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Optimization of Laccase Production from Marasimus Palmivorus

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Abstract. Laccase is an enzyme that has the ability to oxidize substances. It is one of those enzymes that has innate qualities of reactive radical generation, and its use in many domains has been overlooked due to its commercial unavailability. The ability of *Marasimus palmivorus* MG717877.1 to produce extracellular enzymes (Laccase enzyme) utilizing media containing substrate, named Guaiacol agar medium, was tested. In submerged culture, the ideal pH, incubation duration, and temperature for laccase synthesis were examined. The maximum enzyme activity was reported when the pH of the media was 5.5; laccase activity was (1.03U/ml). The maximum enzyme activity for laccase enzyme was 1.040 U/ml when the temperature was 25°, and (0.922U/ml) after the third day of incubation.

INTRODUCTION

The use of natural catalysts, such as enzymes, to accomplish chemical transformations of organic substances is known as bio catalysis. In bio catalysis, whole-cell generating enzymes or isolated enzymes are employed. The specificity of the biocatalyst is a major advantage of bio catalysis, since it can result in an increased rate of a single product [1]. New bio catalytic methods are dependent on the availability of novel, useful new enzymes, which are typically discovered by screening bacteria that perform the necessary target reaction. Exploration of harsh settings can yield unique microbial culture collections that can be utilized to screen for enzymes that can execute a specific bio catalytic process. These enzymes could then be employed as bioprocesses in biotechnologies that are relevant to industry [2]. Laccases are multicopper-containing enzymes that reduce molecular oxygen to water and belong to the polyphenol oxidases family [3]. This enzyme is an intensity of the color protein that catalyzes the oxidation of a wide range of organic and inorganic compounds using molecular oxgen as an electron acceptor [4]. Laccase prefers aromatic molecules with hydroxyl and amine groups, including diphenols, polyphenols, diamines, and aromatic amines, as a substrate [5]. The substrate oxidized only by laccase enzyme is syringaldazine [4-hydroxy-3,5-dimethoxy benzaldehyde azine] [6]. Laccases are produced by microorganisms, insects, higher plants, and fungi, among other living creatures. Only a few laccase enzymes from bacteria have been isolated and described. The initial inquiry on this topic was the bacterial laccase, which was produced by the rizospheric bacterium Azospirillum lipoferum [7]. Bacterial laccases have received increased attention in recent years as a result of their capacity to overcome the limitations of instability when compared to fungal laccases. At high temperatures and pH conditions, they are extremely active and much more stable. Bacterial laccases have evolved into an important industrial enzyme that is used in a variety of processes, including the detoxification of industrial effluents, primarily from the paper and pulp, textile, and petrochemical industries, as well as a diagnostic tool, a cleaning agent for certain water purification systems, and a catalyst for the production of anticancer drugs. In cureent study, the objective of this research was to screen laccase enzyme production in *M. palmivorus* MG717877.1 and establish the best conditions.

MATERIALS AND METHODS

Fungal Strains

The advanced / Mycology / Department of Biology / College of Science / University of Babylon provided *Marasimus palmivorus* (MG717877.1).

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Culture Media

PDA (potato dextrose agar): This media was made following to the instructions of an Indian company (HEMIDIA) by dissolving 39 grams of medium in 1 liter of distill water, mixing carefully, and then adding 250 mg/ml chloramphenicol. It's then autoclaved(Labtech/ Korea) for 15 minutes at 121°C, then cooled to 45°C and put into sterile petri dishes.

Laccase Production Medium

This medium was made with the following modified medium components (g/l): urea, 0.14; sucrose, 2.0; yeast extract, 0.34; MgSO4 7H2O, 0.07; CaCl2 2H2O, 0.004; NiSO4 7H2O, 0.003; KH2PO4, 0.1; and Na2HPO4, 0.3) in a liquid culture media. For the production of urease, sequential optimization studies were conducted using various organic nitrogen sources (yeast extract, beef extract, meat extract, peptone, soybean meal, and tryptone, 0.5–2.0 percent), inorganic nitrogen sources (NH4NO3, (NH4)2SO4, NH4Cl, KNO3, and NaNO3, 0.5–1.0 percent), and carbon sources (glucose, fructose, sucrose, maltose, lactose, and starch, After adjusting the pH of this media to 5.0, fifty mL were dispensed into a 250 Erlenmeyer flask and autoclaved [8].

Primary Screening for Laccase Producing Fungi

Primary Screening Using Guaiacol

The formation of reddish-brown zones in the media indicated a favorable result when agar disk (5mm) plugs of species were cultured on production medium for seven days at 25 °C [9].

Producing laccase enzyme in liquid media (secondary screening)

These reactions were revealed by putting 50 mL of enzyme production media into 250 mL Erlenmyer flasks and inoculating each of the investigated fungal isolates with a 5mm agar disc plug. After a 7-day incubation period at 28°C with a rotary shaker at 150 rpm. Gauze was used to extract laccase-containing culture fluid from mycelium, which was then centrifuged for 15 minutes at 6000 rpm. Enzyme activity was estimated using the procedure given by [10].

Determination of laccase activity

The enzymatic activity was determined by detecting the oxidation of Guaiacol for 3 minutes at 525nm. 1ml crude enzyme filtrate, 1.5 ml guaiacol(1mM), 2ml (0.1M) citrate-phosphate buffer (pH5) [11]. the following formula was used to calculate laccase activity:

$$\text{Enzyme activity U/ml} = \frac{0.1 * \text{Time} * \text{volume (crude enzyme)}}{\text{O.D}}$$

Optimization Conditions for Enzyme Production (Laccase Enzyme)

Incubation Period

The preparation media was produced and poured into four flasks (volume 250 mL), each comprising 100 mL. Taking a 10mm disc from the fungi's margins was used to inoculate the production media (*Marasimus palmivorus*, MG717877.1). Then it was incubated for 5 days at 27°C (120 hours). Every 24 hours, samples were taken and enzyme activity was measured.

pH

In 24 flasks, the production media were prepared (twelve flasks for each isolate, volume 250 ml, each flask containing 100 ml). By taking a disc (10mm) from the margins of colonies *(M. palmivorus* MG717877.1), growth media with various pH (3.5, 4.5, 5.5, 6.5, 7.5, and 8.5) were inoculated and incubated at 27°C for 96 hours (2 replicates for each isolates). Samples were obtained and enzyme activity was estimated.

Temperature

24 flasks were used to prepare the production media (twelve flasks for isolate, volume 250 ml, each flask comprising 100 ml). Media were containing (pH 5.5 for *M. palmivorus* MG717877.1) for different temperature (10,15,20,25,30 and 35) disc (10mm) taken from the edges of isolate and used the same method that used in PH.

RESULTS AND DISCUSSION

Primary and Secondary Screening of Laccase Producing Fungi

Laccase-producing fungi were detected using a simple screening procedure on solid media containing 0.02 percent guaiacol as substrate. Fungal species *(M. palmivorus* MG717877.1) was tested and found to be positive when a reddish-brown zone appeared around colonies (figure.1)

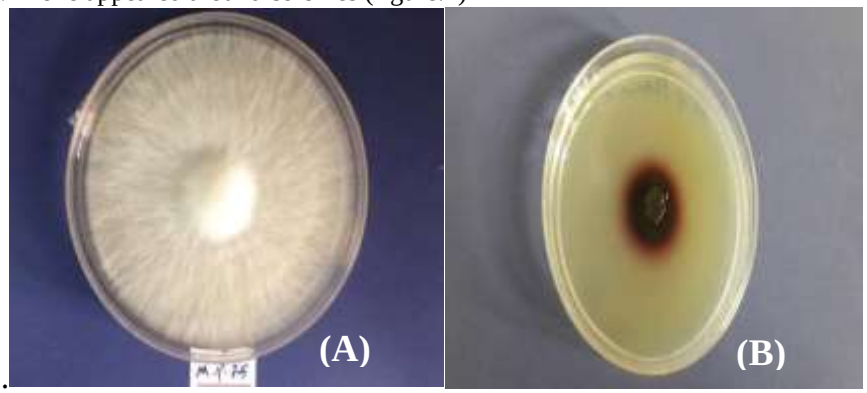

FIGURE 1. Primary screening of laccase producing fungi (A: *M. palmivorus* as control negative (B) M. palmivorus with substrate).

The oxidative polymerization of guaiacol resulted in the appearance of a reddish-brown zone in the medium [12]. On a solid media comprising 0.5 percent substrate for enzyme(guaiacol), 12 fungal species of white rot fungi generated a reddish-brown hue, whereas 6 fungal species of brown rot fungi formed a very light reddish-brown color and seven isolates gave a negative reaction, according to [13]. Using 0.02 percent guaiacol, twelve isolates of white rot fungi were evaluated. *M.palmivorus* MG717877.1 was chosen as the strain to improve laccase production based on these findings. According to [14], the delicious fungs *P. sapidus*, which grows on liquid medium, was chosen since the most active oxidation appeared only with six fungal isolates . Submerged culture was used to assess this fungal isolate's ability to produce laccase. The activity of enzymes was measured using Guaiacol as a substrate. *M.palmivorus* MG717877.1 had the highest laccase production (0.166U/ml), according to these findings. Basidiomycota are laccase producers. These findings are consistent with our research. Figure (2)

FIGURE 2. Production of laccase enzyme using liquid media containing 0.02% guaiacol as indicator compound (A: M. palmivorus as control negative B: M. palmivorus with substrate).

Optimization for Laccase Enzyme Production

Determination of the Optimum Hydrogen Concentration (pH)

M.palmivorus MG717877.1 was cultured on submerged liquid medium with various pH values to explore the influence of the primary medium pH on laccase synthesis (3.5, 4.5, 5.5 ,6.5,7.5, and 8.5). The maximum enzyme activity was found at pH of this media was equal to 5.5; laccase activity was (1.03U/ml), as shown in figure (3). The influence of (PH) can be characterized by two factors: the first is its ability to affect medium characteristics such as nutrient solubility and transportation, affecting nutrient availability to the growing microorganism, and the second is the effect of pH on enzyme ionizable group, affecting the stability of the enzyme . Over a given pH range, enzymes are known to be active. This could be due to the fact that changes in pH can cause the enzymes' three-dimensional structure to shift [15]. [16]. found that effective laccase yield by *P. ostreatus* was showed in a pH range of (5.0–5.5) in submerged culture, while [17], discovered that the good pH for growth fungi and laccase activities in Schizophyllum commune was (5.5), all of which are consistent with the current study. The pH variation is an important factor for the improvement of laccase quantity. Fungal laccase exhibited higher stability in acidic pH (pH 4–6/3.6–5.2) to promote the catalytic efficiency. The mobility of polypeptide chain increased electrostatic interactions at pH 3.0[18]. Low pH causes the unfolding of protein due to accumulation of hydrophobic surfaces. This was a logical hint to loss the laccase activity. The maximum laccase quantity in a shorter period is advantageous in industrial applications, while fungal species require longer period for laccase production. In the same way, researchers prefer 14 to 20 days for maximum laccase secretion from wood rotting fungi [19].

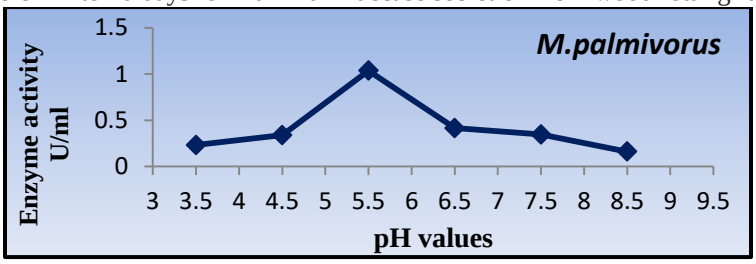

FIGURE 3. Effect of pH in laccase production from M.palmivorus

Determination of the Optimum Temperature

Different incubation temperatures were employed to examine the function of temperature on the generation and activity of laccase (10, 15, 20, 25, 30, and 35°C) in order to analyze the role of temperature on the growth and metabolism of microorganisms. The current experiment **(**Figure 4) investigated the influence of various temperatures on laccase production. Maximum enzyme activity for laccase enzyme 1.040 U/ml for *M. palmivorus* MG717877.1 was obtained at 25˚C. The temperature effect is important in the production of the enzyme from microorganisms through their effects in the solubility of oxygen in the medium, kinetic energy and the speed of enzymatic reactions. The results are consistent with the majority of prior research that have been conducted to illustrate the influence of temperature on enzyme production. [20] were found that maximal enzyme production was seen at 28°C in *Marasmiellus palmivorus*, while 30°C produced more laccase in *Pleurotus osteatus* [21]. The results obtained are comparable to those obtained by [22] who reported maximal laccase production by *Marasmium sp.* under solid state fermentation at temperatures ranging from 25 to 31 degrees Celsius. Fermentation procedures (SSF and SmF) are normally developed with mesophilic microbial strains. Scytalidium lignicola is a mesophilic organism, thus its growth temperature is between 20 and 40 degrees Celsius. This had an effect on the enzyme's production, either positively or adversely.

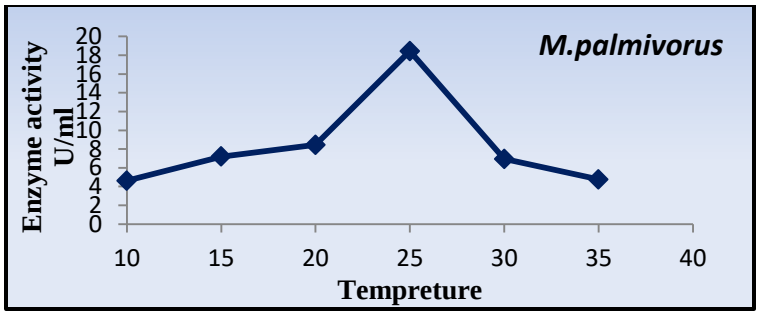

FIGURE 4. Effect of temperature in laccase production from M.palmivorus

Determination of the Optimum Incubation period

M.palmivorus MG717877.1 was cultured on laccase production medium for various incubation durations in order to determine the best incubation period for laccase synthesis (24,48,72,96,120, and 144 hr). The optimum incubation duration was 96 hours, and during that laccase activity reached (0.922U/ml), but after 120 hours, enzyme activity began to fall sharply, reaching (0.46875U/ml). According to [23], the best level of enzyme production for *M.palmivorus* MG717877.1 was obtained after 96 hours, while the optimum laccase production for the same strain occurred after 72 hours [24]. The optimal laccase synthesis by *Ganoderma sp* was seen after 10 days of incubation, according to [5]. These results was showed in (Figure 5).

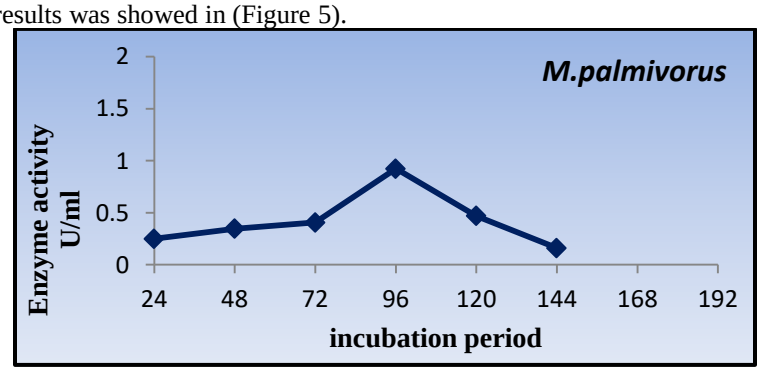

FIGURE 5. Effect of temperature in laccase production from M. palmivorus

CONCLUSION

The primary and secondary screening methods were utilized to identify factors that were used in optimizing laccase production by *Marasmiellus palmivorus* under sold state fermentation, and this study contributed in the knowledge of some of the factors that were used in laccase enzyme optimization.

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REFERENCES

1. Burton, S. G. (2003). Laccases and Phenol Oxidases in Organic Synthesis – a Review. Current Organic Chemistry. 7:1317-1331.
2. Patrik, F., Mtui, G., Mshandete, A.M., Johansson, Livaisi A. 2009. Purification and characterization of a laccase from the basidomycete *Funalia trogii* (Berk.) isolated in Tanzania. African J. Biochem. Research. 3(5),250-258.
3. Elshafei, A.M.; Hassan, M.M.; Haroun, B.M.; Elsayed, M.A. and Othman A.M. (2012). Optimization of laccase production from *Penicillium martensii*

4. Imran, M.; Asad, M.J.; Hadri, S.H and Mehmood, S. (2012). Production and industrial applications of laccase enzyme. J Cell Mol. Biol, 10(1), pp:1-11. NRC345. Advances in Life Sciences, 2(1), 31-37.
5. Sivakumar, R.; Rajendran, R.; Balakumar, C. and Tamilvendan, M. (2010). Isolation, Screening and Optimization of Production Medium for Thermostable Laccase Production from *Ganoderma sp*. Int.J.Eng. Sci, 2(12), pp:7133-7141.
6. Thurston, C. F. (1994). The structure and function of fungal laccases. Microbiology, 140: 19-26.
7. Verma, A. and Shirkot, P. (2014). Purification and Characterization of Thermostable Laccase from Thermophilic *Geobacillus thermocatenulatus* MS5 and its applications in removal of Textile Dyes. Sch. Acad. J. Biosci. 2(8): 479- 485.
8. Birhanli, E. Selim Erdogan, Ozfer Yesiladaa, Yunus Onal, Laccase production by newly isolated white rot fungus Funalia trogii: Effect of immobilization matrix on laccase production, Biochemical Engineering Journal 71 (2013) 134–139.
9. Viswanath, B.; Chandra, M. S.; Kumar, K. and Reddy, B.R. (2008a). Production and Purification of Laccase from *Stereum ostrea* and its Ability to Decolorize Textile Dyes. Dynamic Biochemistry, Process Biotechnology and Molecular Biology 2 (1), 19-25.
10. Sidhu, A.K., Agrawal, S.B., Sable, V., S., Patil, S.N., Gaikwad, V.B., 2014.Isolation of *Colletotrichum gloeosporioides gr.*, a novel entophytic Laccase -producing fungus from the leaves of a medicinal plant, *Piper betle.* Int. J. Scientific and Engi. Research 5(2)1087-1098.
11. Jhadav, A. *et al.* Optimization of production and partial purification of laccase by *Phanerochaete chrysosporium* using submerged fermentation. *Int. J. Microbiol Res.* **1**(2), 09–12 (2009).
12. Mabrouk, A.M.; Kheiralla, Z.H.; Hamed, E.R. and Youssry, A.A. (2010). Screening of some marine-derived fungal isolates for lignin degrading enzymes (LDEs) production. Agric. Biol. J. N. Am., 2010, 1(4): 591-599.
13. Artiningsih, T.; Tuah, S.J.; Mirmanto, E. and Osaki, M. (2000). Survey of peat soils in sebangau-sahayan water-catchment, central kalimantan for laccase producing fungi and their organic decomposing Ability. Hokkaido University & indonesian institute of sciences pp. 61-67.
14. Linke, D.; Bouws, H.; Peters, T.; Nimtz, M.; Berger, R. and Zorn, H. (2005). Laccases of *Pleurotus sapidus:* Characterization and Cloning. J.Agric. Food Chem. 53: 9498-9505.
15. Pclczar, M.J.; Chan, E.C.S and Kring, N.R. (2004). Microbiology. 5th Ed, Tata McGraw-Hill Publishing Co. Ltd, New Delhi, India.
16. Prasad K K,Mohan S V, Rao R S,Pati B R& Sarma P N (2005), laccase production by plerotus ostreatus 1804, Optimization of submerged culture condition by Taguchi DOE methodology. Biochem Eng J,24(1),17.
17. Adejoye, O. D. and Fasidi, I. O. (2009). Effect of culture conditions on biomass and laccase production in submerged medium by *Schizophyllum commune* (FR.), a Nigerian edible mushroom. Elec. J. Envi., Agri. Food Chem., 8: 1186 1193.
18. Wang, H., Tang, C., Yu, G. & Li, P. A novel membrane-surface liquid co-culture to improve the production of laccase from *Ganoderma lucidum*. *Biochem. Eng. J.* 80, 27–36 (2013).
19. Wehaidya, H. R., Heba, M. E., Samia, A. A. & Mohamed, A. A. Comparative study on crude and partially purified laccase from *Polyporus durus* ATCC 26726 in the decolorization of textile dyes and wastewater treatment. *Egypt. Pharm. J.* **17**, 94–103 (2018).
20. Chenthamarakshan, A., Parambayil, N., Miziriya, N., Soumya, P.S., Laksmi, M.S.K., Ramgopal, A.,Dileep, A., Nambisan, P.,. Optimization of laccase production from *Marasmiellus palmivorours* LA1 by Taguchi method of design of experiments.BMC Biotechnol.17:12. 2017
21. Nadeem, A., Baig, S., Sheikh, N.,.Mycotechnological production of laccase by *Pleurotus ostreatus*-P1 and its inhibition study.J.Ani.Plant Sci.24(2),492-502. 2014.
22. Risdianto, H., Suhardi, S.H., Setiadi, T., Kokugan, T., 2010.The influence of temperature on laccase production in solid state fermentation by using white rot fungus *Marasimus* sp.The 1st International Seminar on Fundamental and Application of Chemical Engineering. A009:1-6.
23. Medeiros, M.B.; Bento, A.V.; Nunes, A.L.L. and Oliveira, S.C. (1999). Optimization of some variables that affect the synthesis of laccase by *Pleurotus ostreatus*. Bioprocess Engineering 21:483-487.
24. Palmieri, G; Giardina, P; Bianco, C; Fontanella, B; Sannia, G. (2000). Copper induction of laccase isoenzymes in the ligninolytic fungus *Pleurotus ostreatus*. Appl Environ Microbiol 66(3): 920_924.