Optimization of process parameters for maximum laccase production by “one-factor-at-a time” approach

3.1 INTRODUCTION

Laccases are produced by wide spectrum of microorganisms. Among them fungal laccases gained more industrial demand due to their broad substrate specificity and wide reaction capabilities. *Pleurotus* has been extensively used for the production of laccase enzyme. Increasing the yield and reducing the cost of production are the major goals in current studies on laccase production.

Both the composition and concentration of fermentation medium significantly affect the growth of the organism and yield of enzyme. So there is a need for the optimization of culture conditions for the higher enzyme yield and consistent product quality during industrial fermentation process (Bozic *et al.*, 2011; Srivastava and Baruah, 1986; Rao and Satyanarayana, 2003). Each component of the media, especially the nutritional components such as carbon and nitrogen sources as well as the physicochemical conditions such as pH, temperature, incubation time and inoculum concentration play significant role in maximizing enzyme production (Oshoma *et al.*, 2010; Rezaei *et al.*, 2010).

The main objective of the fermentation industry is the large scale production of a particular product from an organism. This can be achieved either by modifying the organism or by customizing the production medium. This medium optimization can be achieved by varying the concentration or changing the medium components, which leads to the better growth of the organism. This involves investigation of different combinations of medium components and culture conditions for the better production of specific product of interest. This
optimization process is an expensive, time consuming, laborious and open ended process.

According to Kennedy and Krouse (1999) many techniques are available for process optimization, including both close-ended systems and open-ended systems. In close-ended system, a fixed number and type of parameters are analyzed whereas in an open-ended system, any number and type of parameters are analyzed for optimization of fermentation process. An ideal method will start with an open-ended system, select the best parameters for optimization of fermentation process and then move to the close-ended system (Panda et al., 2007).

The present study started with the use of burrowing technique, which is an open-ended system, for process optimization. In this the process parameters are obtained from the literatures on the basis of what other researchers used for same genus and species. This resulted in numerous options, so the factors were short listed on the basis of their significance in laccase production. Subsequently these factors were subjected to further optimization by “one-factor-at-a time” approach as well as statistical approach.

One-factor-at-a time is a close-ended system for medium optimization. It is a simple and classical approach for medium optimization. In this method the concentration of only a single independent variable is changed over a desired range at a time while keeping all the other variables at constant concentration (Ahamad et al., 2006; Alexeeva et al., 2002; Patidar et al., 2005). Due to its easiness to conduct the experiments, this approach has gained tremendous popularity and wide application (Bajpai et al., 1992; Poddar et al., 2012). Main advantage of this technique is that, the individual effects of bioprocess variables can be observed on a graph and there is no need for statistical analysis.
3.2 MATERIALS AND METHODS

3.2.1 Laccase production by *Pleurotus ostreatus* NCIM 1200 under solid state fermentation (SSF)

3.2.1.1 Microorganism

*Pleurotus ostreatus* strain NCIM 1200 obtained from National Collection of Industrial Microorganisms (NCIM), Pune, was used in this study. Stock cultures were maintained on Potato Dextrose Agar (PDA) plates and were sub cultured periodically. They were grown at 28 °C for one week and stored at 4 °C.

3.2.1.2 Culture medium

Potato dextrose agar medium obtained from HIMEDIA laboratories, India and prepared according to the manufacturer’s instruction was used throughout the study for the fungal cultivation.

3.2.1.3 Laccase production potential

The ability of *Pleurotus ostreatus* strain NCIM 1200 to produce laccase enzyme was confirmed by growing them on PDA plates containing 0.02% guaiacol (Coll *et al.*, 1993). The plates were inoculated with mycelial plugs and incubated at 30 °C for seven days. The plates were regularly checked for the presence of brick red colour around the mycelium indicating the presence of guaiacol oxidizing laccase enzyme.

3.2.1.4 Culture revival

The pure culture obtained from NCIM, Pune was subjected to culture revival through one round mushroom cultivation before the laboratory scale laccase production studies. For this the fungal spawn was formed using the mycelial plugs of *Pleurotus ostreatus*. *Sorghum vulgare* grains mixed with
calcium carbonate were used as the carrier medium for spawn production. Mushroom cultivation was carried out using these spawns in polypropylene bags. Pure cultures were freshly isolated from the newly formed fruiting bodies.

A well grown mushroom was selected from the mushroom bed and the cap (pileus) of the mushroom was surface sterilized with 70 % ethanol. A piece of mushroom (2 sq. cm size) was cut and surface sterilized using 0.5 % HgCl$_2$ for 30 sec followed by washing in sterile water 2-3 times to remove traces of HgCl$_2$. The edges were cut off and a piece of 1 sq. cm size was placed at the centre of a PDA plate under aseptic condition, incubated for 7-10 days at room temperature. These freshly isolated pure cultures were subjected to further studies on laccase production under solid state fermentation condition (SSF).

### 3.2.1.5 Substrate preparation

Pineapple leaves were selected as the substrate for SSF. Pineapple leaves were cut to 1 cm$^2$ size and autoclaved at 121 °C for 20 minutes. The moisture content was determined by drying the pineapple leaves to constant weight at 110°C in a hot air oven and was found to be 90%.

### 3.2.1.6 Inoculum preparation

Fungal mycelial plugs were used as the inoculum for solid state fermentation. Agar plugs (1cm$^2$ size cut from outer edges of the colonies in petriplate) were prepared by growing fungus on potato dextrose agar plates at room temperature (28± 2 °C) for five days.

### 3.2.1.7 Inoculation and incubation

250mL erlenmeyer flasks with measured quantity(5-25g) of autoclaved pineapple leaves were inoculated with required numbers of mycelial plugs of 1cm$^2$ cut from
actively growing fungal colonies on potato dextrose plate and incubated at room temperature (28± 2 ºC). After the desired period of incubation, the enzyme was extracted from the fermented biomass.

### 3.2.1.8 Extraction and recovery of enzyme

Recovery of enzymes from fermented matter is an important factor that affects the cost-effectiveness of the overall process. Laccase enzyme was extracted from the fermented biomass using 0.1 M citrate buffer of pH 5.0. 50 ml of ice cold citrate buffer was added to the fermented substrate, the contents of the flasks were mixed thoroughly by agitation at room temperature in a shaker for 15 minutes at 100 rpm. The crude extracts were passed through 0.22 µm filter (Millipore). The culture filtrate was harvested by centrifugation (Sigma-3K30) at 10,000 rpm for 10 min at 4 ºC. The clear supernatant was used as the crude enzyme sample for further studies.

### 3.2.2 Analytical methods

#### 3.2.2.1 Enzyme activity assays

Lignin peroxidase activity was assayed according to the method of Tien and Kirk (1983) in which H$_2$O$_2$ dependent oxidation of veratryl alcohol (3, 4-dimethoxybenzyl alcohol) to veratraldehyde was observed. The increase in absorbance was checked at 310 nm. The reaction mixture contained 250 µL of enzyme sample, 250 µL of 1mM veratryl alcohol, 500 µL of 0.1 M citrate buffer (pH 5.0) and 100 µL of 0.2 mM H$_2$O$_2$.

Manganese peroxidase activity was determined using phenol red as substrate by the method of Kuwahara et al (1984). The absorbance at 610 nm was checked. The reaction mixture contained 250 µL of enzyme sample, 50 µL of 0.1% phenol red, 100 µL of 250 mM sodium lactate, 25 µL of 2mM manganese
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sulphate, 100 µL of 0.5% BSA, 500 µL of 0.1 M citrate buffer (pH 5.0) and 25 µL of 0.2 mM H₂O₂.

Laccase activity was determined by the oxidation of ABTS (2, 2-azino-bis (3-ethylbenzthiazoline)-6-sulfonate) by the method of Papinutti et al (2003). The reaction mixture consists of 100 µL of 0.3 mM ABTS, 300 µL citrate buffer and 600 µL of enzyme sample. ABTS oxidation was calculated by monitoring the increase in absorbance at 420 nm (Shimadzu UV-1601, Japan).

Enzyme activity was expressed in international units (IU/mL). One international unit (IU) of enzyme activity can be defined as the amount of enzyme that oxidizes 1µM of substrate per minute under standard assay conditions.

All experiments were performed in triplicate and results are mean ± SD of triplicate experiments.

3.2.2.2 Protein estimation

Protein concentration of the sample was determined according to Lowry et al (1951) using bovine serum albumin (BSA) as standard and was expressed as mg/mL.

Reagent

(a) 0.2N sodium hydroxide
(b) 4% sodium carbonate
(c) 2% sodium potassium tartarate
(d) 1% cupric sulphate
(e) *Working reagent: 100 mL of working reagent was prepared by mixing solutions a, b, c and d in 49:49:1:1 proportions respectively.
(f) *1:1 Folin and Ciocalteau’s phenol reagent diluted with distilled water

* Freshly prepared before use


**Estimation**

1mL of freshly prepared working reagent (e) was added to 200 µL of the sample, mixed well and then 100 µL of reagent (f) was added and incubated for 30 min at room temperature followed by measuring the absorbance at 640 nm in a UV- Visible spectrophotometer (Shimadzu, Japan).

### 3.2.2.3 Specific activity

Specific activity of the enzyme sample was calculated by dividing the enzyme activity value with the protein concentration of same sample and it was expressed as IU/mg of protein.

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\text{Specific activity (IU / mg)} = \frac{\text{Enzyme activity (IU / mL)}}{\text{Protein content (mg / mL)}}
\]

The influence of various bioprocess variables on laccase production by *Pleurotus ostreatus* NCIM 1200 under solid state fermentation condition were optimized towards its maximum production. The strategy adopted for the enhanced production of laccase includes the combination of ‘one-factor-at-a-time’ approach and statistical approach involving Plackett-Burman experimental design and Box Behnken design.

### 3.2.3 Optimization of process parameters for laccase production by *Pleurotus ostreatus* - ‘one-factor-at-a-time’ approach

The effect of various nutritional and physical parameters on production was examined initially using ‘one-factor-at-a-time’ method followed by a time course experiment under the optimized conditions. This method serves as a platform to choose the effective range of values for each parameter on laccase production for further statistical optimization studies.
The parameters selected for optimization included incubation period, inoculum concentration, substrate size, substrate concentration per flask, incubation temperature, initial pH of the medium, carbon source, inorganic nitrogen source, organic nitrogen source, surfactants and additives.

Medium preparation for SSF, inoculum preparation, inoculation and incubation, extraction and recovery of enzyme were done according to the methods described earlier unless otherwise mentioned. In each case enzyme samples were subjected to enzyme activity, protein content and specific activity analysis using standard assay procedures as mentioned earlier.

All experiments were carried out in triplicates. The results are an average of triplicate experiments and standard deviation was determined using Excel 2007 (Microsoft Corporation, Redmond, USA). The graphs were also plotted with Excel 2007.

3.2.3.1 Incubation period

Optimum period of incubation for maximum laccase production was determined by incubating the inoculated media for a total period of 13 days. Samples were analyzed at regular intervals for enzyme activity, protein content and specific activity using standard assay procedures.

3.2.3.2 Inoculum concentration

Optimum inoculum concentration that supports the maximal laccase production during SSF was determined by varying the number of fungal mycelial plugs per flask. 1-10 number of mycelia plugs were added to different culture flasks and subjected to SSF for 11 days. Samples were collected and analyzed for enzyme activity, protein content and specific activity under standard assay procedures.
3.2.3.3 Substrate size

Pineapple leaves were selected as the substrate and the optimal particle size for maximum laccase production was evaluated by growing the fungal culture on pineapple leaves with length ranging from 1.27 to 6.35 cm and analyzed for enzyme activity, protein content and specific activity under standard assay procedures. The SSF was carried out for a period of 11 days using 3 mycelial plugs as inoculum.

3.2.3.4 Substrate concentration

The effect of substrate concentration on enzyme production was studied by conducting SSF with varying amounts of pineapple leaves per flask (5 g to 25 g) for 11 days and samples were subjected to enzyme activity, protein content and specific activity analysis under standard assay procedures.

3.2.3.5 Incubation temperature

The optimum temperature for laccase production using *Pleurotus ostreatus* was determined by incubating the inoculated culture flasks at temperatures of 20°C, 22°C, 24°C, 26°C, 28°C and 30°C for 11 days and analyzing enzyme activity, protein content and specific activity under standard assay procedures.

3.2.3.6 Initial pH of the medium

The effect of initial pH of the medium on maximal laccase production was studied by conducting SSF at varying pH levels of 3-8 by adjusting the pH of the moistening solution using citrate phosphate buffer (pH 3-8). The studies were carried out at 28 °C for 11 days and the samples were subjected to enzyme
activity, protein content and specific activity analysis under standard assay procedures.

### 3.2.3.7 Additional carbon source

The effect of additional carbon sources on maximum laccase production by the organism was evaluated by adding various sugars at a concentration of 1% (w/v) to the solid substrate medium. The carbon sources tested included glucose, maltose, lactose, starch and sucrose. The optimal concentration of the selected carbon source for maximal laccase production was further analyzed by varying its concentration from 1-5 % in the medium. The studies were carried out at an initial pH of 5 and the samples were collected after 11 days of incubation at 28 °C and subjected to enzyme activity, protein content and specific activity analysis under standard assay procedures.

### 3.2.3.8 Inorganic nitrogen source

The effect of additional inorganic nitrogen sources on maximum laccase production by the organism was evaluated by adding various inorganic nitrogen sources at a concentration of 1% (w/v) to the solid substrate medium. The inorganic nitrogen sources tested included ammonium nitrate, ammonium chloride, ammonium sulphate, ammonium acetate and ammonium dihydrogen ortho phosphate. The optimal concentration of the selected inorganic nitrogen source which supported the maximal laccase production was further analyzed by varying its concentration from 1-5 % in the medium. The studies were carried out at an initial pH of 5, maltose as additional carbon source and the samples were collected after 11 days of incubation at 28 °C and subjected to enzyme activity, protein content and specific activity analysis under standard assay procedures.
3.2.3.9 Organic nitrogen source

The effect of additional organic nitrogen sources on maximum laccase production by the organism was evaluated by adding various organic nitrogen sources at a concentration of 1% (w/v) to the solid substrate medium. The organic nitrogen sources tested included peptone, tryptone, beef extract, yeast extract and malt extract. The optimal concentration of the selected organic nitrogen source which supported the maximal laccase production was further analyzed by varying its concentration from 1-5 % in the medium. The studies were carried out at an initial pH of 5, maltose as additional carbon source and ammonium dihydrogen ortho phosphate as inorganic nitrogen source. The samples were collected after 11 days of incubation at 28 °C and subjected to enzyme activity, protein content and specific activity analysis under standard assay procedures.

3.2.3.10 Surfactants

The effect of different surfactants at varying concentrations ranging from 0.01%- 0.05% on enzyme production was also studied. The tested surfactants included Tween 80, Tween 20 and SDS. The studies were carried out at an initial pH of 5, maltose as additional carbon source, ammonium dihydrogen ortho phosphate as inorganic nitrogen source and tryptone as organic carbon source. The samples were collected after 11 days of incubation at 28 °C and subjected to enzyme activity, protein content and specific activity analysis under standard assay procedures.

3.2.3.11 Additives

Various compounds which were already known to be potent enhancers for laccase production were also tested at varying concentrations to determine their individual effect on maximal laccase production by Pleurotus ostreatus NCIM
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1200 using pineapple leaves as substrate. The selected compounds included veratryl alcohol, copper sulphate, gallic acid, ABTS and guaiacol at 0.5- 1.5 mM concentrations. The medium without any additives was used as the control. The studies were carried out at an initial pH of 5, maltose as additional carbon source, ammonium dihydrogen ortho phosphate as inorganic nitrogen source, tryptone as organic carbon source and the surfactant, Tween 20. All the additives were filter sterilized and added at the time of inoculation. The samples were collected after 11 days of incubation at 28 °C and subjected to enzyme activity, protein content and specific activity analysis under standard assay procedures.

3.2.4 Time course study

After completing the optimization of all process variables using ‘one-factor-at-a-time’ method a time course experiment was conducted with the optimal values obtained for each variables. The conditions selected included the following:

- 3 mycelial plugs as inoculum
- 10 g pineapple leaf per flask with a length of 2.54 cm as substrate
- pH 5.0
- Incubation temperature of 28 °C
- 4% maltose
- 2% ammonium dihydrogen ortho phosphate
- 1% tryptone
- 0.01% Tween 20
- 1 mM gallic acid

Inoculated flasks were incubated for 13 days and samples drawn on each day were subjected to enzyme activity, protein content and specific activity analysis under standard assay procedures.
3.3 RESULTS AND DISCUSSION

Pure cultures of *Pleurotus ostreatus* strain NCIM 1200 obtained from NCIM, Pune were grown on potato dextrose agar (PDA) plates showed rich white aerial mycelium (Fig. 3.1). The reverse side of the mycelia was also colourless, pigmentation was also not observed even after two weeks of growth on PDA plates.

Fig. 3.1: Growth of *Pleurotus ostreatus* on PDA plates

3.3.1 Laccase production potential

Okino et al (2000) reported the use of guaiacol which is a very sensitive chromogen substrate for rapid screening of extracellular production of guaiacol oxidizing enzymes by fungal strains. The oxidative polymerization of guaiacol leads to the formation of reddish brown zone around the fungal colonies. The *Pleurotus ostreatus* strain NCIM 1200 growing on guaiacol containing PDA plates showed its potential for extracellular laccase production by the formation of reddish brown zone (Fig. 3.2) around the fungal mycelia.
Fig.3.2: Qualitative assay indicating laccase production on guaiacol containing PDA plates

The organism was also checked for its ability to produce manganese pexroxidase (MnP) and lignin peroxidase (LiP) and it was found to lack both these enzymes.

3.3.2 Culture revival

Mushrooms were produced on paddy straw substrate and it gave milky white fruiting bodies (Fig.3.3). They were collected, surface sterilized and pure cultures were freshly isolated. The pure cultures thus obtained were subjected to laccase production studies under solid state fermentation condition.

Fig.3.3: Fruiting bodies of Pleurotus ostreatus NCIM 1200
3.3.3 Optimization of process parameters for laccase production by *Pleurotus ostreatus* - ‘one-factor-at-a-time’ approach

3.3.3.1 Optimization of incubation time

In a fermentation condition the effect of incubation period on enzyme production is mainly governed by the characteristics of the culture and is also based on its growth rate. The organism was subjected to SSF using pineapple leaf substrate for a period of 13 days and the results documented in Fig.3.4 shows that the organism gave maximum laccase production on 11\(^{th}\) day of incubation with laccase activity of 261.6 IU/mL.

![Graph](https://via.placeholder.com/150)

**Fig.3.4:** Optimization of incubation time for laccase production by *P. ostreatus* under SSF. Data points are arithmetic means of triplicates, while error bars denote the standard deviation from the mean.

The organism started laccase production only after 24 hours of incubation. Even though the organism showed fluctuations in laccase production during its growth, maximum specific activity was also obtained on the 11\(^{th}\) day of incubation. Hence, 11 days of incubation was considered as optimum during the later optimization studies. Previous studies report different incubation period for maximum laccase production using different substrates. Hashim in 2012 reported maximum laccase production by *P. ostreatus* on 15\(^{th}\) day on wheat straw, whereas
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Gupte et al (2007) reported maximum laccase production on 8\textsuperscript{th} day using same substrate. These reports accounts for the different pattern of laccase production by white rot fungi depending on the type of substrates and other culture conditions.

3.3.3.2 Optimization of inoculum concentration

Mycelial plugs with a size of 1 cm\textsuperscript{2} cut from actively growing fungal plates were used as the inoculum. The studies performed for the optimization of inoculum concentration revealed maximum laccase production (265.93 IU/mL) with 3 mycelial plugs as inoculum. Fig.3.5 diagrammatically represents the effect of inoculum concentration on laccase production.

![Fig.3.5: Optimization of inoculum concentration for laccase production by *P. ostreatus* under SSF. Data points are arithmetic means of triplicates, while error bars denote the standard deviation from the mean.](attachment:image)

In previous studies, different inoculum sizes have been used for laccase production, for example mycelial plugs measuring 10mm, 3mm and 8mm in diameter (Hashim, 2012). Further studies were conducted using 3 mycelial plugs as inoculum.
3.3.3.3 Optimization of substrate size

The adherence and the growth of microorganisms on the solid substrate as well as the enzyme action on it mainly depend upon the physical properties of the substrate including porosity, surface area and substrate size. Among these properties the substrate size plays the crucial role since all other properties depend on this. Data obtained for the present study suggests that pineapple leaf with 2.54 cm length leads to maximum laccase production under SSF (Fig. 3.6).

![Graph showing optimization of substrate size for laccase production by P. ostreatus under SSF](image)

**Fig.3.6: Optimization of substrate size for laccase production by P. ostreatus under SSF.** Data points are arithmetic means of triplicates, while error bars denote the standard deviation from the mean.

Under this condition the organism showed an enzyme activity of 230.57 IU/mL. Further increase in substrate size led to decreased enzyme production. The enzyme yield was low in the case of substrates with both lower and higher particle size. The result showed good agreement with the general concept that lower particle size results in substrate agglomeration, decreased heat transfer and enhanced channeling problems while larger particles reduce the enzyme yield due to limited surface area for microbial attack (Pandey et al., 2000).
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3.3.3.4 Optimization of substrate concentration

In SSF, the surface-to-mass ratio of solid substrate was one of the important factors which directly related to the surface area available for the growth of cells. Present study varying the pineapple leaf weight used per flask revealed the maximum laccase production of 255.53 IU/mL (Fig.3.7) at 10 g per flask. Further increase in substrate concentration decreases the enzyme production.

![Graph showing laccase activity and specific activity vs. substrate concentration](image)

Fig.3.7: Optimization of substrate concentration for laccase production by *P. ostreatus* under SSF. Data points are arithmetic means of triplicates, while error bars denote the standard deviation from the mean.

3.3.3.5 Optimization of incubation temperature

In the SSF systems during fermentation there is a general increase in the temperature of the fermenting mass due to respiration (Pandey and Radhakrishnan, 1992). The impact of temperature is more prominent in the scale up processes and it remains an inevitable factor due to its impact on microbial growth and metabolite production. Fig.3.8 shows the effect of incubation temperature on laccase production. The optimum laccase production (762.7 IU/mL) was obtained at a temperature of 28 °C.
While considerable level of laccase production was found at lower temperatures, incubation temperature above 30 °C decreased the laccase production. No fungal growth was observed at higher temperatures, i.e above 60 °C, due to the drying of the substrate. In SSF, incubation temperature is a critical parameter which has to be controlled and it varies among different organisms. Temperature plays a role in changing the physical properties of the cell membrane and thereby influences the secretion of extracellular enzymes and the time course activities of the enzyme. Lang et al (2000) has demonstrated the influence of incubation temperature on the activity pattern of lignin modifying enzymes (LMEs), as temperature directly influences the growth and enzyme production. The effect of temperature on the growth of \textit{P. ostreatus} as well as laccase production was studied extensively by many researchers and they came to a conclusion that temperature exerts a similar effect on growth and enzyme production despite of the mode of fermentation. Patel et al (2009) reported the same temperature optima of 28 °C for maximum laccase production. Many researchers have reported an optimum temperature between 25 and 30 °C for
laccase production using various white rot fungi (Lang et al., 2000; Patel and Gupte, 2016; Ravikumar et al., 2012; Chhaya and Gupte, 2013; Elsayed et al., 2012). Declined enzyme activity and growth of the organism at high temperatures is due to the reduction of dissolved oxygen in the reaction system, which is adverse for enzyme catalysis.

### 3.3.3.6 Optimization of initial pH of the fermentation medium

The initial pH of the culture medium was varied from 3 to 8 and the results depicted in Fig.3.9 indicate maximum laccase production (486.93 IU/mL) at an initial pH of 5.

![Graph showing laccase activity and specific activity vs initial pH of the medium](image)

**Fig.3.9:** Optimization of initial pH of the medium for laccase production by *P. ostreatus* under SSF. Data points are arithmetic means of triplicates, while error bars denote the standard deviation from the mean.

Further rise in pH drastically reduced the enzyme production. This may be due to the poor mycelial growth of the organism restricting the enzyme production at these elevated pH levels. Similar results showing maximum laccase production at pH 5 has been reported earlier by Patel and Gupte (2016), Chhaya and Gupte (2013) and Ravikumar et al (2012) using one-factor-at-a-time...
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approach for medium optimization under SSF. The pH of culture medium is critical for growth of the organism and lignolytic enzyme production. According to Sivakumar et al (2010) the optimum pH for laccase production falls between 4.5 and 6, in many white rot fungi. Li et al., 2010 have also reported optimum laccase activity at pH 4 and further decrease or increase in the pH value rapidly decreases the activity and was almost nil at pH 7. The present results are in agreement with previous reports as most of the fungal enzymes especially laccases have maximum activity when the pH of the medium ranges between 4 – 6 (Schliephake et al., 2000; Galhaup et al., 2002; Jang et al., 2002; Chen et al., 2003a; Patel and Gupte, 2016; Chhaya and Gupte, 2013). Further, Patel et al., 2009 reported the exponential increase in laccase activity from pH 3 to 5, with maximum at pH 5, the observation is in accordance with the present results. However, Patrick et al (2011) have reported optimum laccase activity at pH 6 and 5.5 by Pleurotus sajor – caju, which may be due to the fact that the changes in pH may alter the three dimensional structure of the enzymes (Shulter and Kargi, 2000), the electrostatic properties of the protein surface and the reaction centre or by influencing the stability of the enzyme (Patrick et al., 2011).

3.3.3.7 Optimization of additional carbon source

Selection of an appropriate carbon source is important in growth and metabolism of fungi, hence affecting enzyme production. The effect of various carbon sources on laccase production is as shown in the Fig. 3.10. It was found that the presence of maltose as additional carbon source showed maximum laccase production (309.33 IU/mL). The results clearly show that all the carbon sources tested in the present study favoured the extracellular production of laccase. But higher specific activity was also obtained with the addition of
maltose. This makes it an ideal choice for additional carbon source for better laccase production.

Fig. 3.10: Effect of various carbon sources on laccase production by *P. ostreatus* under SSF. Data points are arithmetic means of triplicates, while error bars denote the standard deviation from the mean.

The laccase activity increased exponentially along with increase in maltose concentrations up to 4% but further increase lead to decrease in activity (Fig. 3.11). Hence 4% maltose was considered as the optimal concentration of additional carbon source for maximum laccase production under SSF.

Fig. 3.11: Optimization of maltose concentration for laccase production by *P. ostreatus* under SSF. Data points are arithmetic means of triplicates, while error bars denote the standard deviation from the mean.
Incorporation of additional carbon source to the fermentation medium showed an increased enzyme yield compared with fermentation using pineapple leaves alone. Nature and type of carbon and nitrogen sources are the most important factors for any fermentation process (Pandey and Radhakrishnan, 1992). According to Mikiashvili et al., (2006) carbon sources, such as mannitol, glucose and cellobiose usually give higher laccase activity compared with other sources like cellulose and lactose that are assimilated more slowly. At the same time glucose acts as a typical repressor of laccase production in many species. Mikiashvili et al., described two different laccase activity in the medium by two different strains of Pleurotus ostreatus (2006) and Trametes versicolor (2005) depending on the type of carbon supplement. Therefore carbon sources are species and strain specific and its concentration is also particularly important. Excess concentration of carbon source usually represses laccase expression, whereas certain minimal carbon source levels appear to be essential to maintain the culture viability and to give sustained laccase production over time.

3.3.3.8 Optimization of inorganic nitrogen source

There are many previous reports on the powerful effect of nitrogen source in regulating the ligninolytic enzyme production by wood rotting basidiomycetes (Galhaup et al., 2002). Both the nature and concentration of nitrogen source play a role. In the present study, better laccase activity was observed with ammonium dihydrogen ortho phosphate compared with other inorganic nitrogen sources tested such as ammonium nitrate, ammonium chloride, ammonium sulphate and ammonium acetate (Fig.3.12). Maximum laccase activity was obtained with 2 % (NH₄) H₂ PO₄ (881.53 IU/mL) as shown in Fig.3.13.
3.3.3 Optimization of organic nitrogen source

It is evident from the results in Fig.3.14 that among the various organic nitrogen sources tested, peptone, tryptone, beef extract, yeast extract and malt extract, tryptone gave maximum laccase activity (876.2 IU/mL) at a concentration
of 1%. Further increase in tryptone concentration decreased the enzyme production (Fig.3.15).

![Graph](image1.png)

**Fig.3.14:** Effect of various organic nitrogen sources on laccase production by *P. ostreatus* under SSF. Data points are arithmetic means of triplicates, while error bars denote the standard deviation from the mean.

![Graph](image2.png)

**Fig.3.15:** Optimization of tryptone concentration in the medium for laccase production by *P. ostreatus* under SSF. Data points are arithmetic means of triplicates, while error bars denote the standard deviation from the mean.

Many controversial reports exist on the effect of nitrogen source on ligninolytic organisms. For example, the ligninolytic enzyme production by the best studied ligninolytic fungus, *Phanerochaete chrysosporium*, in the nitrogen
limiting medium (Hamman et al., 1997) as well as in nitrogen containing medium (Srinivasan et al., 1995) are reported. These reports show the differential effect of nitrogen source on laccase production. In nature, ligninolytic enzyme production is enhanced due to nitrogen limitation, i.e nitrogen represses laccase expression. According to Kachlishvili et al (2006) some laccase enzymes are less sensitive to this nitrogen repression. Hou et al., (2004) reported laccase production by *P. ostreatus* in nitrogen limiting medium. He also explained the contradictory results with two different strains of *Pleurotus ostreatus*.

Present study shows the combination effect of both organic and inorganic nitrogen source on laccase production. Here both these sources are found to be optimum at its lower concentration. This adds to the previous report on the enhanced production of laccase by *P. ostreatus* HP-1 (Patel et al., 2009) by the combined use of two different organic and inorganic nitrogen sources. Prasad et al (2005) reported the enhanced laccase production by *P. ostreatus* at lower nitrogen source concentration. It was demonstrated that the use of lignocellulosic waste needed higher energy input for fungal growth due to its complex structure. Mycelium must grow on waste particles to produce an array of enzymes to liberate nutrients and inducers. This positive effect of additional nitrogen sources may be due to this higher biomass formation. As biomass growth may be speeded up by the additional nitrogen source, improving laccase activity, fungal laccases that are not inhibited by nitrogen are more attractive for scale-up.

3.3.3.10 Effect of different surfactants on laccase production

It is well documented that surfactants can increase the cell permeability facilitating the export of several molecules across the membrane leading to increased protein secretion. Among the various surfactants studied for their effect on laccase production, 0.01 % Tween 20 gave maximum laccase activity (800.23
IU/mL) (Fig.3.16). This adds to several previous reports on induced laccase production by *P. ostreatus* by the addition of surfactants (Patel *et al.*, 2009; EI-Batal *et al.*, 2015).

**Fig.3.16**: Effect of various surfactants on laccase production by *P. ostreatus* under SSF. Data points are arithmetic means of triplicates, while error bars denote the standard deviation from the mean.

Zheng and Obbard (2001) reported the ability of surfactants to increase the bioavailability of less soluble substrate to the fungi thereby stimulating the growth and enzyme production. Dombrovskaya and Kostyshin (1996) demonstrated the effective induction of laccase in *Pleurotus floridae* with anionic and cationic surfactants.

### 3.3.3.11 Effect of various additives on laccase production
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There are many previous reports on the induced or enhanced production of laccase by white rot fungi in the presence of certain aromatic compounds as well as Cu$^{2+}$ ions in the fermentation medium (Palmieri et al., 2000; Ikehata et al., 2004). The results depicted in the Fig.3.17 clearly show that all the selected additives were acting as inducers of laccase enzyme.

Fig.3.17: Effect of various additives on laccase production by *P. ostreatus* under SSF. Data points are arithmetic means of triplicates, while error bars denote the standard deviation from the mean.
Chapter 3

The addition of an appropriate inducer to the fermentation medium can significantly enhance laccase production and it can be a prerequisite for effective large scale enzyme production. The inducer concentration has important role on laccase production and is specific to species or strains (Minussi et al., 2007). In the present study maximum laccase production (1009.33 IU/mL) was obtained with the addition of 1mM gallic acid to the fermentation medium. In the case of both ABTS and veratryl alcohol almost same enzyme activities were obtained at three different concentrations, whereas in the case of CuSO$_4$ and guaiacol, increase in concentration lead to decrease in enzyme production. This stimulatory effect of copper on laccase production could be explained as a role for this enzyme in defense mechanism against oxidative stress (Trupkin et al., 2003). The present result of the inhibitory effect of higher CuSO$_4$ concentration on laccase production adds to the already existing report on significant decrease in fungal growth and laccase production by *P. ostreatus* HP-1 at a Cu$^{2+}$ concentration beyond 0.28 to 0.6 mM (Patel et al., 2009). Eggert et al., (1996) reported laccase suppression by guaiacol in *Pleurotus florida* and *Pycnoporus cinnabarinus*.

### 3.3.4 Time course study of laccase production under SSF

The time course of laccase enzyme production by *Pleurotus ostreatus* was studied. The results depicted in Fig.3.18 for the time course study conducted for 13 days under optimal conditions shows that maximum laccase production (1018.3 IU/mL) was observed on 8$^{th}$ day using 3 mycelial plugs as inoculum.
The present study showed a 4 fold increase in laccase production compared to that of unoptimized medium. Similar results were obtained with Phlebia floridensis, which showed maximum laccase production on the 8th day of incubation by Arora and Gill (2000). During the time course study enhanced laccase production was obtained with these optimized conditions and the incubation period was reduced to 8 days from 11 days for maximum laccase production, where as the inoculum concentration remains the same.

3.4 CONCLUSION

The optimization studies on various process parameters for maximum laccase production by “one-factor-at-a-time” approach ended up with a promising result of 4 fold increase in laccase production by Pleurotus ostreatus NCIM 1200. The optimum values obtained for each variable after the study is listed in the Table. 3.1.
Table 3.1: Optimized values for various process parameters after one-factor-at-a-time approach

<table>
<thead>
<tr>
<th>Bioprocess variables</th>
<th>Optimized condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubation period</td>
<td>8 days</td>
</tr>
<tr>
<td>Inoculum</td>
<td>3 mycelial plugs per flask</td>
</tr>
<tr>
<td>Pineapple leaf length</td>
<td>2.54 cm</td>
</tr>
<tr>
<td>Pineapple leaf weight</td>
<td>10 g</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>28 °C</td>
</tr>
<tr>
<td>pH</td>
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</tr>
<tr>
<td>Maltose</td>
<td>4 %</td>
</tr>
<tr>
<td>Ammonium dihydrogen orthophosphate (%)</td>
<td>2 %</td>
</tr>
<tr>
<td>Tryptone (%)</td>
<td>1 %</td>
</tr>
<tr>
<td>Tween 20 (%)</td>
<td>0.01 %</td>
</tr>
<tr>
<td>Gallic acid (mM)</td>
<td>1 mM</td>
</tr>
</tbody>
</table>