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# Production parameter optimization for laccases by *Aspergillus niger*

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**ABSTRACT**

Laccase belongs to the family of blue multi-copper oxidases (MCOS) that has three domain structures and usually contains four copper atoms. *Aspergillus niger* species are reported to produce laccase. An attempt was made to screening, optimization, production and partial purification of laccase enzyme produced from *Aspergillus niger.* Guaiacol and acetate buffer were used to assay laccase production. Laccase activity was highest when operated at the following conditions, 120 h incubation, 30°C temperature, pH-5, 2% glucose as carbon source and 2% peptoneas nitrogen source in the production medium. The enzyme was partially purified by ammonium sulfate precipitation and dialysis method.

***Keywords:*** Laccase, *Aspergillus niger,* enzyme activity, optimization.

Laccases is a multicopper blue oxidase capable of oxidizing ortho- and para-diphenols and aromatic amines by removing an electron and a proton from a hydroxyl group to form radicals. First isolated in 1883 from Rhus venicifera, the Japanese lacquer tree, laccases are commonly found in fungi (Thurston, 1994). Extracellular laccases purified and characterized from various fungal sources but majority of the studies have been focused on relatively few species such as Trametes versicolor, Ganoderma lucidium, Neurospora crassa, Agracius bisporus, Aspergillus nidulans, Pleurotous ostreatus, Panus trigrinus Trametes villosa (Ko et al., 2001).

Laccases are of particular interest with regard to potential industrial applications because of their capability to oxidize a wide range of industrially relevant substrates. Their substrate versatility makes laccases highly interesting for various applications, including textile dye bleaching, pulp bleaching and bioremediation where enzymatic catalysis could serve as a more environmentally bening alternative than the currently used chemical processes. Its potential application

extends in biosensors also (Grac et al., 2002; Duran and Eposito, 2000)labelling in immunoassays, drug analysis, clarification of juices and wines, design of laccase fungicidal and bactericidal preparations (Jahansen, 1996).

Other organic compounds, such as nitroso compounds ortriphenylamine and phenothiazine derivates (i.e. promazine, phenothiazine- 10-propionic acid or 2-nitro-1-naftol-4-sulfonic acid), have been tested as laccase mediators for the oxidation of lignin dimers, kraft paper pulp or textile dyes (Bourbonnais et al., 1997a; Camarero et al., 2005; Fabbrini et al., 2002; Paice et al., 1997). More recently, phenylpyrazolones have been demonstrated to mediate the oxidation of veratryl alcohol or xenobiotics by laccase (Shleev et al., 2003; Shleev et al., 2004). On the other hand, metal ions, such as Mn2+ could act as laccase mediators when they are oxidized by the enzyme (Muñoz et al., 1997). In addition, metallic complexes, the so-called polyoxometalates, have been described as laccase mediators for the degradation of lignin (Gamelas et al., 2005; Gamelas et al., 2007). The versatility of laccase-mediator systems for oxidative transformation of aromatic compounds is evidenced by the variety of possible applications such as: delignification and bleaching of paper pulp decolorization of industrial dyes or detoxification of pollutants (Bajpai, 2004; Barreca et al., 2004; Claus et al., 2002; Husain and Husain, 2008; Morozova et al., 2007a).

## Material and Methods

*Optimization of culture conditions for enzyme production (Ding et al., 2012)*

This was done by varying culture conditions that effect laccase production to determine the optimum conditions for the production of laccase from *Pseudomonas aeruginosa*. Production medium (Dipotassium hydrogen phosphate-2.75g, Potassium hydrogen phosphate-2.25g, Ammonium sulfate-1.00g, Magnesium Chloride-0.20g, Sodim Chloride-0.10g, Ferric Chloride-0.02g, Calcium Chloride-0.01g, Distilled water-1000ml, pH-7.0) was used for analysis.

*Effect of temperature*

Effect of temperature on laccase production was studied. The production medium was inoculated with 1 ml culture of *Aspergillus niger*and incubated at different temperatures *viz.* 20, 30, 40 and 50ºC for 7 days with pH 5.6.

*Effect of pH*

The effect of pH on laccase production was carried out by incubating the flasks containing 100 ml of production media was inoculated with 1 ml of culture

*Aspergillus niger*and incubated at optimized temperature for 7 days at different pH such as 5, 7, 9 and 12.

*Effect of incubation period*

In order to find the optimal time of incubation for the maximum laccase production. 100 ml production medium was inoculated with 1 ml culture of *Aspergillus niger*and incubated at optimized temperature. The sample was withdrawn at different time intervals *viz.* 0, 24, 48, 72, 96 and 120, 144,168, 192 h each time.

*Effect of carbon source*

To analyse the effect of carbon sources on laccase production100 ml production medium supplemented with 2% different carbon sources *viz.* glucose, sucrose, mannitol, maltose was prepared in Erlenmeyer flask (250 ml) with optimized pH and autoclaved. The production media was inoculated with 1 ml culture of*Aspergillus niger* followed by incubation at optimized temperature at optimized time interval.

*Effect of nitrogen source*

In order to find the suitable nitrogen sources for maximum laccase production, 100 ml production medium supplemented with optimized carbon source and 2% different nitrogen sources *viz.* peptone, ammonium chloride, sodium nitrate, ammonium sulfate was prepared and inoculated with 1 ml culture of*Aspergillus niger*followed by incubation at optimized temperature for optimized time interval.

*Production and Partial purificationat optimized culture conditions*

For laccase production, 100 ml Nutrient broth media was prepared. A loopful culture of Aspergillus nigerwas inoculated using a sterile loop and incubated at 30ºC at 120 rpm. The culture was served as seed culture after 72 h for further inoculation. A 100 ml sterile production media containing 2% optimized carbon and nitrogen source was prepared with optimized pH according to the composition given by Unyayar et al., (2005) sterlized and inoculated with 5% seed culture (v/v) and was incubated at optimized temperature for optimized time interval.

*Extraction of crude enzyme*

After incubation the cells were harvested by centrifugation at 10,000 rpm at 4ºC for 10 min. The clear supernatant obtained was used as crude enzyme and was stored in vials for further use.

*Laccase assay*

Laccase activity was determined by measuring the oxidation of guaiacol at 530 nm. The reaction mixture was containing 10 mM guaiacol and 100 mM acetate buffer (pH 5). Absorbance for blank was measured at 470 nm while that of test samples was measured at 530 nm. The change in the absorbance of the reaction mixture with guaiacol was monitored for 10 min of incubation. Enzyme activity was measured in U/ml which is defined as the amount of enzyme catalyzing the production of one micromole of coloured product for min per ml (Jhadav et al., 2009).

Calculation

$$\text{Enzyme activity (u/ml)} \frac{\Delta A_{530}\ \text{nm/min x } V_t \text{ x dilution factor}}{€\ \text{x } V_s}$$

Where,

Vt = final volume of reaction mixture (ml)

Vs = sample volume (ml)

€ = extintion co-efficient of guaiacol = 6740/M/cm.

*Partial purification of Laccases*

Partial purification of laccase enzyme was done through Ammonium sulfate precipitation and Dialysis. Protein content was estimated at each step of purification.

*Ammonium sulfate precipitation*

The crude laccases was filtered through Whatman No.1 filter paper and the resulting filtrate was concentrated by freeze-drying and kept at 4ºC. Protein was precipitated by ammonium sulfate from the filtrate obtained. Solid ammonium sulfate was added slowly to the crude extract isolate to give 70% saturation and the solution was stirred gently for at least 1 h at 4ºC and was left to stand overnight. The precipitate was collected by centrifugation at 8000 rpm for 1 h at 4ºC. The supernatant was discarded and the pellet was dissolved in 0.01 M phosphate buffer (pH 7) (Barda and David, 1949).

**Dialysis**

Pellet was dissolved in 0.01 M phosphate buffer with pH-7 and was dialysed against the same buffer overnight at 4ºC.

**Estimation of protein content of partially purified laccases**

Protein content of the sample was measured by Lowry's method and bovine serum albumin (BSA) was used as standard (Lowery et al., 1951).

**Results and Discussion**

*Optimization of culture conditions for laccase production*

*Effect of temperature on laccase activity*

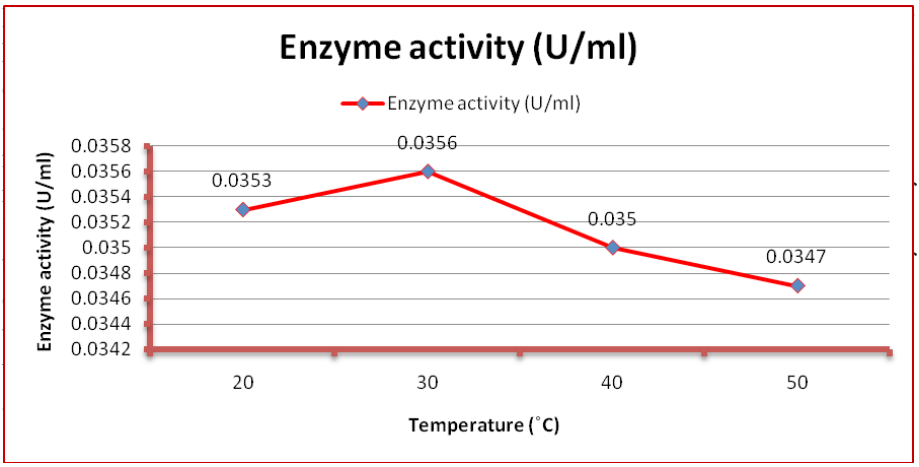


**Fig: 1 Effect of temperatureon laccase activity by** *Aspergillus niger*

*Effect of pH on laccase activity*

Highest laccase activity was observed at pH 7 (0.0341 U/ml), while lowest laccase activity was observed at pH 12 (0.0287 U/ml). (Fig:2).Similar kind of study conducted Sivakumar et al. (2010) and the maximum release of laccase activity of 0.24 U/ml was reported at pH 6.0 by Ganoderma sp. Study conducted by Adejoye and Fasidi, (2009) in which the highest laccase activity of (2.86 U/min) was recorded at pH 5.5.Rajeshwari and Parvantham, (2011) reported maximum laccase production at pH 5. pH is one of the important factor for the

growth and morphology of microorganisms, they are sensitive to the concentration of hydrogen ion present in the medium. The optimal value of pH varies according to the substrate because different substrate causes different reaction for laccases. Since each substrate supported a particular pH for maximum enzyme secretion and the enzyme instability at very high or very low pH.

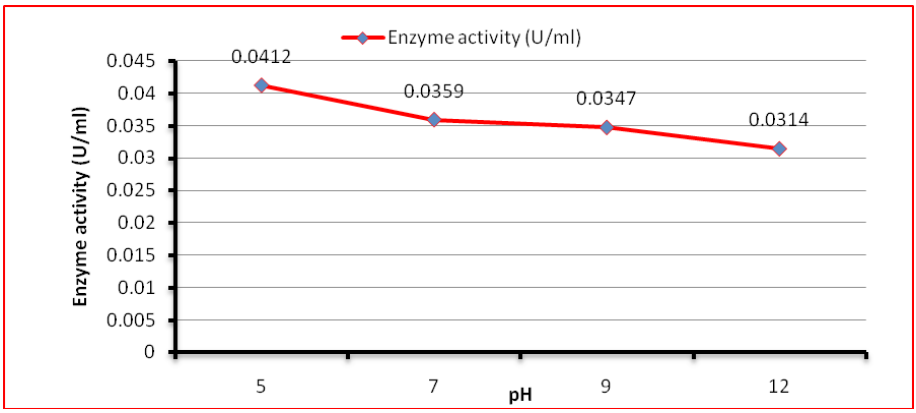


**Fig: 2 Effect of pH on laccase activity**

*Effect of incubation timeon laccase activity*

Laccase activity was observed maximumat 120 h (0.0842 U/ml) (Fig: 3).The present study is in contrast with the finding of Sivakumar et al., (2010) in which effect of incubation time on laccase activity was studied and highest activity was observed on 10th day of incubation with Ganoderma sp. The similar kind of study was conducted by Desai et al., (2011) in which highest activity by fungi was

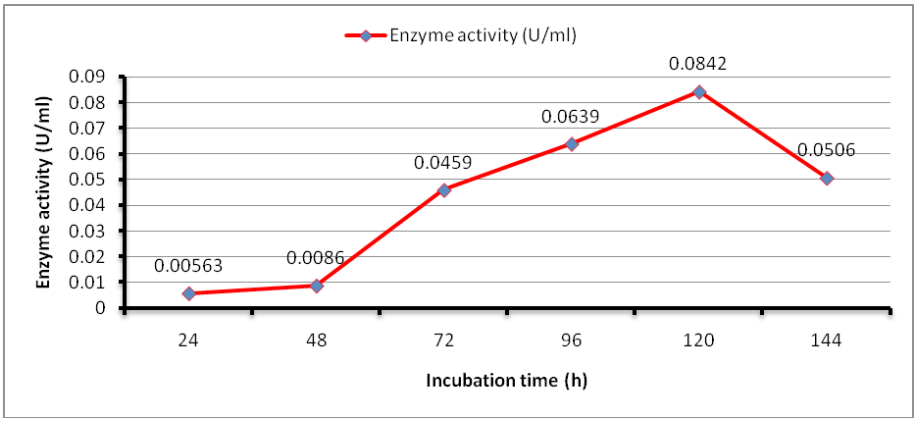


**Fig: 3 Effect of incubation time on laccase activity**

observed on 6th day of incubation. The incubation time plays an important role in the growth of microorganisms and enzyme secretion. Enzyme production increases with time till 72 h after that enzyme production decreased due to depletion of macro and micronutrients in the production medium.

*Effect of carbon source (2%) on laccase activity*

Laccase activity was observed maximum with 2% glucose as carbon source (0.0418 U/ml).The present study is in agreement with study conducted by Ding et al. (2012) in which 20 g/l glucose was most as effective sole carbon source, resulting in the highest laccase production(Fig: 4). In a similar kind of study conducted by Sivakumar et al., (2010)reported that starch supported for the high laccase production. Study conducted by Adejoye and Fasidi, (2009) in which the best laccase activity of (47.5 ± 1.85 U/ml) was induced by mannose. It has been reported that the carbon source is the most important factor in laccase production, and that the addition of suitable amount of other sugar to the culture media has a benign influence on laccase synthesis. Among the carbon source, glucose is readily utilizable substrate which would promote laccase production. Medium containing glucose showed the highest laccase activity as enzymes are substrate specific. Since, glucose is a monosaccharide which is easily broken down, quickly utilized by the microorganisms. It has already been demonstrated that substrates that are efficiently and rapidly utilized by the organism results in high levels of laccase activity.

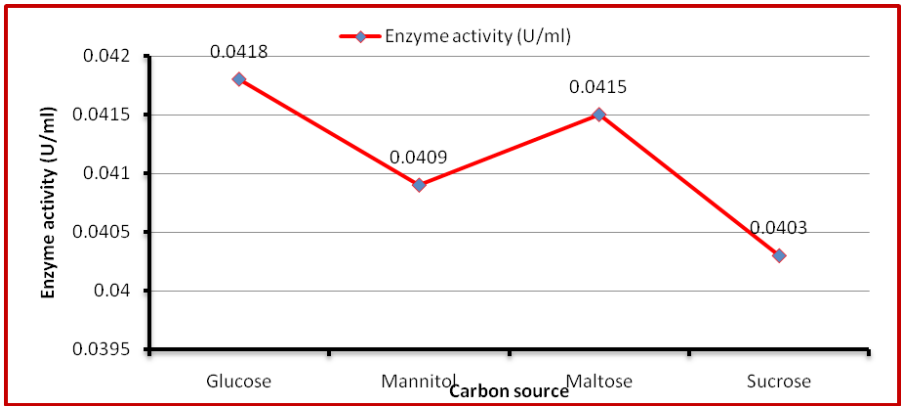


**Fig: 4 Effect of carbon source (2%) on laccase activity**

*Effect of nitrogen source (2%) on laccase activity*

Laccase activity was observed maximum with 2% peptone as nitrogen source (0.0418 U/ml) (Fig: 5).The present study is in contrast with Ding et al. (2012), Sivakumar et al. (2010) and Adejoye and Fasidi (2009). Ding et al. (2012)

reported that yeast extract supported for the highest laccase activity from Ganodermalucidum. Sivakumar et al.(2010) study reveals that yeast extract supported the highest laccase activity of 0.18 U/ml. Nitrogen plays key role in laccase production, while the organic nitrogen source gave high laccase yields. Nitrogen plays key role in laccase production, the nature and the concentration of

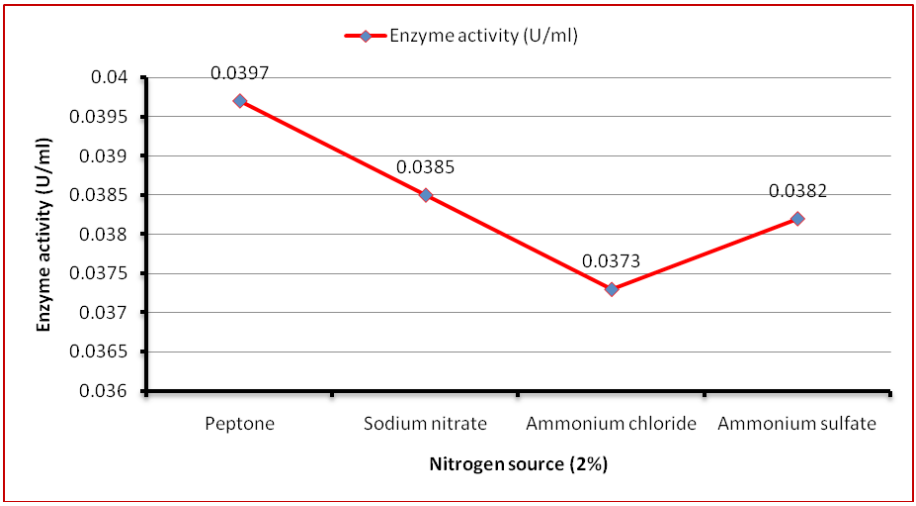


**Figure 5. Effect of nitrogen source (2%) on laccase activity**

nitrogen in the culture medium for growing the organism are essential for laccase production. Medium containing peptone showed the highest laccase activity as enzymes are substrate specific. Peptone is the simplified source of protein and can be readily uptake by the microorganism. It is asource of protein, amino acids for microbial growth.

*Partial Purification of laccases*

Culture filtrate of Aspergillus nigerwas obtained from the medium. Partial purification of laccase was carried by two method ammonium sulfate and dialysis. The crude enzyme was precipitated by ammonium sulfate precipitation up to 70% saturation with a total activity of 0.096 U/ml with 0.82 mg/ml of protein. After ammonium sulfate precipitation, the final purification by dialysis the fraction showed 0.068U/ml enzyme activity 0.55 mg/ml protein. As in present study, similar laccase activity was reported by several workers (Abou-Mansour et al. 2009; Jhadav et al. 2009) and purification of laccase enzyme was done by using ammonium sulfate and dialysis method.

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