potato extract 200 g/l, glucose 20 g/l, KH₂PO₄ 3.0 g/l, and MgSO₄·7H₂O 1.5 g/l) were inoculated with 20 ml of the mycelial suspension and incubated at 28°C on a rotary shaker at 120 rpm for 3 days for the formation of mycelial pellets. The homogenized mycelia obtained by crushing the mycelial pellets in a Waring blender were inoculated into a 7.23 L bioreactor containing 300 g of solid substrate and 300 ml of water (the water holding capacity was 1 ml/g (1 ml water per gram dry medium)). Fermentations were performed at 28°C for 7 days.

**Bioreactor Configuration and Operating Conditions**

The designed tray bioreactor used in this study contains a cylindrical glass vessel with 16.0 cm diameter, 36.0 cm height, and 7 L working volume (Fig. 1). The outer frame of the bioreactor and the air-intake casing were made of glass. The bioreactor was vertically divided into three layers: top (10 cm height), middle (10 cm height), and bottom (6 cm height).

![Fig. 1](image-url)
Biomass Analysis
In the present study, the biomass produced during SSF was quantitatively analyzed each day by measuring the nucleic acids present in the fermentation medium [20]. Samples of the medium (0.4 g) from each layer were collected every 24 h, except that the first sample was taken at 12 h after the start of SSF. After pulverizing the sample in a white porcelain mortar, 25 ml of 5% trichloroacetic acid was added and the mixture was constantly stirred for 25 min at 80°C. Subsequently, the mixture was cooled in an external ice bath. After centrifugation of the mixture (for 15 min at 5,653 × g and 4°C), the absorbance of the supernatant at 260 nm was measured, and the medium without inoculation through the same treating process was used as the blank control. Different amounts of mycelia were weighted precisely and dealt with the method as mentioned above. The linear relationship between the weight of mycelia and the absorbance of the supernatant at 260 nm was calculated. The biomass was expressed as grams per gram matrix. Furthermore, CO2 emission during SSF was also tested by carrying out acid–base titration at every 12 h to analyze the fungal growth condition. All the experiments were performed in triplicate, and the results were expressed as the mean values. Deviation values are standard deviations based on triplicate determinations.

Scanning Electron Microscopy
The status of I. lacteus F17 mycelium growth in the tray bioreactor was observed by scanning electron microscopy (SEM). The mycelium samples collected from the middle layer of the bioreactor at different fermentation periods were fixed in 0.1 M phosphate buffer (pH 7.4) with 5% (v/v) glutaraldehyde for 2 h at 4°C. Then, the samples were dehydrated sequentially using 20%, 40%, 60%, 80%, and 100% ethanol [10]. The samples were coated with gold before examination under a scanning electron microscope (Hitachi S-4800 SEM, Japan). The substrate without fungus was considered as the blank. All the images were taken at a magnification of 500×.

Enzyme Activity Assays
To obtain enzyme extracts, 5 ml/g dry matrix of 10 mM sodium acetate buffer (pH 4.5) was added to the sample in an Erlenmeyer flask, and the flask was incubated on a rotary shaker at 120 rpm for 30 min. Then, the supernatants were used for enzyme activity assays.

The MnP activity in the supernatant was measured with Mn2+ in 0.11 M sodium lactate buffer. Oxidation of Mn2+ to Mn3+ was determined by the increase in absorbance at 240 nm [14]. One unit (U) of MnP activity was defined as the oxidation of 1 µmol of Mn3+ per minute. The enzyme activities were expressed in U/L.

The lignin peroxidase (LiP) assay was based on the oxidation of veratryl alcohol to veratraldehyde, determined by the increase in absorbance at 310 nm [12]. One unit (U) of LiP activity was defined as the oxidation of 1 µmol of veratryl alcohol per minute.

The lactase (Lac) assay was based on the oxidation of ABTS, determined by the increase in absorbance at 420 nm [35]. One unit (U) of Lac activity was defined as the oxidation of 1 µmol of ABTS per minute. All the experiments were performed in triplicate, and all the enzyme activity assays were carried out at 25°C.

Enzyme Purification
In order to investigate the enzyme production by I. lacteus F17 during SSF in the bioreactor, enzyme activities were analyzed daily. The enzyme extracts containing the highest MnP activity were concentrated by ammonium sulfate (70%) and dialyzed against 10 mM of sodium acetate buffer (pH 4.5). After this, the crude enzyme was loaded onto a DEAE-cellulose column (Whatman DE52, England) (2.6 × 30 cm), which had been equilibrated with 10 mM of sodium acetate buffer (pH 4.5) at 4°C. Elution of the proteins was achieved with a flow rate of 100 ml/h by establishing a linear gradient first with 10 mM of sodium acetate buffer (pH 4.5) and then with 0–0.5 M of NaCl at 4°C. Fractions with MnP activity were pooled, concentrated by a Stirred Ultrafiltration Cell Model 8010 system (10 kDa cut-off polyethersulfone membranes; Millipore Corporation, USA) to approx. 1 ml, and then loaded to a Sephadex G-75 gel filtration column (Fluka, USA) (1.7 × 100 cm). Proteins from this column were eluted with 10 mM of sodium acetate buffer (pH 4.5) at a flow rate of 20 ml/h and temperature of 4°C. The peak containing MnP was collected, concentrated, and then applied to FPLC (FPLC with Superdex 75 10/300 column (Pharmacia)), which was previously equilibrated with 10 mM of sodium acetate buffer (pH 4.5). Elution of proteins from this column was detected by continuous recordings of absorbance at 280 and 406 nm. The enzyme solution was stored at
4°C for the characterization and decolorization experiments.

**Enzyme Characterization**

**Enzyme electrophoresis and mass spectrometry.** Electrophoresis on polyacrylamide gel in denatured conditions (SDS-PAGE) was carried out using a 10% Tris/HCl separation gel and 3% Tris/HCl stacking gel. After SDS-PAGE, the gels were stained with Coomassie Brilliant Blue R-250. The protein was excised from the gel and digested with trypsin. Then the resulting peptide mixture was analyzed by LC/MS (liquid chromatography–mass spectrometry) with a mass spectrometer, ProteomeX-LTQ (ThermoFisher Scientific, USA). All complete or partial sequences of this protein were searched against those of the proteins from BLAST similarity search to obtain the identifications with higher confidence.

**Determination of Kinetic Parameters of MnP**

The K_m and k_cat values of MnP were calculated by the hyperbolic non-linear least-square method, using MnSO_4 as the substrate at varying concentrations (0.025–0.2 mM). The reactions were carried out in 0.11 M of sodium lactate buffer (pH 4.5) with 0.1 mM of H_2O_2.

**Effects of Temperature and pH on MnP Activity and Stability**

Determination of the optimum temperature for MnP activity was carried out in 0.11 M of sodium lactate buffer over temperatures from 15°C to 65°C at pH 4.5. To evaluate the thermal stability of the purified MnP, the enzyme was incubated at different temperatures ranging from 25°C to 65°C for 1–3 days and aliquots of samples were removed at every 24 h for analysis.

The effect of pH on MnP was determined at 25°C using citrate–phosphate buffer with a pH range of 2.2–8. The pH stability was assayed by incubating the enzyme at different pH values (pH 3–7, citrate–phosphate buffer) at 25°C for 0–35 h. The remaining activities were measured using the enzyme activity assays mentioned above.

**In Vitro Decolorization of Poly R-478 by MnP**

Dye decolorization was measured spectrophotometrically at 520 nm (WFV UV-2100 Spectrophotometer; Shanghai, China), which is the maximum visible absorbance of the dye Poly R-478.

The reaction mixture contained citrate-phosphate buffer (62.8 mM), MnSO_4 (1 mM), H_2O_2 (0.2 mM), Poly R-478 dye (0.001%), and 200 µl of the purified MnP or 100 µl of crude MnP in a total volume of 1 ml. The MnP activity employed was 900 U/l. The decolorization of Poly R-478 represents the percentage decrease in the peak of absorbance at 520 nm. Control samples, without enzyme or H_2O_2, were done in parallel under identical conditions. The optimal temperature and pH conditions for dye decolorization were carried out in vitro with purified MnP and crude enzyme preparations.

The decolorization efficiency was calculated according to the following Eq. (1):

\[
\text{Decolorization (\%)} = \frac{A_0 - A_t}{A_0} \times 100\%
\]  

where A_0 is the initial absorbance at 520 nm and A_t refers to the absorbance at 520 nm at reaction time t. The data were the mean values of triplicates.

**Results**

**Production of Biomass in the Deep Tray Bioreactor**

In SSF, the mycelium cannot be directly quantified because of its strong adherence to the solid matrix; hence, indirect methods such as analyses of ATP, enzymatic activity, respiration rate, nutrient consumption, and cell constituents are employed to evaluate the biomass. Nucleic acids evolution could be used as an indirect measurement of the growth of the white-rot fungus. The biomass production profile of *I. lacteus* F17 in the tray bioreactor is depicted in Fig. 2A. In SSF using agricultural waste as the substrate, the biomass of the fungus increased rapidly in the bioreactor at the beginning of fermentation, peaked at 60 h, and then reached a plateau until 156 h. The maximum biomass production at the top, middle, and bottom layers of the bioreactor was 0.25, 0.23, and 0.24 g per gram matrix, respectively. The rate of CO_2 released by *I. lacteus* F17 under SSF is shown in Fig. 2A, which indicated the metabolic activity of the fungus in the tray bioreactor. The release rate rapidly increased for 36 h, reached to the highest value at 60 h, and then reduced significantly. When compared with biomass production, the fungus grew rapidly by consuming carbon sources at the beginning of the 60 h fermentation, leading to high rate of CO_2 release. After 60 h, the biomass production remained almost constant and the CO_2 release rate decreased, indicating that the respiratory metabolism of fungus was becoming increasingly stressed.

**Production of MnP in the Deep Tray Bioreactor**

Fig. 2B presents the MnP production by *I. lacteus* F17 during SSF in tray bioreactor. MnP appeared after 36 h of fermentation, and then started to increase rapidly to a high level. The activity of MnP was approx 950 U/l, with maximum activity observed at the bottom layer of the bed at 84 h, along with LiP and Lac activities of about 31 U/l and 17 U/l, respectively. After that, the MnP activity decreased drastically until the end of cultivation. When compared with the biomass, the MnP activity started to increase and gradually reached the maximum value while the biomass remained...
stable, indicating the production of a secondary metabolite by the fungus when its growth rate was zero.

**SEM Observation**

The morphology of *I. lacteus* F17 grown on support matrix was examined by SEM. Fig. 3 shows the SEM images of the mycelium growth status after 0, 24, 48, and 60 h of SSF. The mycelia could be easily observed on the surface of the medium mixed with pine sawdust, rice straw, and soybean powder. They were densely entangled in the support matrix after 60 h of SSF (Fig. 3D). These results were consistent with the data regarding *I. lacteus* F17 biomass production in the bioreactor, indicating that the local tray bioreactor provided an appropriate growth environment for the fungus.

**Fermentation Kinetics Model Fitting**

A logistic model can be used as the mathematical model to reveal the fungal growth condition [7, 36], which can be defined as follows:

$$N_t = \frac{N_m}{1 + (N_m/N_0 - 1) \exp^{-\mu t}}$$  \hspace{1cm} (2)

The Luedeking–Piret equation has also been used for the kinetic analysis of extracellular enzyme production. According to this model, the product formation rate depends on both the instantaneous biomass concentration $N$ and the growth rate $dN/dt$:

$$\frac{dP}{dt} = \frac{\alpha N}{\beta N + \mu}$$  \hspace{1cm} (3)

By substituting Eq. (2) into Eq. (3) and integrating it, the following equation could be obtained:

$$P_t = N_0 \left[ \frac{\alpha}{(N_m/N_0 - 1) \exp^{-\mu t} + \frac{\alpha}{N_m/N_0} \exp^{-\mu t}} \right] \left[ 1 + \frac{\exp^{-\mu t}}{N_m/N_0} \right]$$  \hspace{1cm} (4)

where $N_t$ is the biomass synthesized during the time
interval considered (g per gram matrix), $N_0$ is the initial biomass of the inoculum (g per gram matrix), $N_m$ is the maximum biomass during fermentation (g per gram matrix), $\mu$ is the maximum specific growth rate (h$^{-1}$), $t$ is the differential time interval (h), $\alpha$ is the growth-associated product formation, and $\beta$ is the non-growth-associated product formation coefficient and other variables.

The average biomass and MnP production evaluated at the three layers can be used as a parameter to measure the ability of the entire bioreactor to enhance fungal growth and MnP production. Eqs. (2) and (4) were solved by a data processing software, Origion 8.0. Through the nonlinear least-squares fitting technique, the program provided the result as the output. The fitted equation can be given as follows:

$$N_t = \frac{0.23575}{1 + 3.37869\exp^{-0.06681t}} \quad (5)$$

$$P_t = \frac{0.23575\left[\frac{-4569.29145}{3.37869\exp^{0.06681t}} + 1043.52993}{1 + 1737.20581\ln\left(1 + \exp^{0.06681t}\right)\right]} \quad 12h \leq t \leq 84h \quad (6)$$

The Adj. $R^2$ of the nonlinear regression of the biomass and MnP production was 0.95 and 0.96, respectively, indicating that Eq. (5) can be used to determine the relationship between production of biomass and time of fermentation. Eq. (6) represents the relationship between MnP production and time of fermentation.

### Purification and Identification of MnP

The MnP was isolated and purified for further examination of its enzyme properties. Crude enzyme was extracted from SSF culture of I. lacteus F17 in the tray bioreactor at 84 h, corresponding to the maximum MnP activity, and concentrated by ammonium sulfate precipitation. Fractionation of MnP was performed by DEAE-cellulose anion-exchange chromatography, followed by Sephadex G-75 gel filtration chromatography. SDS-PAGE (Fig. 4) revealed that the molecular mass of the purified MnP was about 43.2 kDa, which agreed well with the molecular mass range of the MnP family.

Four peptides with $m/z$ of 689.33, 979.52, 1,054.53, and 1,625.74 corresponding to the amino acid sequences VACPDGK, PNIVAAAPDK, LQSDHDLAR, and TACEWQSVNNQAK, were obtained by LC-MS. Then, they were searched against a nonredundant protein database using BLASTp in NCBI. Table 1 shows the homology of the internal sequences of I. lacteus F17 MnP with those of representative MnPs. The amino acid sequences of four peptides showed low levels of identity with MnP from P. chrysosporium (57%, 30%, 67%, and 71%, respectively). It also showed relatively high levels of identity with MnPs from P. ostreatus and T. versicolor, ranging from 42% to 100%. Besides this, the amino acid sequences of these four peptides were in full accordance with those of the MnP of I. lacteus F17 (AGO86670.2), which was predicted by the full-length cDNA sequence obtained by our laboratory. Namely, the purified MnP in this work may be the deduced MnP (AGO86670.2) or its isozymes from I. lacteus F17.

### Kinetic Parameters of the Purified MnP

The kinetic constants of the purified MnP were determined based on typical Michaelis–Menten behavior. The $K_m$ and $k_{cat}$ values of MnP for Mn$^{2+}$ were found to be 80 $\mu$mol/l and 273.9 s$^{-1}$, respectively. Furthermore, the catalytic efficiency (specificity constant, $k_{cat}/K_m$) of the purified enzyme was $3.4 \times 10^6$ mol$^{-1}$ l s$^{-1}$.

### Effects of Temperature and pH on the Activity and Stability of Purified MnP

As illustrated in Fig. 5A, the purified MnP showed its maximal activity at 25°C, and had a relatively high activity at 25–55°C. Furthermore, the enzyme was stable at low and moderate temperatures, maintaining more than 90% of its maximal activity after incubation at temperature ranging from 25°C to 45°C for 24 h. However, a rapid loss of stability occurred at temperatures higher than 55°C. The half-lives of MnP at pH 4.5 were 19, 18, 18, and 5 days, and
2.1 h at 25°C, 35°C, 45°C, 55°C, and 65°C, respectively.

In addition, the effect of varying the pH on the activity of the purified MnP was also examined. Fig. 5B shows the influence of pH in the range of 2.2-8 on MnP activity. The enzyme was stable in the pH range of 4-7, exhibited an optimum pH of about 7, and retained more than 90% of the maximal activity after 24 h incubation at 25°C in citrate–phosphate buffer with different pH values. In addition, the

**Table 1.** Sequence homology for internal peptides of *I. lacteus* F17 MnP by NCBI BLAST short sequence search.

<table>
<thead>
<tr>
<th>[M+H]^+</th>
<th>Amino acid sequences</th>
<th>Similar sequences in the database of the NCBI (Accession No.)</th>
<th>Identity (%)</th>
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<td>71</td>
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</table>

AGO86670.2: MnP of *I. lacteus*; BAA33009.2: MnP of *Pleurotus ostreatus*; CAA83148.1: MnP of *Trametes versicolor*; AAA33744.1: MnP of *Phanerochaete chrysosporium*.

**Fig. 5.** Effects of temperature and pH on the activity and stability of purified MnP from *I. lacteus* F17.

(A) The optimum temperature and thermostability of purified MnP. For thermal stability, the enzyme was incubated for 24 h at various temperatures. The residual MnP activity was assayed under standard assay conditions (maximum activity = 100%). (B) The optimum pH and pH stability of purified MnP. For pH stability, the purified MnP was incubated for 24 h at 25°C in various pHs in citrate–phosphate buffer. The residual MnP activity was assayed under standard assay conditions (maximum activity=100%). Deviation values are standard deviations based on triplicate determinations.
half-lives of MnP at 25°C were 3, 14, 28, 28, and 9 days at pH 3, 4, 5, 6, and 7, respectively. Therefore, the purified MnP exhibited great stability in these conditions of the temperature ranging from 25°C to 55°C and the pH range of 3–7.

**In Vitro Decolorization of Poly R-478**

Poly R-478 is a charged, sulfonated, anthraquinone polymeric dye, which has been shown to be useful for the screening of fungi to degrade lignin. This dye was found to be one of the recalcitrant organic compounds and could remain colored for a long time [18]. Thus, it was chosen to examine the degrading capability of both crude and purified MnP.

The optimal temperature and pH for crude MnP to decolorize Poly R-478 was first evaluated (Fig. 6A). Efficient decolorization was only observed at pH 4.0 and decreased rapidly at other values. Thus, for practical application, an acidic pH system is required for the enzymatic decolorization. The optimal temperature for decolorization was around 45°C. However, the effective range of temperature was broader compared with pH. Furthermore, the effects of temperature and pH on decolorization efficiency by the purified MnP were evaluated, and optimum values of 35°C and 4.0 were detected (Fig. 6B). The corresponding maximum decolorization percentages were 21% for crude enzyme and 14% for purified enzyme after 1 h of reaction (Fig. 6). Then, the decolorization of Poly R-487 was enhanced slowly along with the reaction time extension. The decolorization for prolonged times is in accordance with the thermostability of this enzyme.

**Discussion**

For the cultivation of *I. lacteus* F 17 on a solid substrate, a cylindrical static bed bioreactor, aerated from the two sides with moist air, was set up in this study for batch SSF at a laboratory scale (see Fig. 1). The design of this deep tray bioreactor combined ideas of both the solid-state tray reactor and packed bed reactor to overcome some problems, such as the requirement of large areas and the occurrence of a dry bed, as well as mycelium damage. Three perforated trays in the bioreactor formed a thin layer, allowing good attachment of the fungus to the support composed of agro-industrial residues, as well as facilitating appropriate oxygen and nutrient diffusion. The support material for the attachment and growth of *I. lacteus* F 17 was a mixture of pine sawdust, rice straw, and soybean powder. After 60 h of incubation, the support was completely penetrated and its surface was covered with a layer of fungal hyphae (see Fig. 3D). The major components of rice straw are cellulose and hemicelluloses, which are macromolecules composed of different sugar monomers. Pine sawdust is not only a carbon source but also an important inducer for ligninolytic enzymes production by white-rot fungi. Thus, the support...
material used in this study is not only a good immobilization matrix with very low cost but also provides all the nutrients needed for fungal growth and enzyme production. Taken together, the main advantage of this bioreactor is the reduction of MnP production costs as well as the ability to mimic the natural environment for rapid mycelium growth. In addition, this bioreactor is simple and has less stringent control of operative conditions.

Moilanen et al. [16] reported that the maximum MnP activity (340 nkat/g DM) occurred after 14 days of fermentation in a solid-state reactor with an internal conveyor, but their MnP activity stayed at almost the same level at 100 g-scale in plastic jars and 4 kg-scale in bioreactor cultivation. A similar study was carried out by Vivekanand et al. [32] using a bioreactor that contained four circular aluminum trays, and the maximum MnP activity (1,339.0 ± 131.23 U/l) was obtained after 6 days of SSF by Aspergillus fumigatus VkJ2.4.5. Phanerochaete chrysosporium BKM-F-1767 had also produced 1,293 U/l of MnP activity, in a fixed-bed bioreactor on the 7th day [17]. Compared with these reports, 950 U/l of MnP activity was achieved after 3.5 days of SSF in our bioreactor, indicating a higher MnP activity obtained from our bioreactor in a relatively short time. In addition, the MnP activity was 1.68-fold higher than that observed in flask cultures under the same conditions. In fact, the tray bioreactors have been proposed as an appropriate type of bioreactor for enzyme production through solid-state fermentation [31, 24]. Couto et al. [4, 22] demonstrated that the tray bioreactor is an ideal configuration for the production of laccase by Trametes versicolor and Trametes hirsuta under solid-state conditions when compared with other types of configurations. Rosales et al. [23] focused on obtaining high laccase activity levels in T. hirsuta in a 1.8 L static tray bioreactor under solid-state conditions, and they exhibited a very high laccase activity of around 12,000 U/l. From these research results, it can be indicated that the tray bioreactor is a successful scale-up for the production of enzymes from filamentous fungi under SSF conditions and it is economical and accessible.

Nucleic acid, ergosterol, and glucosamine have been used to evaluate the biomass of fungal Aspergillus terreus in SSF [34]. Nucleic acid proved to be a good biomass indicator because its content changed only with mycelial growth during the cultivation period, whereas the amount of ergosterol and glucosamine could not accurately represent the biomass changes. In the present study, the nucleic acid content of the mycelium was chosen as the indicator to estimate the biomass of I. lacteus F17 during the cultivation period in the deep tray bioreactor. Furthermore, CO₂ measurements also provided valuable information about the progress of mycelium growth and metabolism on the solid medium tested in the bioreactor. The profile of CO₂ emission is shown in Fig. 2A, which was noted to be in agreement with the amount of I. lacteus F17 biomass produced. During early growth, the nucleic acid content was higher and there was a strong increase in CO₂ production before 60 h of fermentation, indicating aerobic metabolism in the tray bioreactor. During the late growth phase, the nucleic acid content remained almost constant, and the MnP activity of I. lacteus F17 grown on the solid medium was high, while the CO₂ production decreased consistently through the end of fermentation, indicating that the digestion of carbon sources present in the growth medium gradually became more difficult and that secondary metabolism was initiated. The causes of deceleration of growth may be the accumulation of inhibitory metabolites in the medium, exhaustion of readily utilisable nutrients, or the onset of oxygen limitation. To analyze the bioreactor performance, logistic and Luedeking–Piret equations were applied to determine the different kinetic parameters related to fungal biomass production and MnP synthesis. The results calculated using the models were comparable with the experimental data, and the model equations could actually reflect the enzymatic fermentation process and kinetic mechanism.

The purified MnP showed an optimum activity at 25°C, which is slightly lower than that of MnP obtained from other organisms such as Pleurotus ostreatus (30°C) [1], I. lacteus (35°C) [26], and Lentinula edodes (40°C) [3]. Moreover, the enzyme was quite stable up to 55°C, and did not lose activity for at least 24 h. Furthermore, it is worth noting that this MnP had stronger half-lives within a broad temperature range of 25–55°C among MnPs reported so far [3, 15, 28]. In fact, good thermostability is a desirable feature of an enzyme for enzymatic industrial applications. The purified MnP obtained in the present study was stable in the pH range of 3–7, with an optimum pH of 7 at 25°C, whereas most of the MnPs reported previously exhibited maximum activity at an acidic pH ranging from 4.5 to 5.0 [3, 21, 28].

In the present study, Poly R-478 was selected as the lignin model compound, whose strong structure is especially difficult to degrade through most of the available technologies [5]. The effects of temperature and pH on the decolorization of Poly R-478 by both crude and purified MnP produced from I. lacteus F17 were analyzed. It was noticed that the
optimal temperature for crude and purified MnP to decolorize was 45°C and 35°C, respectively, greatly different from the optimum temperature of 25°C for the MnP activity. It is probably attributed to the strong thermal stability of the MnP at a broad temperature range of 25–45°C, and the enzyme retained more than 90% of its original activity for at least 3 days, and exhibited a half-life of 18 days at 45°C. However, the crude enzyme lost its ability to decolorize with further increases of the reaction temperature to 55°C. The purified MnP was effective for decolorization of the polymeric dye, but the decolorization rate was a little low when compared with the crude MnP (Fig. 6), even though the concentration of the purified MnP was 2-fold higher than that of the crude enzyme. From a practical point of view, crude enzyme is preferred owing to its low cost. Therefore, the use of crude enzyme for in vitro decolorization may be a viable technology for the degradation of some recalcitrant organic compounds. Furthermore, the decolorization of Poly R-478 was found to be strictly dependent on the pH of the reaction system, and the optimum pH for the decolorization by both types of MnP from I. lacteus F17 was detected at pH 4. The acidic optimum pH in dye oxidation by MnP has already been reported and was suggested to be due to the increased redox potential of the oxidized heme at low pH [15].

A systematic investigation on the growth of I. lacteus F17 and MnP production in the designed bioreactor was presented, and MnP purification as well as its kinetic properties and application in decolorization of the polymeric model dye were reported in this study. The results indicated that the proposed bioreactor is an effective system for MnP production by I. lacteus F17 at laboratory scale. The experiments of in vitro decolorization of Poly R-478 by MnP indicate this enzymatic degrading ability. Thus, if the conditions permit the scale-up of enzyme production through an efficient production system without operational problems, the enzymatic biodegradation can be selected as a viable option for treatment of dye wastewater at an industrial scale. Therefore, further studies are needed to optimize the bioreactor configuration and environmental factors, as well as to further enhance the enzyme product yield.

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References


