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ENHANCEMENT OF LIGNIN PEROXIDASE AND MANGANESE PEROXIDASE PRODUCTION BY *HUMICOLA GRISEA* IN A TUBULAR AIR-LIFT FERMENTER

Anuradha B and Darah I*

Fermentation and Enzyme Technology Laboratory, School of Biological Science, Universiti Sains Malaysia, 11800 USM, Pulau Pinang, Malaysia

Abstrak: Penghasilan enzim lignin peroksidase (LiP) dan mangan peroksidase (MnP) oleh *Humicola grisea* di dalam fermenter angkut udara diselidiki. Penghasilan optimum parameter yang digunakan pada fermenter ini ialah kadar pengudaraan 1.0 vvm, 1.0% (b/i) glukosa, 10.5 mM $(NH_4)_2HPO_4$ dan kepekatan inokulum 1.0% (i/i; 6×10^6 spora/ml). Peningkatan sebanyak 52% dalam penghasilan LiP dan 49% dalam MnP diperoleh selepas semua parameter dioptimumkan.

Abstract: The production of lignin peroxidase (LiP) and manganese peroxidase (MnP) by *Humicola grisea* in a tubular air-lift fermenter were studied. The optimized production parameters used in the fermenter were aeration rate of 1.0 vvm, 1.0% (w/v) glucose, 10.5 mM $(NH_4)_2HPO_4$ and inoculum concentration of 1.0% (v/v; 6×10^6 spores/ml). The increment of about 52% of LiP and 49% of MnP productions were obtained after optimization of the parameters.

Keywords: Lignin Peroxidase, Manganese Peroxidase, *Humicola grisea*

INTRODUCTION

Lignin-degrading enzymes are enzymes that are extensively studied in lignin biodegradation. It is well known that lignin-degrading enzymes have several potential in industrial applications such as in biopulping (Hakala *et al.* 2005; Souza-Cruz *et al.* 2004), detoxification of recalcitrant organopollutants, decolourization of textile effluents (Selvam *et al.* 2003), and degrading of pulp and paper mill effluents (Anggelis *et al.* 2003; Sahoo & Gupta 2005). The main limitation in the development of these enzymes is its low production activity by microorganisms. This could be due to the fact that biosynthesis by microorganisms in different culture systems are highly influenced by the age and size of the inoculum, medium compositions, and physical cultural conditions. In fact, many contradictory findings on the growth characteristics of microorganisms in a submerged culture system were highlighted.

*Corresponding author: darah@usm.my

To increase the production of these enzymes, optimization through nutritional supplement, addition of detergents, inducers and also variation of cultural conditions have been carried out. In our previous study, we found that aeration and agitation were important parameters that determined the success of scaling up the fermentation for lignin-degrading enzymes production in submerged cultures. However, our finding also showed that vigorous agitation might inhibit the growth as well as enzymes production. This was due to the high shear force that occurred when high agitation was employed. Therefore to enhance the production of lignin peroxidase (LiP) and manganese peroxidase (MnP) by *Humicola grisea* in submerged culture, we proposed th usage of a tubular air-lift fermenter which does not employ mechanical agitation. Darah and Ibrahim (1998) used the tubular type air-lift fermenter for the cultivation of *Phanerochaete chrysosporium* and for the production of lignin-degrading enzymes. In fact, Trager *et al.* (1989) also employed the tower type air-lift fermenter for the cultivation of fungi to reduce the shear force. These types of fermenters are extremely powerful in maintaining the homogeneity of oxygen distribution without exerting physical stress on the growing cells.

In an air-lift fermenter, air that flow from sparger into the cultivation medium is soft without high shear force. The air flow pushes air bubbles towards the upper part of the medium where transmission force is created. This force enables solid particles such as mycelium pellets to float or be suspended in the medium. This environment is needed for cultivation of filamentous fungi such as *H. grisea*. Heck and Onken (1987) showed that solid particles at a concentration of 2800 kg/m^3 and a solid content of 30% (w/w) could be suspended in an air-lift bioreactor. In this paper, we described the results obtained from an air-lift fermenter that was used for the production of lignin-degrading enzymes by *H. grisea*. Some of the important parameters which are involved in the fermentation using an air-lift fermenter had been optimized and the results obtained have been evaluated for further scale-up processes of lignin-degrading enzyme production.

MATERIALS AND METHODS

Fungus and Inoculum Preparation

H. grisea (DSM 2641) was maintained on 5% malt extract agar slant and the incubation was performed at 37°C until sporulation (5 days). The cultures were stored at 4°C and were subcultured every 4 weeks. A volume of 4 ml of sterile distilled water was added to an agar slant and shaken. The resulting spore suspension at a concentration of 1×10^6 spores/ml was used as the inoculum.

Design of a Tubular Air-Lift Fermenter

The fermenter used was produced by the Glass Centre, School of Chemical Sciences, USM. It consists of a vertical cylindrical glass column (Fig. 1) with a working volume of 2L, height of 44 cm and diameter of 10 cm. The sparger used was a multi-hole type with a filtered sterile (Millipore membrane filter, 0.22 μm) air supply of 1.0 vvm, unless otherwise stated. Effluent gas was removed through

exit via headspace leading to a vent of 100 mm length. The sampling was assisted by internal pressure.

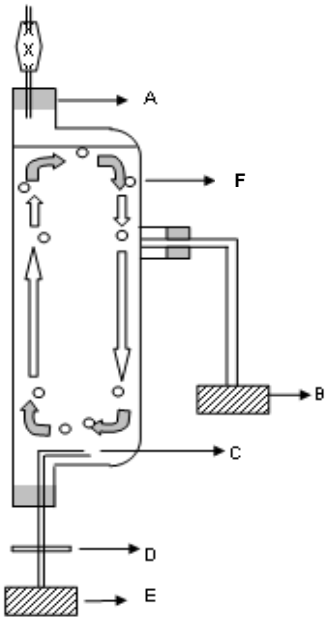

Figure 1: Schematic diagram of a tubular air-lift fermenter: A – gas exit (vent), B – sampling port, C – sparger, D – air filter, E – air pump, F – turbulent/direction of medium flow. The arrows indicate the flow of the medium.

Medium Compositions and Cultivation Conditions

The optimized cultivation medium of Tien and Kirk (1988) containing (g/l): 0.10 % (w/v) glucose, 0.23 g $(NH_4)_2HPO_4$, 10 mM veratryl alcohol, 0.2 g KH_2PO_4, 0.01 g $CaCl_2.2H_2O$, 0.05 g $MgSO_4.7H_2O$, 0.005 g $MnSO_4.7H_2O$, 0.001 g thiamine and 7 ml mineral solution [which contained (g/l): 3.0 g $MgSO_4.7H_2O$, 0.5 g $MnSO_4.7H_2O$, 1.0g NaCl, 1.0g $FeSO_4.7H_2O$, 0.001 g $Al.K(SO_4)_2.12H_2O$, 0.01 g H_3BO_3, 0.01 g $NaMoO_4$ and 1.5 g nitrilotriacetate] was used. About 0.25 mM of p-chloromercuribenzoic acid (pCMB) was added aseptically to the medium to prevent destruction of lignin-degrading enzymes by thiol proteinase, which was produced simultaneously into the medium (Anuradha 2002). The inoculum size was 1.0% (v/v) spore suspension containing 6×10^6 spores/ml. Cultivation was performed at room temperature (30 ± 2ºC) for a period of 10 days. The sampling was prepared every 24 hours. The LiP and MnP activities as well as the residual ammonium and glucose concentrations were determined.

Enzyme Extraction
The culture broths were filtered through Whatman No.1 filter paper. The culture filtrates were then used as the crude enzyme source.

Growth Determination
Fungal growth was determined based on the mycelial dry weight after drying at 65°C for 24 hours or until a constant dry mass (Darah & Ibrahim 1998).

Analyses
LiP activity was assayed using the method described previously by Tien and Kirk (1988). An aliquot of 1200 μl crude enzyme, 612 μl of 100 mM sodium tartrate buffer (pH 3.0) and 80 μl of 10 mM veratryl alcohol were added sequentially into a quartz cuvette. About 108 μl of 5 mM hydrogen peroxide were added to initiate the reaction. The total reaction volume was 2 ml. The increase in absorbance at 310 nm as a result of the oxidation of veratryl alcohol to veratryl aldehyde by LiP over 1 minute interval at room temperature was monitored using a spectrophotometer. An increase of 1.0 in absorbance corresponds the production of 179 nmols veratryl aldehyde formed from the oxidation of veratryl alcohol per minute under the assay conditions.

MnP activity was assayed using the method of Glenn *et al.* (1986). An aliquot of 100 μl crude enzyme, 400 μl of 0.25 mM sodium tartrate (pH 5.0), 80 μl of 1.25 mM vanillyacetone (in 50% aqueous dimethyl-formamide), 400 μl of 0.25 mM $MnSO_4$ and 20 μl of 5 mM hydrogen peroxide were added sequentially into a quartz cuvette. The results were corrected for manganese independent peroxides by repeating the assay without $MnSO_4$. A decrease of 1.0 absorbance at 336 nm corresponds the oxidation of 546 nmols vanillyacetone per ml crude enzyme. One unit of MnP activity was defined as the activity that produces 1μmol of product per minute under the assay conditions.

The residual ammonium concentration was determined with Nessler's reagent (Bonnarme *et al*. 1993) while glucose concentration was estimated by using the method of Somogyi (1952).

RESULTS AND DISCUSSION

The production of LiP and MnP by *H. grisea* has been studied extensively using a shake-flask system (Anuradha & Darah 2005). However, such a system has limitations which prevented the effective diffusion of substrate and oxygen, thus hindering further improvement of enzyme productivity. Therefore, the culture was shifted from a shake-flask system to a tubular air-lift fermenter, which was able to provide adequate oxygen supply and at the same time, reduced frictional stress, abrasion or shear force.

The fungal growth, LiP and MnP were determined in the course of the cultivation (Fig. 2). Aeration supplied to a fermenter plays a crucial role in fungal growth, therefore a suitable aeration rate is needed to enhance the fungal growth as well as the enzyme activities. It was found that the optimal aeration rate for maximum lignin-degrading enzymes production was 1.0 vvm with about 65.20

mU/ml LiP and 13.08 mU/ml MnP, and also with 17.30 g/l of fungal dry weight. At aeration rate of 0.5 vvm, the production was low. On the other hand, at high aeration rate excessive foaming was observed, resulting in poor growth and low enzyme production. An optimal aeration rate of 1.0 vvm was obtained which corresponded to maximum enzymes production and growth [Fig. 2(A)].

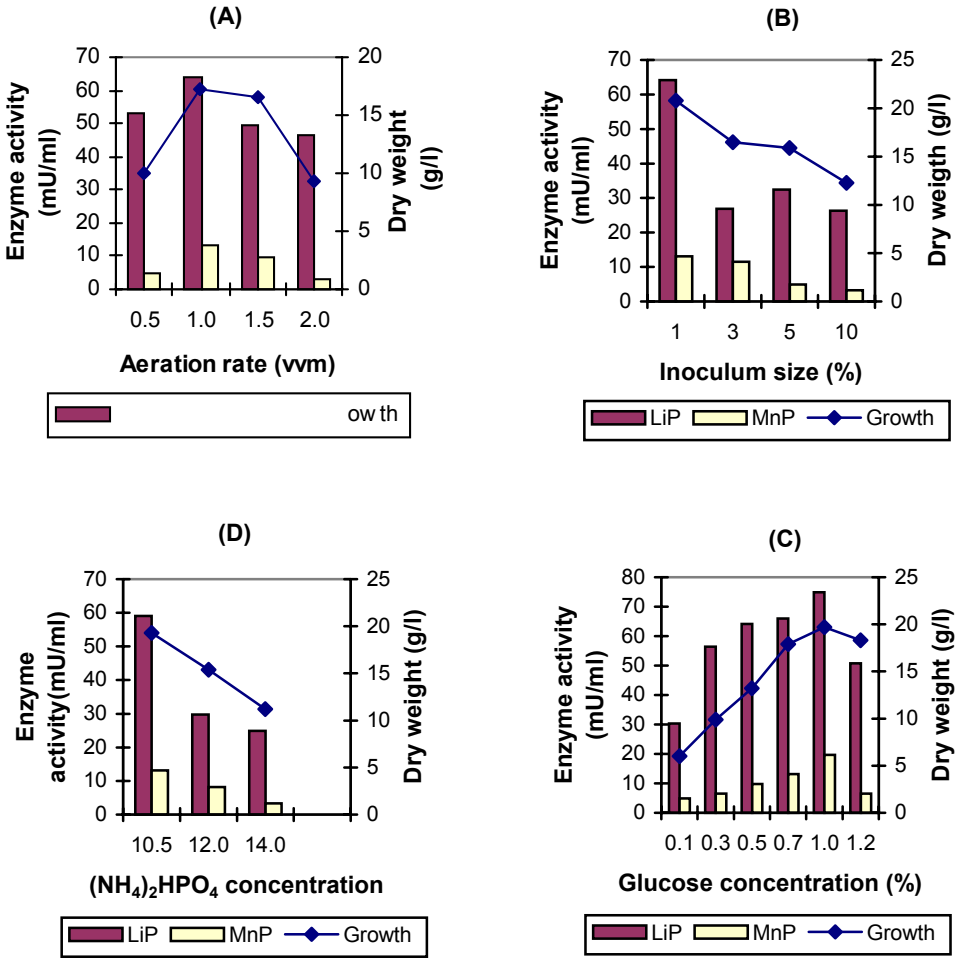

Figure 2: Effects of (A) aeration rate, (B) inoculum size, and (C) $(NH_4)_2HPO_4$ and (D) glucose concentrations, on the production of LiP and MnP by *H. grisea* in a tubular air-lift fermenter system.

The effect of various inoculum sizes on the enzyme production was determined. The fungal growth decreased significantly with the increase of inoculum size. The maximum growth of about 20.50 g/l, and LiP and MnP production of about 64.00 mU/ml and 13.08 mU/ml, respectively, were achieved

when 1.0% (v/v); 6×10^6 spores/ml was used [Fig. 2(B)]. The results revealed that higher inoculum size resulted in lower enzyme production. At high inoculum size, the growth was usually rapid, resulting in a higher rate of substrate and oxygen consumption rate, a phenomenon that was termed as "fast metabolism" (Darah & Ibrahim 1998).

Different concentrations of $(NH_4)_2HPO_4$ were studied to clarify if a higher concentration was needed for cultivation in air-lift fermenter compared to a shake-flask system. The results revealed that the optimal concentration of nitrogen source needed was 10.50 mM for maximum activities of LiP and MnP, which produced about 59.00 mU/ml and 13.08 mU/ml, respectively [Fig. 2(C)]. At a higher concentration, both LiP and MnP activities decreased about 50% from the maximum production. The best growth was also obtained at the same concentration. According to Kirk *et al*. (1978), metabolism of nitrogen source always compete with lignin metabolism and it was due to the demand of the same cofactor. In this condition, ammonium was not only consumed as a nitrogen source but also as a substance to maintain acid concentration level in the medium during a fermentation process (Stanbury & Whitaker 1984).

In the initial study, glucose concentration of 0.1% (w/v) was used to produce a maximum production of lignin-degrading enzyme in a shake-flask system. However, cultivation using an air-lift fermenter may need a higher concentration of glucose as a carbon source. Therefore, a range of different concentrations of glucose was studied. The results obtained showed that glucose concentration of 1.0% (w/v) was an optimal concentration for a maximum production of LiP and MnP in the air-lift fermenter with about 74.2 mU/ml and 19.7 mU/ml, respectively. The fungal growth was also maximum with 1.0% of glucose with dry weight of 19.7 g/l [Fig. 2(D)].

Detailed time course of LiP and MnP production in an air-lift fermenter before and after optimization is shown in Figure 3. Figure 3(A) shows the production of LiP before and after optimization, whereas Figure 3(B) shows the results of MnP before and after optimization. Both profiles exhibited almost the same pattern of production, however, the maximum production of LiP was achieved on day 4 of cultivation. There was about 52% increment obtained after optimization. In MnP production, there was a difference in the profiles of enzyme production. A maximum MnP production was achieved on day 5 of cultivation after optimization compared to day 4 before optimization. There was an increment of about 49% obtained after optimization. For both of the enzymes, their activities dropped or decreased after reaching the maximum production.

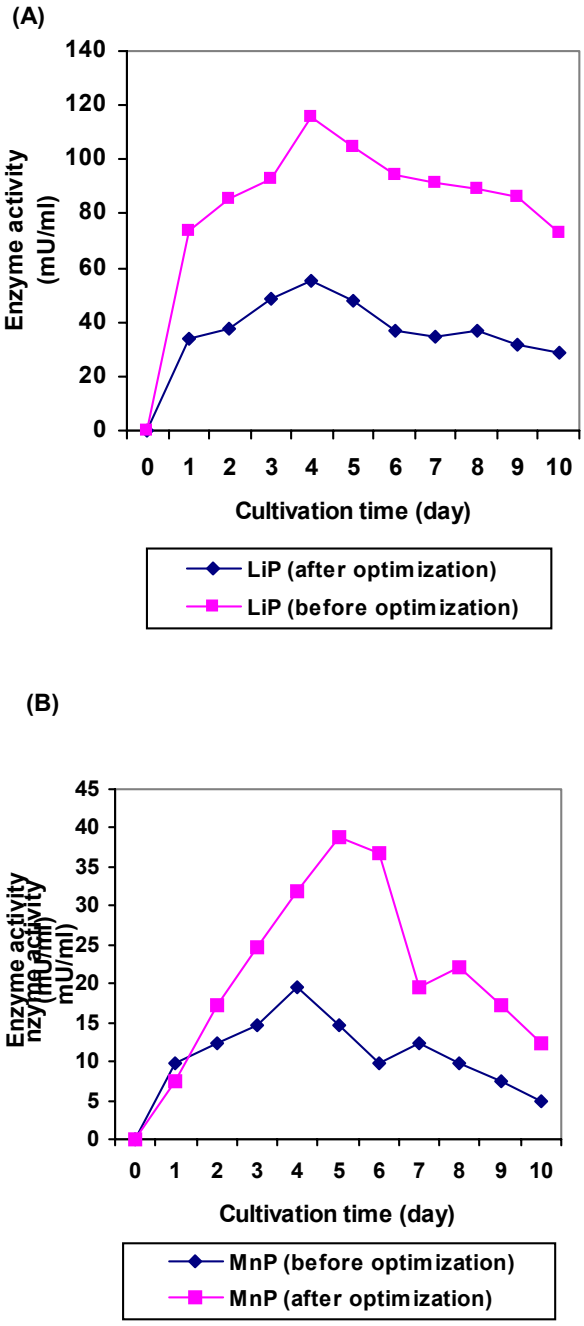

Figure 3: Time course of (A) LiP and (B) MnP production by *H. grisea* before and after optimization in a tubular air-lift fermenter.

The correlation between glucose (as a carbon source) and ammonium (as a nitrogen source) consumption by *H. grisea* in an air-lift fermenter are shown in Figure 4. If we compare Figures 3 and 4, there were significant correlation between the enzymes production, and the level of glucose and ammonium in the culture medium. As the LiP and MnP production increased, the glucose and ammonium concentrations decreased gradually. However, there was about 26% of residual glucose found in the growth medium after ninth day of cultivation. A fast depletion of ammonium concentration was obtained but it was almost constant for the last three days. The maximum growth obtained coincided with the consumption of glucose and ammonium by *H. grisea* (Fig. 5). Almost 50% of increment was obtained in fungal growth after optimization. Before optimization, the maximum growth was achieved on day 6 of cultivation with about 11.10 g/l of dry weight, whereas after optimization, the maximum growth was detected on day 5 with about 21.80 g/l of dry weight. Therefore, the production profile of LiP and MnP exhibited a close association with fungal growth, and coinciding with the depletion of glucose and ammonium concentration in the medium. Glenn *et al.* (1986) and Kirk *et al.* (1978) also found that the production of MnP and LiP were triggered by the depletion of glucose (a carbon source) and ammonium (a nitrogen source) in the cultivation medium and this condition usually coincided with the secondary phase of growth. Our findings demonstrated some behavior of *H. grisea* in a tubular air-lift fermenter. The suitable ratio of fungal growth and enzyme production were attributed to the mild conditions provided by the tubular air-lift system.

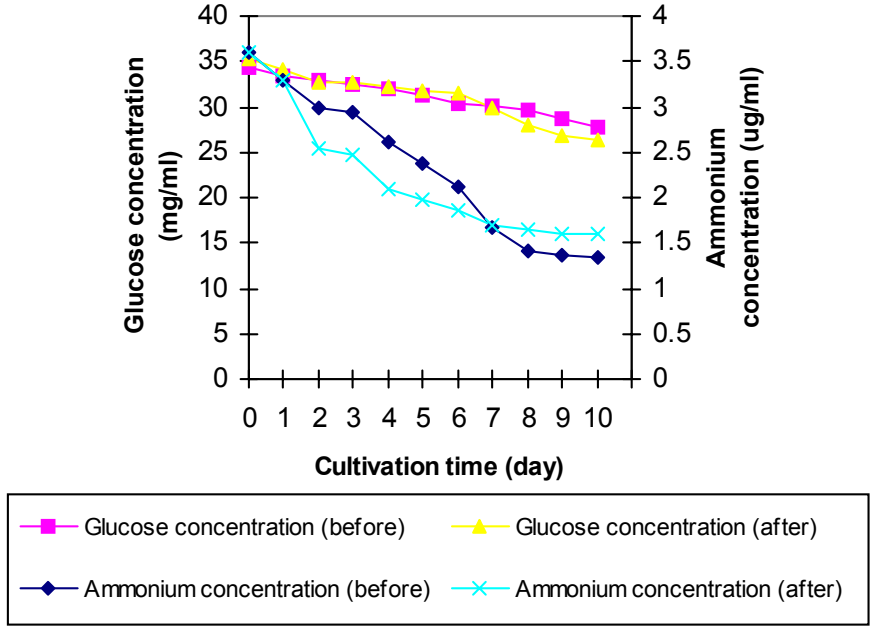

Figure 4: Time course of glucose and ammonium concentrations before and after optimization in a tubular air-lift fermenter.

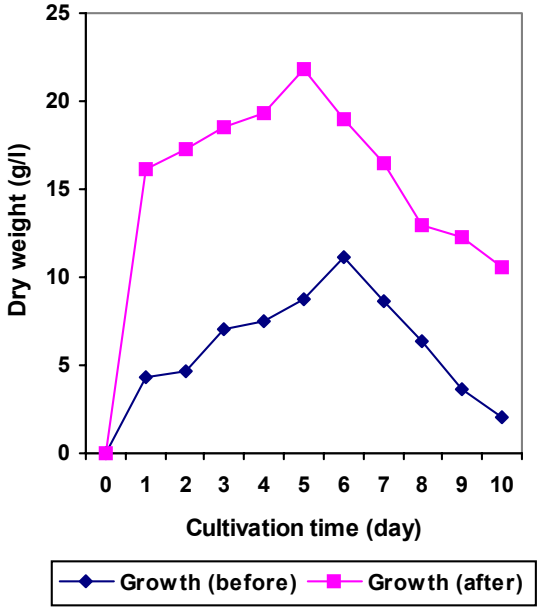

Figure 5: Time course of fungal growth of *H. grisea* before and after optimization in a tubular air-lift fermenter.

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