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Effects of Pluronic F68 on Manganese Peroxidase Production by Pelletized *Phanerochaete chrysosporium*

Zhi-min Li · Yan Liu · Zhan-you Chi · Shu-lin Chen

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Abstract In this study, a new process was developed for manganese peroxidase (MnP) production by *Phanerochaete chrysosporium* under an agitated and aerated cultivation condition. It was found that change of the inoculum from spore suspension to pellets resulted in enhanced MnP production of 200 U/L in rotated shake flasks. Several additives, including Pluronic F68, Tween 80, and PEG8000, significantly increased the enzyme production. With an optimal concentration in 125 mL flasks, Pluronic F68 increased MnP productivity by 180%. Moreover, successful enzyme production was achieved in a 5-L fermentor at an agitation speed of 300 rpm with the addition of 0.1% Pluronic F68.

Keywords *Phanerochaete chrysosporium* · Pluronic F68 · Enzyme · Biomass · Submerged · Optimization

Introduction

Phanerochaete chrysosporium is one of the well-known filamentous fungi that can produce extracellular oxidative enzymes such as lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase (Lac) [1–3]. These enzymes are the prominent catalysts that have great potential in biodegradation of lignocellulosic materials [4, 5]. Additionally, they have wide applications in many other industrial and environmental fields such as fiber bleaching and bioremediation of organic pollutants [1, 6–8]. However, the low yield and productivity

Z.-m. Li
State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, 130 Meilong Road, Shanghai 200237, China

Y. Liu
Biosystems and Agricultural Engineering, Michigan State University, East Lansing, MI 48824, USA

Z.-y. Chi · S.-l. Chen (✉)
Department of Biological Systems Engineering, Washington State University, L.J. Smith 213, Pullman, WA 99164, USA
e-mail: chens@wsu.edu

limited their industrial implementation [9]. It has been reported that mechanical stress led to inactivation of LiP, and agitation seriously suppressed ligninolytic enzyme production [10, 11]. Additionally, biosynthesis of ligninolytic enzymes requires high oxygen tension, which needs strong agitation and more aeration.

Shear protective additives have been used to improve animal cell viability in gas sparging cultures [12]. The most widely used additives for shear protection include derivatized celluloses, derivatized starches, dextrans, pluronics, polyvinyl alcohols, polyvinyl pyrrolidone, polyethylene glycols (PEG), proteins, and serum [13]. Among them, Pluronic F68 has been used in large-scale mammalian, insect, and plant cell cultures to achieve great performance [14, 15]. The possible protective function of Pluronic F68 has been commonly believed as its ability to suppress the attachment of cells to bubbles and reduce the plasma membrane fluidity of cells [13]. In addition to the physical factors, Pluronic F68 has been known to produce physiological and biochemical effects in some cases [16].

Several processes have been studied to increase lignolytic enzymes production in submerged liquid culture. Venkatadri reported that detergents including Tween series (Tween 20, 40, 60, and 80) and cholamidopropyl dimethylammonio propanesulfonate were able to protect both purified ligninase and crude ligninase in a cell-free culture against mechanical inactivation [11]. Grgic and Perdih demonstrated that addition of PEG and other polyoxyalkanes stimulated ligninase production [17]. Oleic acid and some phospholipids had a similar effect [18–20]. However, there was few research of MnP improvement by these additives. Addition of Pluronic F68 to fungal culture and ligninolytic enzyme production has not been reported. In this study, Pluronic F68 was hypothesized as an effective shear protective additive for improving MnP productivity in an agitated and aerated culture of *P. chrysosporium*. The effects of pellet inoculation, comparison of different additives including Pluronic F68, and concentration of Pluronic F68 on MnP production were investigated in flask cultures. The optimal conditions obtained from flask culture were tested in a 5-L fermentor as well.

Materials and Methods

Microorganism and Seed Culture

P. chrysosporium (ATCC 24725) was obtained from the American Type Culture Collection (Manassas, VA, USA). This strain was first cultured on solid potato dextrose agar medium to produce spores at 30 °C. After 7 days cultivation, the spores were washed from the agar with sterile distilled water and collected as a spore solution at a concentration of 1×10^9 spores/mL. One milliliter of spore solution was inoculated into 150 mL potato dextrose broth (PDB) medium with 5 g/L $CaCO_3$ to produce seed pellets. The cultures were performed at 30 °C for 24 h on a rotary shaker at 170 rpm.

Enzyme Production in Flask Culture

The production medium was similar to Kirk's chemically defined standard medium, but without addition of 2,2-dimethyl succinate, and substituted Tween 80 or Tween 20 with four additives, Pluronic F68, Tween 80, PEG 8000, and bovine serum albumin (BSA) [3]. Ten percent of the additive solutions were autoclaved and added at the beginning of fermentation. The concentrations of Pluronic F68, Tween 80, PEG 8000, and BSA were set at 0.1%, 0.05%,

0.1%, and 0.04%, respectively, according to literatures [17, 18]. The cultivations were carried out at different medium sizes with three replicates (50 mL in 125-mL Erlenmeyer flask, 100 mL in 250-mL Erlenmeyer flask, and 200 mL in 500-mL Erlenmeyer flask) in a rotary shaker with agitation speed at180 rpm and temperature at 37 °C. Ten percent *v*/*v* pellet seed was inoculated into each flask. To investigate the effect of adding time of Pluronic F68 on MnP production, four conditions were studied, which included flasks A, B, C, and D. In flask A, Pluronic F68 was added to 0.05% at 48 h. In flask B, Pluronic F68 was added to 0.05% at the beginning of the culture. In flask C, Pluronic F68 was added twice, once at the beginning of fermentation to the concentration of 0.05% and another time to 0.1% at 24 h. For flask D, Pluronic F68 was added into seed cultivation about 0.05%, except of the similar additive addition to flask B.

Enzyme Production in a 5-L Fermentor

The fermentation in a 5-L bioreactor (New Brunswick Scientific Co.) was carried out with a working volume of 3 L operated at 37 °C, 300 rpm, and an aeration rate of 0.5 vvm. The medium composition was the same as above and with 0.1% Pluronic F68 addition.

Analysis

MnP activity was determined by a modified method developed by Ha [21]. Culture filtrate/enzyme solution (100 μL) was added into 1 mL of sodium tartrate buffer (0.2 M, pH 4.5) containing 0.2 mM $MnSO_4$ and 8 mM guaiacol, and the mixture was incubated at room temperature (22 °C). Light absorbance values at 465 nm were determined using a Spectrascan UV 2700 (Thermo Scientific Co., USA). One unit of MnP activity is defined as the amount of enzyme to convert 1 μmol guaiacol per minute under these assay conditions.

An Olympus microphotograph (Tokyo, Japan) was used to observe the pellet morphology and measure the size of the pellets. The pH value was measured with a Fisher portable pH meter (Fisher Scientific, USA). Dry biomass was determined by first washing the pellet mycelia with 6 M HCl to neutralize excess $CaCO_3$ attached in the pellets, and then washing the pellets twice with distilled water. The washed biomass was dried in an oven at 105 °C overnight before weight analysis.

Glucose was analyzed using enzymatic assay kit (Megazyme International Ireland Ltd., Ireland).

Results

MnP Production Using Pelletized *P. chrysosporium* in Chemical Defined Medium

In the PDB medium with 0.5% $CaCO_3$, *P. chrysosporium* formed small pellets with a uniform diameter of about 0.45–0.60 mm after 24 h cultivation (Fig. 1). The average biomass in seed culture was over 2.00 g/L, the number of pellets was 205/mL, and the final pH was 6.90 after seed cultivation. Glucose in PDB was consumed very little, as the residue glucose in seed was still over 19 g/L. These pellets grew to larger size of 2–3 mm in the production medium, but no large clumps were formed. The maximum MnP activity about 200 U/L was achieved on the fourth day (Fig. 2). However, the large and irregular clumps appeared, and little MnP activity could be detected under the condition of spore inoculation (data not shown).

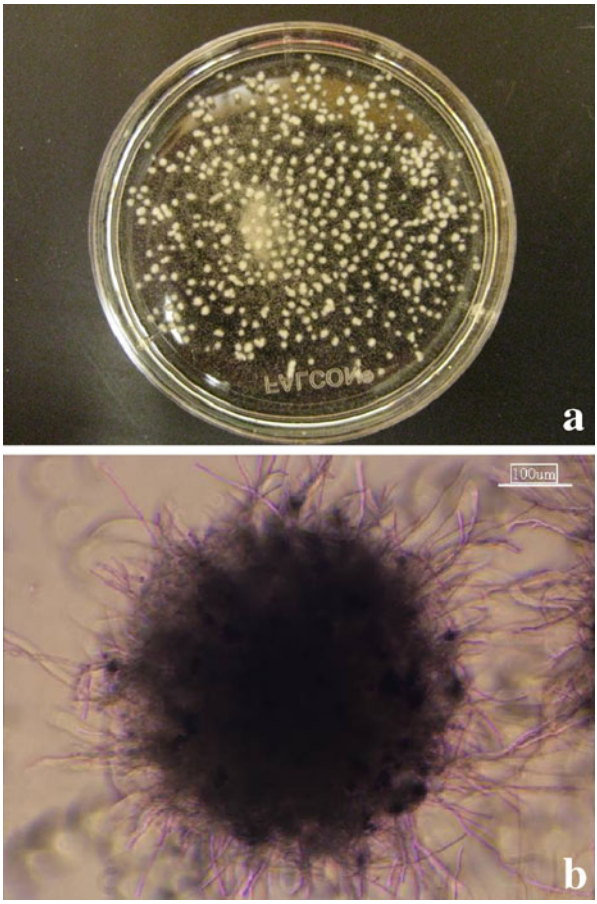

Fig. 1 Morphology of seed pellets cultivated at 24 h: (**a**) general morphology of inoculums; (**b**) microscope observation of pellet. *Bar*=100 μm

Effect of Pluronic F68 on Growth and Metabolism

Figure 3 illustrates the growth of pelletized *P. chrysosporium* with the addition of 0.05% Pluronic F68. A similar trend was observed in biomass, glucose, and pH between cultivation with and without Pluronic F68. The growth process can be divided into two phases. In phase

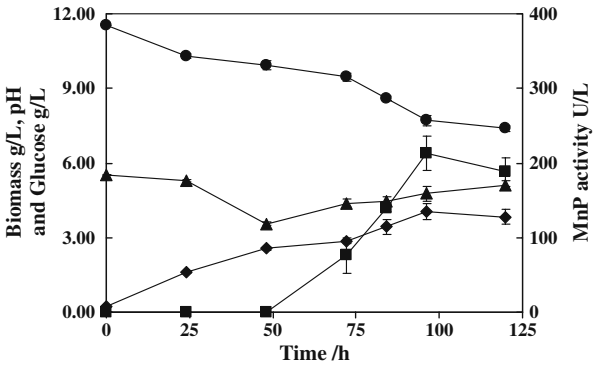

Fig. 2 MnP production with pellet inoculum in shake flask. Biomass (*diamonds*), pH (*triangles*), glucose concentration (*circles*), and MnP activity (*squares*)

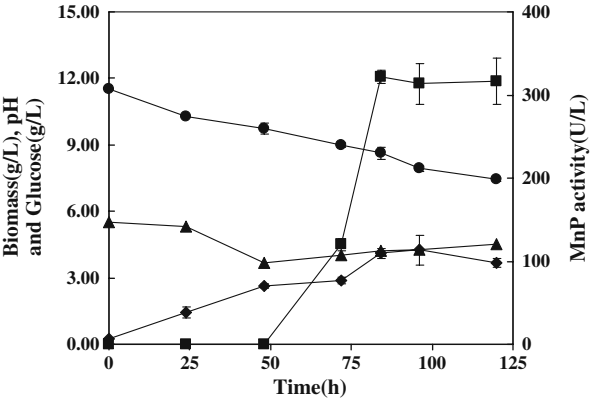

Fig. 3 The time course of biomass, pH, and glucose concentration with pellet inoculum and Pluronic F68 addition in shake flask. Biomass (*diamonds*), pH (*triangles*), glucose concentration (*circles*), and MnP activity (*squares*)

I (the first 48 h), glucose and pH declined, and biomass quickly increased. In phase II, pellets grew slowly, and pH began to rise. During phase I, biomass and glucose concentration and pH variation were nearly identical between the treatments with and without additives. Some small differences in glucose and pH appeared between these two cultures after 48 h. The pH was higher, and the biomass was less in the culture without Pluronic F68 than the results with Pluronic F68. As for MnP production, there was significant difference. Although enzyme activity was detected at 72 h for both cultivations, the enzymatic specific production rate with Pluronic F68 was much higher than that without Pluronic F68. Also, it took only 84 h to achieve maximum enzyme activity in the culture with presence of Pluronic F68, whereas 96 h was needed in the culture with absence of Pluronic F68.

Optimization of Addition Time and Pluronic F68 Concentration

The effect of adding time of Pluronic F68 on MnP production was conducted as well (Fig. 4). The maximum MnP activity in flask A was less than that in flask B, although it was similar in the first 72 h. In flask C, Pluronic F68 was added twice, which delayed

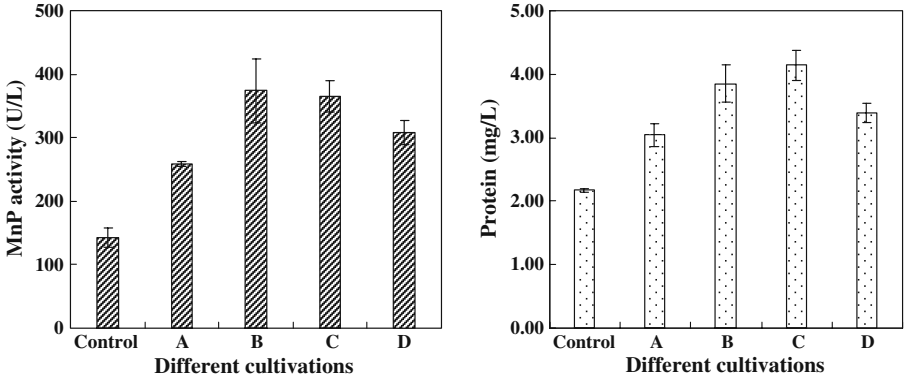

Fig. 4 Effects of adding time of Pluronic F68 on MnP production and protein: *Control*, without Pluronic F68 addition; *A*, Pluronic F68 was added to 0.05% at 48 h; *B*, Pluronic F68 was added to 0.05% at the beginning of fermentation; *C*, Pluronic F68 was added to 0.05% at the beginning of fermentation and again at 24 h; and *D*, Pluronic F68 was added in seed culture except of the addition at the beginning of fermentation

enzyme production slightly, whereas the maximum activity was similar to the activity in flask B. Also, the addition of Pluronic F68 in seed culture (flask D) had no more benefit to enzyme production even with an obvious decline, compared with the results in flasks B and C. However, the enzyme activity in flask D was higher than that in flask A, which indicated that the time of addition should be at the beginning of fermentation. The effect of Pluronic F68 concentration on enzyme production was shown in Fig. 5. The concentration of 0.05% improved extracellular MnP activity by 167%. With the increased concentration of Pluronic F68, MnP activity slightly decreased, but was still significantly higher than that in the control. However, the higher content of Pluronic F68 delayed MnP production, where the maximum activity with 0.3% Pluronic F68 was obtained at 111 h. All tested concentrations of Pluronic F68 showed no effect on cell growth and pellet formation. Results of extracellular protein (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) assay indicated that protein MnP was the major band. Similar protein bands could be observed from culture samples both with and without the additive. The results indicated that the treatment of 0.05% Pluronic F68 resulted in the highest enzyme activity and productivity.

Comparison of Different Shear Protective Additives on MnP Production

In all of these experiments, with or without additives, similar-sized pellets (2–3 mm) were formed, and the pellet biomass concentration reached 3.5–4.0 g/L. With the exception of BSA, all other additives significantly improved enzyme production (Table 1). The MnP activity reached its maximum at different times for different additives. Pluronic F68 presented the best performance among these additives, in which the MnP production was increased by 140%, whereas PEG 8000 and Tween 80 had an improvement of 80%. The maximum enzyme activity was detected at 87 h for both cultures supplemented with Pluronic F68 and control. The addition of PEG 8000 and Tween 80 delayed the peak time of enzyme production for 24 h. The addition of BSA decreased enzyme production more than 50% compared to that in control.

MnP Production in 5-L Fermentor

Although MnP activity could be detected in a 125-mL flask by pelleted *P. chrysosporium* without any additives, there were no detectable MnP in cultures using a 5-L stirred tank

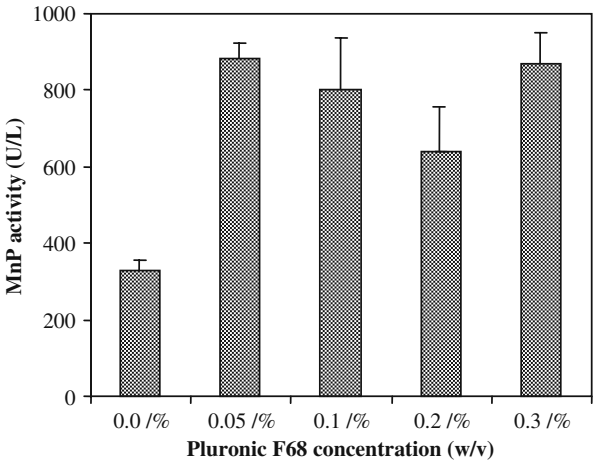

Fig. 5 Maximum MnP activity under different Pluronic F68 concentrations

Table 1 Effects of different additives on MnP production in agitated submerged culture

Additive	Concentration of additive, %	MnP activity, U/L	Hours of maximum activity
Control	0	331±25	87
Pluronic F68	0.1	803±133	87
PEG 8000	0.1	606±70	111
BSA	0.04	203±29	111
Tween 80	0.05	620±70	111

fermentor; even the pellet formation and cell growth were achieved. However, the use of shear protective additives made it possible to scale up from 125, 250, to 500 mL flasks and 5-L bioreactor. In both 250 and 500 mL flasks, the maximum MnP production was above 800 U/L at 80–90 h with 0.05% Pluronic F68 addition, which nearly doubled the production of the control. Further increase in Pluronic F68 supplement did not improve enzyme productivity compared with that of 0.05% of Pluronic F68 concentration.

Cell growth, glucose consumption, and pH decrease in the 5-L bioreactor culture had the same trend as cultures in the flasks at the initial 48 h (Fig. 6). There was a substantial difference between the culture in flasks and that in the 5-L reactor in terms of pH profile after 48 h cultivation. The pH increased slowly in the bioreactor compared to flask cultures. Only a small amount of glucose was consumed in both 5-L fermentor and flask cultures. The residual glucose concentration in both cultures was 7 g/L after 120 h cultivation. In terms of MnP production, the final MnP activity was low (320 U/L), and the initial production time was delayed about 12 h in fermentor, compared with that in 125 mL flasks (Figs. 3 and 6).

Discussion

Inoculum type and size have great effect on fungal morphology, growth, and metabolite production [22]. The results in this study clearly demonstrated benefits of pelletized biomass as fungal inoculum. Although there are many advantages for spore inoculum, such as convenient inoculum preparation and prolonged storability, longer lag time and larger

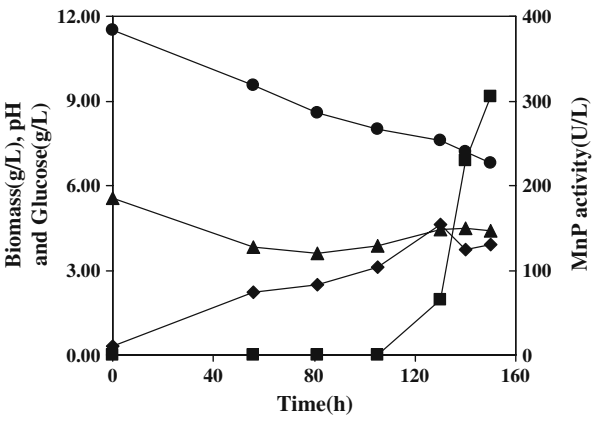

Fig. 6 The time course of *Phanerochaete chrysosporium* cultivation with pellet inoculum and Pluronic F68 addition in 5-L bioreactor. Biomass (*diamonds*), pH (*triangles*), glucose concentration (*circles*), and MnP activity (*squares*)

inoculum size are the major problems because the spores are metabolically dormant and need to be induced to vegetative growth [23]. Mycelial inoculum has been found to yield higher protein content and biomass formation in *Aspergillus niger*, but the blended mycelial inoculum still requires 5–7 days to achieve maximal MnP activity in a culture of *P. chrysosporium* [3, 22]. Mycelial pellet inoculum has more inoculum biomass and vegetative cells, which will retain metabolic robustness while shortening the lag phase. The initial pellet seeds also provide protection to the microorganisms from shear force. Once pellets are formed in seed culture, they can withstand mechanical stress and benefit from their own immobilization. Pellet growth stemming from cultivation creates totally different rheology in the environment and helps to achieve more biomass. The extracellular protease activity of fungal cultures has been studied and concluded to be related with inoculum type and pellet size and decreased dramatically when the morphology was changed from free mycelia to pellets. It was also discussed that pellet formation could induce glucoamylase formation and direct cell metabolism towards the specific metabolite biosynthesis to minimize the production and release of protease in *A. niger* [22, 24]. Therefore, the change of inoculum type from spore or filamentous mycelium to pellet had positive effects on MnP production, which is consistent with the results from some enzyme production by fungi [25].

Pluronic F68 is typically added to cell culture media at 0.5–3 g/L. The protective effect of Pluronic F68 on animal cell cultures is concentration dependent, increasing with concentration but leveling off at 0.05% [12]. This is also the case in its application to MnP production. When the additive concentration was over 0.05%, there was no additional activity improvement. Adding Pluronic F68 in the later phase of the culture had less effect than in initial phase, although it resulted in higher enzyme activity than the control. This phenomenon indicated that the shear force had effects on MnP production at the beginning of fermentation.

This study demonstrated that Pluronic F68 can be used as a shear protective additive to enhance MnP production by pelletized *P. chrysosporium* for the first time. Although Pluronic F68 has been added into animal and plant cell culture to avoid and minimize the shear stress, its protection on fungi pellet was not reported before. Applying 0.05–0.1% of Pluronic F68 could not only improve the MnP volumetric enzymatic activity significantly but also shorten the time to achieve maximum activity. It has been reported that the ligninase production in the culture supplemented with Tween 80 had the maximum activity at 5 days with the concentration of 0.01%, whereas it needed 5–6 days at a concentration of 0.05% and 6 days at 0.1% to reach the maximum activity. Higher concentrations of additives would delay LiP and MnP production [10, 11, 17]. A similar result was obtained in the current study of MnP production with the addition of Tween 80 and PEG 8000. However, production of MnP was not affected but rather stimulated at low concentration of Pluronic F68 such as 0.05%. This is the unique character of Pluronic F68, compared to other additives, although MnP formation was still significantly delayed at concentrations of 0.2% and 0.3%. As for the BSA's effect, decreased MnP production is probably caused by the responsive mechanism of nitrogen starvation in MnP production by *P. Chrysosporium*. It is obvious that BSA was taken into cells as a nitrogen source, and MnP formation was deferred.

There have been many reports that discussed the physiological mechanism of Pluronic F68 and its shear stress protection in plant/animal cell culture, but the exact mechanism has remained unclear and may be specific to cell type and metabolites. The possible mechanisms include (1) the ability to suppress the attachment of cells to bubbles by reducing surface viscosity, decreasing foam stability, and modifying the surface

hydrophobicity; (2) the ability to strengthen cells from incorporation of the surfactant into the cell membrane; and (3) the ability to improve nutritional transport because of a reduced cell-fluid interfacial tension. Although fungal mycelial pellet should be different from single animal or plant cells in resistance to shear damage, Pluronic F68 did improve MnP production in our study. Moreover, the ligninolytic enzymes have been reported to be located in the perioheral regions of the fungal cell cytoplasm in association with the cell membrane, fungal cell wall, and extracellular slime materials, which may indicate the original secret pathway of these enzymes. Additionally, the slime part was reported to be sensitive to shear force [26]. It was possible that Pluronic F68 has protected this whole pathway from shear stress via the similar mechanisms to single cell. According to the process development with or without Pluronic F68, low concentration of Pluronic F68 seemed to benefit the biomass and smooth the pH variation, which is better for MnP production because the optimal pH for production was 4.5 [3]. The pH variation could be associated with microbial metabolism, which could lead to the speculation that Pluronic F68 interfered with cellular metabolism. More extracellular proteins existed in the broth with Pluronic F68 addition, although the specific activity of MnP was still higher than that in fermentation without Pluronic F68. In addition, the specific activity of MnP with Pluronic F68 addition was not proportional to volumetric MnP activity, which illustrated that Pluronic F68 could facilitate releasing the produced enzyme into the extracellular environment (Fig. 4).

MnP can be generated with Pluronic F68 addition in the 5-L stirred tank reactor, even at agitation speed of 300 rpm. However, only half of the maximum activity in the flask occurred in the fermentor, and the initial production time was not the same as that in the flasks. The adverse effects from both mechanical agitation and sparging could have contributed to these results. MnP enzyme production depends on strain, cultivation condition, bioreactor, etc. In a culture with agitation and aeration, enzyme production was very little even with the addition of Tween 80 and PEG. The maximum MnP activity was 120 U/L with PEG stimulation, but the LiP activity was 1,180 U/L, which was improved significantly. Meanwhile with Tween 20 or 80 addition, LiP production was shown to be stimulated, whereas its effect on MnP was not reported.

Pluronic F68 was successfully used to improve the MnP production in agitated and aerated cultures. Activity level of MnP obtained was over 600 U/L by pelleted *P. chrysosporium* and was increased further to 1,000 U/L with the addition of Pluronic F68 in the shaken flask culture. MnP production was also achieved in a 5-L fermentor, although the activity level was lower than that in flasks. However, there are some differences on production of MnP between different batches either in flask or reactor cultures which should be further studied.

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