LARGE SCALE PRODUCTION OF LACCASE BY PLEUROTUS OSTREATUS IMI 395545 IN BIOREACTOR

ABSTRACT

In this chapter large scale laccase production from Pleurotus ostreatus IMI 395545 in a bench top bioreactor was studied. Basic culture parameters influencing the laccase synthesis in aerated bioreactor cultures were evaluated to improve the yield. In the bioreactor, the influences of temperature, stabilization of medium pH, effect of dissolved oxygen tension (DOT) percentage were examined in three levels. A marked enhancement in laccase production was observed when the optimized conditions were employed in the bioreactor. A total of 880±4.8 U/l of laccase was produced in 10 days. The yield was increased to 2 fold, when compared to the initial un-optimized culture conditions in the shaker flask (410±1.6 U/l). This enhancement in laccase yield may be due to the maintenance of controlled temperature at 30 °C, stabilized pH at 6.0 and 40 % DOT. The optimized culture condition in the bioreactor resulted in the reduction of the production time by 48 h. The study provides useful information to the industrialists seeking environmentally benign technology for the production of enzyme in large quantity.
3.1. INTRODUCTION

Laccases specifically are versatile and active in a broad range of substrates. The versatility of laccase allows the biocatalyst to be suitable for several processes such as biopulping, biobleaching, textile dye decoloration, treatment of industrial wastewater and a wide range of other applications. Indeed, they could become one of the most important biocatalysts in fungal biotechnology [Schauer and Borriss, 2004]. Its unique property of oxidizing substrates using molecular oxygen also makes it useful for the analysis of drugs and biomolecules of diagnostic importance [Bauer et al., 1999; Ferry and Leech, 2005]. The production of laccase by fungi is associated with secondary metabolism, the main drawback of which is limited yield by the enzyme under the growth limiting conditions [Moreira et al., 2000]. At present, research and application are limited by low yield of the enzyme in an active form [Jonsson et al., 1997]. The successful applications of laccase require large quantities of the enzyme. Various studies have demonstrated small-scale production of laccase in both shake-flask and solid-state fermentations [Hou et al., 2004; Stajic et al., 2010]. Large-scale production of the enzyme in bioreactors was also discussed by Couto and Herrera [2007]. However, the quantities of laccase reported in earlier studies are too low for industrial applications.

A fermentation system is a complicated multi-phase, multi-component system. Growth and production of the organism are affected by a wide range of parameters, including cultivation medium, inoculum, pH, temperature, aeration agitation, shear stress, etc. Compared to many unicellular microbes, filamentous fungi fermentation processes present special challenges in optimization and scale-up because of the varying fungal morphological forms [Wang et al., 2005].
The production of ligninolytic enzymes under bioreactor conditions has met with limited success for two main reasons (i) under agitated culture conditions, there is a loss in the production capability due to a change in the morphology of the fungus [Venkatadri and Irvine, 1990; Cui et al., 1998; Nakamura et al., 1999] (ii) the agitation may lead to mechanical inactivation of the enzyme of interest [Venkatadri and Irvine, 1990]. These two problems can be overcome by low shear bioreactors such as those employing airlift or with immobilized fungus [Miura et al., 1997; Nakamura et al., 1999].

The aim of this chapter was to investigate the influence of different parameters on growth of organism and the production of laccase in the bioreactor by Pleurotus ostreatus IMI 395545. The main parameters investigated for the large scale production are temperature, pH and dissolved oxygen tension.

3.2. MATERIALS AND METHODS

3.2.1. Chemicals

Antifoam B emulsion was purchased from Sigma-Aldrich, USA. All other chemicals used in this study were of analytical grade.

3.2.2. Inoculum preparation

The fungal inoculum was prepared as described previously in the chapter 2 and the suspension of fungal mycelium was aseptically transferred to the bioreactor.

3.2.3. Production medium

The composition of the medium used for the study was already optimized by Taguchi DOE methodology which supports a high laccase activity (previously discussed in the second chapter). The composition of the production medium per liter was as follows; glucose – 20 g; yeast extract – 5 g; malt extract –7 g;
mineral solution – 200 ml; inoculum – 5 ml; L-aspargine – 20 mg. Inducer (1 mM CuSO₄) was added to the medium aseptically after 168 h to avoid the potential toxicity of the compound to the organism. Composition of the mineral solution was as given in chapter 2.

### 3.2.4. Bioreactor

The bioreactor consists of ADI 1025 Bio Console and the process regulator, ADI 1010 Bio Controller was used to study the growth and production of laccase by *Pleurotus ostreatus* IMI 395545 (Figure 3.1). The total volume of bioreactor was 3.2 liters fitted with six-bladed turbine impeller which allows working volume of 2.7 liters. Bioreactor provides additional provisions like pH sensor, dissolved O₂ sensor, temperature sensor and foam sensor for controlling the fermentation parameters automatically.

### 3.2.5. Fermentation condition

Fermentation was carried out in a 3.2 liters bench top stirred tank reactor (ADI 1025 Bio Console; Applikon Biotechnology B.V. Netherlands). The reactor was autoclaved for 15 min at 121°C prior to inoculation and then inoculated with 10 ml of homogenized *Pleurotus ostreatus* IMI 395545 mycelial suspension. The DOT was monitored with a polarographic pO₂ electrode automatically at the desired level by changing the aeration rate. Antifoam B emulsion was added to break the foam during the operation. In the reactor, the pH was monitored with a pH electrode and controlled by automatic titration with 2N H₂SO₄ and 2N KOH. The reactor was operated in a batch mode and samples were aseptically removed every 24 h for analysis. Biomass was determined by weighing the mycelial dry weight at the end of the culture.
3.2.6. Conditions investigated

The physiological conditions and stress were mainly investigated for their effects on growth of the fungus and the productions of laccase were as follows: pH of the medium (5.5, 6.0 and 6.5), incubation temperature (25, 30 and 35 °C) and availability of oxygen (5, 20 and 40% DOT). Without maintaining any specific condition both laccase production and biomass were assayed with stirring the culture alone was considered as control.

3.2.7. Laccase assay

Laccase activity was determined using guaiacol as the substrate according to the method of Sandhu and Arora [1985]. Kindly refer the first chapter for details (1.2.6).

3.2.8. Determination of biomass

The mycelial biomass were harvested from the culture, it was washed with distilled water and pre-weighed in a sterilized beaker. Then the biomass was dried at 105 °C for 2 h and it was weighed in g [Haq and Daud, 1995].

3.3. RESULTS

The growth of the fungus and the production of enzyme were highly influenced by effective controlling the factors like pH, temperature and percentage of DOT provision available in the bioreactor (Figure 3.1). Two critical parameters (laccase activity and biomass) were decided to be studied at different time intervals during the growth and production of laccase by the Pleurotus ostreatus IMI 395545. Figure 3.2 shows the growth of organism in the production medium without maintaining any specific parameters and it was considered as control. The maximum
production of laccase and biomass were obtained on 12th day of the incubation (620±1.5 U/l and 17.5±2.1 g/l respectively).

Table 3.1 shows the effect of temperature in the production of laccase and biomass in bioreactor. Experimental data suggest that the increased production of laccase was obtained at 25 °C when the initial pH was 6.0. The maximum production of laccase (680±5.6 U/l) was obtained when the pH of the medium was not stabilized. The volumetric production rate ($Q_p^{a}$) of laccase was determined as 2.36 U/l/h. When the temperature was maintained at 30 °C both the yield of laccase and biomass were reduced to 640±6.3 U/l and 17.4±2.8 g/l respectively. The production of both biomass and laccase were further decreased, when the temperature was raised to 35 °C. Whereas maintaining 25 °C has more practical difficulties, hence further experiments were carried out at 30 °C. On the industrial scale, cooling is more expensive than heating; thus, the slightly lower enzyme production at 30 °C could be compensated by the lower energy and cost required for temperature control.

pH is one of the important factor, which play a major role in the production of laccase. Three levels of pH were studied by maintaining the temperature constantly at 30 °C (Table 3.2). The maximum production of laccase and biomass were attained at pH 6.0, when compared to pH 5.5 and 6.5 respectively. When the pH was stabilized at pH 6.0, the obtained laccase activity and biomass are 740±4.3 U/l and 20.4±3.6 g/l respectively.

To determine the effect of DOT percentage in the production of laccase and growth of Pleurotus ostreatus IMI 395545, three levels of DOT were selected and the results are given in the table 3.3. The highest laccase activity (860±6.3 U/l) was found at 40% of DOT percentage. At the same time yield of biomass was reduced to
16.4±2.3 g/l. When the DOT was reduced to 20% and 5%, the yield of the laccase also reduced to 720±5.4 and 640±4.7 U/l respectively.

To validate the influence of optimum temperature, pH and DOT in the laccase yield and biomass were determined under stabilized control condition. The obtained results show that, the above mentioned parameters play a major role in the production of laccase. At the end of 10th day, the production of laccase reached the maximum activity of 880±4.8 U/l (Figure 3.3), whereas in control cultures it requires 12 days for obtaining the maximum production (640±2.5 U/l).

3.4. DISCUSSION

The yield of extracellular enzymes like laccase is significantly influenced by physicochemical conditions. Figure 3.1 shows the growth of the *Pleurotus ostreatus* IMI 595545 in the bioreactor. In control culture (Figure 3.2) the production of laccase was proportionally increased with the fungal biomass.

The impact of temperature is more prominent in the scale-up processes; it remains an inevitable factor in all systems due to its impact on microbial growth and enzyme production. The optimal temperature of laccase production has been reported to differ greatly from one strain to another [Farnet *et al.*, 2000]. In general, fungi are cultivated at temperatures between 25 and 30 °C for optimal laccase production [Pointing, 2001; Arora *et al.*, 2002]. According to the table 3.1, the production of laccase was less at 30 °C than 25 °C, but the difference in the yield between the temperature ranges was less significant (6.2% reduction in laccase yield). Obtained results showed that, production of laccase at 30 °C was sufficient for scale up process because the yield of laccase was decreased, when the temperature increased to 35 °C. The above statement has good agreement with the report of Nyanhongo *et al.* [2002].
They have reported that when fungi were cultivated at temperature higher than 30 °C the activity of ligninolytic enzyme reduced remarkably. Optimum temperature for laccase production was 30 °C for *Trametes modesta* and *Cyathus bulleri* [Nyanhongo *et al.*, 2002; Vasdev *et al.*, 2005].

It has been already proved that there is high correlation between laccase production and change in the medium pH during cultivation. As per the table 3.2, the production of laccase was increased to 740±4.3 U/l in pH stabilized culture, when compared with production of laccase (410±1.6 U/l) in unstabilized pH condition. For the growth and secretion of laccase, strain needs stable pH 6.0. A pH regulatory system may be especially important. Apart from the regulatory effect on gene expression, cultivation pH can also affect fungal morphology greatly [Whitaker and Long, 1973]. Generally in all the cases, when the enzyme was detected in the medium, fungus alkalinized the medium. The experimental result shows that ligninolytic fungus secrete maximum enzyme, based on the environmental condition of the medium. It has been proved that, most of the fungal laccases reach their maximum activity, when the initial pH of the nutrient medium ranges from 4 to 6 [Galhaup *et al.*, 2002b; Jang *et al.*, 2002; Chen *et al.*, 2003].

Experimental results (Table 3.3) showed that the influence of DOT is linear; at 40% saturation of DOT increased laccase activity (860±6.3 U/l) was observed. Interestingly highest volumetric production rate of laccase activity (2.98 U/l/h) was observed when the DOT was maintained at 40 % in the bioreactor. From the obtained results it was confirmed that the high amount of DOT is essential for growth of the biomass and the production of laccase. Significant change in the laccase production and biomass were observed when the percentage of DOT was increased to 40 %. According to Hess *et al.* [2002] good oxygen transfer and low shear environment are suitable for laccase production by *Trametes multicolor*.
It is important to note that, when the percentage of DOT increased, the specific growth rate was also increased along with laccase production. The above results are highly correlated with Bai et al. [2004], who found that the enrichment of oxygen could result in a change in the respiratory pathway and thus affect protein synthesis. However, the fungal morphology altered simultaneously and the decreased intracellular protein content correlated with the shortened means of both the main hyphal length and total hyphal length, as well as with oxygen enrichment.

When the culture was carried out under optimized culture conditions in bioreactor, the production of laccase has reached maximum of 880±4.8 (10th day of culture) in shorter duration as shown in the figure 3.3. During the cultivation of Pleurotus ostreatus IMI 395545 in a 3-l bioreactor wall growth was minimal. Different process parameters e.g. stirrer speed, reactor type and medium composition are known to influence the growth and morphology of fungi and their product formation [Schugerl, 1997]. Whereas stirrer speed was generally not considered for the optimization process due to the following reason, fungal morphology to a large extent is affected by agitation in a stirred-tank bioreactor. Strong agitation will form free filaments. When pellets are formed, the pellet size, structure and survival are also affected by agitation [Wang et al., 2005]. This was evident from the different pellet sizes obtained in the reactors as well as the varying amounts of biomass and laccase activity produced by Pleurotus ostreatus IMI 395545 under different cultivation condition.

3.5. CONCLUSION

From the study it was found that, maintaining the stable pH 6.0 throughout the culture was most suitable for the growth and laccase production of Pleurotus ostreatus IMI 395545. It was found that, the highest growth rate was recorded, when the production was carried out in a bioreactor with the temperature at 30 °C, stabilized pH at 6.0 and 40 DOT% throughout the culture period. The study also shows that, an
appropriate combination of culture condition i.e., optimized carbon and nitrogen source with effective inducer, stabilized pH, temperature and DOT at the optimized level increase the laccase production within a short period of incubation. These results applied in a large scale bioreactor culture prove to be of an economic advantage for broad application of laccase.
Figure 3.1. Growth of *Pleurotus ostreatus* IMI 395545 in the ADI 1025 Bio Console bioreactor.
Figure 3.2. Production of *Pleurotus ostreatus* IMI 395545 laccase in the optimized medium in bioreactor. Results are the mean ±SD of triplicate experiments.

Figure 3.3. Production of *Pleurotus ostreatus* IMI 395545 laccase in the optimized medium under optimized culture conditions in bioreactor. Results are the mean ±SD of triplicate experiments.
Table 3.1. The influence of temperature on the laccase and biomass production by *Pleurotus ostreatus* IMI 395545

<table>
<thead>
<tr>
<th>S.No</th>
<th>Temperature °C</th>
<th>pH</th>
<th>Biomass (g/l)</th>
<th>Laccase (U/l)</th>
<th>$Q_p^a$ (U/l/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25</td>
<td>6.0*</td>
<td>18.2±3.2</td>
<td>680±5.6</td>
<td>2.36</td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>6.0*</td>
<td>17.4±2.8</td>
<td>640±6.3</td>
<td>2.22</td>
</tr>
<tr>
<td>3</td>
<td>35</td>
<td>6.0*</td>
<td>13.6±1.4</td>
<td>420±4.6</td>
<td>1.45</td>
</tr>
</tbody>
</table>

$Q_p^a$: Maximum volumetric laccase production rate, determined from the maximum slope of the plot of laccase activity versus time.

* Initial pH of the medium

Table 3.2. The influence of pH on the laccase and biomass production by *Pleurotus ostreatus* IMI 395545

<table>
<thead>
<tr>
<th>S.No</th>
<th>Temperature °C</th>
<th>pH</th>
<th>Biomass (g/l)</th>
<th>Laccase (U/l)</th>
<th>$Q_p^a$ (U/l/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>30</td>
<td>5.5</td>
<td>13.6±1.3</td>
<td>590±6.5</td>
<td>2.04</td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>6.0</td>
<td>20.4±3.6</td>
<td>740±4.3</td>
<td>2.56</td>
</tr>
<tr>
<td>3</td>
<td>30</td>
<td>6.5</td>
<td>10.6±1.2</td>
<td>420±5.4</td>
<td>1.45</td>
</tr>
<tr>
<td>4</td>
<td>30</td>
<td>pH NS*</td>
<td>17.4±2.7</td>
<td>630±5.6</td>
<td>2.22</td>
</tr>
</tbody>
</table>

# pH maintained throughout the culture

$Q_p^a$: Maximum volumetric laccase production rate, determined from the maximum slope of the plot of laccase activity versus time.

NS*: Not Stabilized
Table 3.3. Influence of different DOT levels on the laccase and biomass production by *Pleurotus ostreatus* IMI 395545 during batch cultivation in a 3 l bioreactor at 30 °C and pH 6.0 in optimized medium

<table>
<thead>
<tr>
<th>S.No</th>
<th>DOT % of Saturation</th>
<th>Biomass (g/l)</th>
<th>Laccase (U/l)</th>
<th>(Q_p^a) (U/l/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>40</td>
<td>16.4±2.3</td>
<td>860±6.3</td>
<td>2.98</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>14.5±1.8</td>
<td>720±5.4</td>
<td>2.50</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>14.0±1.5</td>
<td>640±4.7</td>
<td>2.22</td>
</tr>
</tbody>
</table>

DOT: Dissolved oxygen tension

\(Q_p^a\): Maximum volumetric laccase production rate, determined from the maximum slope of the plot of laccase activity versus time.