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Research Article

EFFECT OF NUTRITIONAL AND ENVIRONMENTAL CONDITIONS ON PRODUCTION OF EXTRACELLULAR LACCASE UNDER SUBMERGED CULTURE CONDITIONS IN *LENTINUS KAUFFMANII*

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ABSTRACT

The production of laccase by an indigenous strain of *Lentinus kauffmanii* was studied on submerged fermentation. The physical parameters namely, pH, Temperature, the nutritional parameters like suitable carbon and nitrogen sources, metal ions were studied for the higher enzyme production. The optimum pH for the both biomass and laccase production was found to be pH 5.5. Of the different temperature (20°C, 25°C, 30°C, 35°C and 40°C) tested for the optimal laccase production 30°C showed the maximum activity. Among the different carbon sources tested fructose supported maximum (63.51 U/ml) laccase activity, while beef extract supported the maximum activity (64.31 U/ml) among the different nitrogen sources tested. Of the different metal ion tested, copper sulphate enhanced the maximum enzyme activity (75.6 U/ml). The above result indicates that the *L. kauffmanii* can be used as a biotechnological tool.

Keywords: Laccase, *Lentinus kauffmanii*, Physical parameters, Nutritional, Metal ions

INTRODUCTION

Laccases are mostly extracellular, glycoproteins, multinuclear enzymes with molecular weight between 60 and 80KDa. They contain 15-30% of carbohydrates and usually has as acidic iso-electric point. Substrate oxidation by laccase is a one-electron reaction generating a free radical, which usually reacts further through non-enzymatic route. Bourbonnais and Paice[1] have shown that the artificial Laccase substrate ABTS [2,2-azinobis-(3- ethyl benzthiozoline 6-sulphate)] has the capacity to act as mediator enabling the oxidation of non-phenolic lignin model compounds that are not laccase substrates on their own. Current developments in biotechnology are yielding new applications for enzymes. A large number of micro-organisms, plants, roots, tubers, including bacteria, yeast and fungi produce different types of enzymes. One such enzyme known to play a major role in many industrial processes is Laccase (E.C. 1.10.3.2 benzenediol: oxygen oxidoreductase).

Lentinus kauffmanii (A.H. Smith) is an edible mushroom which belong to the phylum basidiomycetes, order agaricales, family Lentinaceae. The fungus usually grows abundantly during the raining season and frequently appears on dead wood. Fungal biomass have been found to be important for several purposes such as process reduction in fermentation technology, food or protein supplement and extraction of metabolites such as polysaccharides and enzymes [2].

The majority of laccase characterized so far have been derived from fungi, especially from white rot basidiomycetes that are efficient lignin degraders. Well known laccase producers includes the fungi such as *Trametes vesicolor, Pleurotus sajor-caju, P. eryngii, Cerrena unicolor*, *Panus rigrinus, Pycnoporous sanguineous*[3,4,5,6,7,8].

In basidiomycetes, extra cellular laccases are constitutively produced in small amounts, and they are affected by many typical fermentation factors such as medium composition, carbon and nitrogen ratio, pH, temperature aeration rate etc[9]. Culture condition and medium composition also play a major role on enzyme expression. Addition of nitrogen to certain fungal cultures increased the degradation of lignin related compounds. Buswell et al.,[10] reported that the production of lignin modifying enzymes is strongly affected by the nature and amount of nutrient especially nitrogen and trace element in the growth medium.

Laccase has been marketed by several companies, although the current prices seen too high for bulk environmental application[11]. Culture condition and medium composition also play a major role on enzyme expression. Physiological demands vary among white rot fungi and considerable research has been done on the influence of agitation, pH, temperature, carbon source, nitrogen sources, and metal ion level. Hence in the present study the test fungus was studied for the optimization of physiological factors and nutritional factors are used for the higher laccase production.

MATERIALS AND METHODS

Organism and inoculum preparation

Fruiting body of the *L. kauffmanii* was isolated from Keeriparai forest of Western Ghats, Tamil Nadu, India, and the culture was maintained on malt-extract agar medium at 4°C. Inoculum of *L. kauffmanii* was prepared from mycelia grown on the same media incubated at 25°C for 4–6 days. From the plate 7 mm diameter mycelial disc were used as the inocula.

Selection of medium for laccase production

The white-rot fungus was grown in a chemically defined medium composed of KH_2PO_4 (1 g L^{-1}), $MgSO_4.7H_2O$ (0.5 g L^{-1}), yeast extract (1 g l^{-1}), $CaCl_2.2H_2O$ (0.14 g1^{-1}), glucose 10g and thiamine (0.0025 g L^{-1})[12]. Incubation was carried out statically at 25 ± 1°C in 125 ml Erlenmeyer flask containing 50 ml of the medium inoculated with 7mm agar plug from 6 day old mycelia grown on malt-extract agar. Periodic harvesting of the mycelia was performed using the filter paper. A aliquots of supernatant was collected aseptically and culture filtrates were used as enzyme sources.

Screening of nutritional factors for increasing laccase activity

After the selection of the best medium for laccase activity, five carbon, nitrogen and metal ion compounds were investigated for their capacity to increase enzyme activity in *L. kauffmanii*. One control treatment was used without the addition of any putative inducer. Various carbon sources such as fructose, sucrose, mannitol, lactose, starch; nitrogen sources such as beef extract, peptone, ammonium chloride, ammonium nitrate and ammonium tartarate; metal ions such as manganous sulphate, aluminium oxide, sodium sulphate, zinc sulphate and copper sulphate were used. Optimization of physiological parameters such as pH (4.0–8.0) and temperature (20–40°C) were carried out. All chemicals used in this research were of analytical grade and were used without further purification.

The compounds were sterilized by filtration using a Millipore membrane (0.45 µm) and added aseptically into the flasks. Alternative days (3-21) after inoculation the laccase activity was determined.

Laccase activity assessment in the filtrate

The laccase activity was determined by measuring the oxidation of 2,2'-azino-bis 3- ethylbenzothiazoline-6-sulphoric acid (ABTS) (Sigma). Increase in absorbance in 3 minutes was measured spectrophotometrically at 420nm[13]. The reacting mixture contained 100µl of 50mM ABTS and 800µl of 20mM Na-acetate buffer (pH4.5) and 100µl of approximately diluted enzyme extract. One unit of enzyme activity is defined as the amount of enzyme that oxidized 1µM of substrate per min. One unit of enzyme is the amount of enzyme required for oxidising 1 mmol ABTS per minute.

The experiments were performed at least two times using three replicates. The data presented in the figures correspond to mean values with three replicates.

RESULTS AND DISCUSSION

There was significant differences in fungal mycelial biomass yield and extracellular laccase activity for all the cultural condition studied. Growth of the fungus was initiated after 3 days of inoculation. Results from this study showed that as the incubation temperature increased, fungal mycelial biomass yield and laccase activity decreased. Fungal biomass yield and activity of laccase was analyzed in alternative days (3 to21) of incubation. Laccase production by fungi has been shown to depend markedly on the composition of the culture medium, carbon, nitrogen content and metal ion compounds[9].

Effect of various pH

pH is one of the important parameter in fungal cultivation. Various pH (4.0-8.0) of the laccase production medium were adjusted with pH meter. Optimal pH for maximum laccase production (56.7U/ml) was observed at 5.5 in 17th day of incubation. Exponential increase in laccase activity was observed from pH 4.0-5.5 but there after laccase production was decreased with increase in pH (Fig 1). This may be attributed to the fact that change in pH may alter the three dimensional structure of the enzymes[14]. A pH between 4.5- 5.0 has been shown to be optimal for laccase activity with marked suppression above 5.5 and below 3.5 [15, 16].

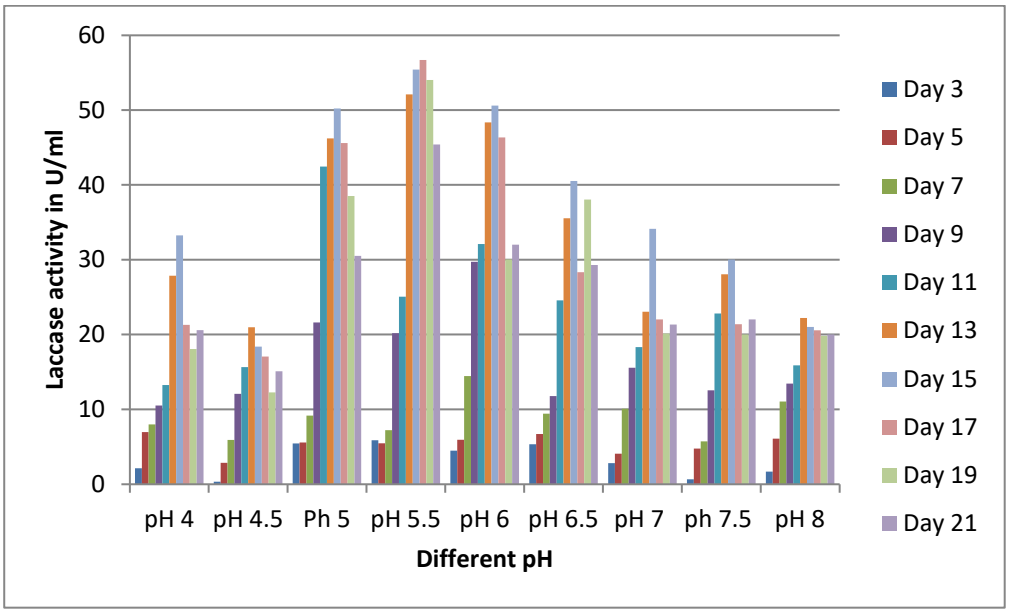

Fig. 1: Effect of different pH on production of Laccase

Results from this study showed that pH value of 5.5 was found to be the optimum for biomass production and laccase activity; however, biomass production and laccase activity was also recorded at pH 4.0 and 8.0. This may be attributed to the fact that change in pH may alter the three – dimensional structure of the enzymes[17]. Safari *et al.* [18] also reported that culture pH is an index of fungi enzyme activity; wherever the pH was low, fungi activity was high. For these reasons, enzymes are known to be active over a certain pH range.

Effect of different Temperature

Temperature is of much significance in the liquid state, even though the impact of temperature is more prominent in the scale up processes, it remains an inevitable factor in all systems due to its impact an microbial growth and enzyme production. Result of the present study showed that the temperature of 25^{o}C was optimum for laccase production (53.4U/ml) and no considerable activity was observed at any of the other temperatures considered (20, 30, 35, 40ºC). The culture of *Cyathus bulleri* showed maximum laccase production at 30ºC[19]. (Fig 2)

The optimal temperature of laccase production is been reported to differ greatly from one strain to another[20]. In general fungi are cultivated at temperatures between 25°C and 30°C for optimal laccase production[21, 22]. When cultivated at temperatures higher than 30°C the activity of the ligninolytic enzyme was reduced [23].

Biomass production and laccase activity was observed to be high at temperature 25-30°C, with temperature 25°C as the optimum. Biomass production and laccase activity of this fungus was not favoured by low nor high temperatures. Similar observations were made by Gbolagade *et al.* [24] on biomass production of *Pleurotus florida*. Zadrazil *et al.* [23] reported that temperatures higher than 30°C reduce the activity of ligninolytic enzymes.

Effect of different carbon and nitrogen sources

The effect of conventional organic carbon and nitrogen sources on adaptation of the fungus for the production of laccase is of importance. Many previous studies have proved that both the nature and concentration of nitrogen sources are powerful nutrition factors regulating ligninolytic enzyme production by wood-rotting basidiomycetes[25]. (Fig 3)

Among the different carbon sources that were tested for biomass production and laccase production, fructose stimulated the highest (63.51 U/ml) laccase production. Mansure et al., [26] showed that the use of fructose instead of glucose resulted in a 100 fold increase in the specific laccase activity of basidiomycetes. Increased enzyme activity in media containing these simple sugars can be explained by the high production rate of secondary metabolites when their producing organisms grow in complex media[27]. Leifa *et al.* [28] reported that monosaccharide in general were better than sugar alcohol and complex sugar (cellulose) for biomass production. This was probably due to ease in polymerization and their simple nature. Vahidi *et al.* [29] reported increased antifungal activity of *M.*

leptocephoda at pH 5.5 and 25°C when simple sugars were used as carbon sources. (Fig 4)

The ligninolytic enzymes have been seen to be regulated by the usable concentration of nitrogen in the medium. The low nitrogen level can stimulate the ligninolytic enzyme production, whereas high nitrogen levels repress it[30]. It has been reported[31] that a higher yield of laccase can be obtained using nitrogen rich media rather than nitrogen limited media.

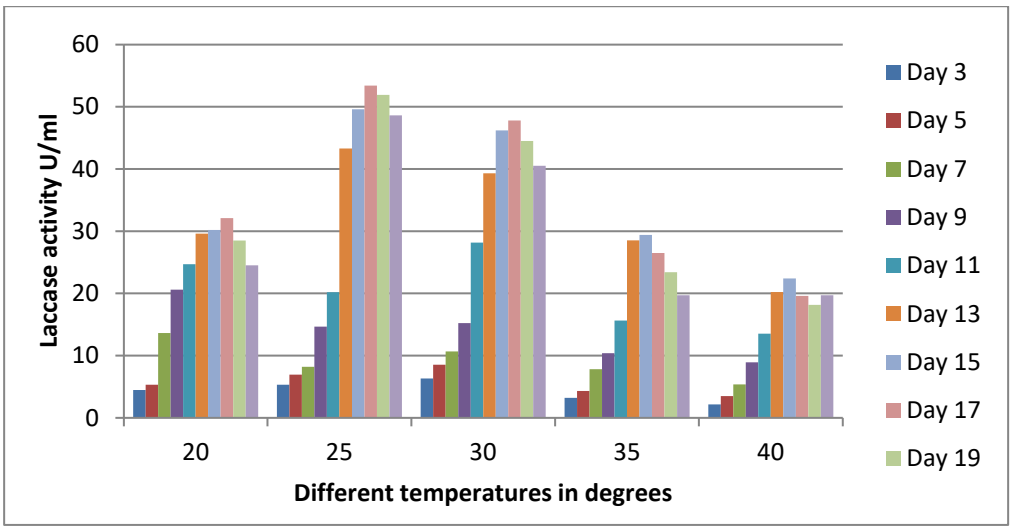

Fig. 2: Effect of different Temperature on production of Laccase

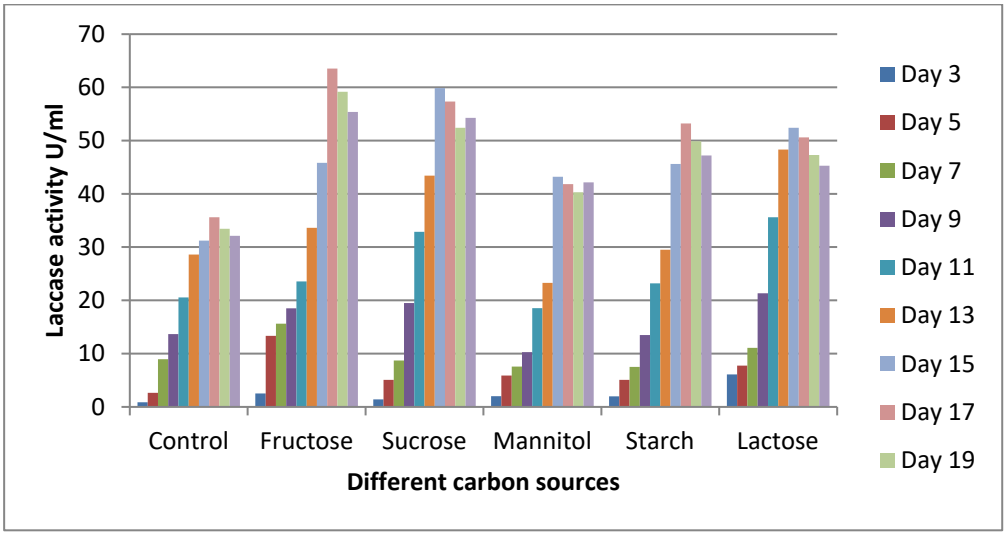

Fig. 3: Effect of different carbon sources on production of Laccase

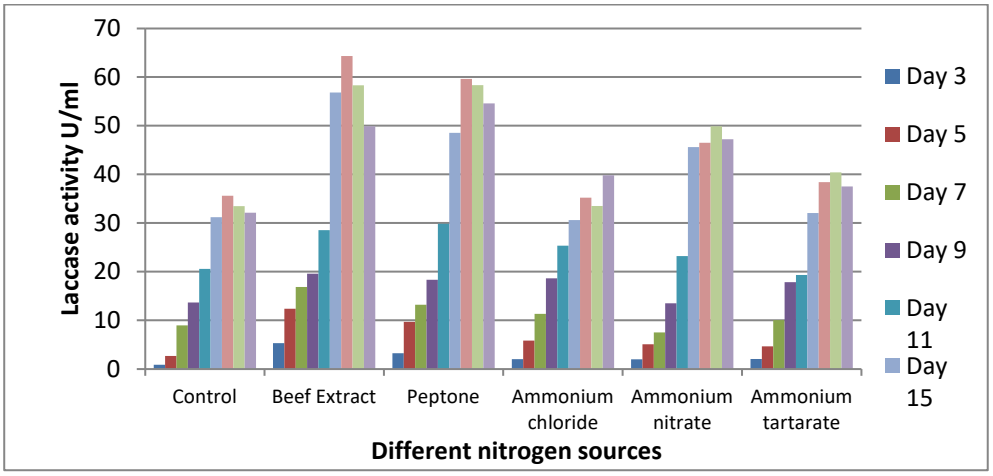

Fig. 4: Effect of different nitrogen sources on production of Laccase

Fungi mycelia biomass production and laccase activity was also obtained in submerged cultures in the study containing different complex nitrogen sources. The differences in nitrogen sources requirements may suggest that biomass production in different fungi may be influenced by different nutritional requirements. Among different complex nitrogen sources used, beef extract stimulated higher (64.31 U/ml) production of laccase while the inorganic nitrogen sources stimulated the least fungi biomass yield and laccase production. These results are in agreement with the findings of Vahidi *et al.* [29] who reported increase in antifungal activity when yeast extract was used as nitrogen source and decrease in growth and fungi activity when media containing NH_4Cl and $NaN0_3$ were used as growth medium.

Effect of different metal ions

Copper as micronutrient has a key role as a metal activator, induces both laccase transcription and plays an important role in laccase production[32]. Among the five metal ions tested copper sulphate showed maximum (75.6 U/ml) laccase production on 15th day of incubation. At maximum laccase production no further increase in fungal biomass was observed, since production of ligninolytic enzymes occurs during their secondary metabolism of the growth phase[33]. A possible explanation for this stimulatory effect of copper on laccase biosynthesis could be a role for this enzyme activity in terms of a defense mechanism against oxidative stress, with laccase involved in the synthesis of pigments to prevent the uptake of metals[34]. Galhaup et al [35] and Stajic et al., [36] reported that the addition of copper sulphate in various concentrations stimulates laccase production in *T. pubuscens, P. eryngii* and *P. ostreatus*. (Fig 5)

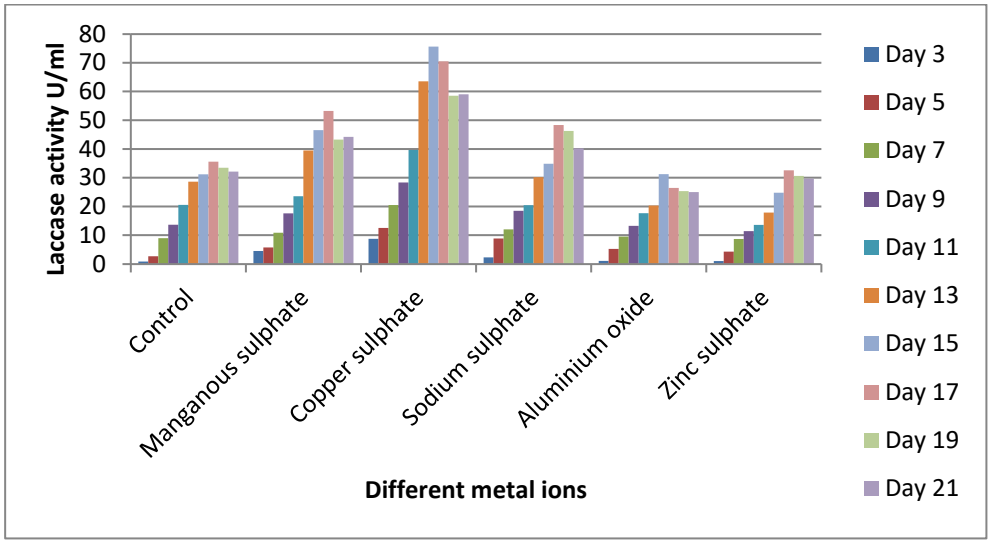

Fig. 5: Effect of different metal ions on production of Laccase

CONCLUSION

The present study which was deal with the isolate *L. kauffmanii* is found to be a very efficient producer of laccase under submerged conditions. Optimization of the fermentation process by conventional method resulted in higher laccase production. The enzyme production ability of this organism can be enhanced by supplementing the basal media with various inducers (carbon, nitrogen and metal ions). An overall two fold increase in laccase production was attained as compared to the control. The substrates and inducers are safe, cheap and could be suggested for prospective application for the higher production of enzyme. From the above study it can be concluded that this non-toxic fungus *L. kauffmanii* was an efficient laccase producer in culture media with the addition of nutritional factors. These finding have implication in the culture condition choice and design for further investigation at large scale.

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