ISSN: 0975 -8542

Journal of Global Pharma Technology

Available Online at: www.jgpt.co.in

RESEARCH ARTICLE

Production, Purification and Characterization of Laccase from *Pleurotus ostreatus*

Noor M. Alwan[1], Abdulkareem J. Hashim[2], Ghassan M. Sulaiman[3*]

1. *Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq.*
2. *Department of Biotechnology, College of Science, University of Baghdad, Baghdad, Iraq.*
3. *Division of Biotechnology, Department of Applied Sciences, University of Technology, Baghdad, Iraq.*

***Corresponding Author: Ghassan M. Sulaiman**

Abstract

Laccases are a model for multi copper oxidases and contribute in several applications such as biopulping textile, bioremediation, food industries and pharmaceutical compounds. In this work, factorial parameter was used to study the optimization of production conditions yielded an enzyme .The fungal laccase which produced by *Pleurotus ostreatus* using barley bran under optimization condition by solid state fermentation was purified by DEAE-Cellulose (IEXC) and this purified laccase enzyme was effected to pH and temperatures to determine the pH optimum and temperatures for laccase activity using syringaldazine as substrate. Results showed that the optimal conditions for enzyme production were incubation temperature: 35 °C, inoculum size: 3mm discs moisture ratio: 1: 10 W\V, Incubation period: 22 days. Whereas the optimal enzyme activity was at temperature 45°c and the enzyme remain stable at the same temperature. Further the optimal pH for enzyme activity at 5.6 and more stable in pH 6.6.

Keywords: *Laccase, Pleurotus ostreatus, Optimization, Laccase production.*

Introduction

Laccases are common enzymes in nature specially, in fungi and plant and has been found also in bacteria and insects [1]. Laccase in most studies is a fungal origin especially those from classes of white-rot fungi such as *Trametes versicolor, Pleurotus sajor- caju, Pleurotus ostreatus and Ganoderma lucidum* [2]. Laccase is classified as P-diphenol: dioxygen oxidoreductases and are multicopper proteins that use oxygen molecule to oxidize varied aromatic and non aromatic compounds like thiols residues, quinone, polaromatic hydrocarbons(PAHs), lignin derivatives, aromatic amines, anilines, dyes, aromatic alcohols, aromatic aldehydes, substituted phenols and phenols by a radical-catalyzed reaction [3].

Pleurotus ostreatus is the fungus belongs to class Basidiomycetes that produces ligninolytic enzymes such as laccases [4]. Several factor such as pH, temperature, inoculum size, type cultivation (SSF or SmF), nutritional requirements and microelements can effect on the level of laccase production [5].

Solid-state fermentation facilitates enzyme production by fungi because it stimulates the growth of the fungi in the natural environment. Extacellular fungal Laccases can be purified from culture medium of the chosen organism by several techniques for protein purification are repeatedly utilized in purifying laccase.

Perfect purification protocols involve ion exchange, ultrafiltration, gel filtration, hydrophobic interaction or other electrophoretic and chromatographic techniques. Laccases are beneficial enzymes for various technological applications: degradation and modification of dyes in textile, cosmetic industry, pharmaceutical compounds, pollutan degradation, biosensors and food manufacturing.

The catalytic activity and the stability of laccase are frequently influenced by diverse environmental condition, such as pH, ionic concentration, inhibitors, which limit their industrial applications [6].

The aim of present work is production, purification and characterization of laccase produced from fungal source using solid state fermentation.

Materials and Methods

Source of Fungal Isolate

Pleurotus ostreatus was obtained from agricultural research directorate and the isolate was activated on potato dextrose agar (PDA) medium. *P. ostreatus* was preserved in slants with PDA as a stock, and then the slants were stored at 4°c.

Solid State Fermentation (SSF)

The experiment was applied in (SSF) culture using barly bran as abasic substrate alone. Five grams of substrate were put in 250 ml Erlenmeyer flasks and moistened with 10 ml of distilled water; the flasks were closed with cotton stoppers and autoclaved at 121°c for 20 min and after sterilization, inoculated with 5mm diameter discs of PDA over gown with mycelia. Incubation of substrates was carried out under static conditions at 27°c for 7 day, after incubation 40 ml of 0.1mM citrate phosphate buffer pH 5.6 was added to each flask, the slurry was filtered through squeezed to maximize the enzyme extraction [7]. The filtered solution was centrifuged for 10 min at 6000 rpm at 4°c. The supernatant was used for enzyme activity and protein assessment [8].

Laccase Activity Assay

Laccase activity in the crude extract was measured by monitoring the oxidation of syringaldazine. The reaction mixture contained 2 ml of 0.1M citrate phosphate buffer pH 5.6, 0.2 ml of syringaldazine (1mM) and 1 ml crude enzyme extract.

The reaction was carried out during 1 min. Oxidation of syringaldazine was monitored spectrophotometrically by determining absorbance at 525 nm. One unit of laccase activity is defined as the amount of enzyme oxidizing 1µmol of syringaldazine per minute under standard assay condition [9].

Protein concentration was estimated spectrophotometrically using Bradford's reagent to determine potein concentration in the crude extract, 2.5 ml of coomassie Blue (G- 250) was added to test tubes contained 0.1 ml of crude enzyme solution and 0.4 ml of Tris- Hcl buffer the mixture was allowed standing for 1 minute then the absorbance was read at 595nm by spectrophotometer against the reagent blank. The proteins concentration was measured using liner equation of the bovine serum albumin standard curve [10].

Optimum Condition for Laccase Production

Optimum Moisture Ratio

Distilled water was added to the growth media (barely bran) at different ratio (1:1, 1:2, 1:3, 1:4 and 1:5 w\v). In the same steps mentioned above.

Optimum Inoculums Size

The medium above used with different inoculums size of 1, 2, 3, and 4 (5mm) from cultures of *P. ostreatus* the flasks were incubated for 7 days at 27 °c the flask control were used without inoculation.

Optimum Incubation Temperature

Pleurotus ostreatus was grown in (5) flasks. Each flask contains (5g) of the barely bran and incubated at different temperatures (20°c- 25°c - 30°c - 35°c - 38°c). The laccase activity was determined in supernatants after extraction and centrifugation at 6000 rpm for 10 minutes for the crude enzyme.

Optimum Incubation Period

The growth culture was incubated for (4, 7, 14, and 22) days and the laccase activity was determined in the same steps mentioned above.

Purification of laccase

The culture filtrates as saturated up to 80% with ammonium sulphate and centrifuged at 10 rpm for 20 min at 4°c. The precipitate was dissolved in citrate phosphate buffer pH 5.6 and extensively dialysed against the same buffer using dialysis bag. Laccase was purified from the culture supernatant of *P. ostreatus* by anion exchange chromatography.

Six ml of the enzyme extract was applied to a DEAE- cellulose colum (23×1.5) equilibrated with buffer 0.1 mM citrate phosphate buffer pH (5.6) and eluted with a linear gradient of (0.1-0.6M) NaCl. Laccase peaks were obtained and the fractions contains resulting solution was subjected to estimate enzyme activity [11].

Effect of Temperature on Activity and Stability of Purified Laccase

The activity of the laccase purified was measured at different temperatures (25°c, 35 °c, 45°c, 50°c, 60°c, 70°c) using syringaldazine as substrate and to determine stability, the purified enzyme solution was incubated for 1 h and the remaining activity was determined.

Effect of PH on Activity and Stability of Purified Laccase

The effect of pH on the activity of purified enzyme was carry out by using different rang of pH: citrate phosphate buffer for pH (2.6–6.6) and carbonate buffer for pH (8-11), the pH stability was studied by incubating the enzyme with the same buffer for 1 h. Then the remaining activity was tested.

Results and Discussion

Pleurotus ostreatus was obtained and activated on potato dextrose agar (PDA) medium and then 5mm diameter of *P. ostreatus* inoculated on barely bran culture media under static conditions: incubation temperature:27°c , moisture ratio : 1:2 (w/v), incubation period 7 days with laccase activity 0.38 U/ml and specific activity 0.69 U/mg.

Optimum Conditions for Laccase Production

Optimum Moisture Ratio

Moisture ratio is factor to control the level of laccase production in SSF, 5gm of barley bran were humidified by distilled water with different moisture ratio (1:1, 1:2, 1:3, 1:4, and 1:5 w\v) the best moisture ratio was 1:4 W\V which gave maximum specific activity 0.78 U/mg compared to other ratios that were given less specific activity (Figure 1). Higher moisture leads to formation suboptimal product due to reduced mass transfer while decrease in moisture level results in reduced solubility and low presence of nutrients to the culture [12].

Inoculum Size

Different inoculum sizes were used to determine the optimum laccase production by *P. ostreatus*. The size of inoculum were ranged between 1-4, (5mm discs) results explain in figure (2) shows the laccase production was affected by the inoculum size and the production was increased slightly with increasing inoculum size to 3mm discs with specific activity 0.83 U/mg.

Increase discs of inoculum will ensure a rapid propagation and biomass synthesis but after long period the production of enzyme could be decreased because of consuming of the nutrients which leads to decreased in metabolic activity [13].When the level of inoculum increase above the optimum, the competition on the nutrients will reduce the yield of enzyme production [14].

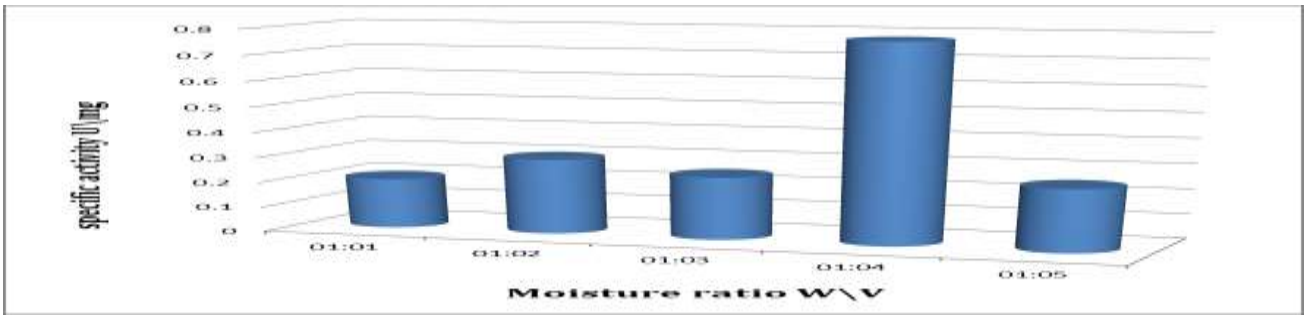

Fig. 1: Effect of Moisture ratio on laccase production by *P. ostreatus* using barly bran as substrate and incubated for 7 days at 27°c with 1 (5mm) inoculum size

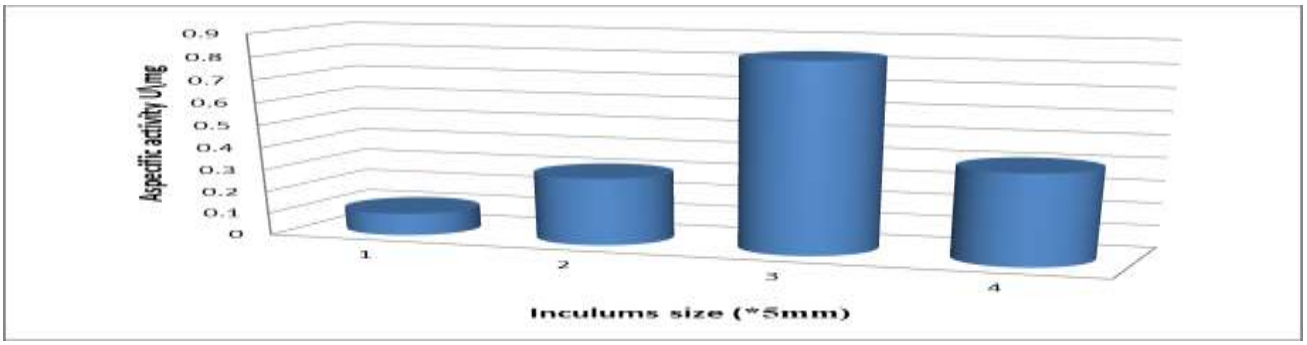

Fig. 2: Effect of inoculum size on laccase production by *P. ostreatus* using barly bran as substrate and incubated for7 days at 27°c, moisture ratio 1:4

Optimum Incubation Temperatures

To determine the optimum temperature for laccase production several incubation temperatures (20°c - 25°c - 30°c - 35°c - 38°c) were used. The highest specific activity of laccase was observed at 35c°, with specific activity 0.87U/mg (Fig. 3).

The temperature was affected on enzyme by effect on the solubility of oxygen in the media, and the speed of enzymatic reactions in the cell that reflect positively or negatively on enzyme production [15]. Laccase supernatant was produced at stationary phase of growth and the best temperature for production was 35°c.

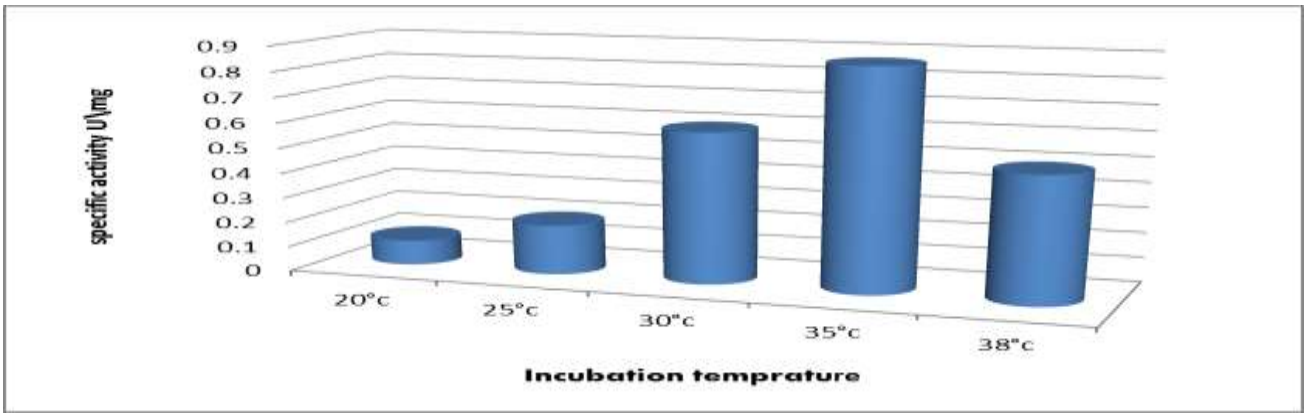

Fig. 3: Effect of incubation temperature on laccase production by *P. ostreatus* using barly bran as substrate and incubated for 7 days with 3 (5mm) inoculum size moisture ratio 1:4

Optimum Incubation Period

Pleurotus ostreatus was grown on barley bran for different incubation periods (4, 7, 14 and 22 days). Figure (4) shows the specific activity reached 0.92U/mg after 22 days up to of incubation. [16].

Showed the highest level of laccase production from *P. ostreatus* was occurred after 28 days by solid state fermentation. Dasai and Nityan, 2011 also reported that *P. florida and P. flabellatus* showed their optimum laccase activities were in the 26th day of culture [17].

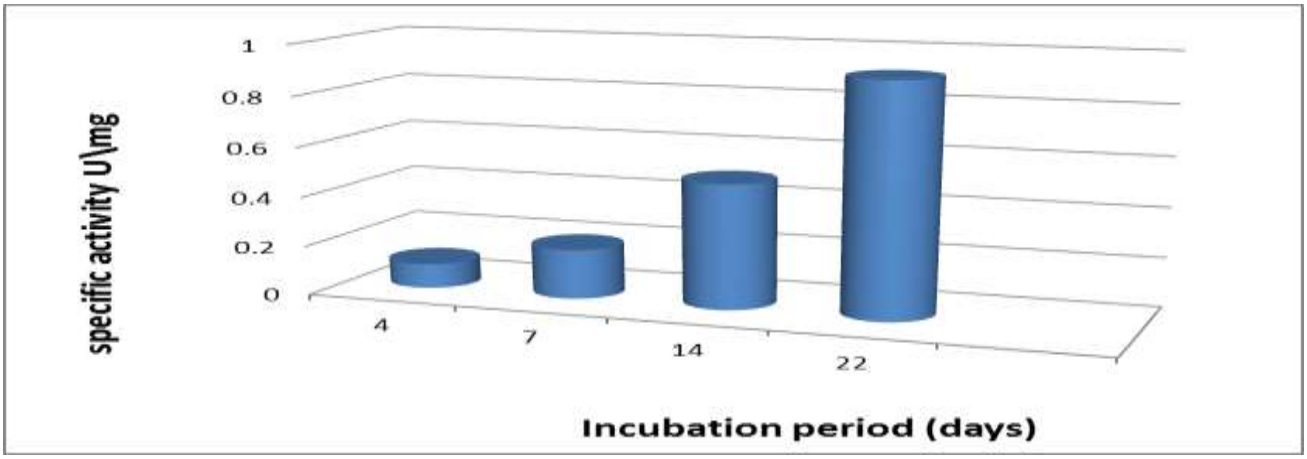

Fig. 4: Effect of incubation period on laccase production by *p. ostreatus using* barly bran as substrate and incubated at 27 °c with 3 (5mm) inculum size, moisture ratio 1:4, incubation temperature 35 °c

Purification of Laccase

The optimum culture conditions were used for the production of laccase from *P. ostraetus*. The isolate was cultured in SSF and supernatant of the culture was used for partial purification of laccase by ammonium sulfate precipitation in 80% saturation and dialysis step .The dialyzed laccase was utilized to DEAE cellulose column . The result of enzyme purification using DEAE-cellulose shows the specific activity was 0.7 U/mg with purification fold 1.2 and Yield reached to 24 as shown in table 1. Ion exchange chromatography has several features like high capacity, high resolution power, good separation which depending on charge differences tendency to concentrate the sample and relatively low cost [18]. Vantamuri and Kaliwal purified laccase from Marasmius sp. using ion exchange chromatography with specific activity of 0.226U/mg and a final yield of 13.5 % [19].

Table 1: Purification steps of laccase from *P. ostraetus*

Purification step	Volume ml	Enzyme Activity u\ml	Protein Concentration mg\ml	Specific Activity u\mg	Total activity	Purification fold	Yield (%)
Crude enzyme	100	0.658	1.0	0.658	65.8	1	100
Saturated Ammonium Sulfate (80%)	30	0.486	0.9	0.54	14.58	0.82	22
dialysis	10	0.274	0.7	0.39	2.74	0.6	4
Ion – exchange DEAE-cellulose	50	0.315	0.4	0.78	15.75	1.2	24

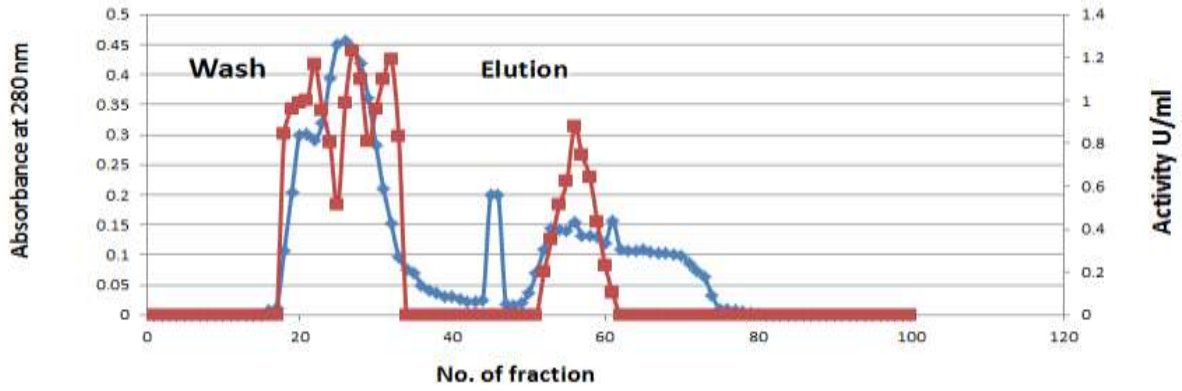

Fig. 5: Ion exchange chromatography for purification of laccase produced *by P. ostreatus* using DEAE-Cellulose column (23×1.5) cm and eluted with citrate phosphate buffer with (0.1 - 0.6M) NaCl

Figure 6 showed three peak of protein with high activity in wash step between (20-35) fraction but elution step shows 2 peaks of protein at fraction between (40-60) and these results indicated that the laccase carry positive charge. If the proteins have the same charge of the resin it will pass through the column without adsorbing while if the proteins with opposite charge it will be bound the concentration of Nacl salt buffer contribute to separate the bounded protein [20].

Effect of Temperature on Activity and Stability of Purified Laccase

Temperature is an important factor which affects enzyme activity. The suitable temperature for laccase activity may differ with different laccase sources the activity of enzyme was assayed at different temperatures values, ranged between 25°c-70°c.Results in figure 6 shows that 45 °c was the optimum temperature for laccase activity 0.847 U/ml, enzyme activity was decreased at less or a higher temperature.

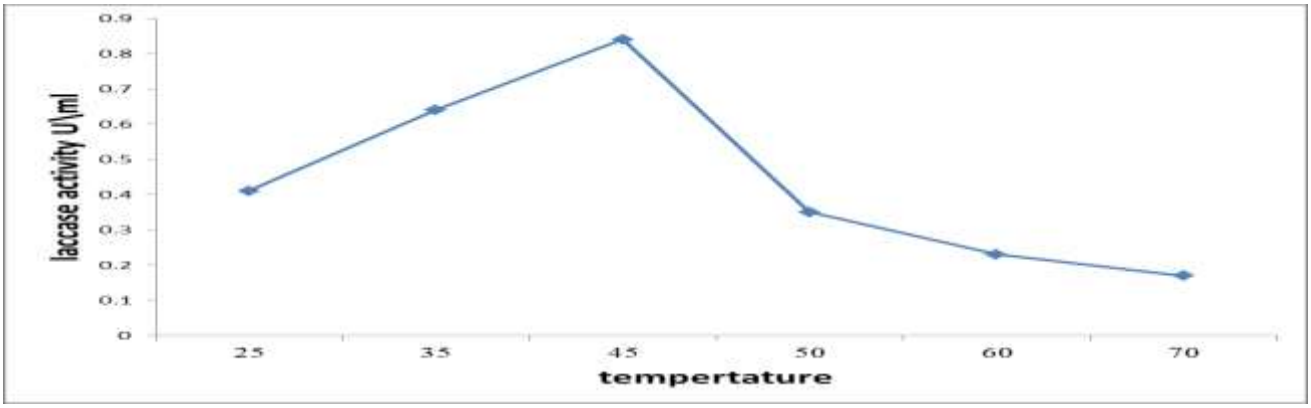

Fig. 6: Effect of temperature on the activity of purified laccase

The activity of enzyme rapidly lost at temperatures greater than its optimum In *P.flavido* – alba the laccase shown to have an optimum activity at 30°c [21].While, in *P. eryngii*, the highest activity was found at 50°c [22].Laccase remain stable in 45°c (Fig. 7). This thermal stability can be attributed to the carbohydrate moiety, which can stabilize the conformation of the protein through

hydrogen bound between the carbohydrates. In *P. cinnabarinus* laccase was very stable below 50°c but was inactivated at higher temperatures [23].

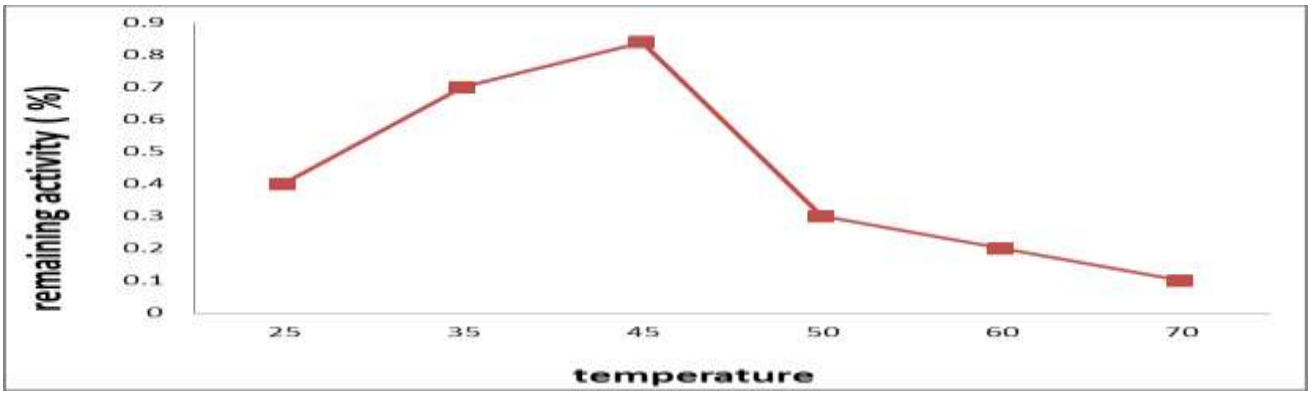

Fig. 7: Effect of temperature on stability of purified laccase

Effect of pH on activity and Stability of Purified laccase

The optimum pH of activity for *P. ostraetus* laccase was 5.6 with activity 0.831 U/ml for the oxidation of syringaldazine as seen in Figure 8.

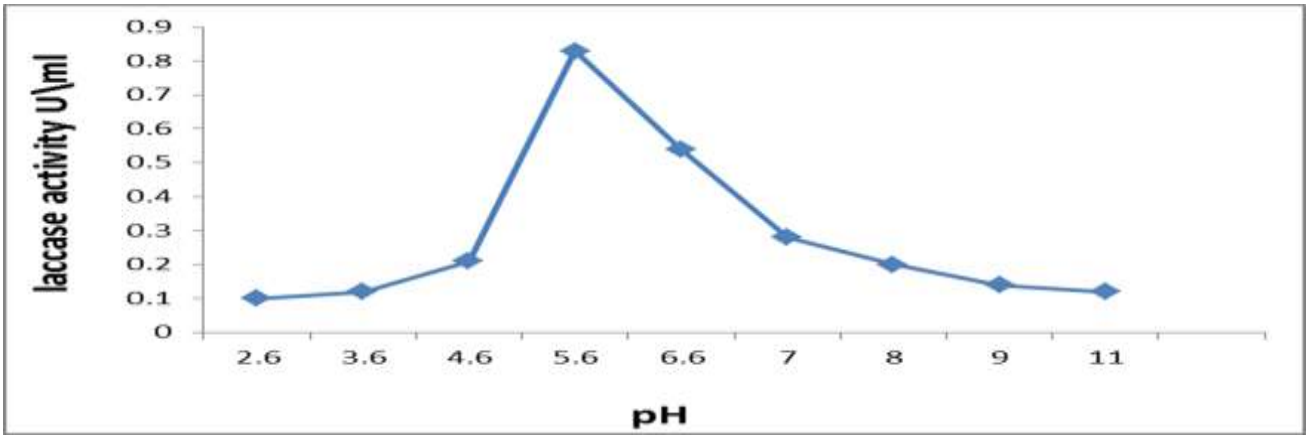

Fig. 8: Effect of pH on the activity of purified laccase

The optimum pH for activity depends on the substrat chosen for example *P.ostreatus* has optimum pH range 5.6 and 6.6 for syringaldazine and guaiacol [24]. The stability of laccase was also studied, the laccase was stable in pH 6.6 and rapid loss of activity higher than pH 6.6 was recorded as shown in figure 9. Typically laccases are stable from pH 4 to 7 for *P. ergynii* laccase was stable from pH3 to 10. The effect of pH on enzyme is due to change in the state of ionization of components of the system as the pH changes. Enzymes are sensitive to changes in hydrogen ion concentration. The extreme levels of hydrogen ion or pH low or high they may be denatured the enzyme. The acidic and basic side groups of enzyme effected through the change in pH this will alters the degree of ionization and. The neutralization of even one of these charges will change the catalytic activity of enzyme.

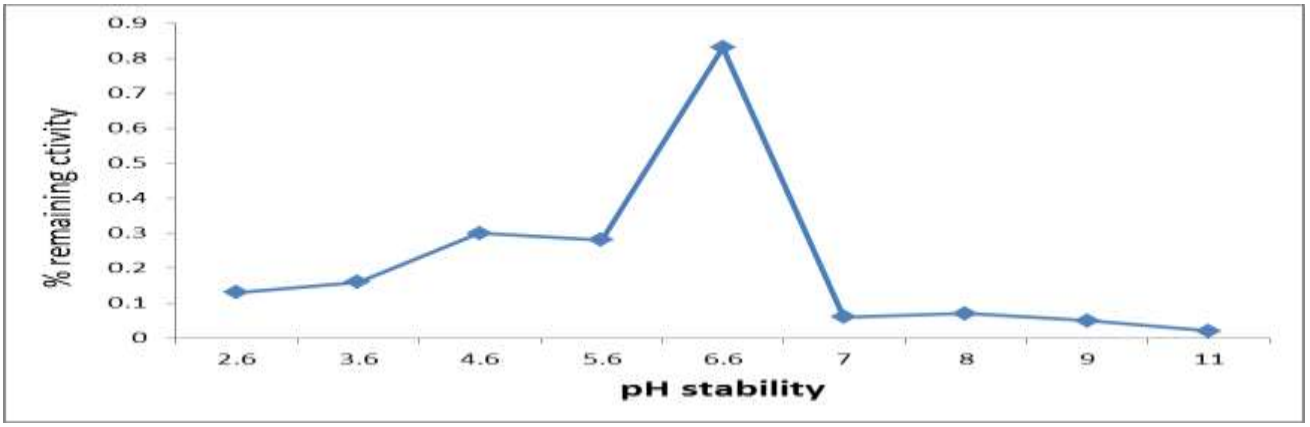

Fig. 9: Effect of pH on the stability of purified laccase activity

Conclusion

We concluded that the *Pleurotus ostreatus* is the best isolate to produce laccase enzyme in solid state medium Supported by barley bran and easily purified by ion exchange chromatography using DEAE cellulose. The optimum temperature for activity was 45°c and the laccase remain stable in 45 °c, while the optimum pH for activity was 5.6 and for stability was 6.6.

Conflicts of Interest

All the authors declare that they have no financial or nonfinancial competing interests.

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