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RESEARCH ARTICLE

PRODUCTION AND OPTIMIZATION OF LACCASE BY *MARASMIUS SP.* BBKAV79 IN SUBMERGED FERMENTATION

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ABSTRACT

The laccase producing novel fungi, *Marasmius sp.* BBKAV79 (Gen Bank accession number KP455496, KP455497) was isolated and subjected to production and optimization of laccase by Submerged Fermentation (SmF). Submerged fermentation involves the nurturing of microorganisms in high oxygen concentrated liquid nutrient medium. The physical parameters namely pH, temperature, inoculum size, incubation time and the nutritional parameters like suitable carbon, nitrogen sources and solvents were studied for the higher laccase production. Yeast extract peptone dextrose-Copper sulphate (YPD-Cu) medium enhanced laccase production. The optimum pH and temperature for laccase was found to be 5.5 and 40° C respectively. The highest production of laccase at pH 6 and the temperature for production was recorded at 40°C. Optimum inoculum size and incubation time for laccase production at 14 mm 0f 6 fungal discs and 9th day respectively. Glucose and peptone were the most suitable carbon and nitrogen sources for laccase production. Ethanol was the most suitable solvent for laccase production. Novel sources of this laccase would be desirable to improve activity yields and substrate specificities.

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INTRODUCTION

Laccase (benzenediol:oxygen oxidoreductase, EC 1.10.3.2), an oxidase belonging to the group of multi-copper proteins of low specificity acting on both o- and p-quinols and often acting also on aminophenols and phenylenediamine, is produced mainly by white-rot fungi to degrade lignin compounds in order to naturally recycle these bio-polymers (Risdianto *et al.,* 2010,Klonowska *et al.,* 2001). The distribution of laccase is widespread among plants (Schückel *et al.,* 2011), fungi (Farnet *et al.,* 2000) and bacteria (Farnet *et al.,* 2004). Laccase finds widespread applications in food industry, pulp and paper industry, textile industry, pharmaceutical industry, nano-biotechnology, soil bioremediation, biodegradation of environmental phenolic pollutants and other related applications (Farnet *et al.,* 1999). These potentials of laccases in biotechnological and environmental applications have stimulated the need to discover promising laccases in huge amount and the demand for this enzyme requires the production process to be economical.

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Submerged fermentation is widely employed for laccase production and for other industrial enzyme production. This process of submerged fermentation involves the growth of microorganisms in a liquid media (Baldrian *et al.,* 2006). Maximizing laccase production can be achieved by optimizing nutritional conditions which includes carbon, nitrogen and physical conditions such as pH, agitation and inoculum size (Dong *et al.,* 2005, Baldrian *et al.,* 2006). SmF, more strongly developed from the 1940s onwards because of the necessity to produce antibiotics on a large scale has been characterized as fermentation in the presence of excess water (Ronak *et al.,* 2013). The claim of laccase in biotechnological processes requires the production of high amount of enzyme at low cost and hence the current focus of research is oriented towards the identification and optimization of such an efficient production system. Submerged Fermentation techniques are common and conventional biotechnology processes in view of production of value-added products such as enzymes, biopharmaceuticals, organic acids, biosurfactants, vitamins, flavoring compounds, biofuels, biopesticidesetc (Ronak *et al.,* 2013). Hence, the present study reports on the production of laccase by isolated *Marasmius sp.* BBKAV79 (Gen Bank accession number KP455496, KP455497) and studied various parameters which

affect the production of laccase enzyme in submerged fermentation.

MATERIALS AND METHODS

Chemicals

The reagent grade chemicals 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), Tannic acid and syringaldazine were purchased commercially from Sigma (St. Louis M.O., U.S.A.). Potato dextrose agar (PDA) and Guaiacol was procured from Hi-Media (Mumbai, India).

Microorganism

Organism Screening for laccase-producing microbes on Potato dextrose agar plates containing indicators resulted in isolation of 8 Fungal Strains. Isolates showing positive reaction were maintained on Potato Dextrose Agar plate at 30 °C and stored at 4°C. The best Laccase producing isolates was identified by 18S ribosomal RNA gene sequence deposited in Gen Bank data base and identified as *Marasmius sp*. BBKAV79 (Gen Bank accession number KP455496, KP455497). This isolate is used here for the optimization study.

Screening of media for laccaseof Production

Laccase production by submerged fermentation (SmF) was studied in five different media. Five media used in the study were those of Olga *et al*. (1998), medium of Slomczynski *et al*. (1995), medium of Coll*et al*. (1993), Yeast extract peptone dextrose-Copper sulphate (YPD-Cu) medium and Glucose Peptone Broth (GPB) media. The medium of Olga *et al*. (1998) contained (in g/l): 3.0 peptone, 10.0 glucose, 0.6 KH_2PO_4, 0.001 $ZnSO_4$, 0.4 K_2HPO_4, 0.0005 $FeSO_4$, 0.05 $MnSO_4$, 0.5 $MgSO_4$; medium of Slomczynski *et al*. (1995) contained (in g/l): 40.00 glucose, 7.00 glycerol, 0.50 L-histidine, 0.10 $CuSO_4$, 1.80 $NaNO_3$, 01.80 NaCl, 0.50 KCl, 0.50 $CaCl_2.H_2O$, 0.05 $FeSO_4.7H_2O$, 1.00 KH_2PO_4, 0.50 $MgSO_4.7H_2O$; Medium of Coll *et al*. (1993) contained (in g/l) 10.00 glucose, 1.00 asparagines, 0.50 yeast extract, 0.50 K_2HPO_4, 1.00 $MgSO_4.7H_2O$, 0.01 $FeSO_4.7H_2O$; Yeast extract peptone dextrose-Copper sulphate (YPD-Cu) medium contained Glucose 20 g/l, Peptone 5 g/l, Yeast extract 2 g/l, Copper sulphate 100mg/l and Glucose Peptone Broth (GPB) media contained (in g/l) Glucose 10.0, Peptone 3.0, KH_2PO_4 0.6, $ZnSO_4$ 0.001, K_2HPO_4 0.4, $FeSO_4$ 0.0005, $MnSO_4$ 0.05, $MgSO_4$ 0.5, $CuSO_4$ 0.01and All these media were adjusted to pH 6.0. Each medium (100 mL) was dispensed into 250 mL conical flask and autoclaved at 121˚C for 15 min. The fungal mycelia disc (6 mm) was inoculated into each of the conical flask, under sterile condition and they were then incubated on a rotary shaker at 120 rpm. 5 mL of the culture filtrate was taken every alternate day and centrifuged at 10,000 rpm for 10 min. The clear supernatant was used as a crude enzyme source for determining laccase activity.

Analytical methods

Extracellular enzyme activity

The Laccase activity was assayed at room temperature by using 10mM Guaiacol in 100 mM sodium acetate buffer (pH 5.0).

The reaction mixture contained 3ml acetate buffer, 1ml Guaiacol and 1ml enzyme source. The change in the absorbance of the reaction mixture containing Guaiacol was monitored at 470 nm for 10 mins of incubation using UV Spectrophotometer. Enzyme activity is measured in U/ml which is defined as the amount of enzyme catalyzing the production of one micromole of colored product per min per ml.

$$\text{Volume activity (U/ml)} = \frac{\Delta A470nm/min \times 4 \times Vt \times \text{dilution factor}}{€ \times Vs}$$

Calculation

Where,

Vt = final volume of reaction mixture (ml) = 5.0
Vs = sample volume (ml) = 1
€ = extinction co-efficient of guaiacol = 6,740/M/cm
4 = derived from unit definition & principle

Physical Parameters effects on laccase production Optimum pH

The influence of pH on laccase activity was studied by recording the absorbance of enzyme catalysed reaction at optimum temperature, using guaiacol as substrate dissolved in different buffers of different pH (pH 4-9) and incubated at 37 ºC for 10min and absorbance were recorded at 470 nm. The buffer systems used were Citrate buffer (pH 4); Citrate buffer (pH 5); Sodium acetate duffer (pH 6); Phosphate buffer (pH 6.8); Tris–HCl buffer (pH 8) and Glycine-NaOH (pH 9).

Optimum Temperature

Effect of temperature can be studied by incubating the enzyme mixture containing enzyme, guaiacol and sodium-acetate buffer at different temperatures, i.e. 10 °C, 20 °C, 30 °C, 40 °C, 50 °C and 60 °C for 15 minutes. After incubation for 15 minutes record the absorbance of enzyme catalyzed reaction.

Time course

In order to find the optimal time of incubation for the maximum laccase production 100 ml production medium was prepared in Erlenmeyer flask (250 ml) and autoclaved. To these five discs (6 mm in diameter) of 5 day old culture was inoculated and incubated at 37 ^{0}C. After5 days the culture was collected at every 5 hours interval. This was used to determine the enzyme activity.

Inoculum Size

The effect of inoculum size was studied by adding different levels of inoculum (1, 2, 3, 4, 5 and 6 mm in diameter) from 5 days old fungal mate from plate and incubated at 30 ºC for 5 days and followed by enzyme extraction and determination of its activity.

Incubation time

To study the effect of incubation period on laccase production, conical flasks each containing 100 mL of production medium were inoculated with *Marasmius sp.* BBKAV79 and incubated

at 30 ºC for various time intervals (6, 7, 8, 9 and 10days). Then the enzyme was extracted and its activity was determined.

pH

The effect of various pH i.e. 3, 4, 5, 6, 7, 8 and 9 on laccase production in YPD-Cu media was done by inoculating *Marasmius sp.*BBKAV79 and incubated the flasks in incubator and assay were done as per guaiacol assay method.

Temperature

The YPD-Cu flasks were inoculated with the well grown *Marasmius sp.* BBKAV79 discs from PDA plates and the flasks were incubated at different temperatures viz., 37^{0}C, 40 °C, 45°C, 50^{0}C and 55 ^{0}C and assay was done as Guaiacol assay method.

Nutritional Parameters effects on laccase production
Carbon sources

Maltose, Sucrose, Starch, Fructose, Glucose and Arabinose were added to YPD-Cu medium at the concentration of 2%. The flasks were inoculated with well grown *Marasmius sp.* BBKAV79discs from PDA plates and the flasks were incubated in 30^{0}C at a pH of 6 and assay were done as guaiacol assay method.

Nitrogen sources

Trypton, Peptone, Sodium nitrate, Ammonium sulphate and Sodium carbonate were added to YPD-Cu media at the concentration of 2% and the flasks were inoculated with well grown *Marasmius sp.* BBKAV79 discs from PDA plates and the flasks were incubated in 30 ^{0}C at a pH of 6 and assay were done as guaiacol assay method.

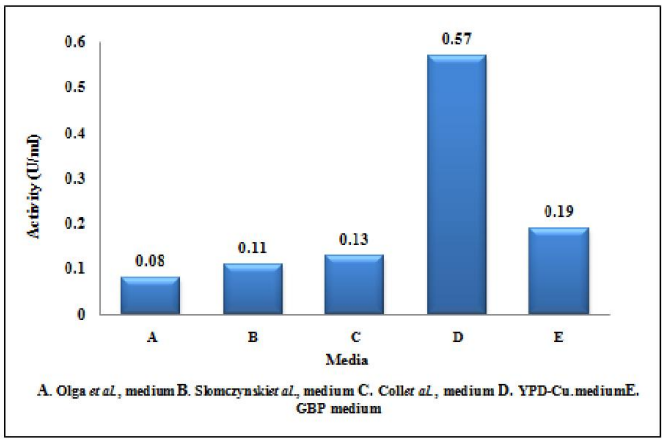
Solvents

The effect of different solvents like ethanol, methanol and isopropanol on the production of laccase from *Marasmius sp.* BBKAV79 was studied. The sources were amended at the concentration of 2% in the production medium. The mycelia disc (6mm diameter) of 5 day old culture was transferred to Erlenmeyer flasks (250ml) containing 100 ml of production medium. Flasks were incubated at 37 ^{0}C and analysed for enzyme activity.

RESULTS

Screening of Media for laccaseproduction

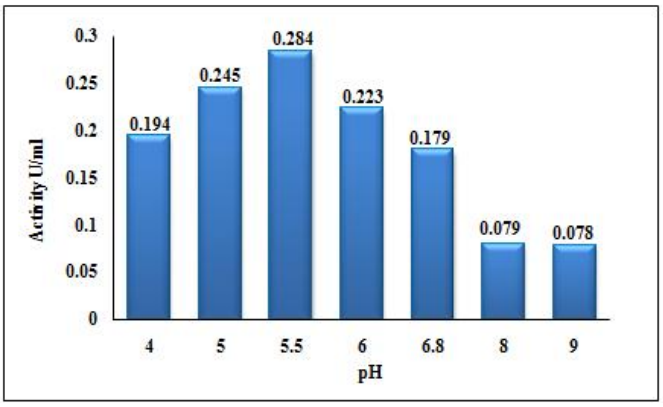
The results indicate that the Yeast extract peptone dextrose-Copper sulphate (YPD-Cu) medium was maximum supported for laccase production (0.570 U/ml) followed by GPB (0.190 U/ml), Coll *et al.,* (1993) medium (0.130), Slomczynski *et al.,* (1995) medium (0.110 U/ml) and Olga *et al.,* (1998) medium (0.080 U/ml). Based on the above studies YPD-Cu media will be used for further study (Graph 1).



Graph 1. Screening of media for laccase production

Influence of Physical Parameters on laccase production
Optimum pH

The results suggested that the Sodium Acetate buffer (pH 5.5) showed the excellent laccase activity (0.284 U/ml) followed by Citrate buffer (pH 5) (0.245 U/ml), Phosphate buffer (pH 6) (0.223 U/ml), Citrate buffer (pH 4) (0.194 U/ml), Phosphate buffer (pH 6.8) (0.179 U/ml), Tris-HCL buffer (pH 8) (0.079 U/ml) and Glycine-NaOH (pH 9) (0.078) (Graph 2).



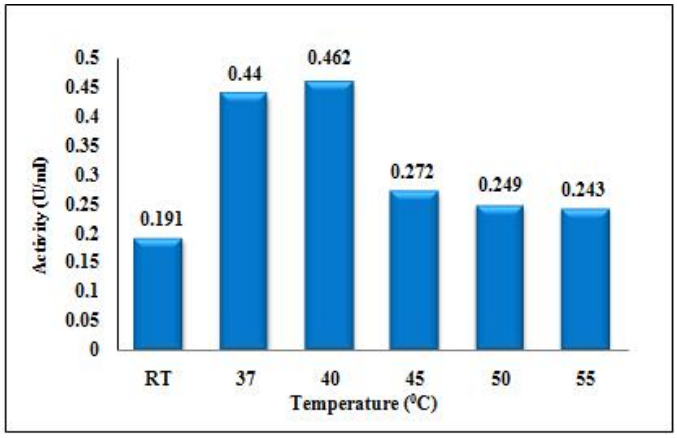
Graph 2. Effect of pH on laccase production

Optimum temperature

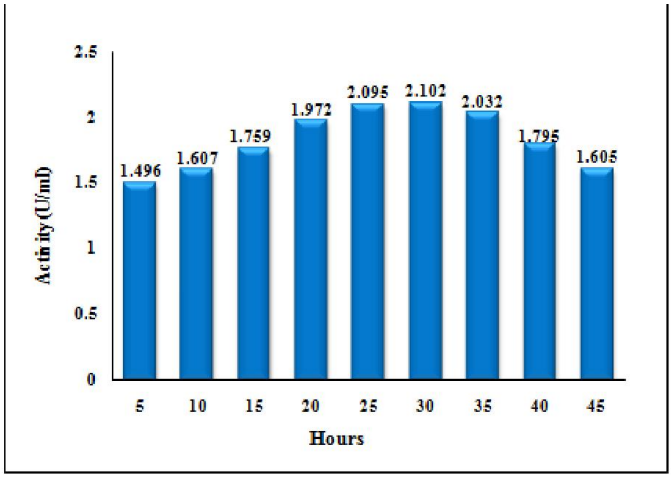
The results showed that the laccase is able to produce optimum temperature at 40 ^{0}C with high laccase activity (0.462 U/ml) followed by 37 °C (0.440 U/ml), 45 °C (0.272 U/ml), 50 °C (0.249 U/ml), 55 °C (0.243 U/ml) and Room temperature (RT) (0.191 U/ml) (Graph 3).

Time course

The results suggested that the extracellular laccase activity of crude enzyme extract was found to increase at initial growth and reached the maximum value of 2.102 U/ml by 30thhour of incubation followed by 25th hour (2.095 U/ml), 30th hour (2.032 U/ml), 20th hour (1.972 U/ml), 40th hour (1.795 U/ml), 15th hour (1.759 U/ml), 10th hour (1.607 U/ml), 45th hour 1.605 U/ml) and 5th hour (1.496 U/ml) (Graph 4).



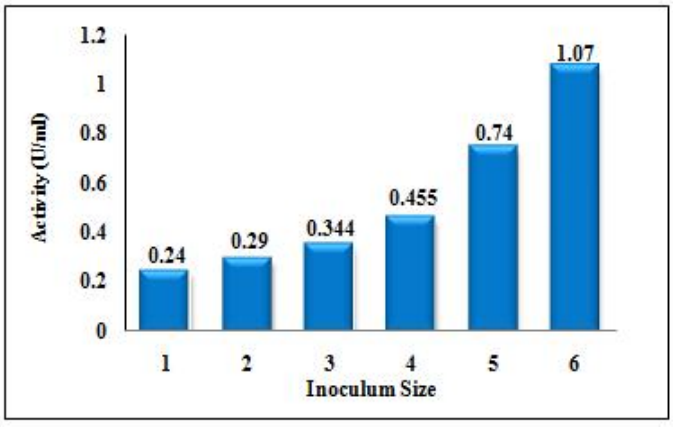
Graph 3. Effect of optimum temperature for laccase production



Graph 4. Time scale for laccase production

Inoculum size

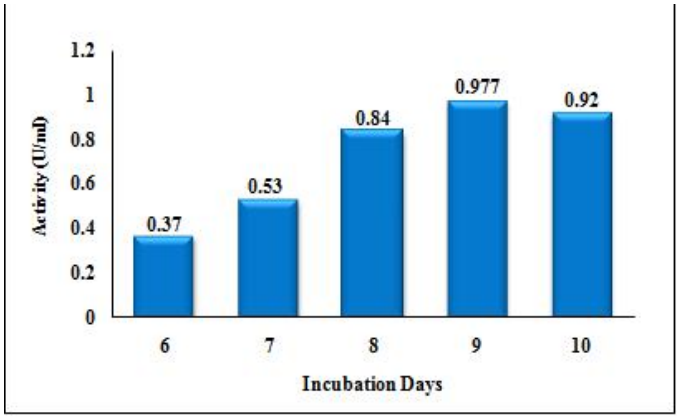
The results showed that the highest laccase specific activity 1.07 U/ml was obtained when using 14 mm of 6 fungal discs followed 14 mm of 5 fungal discs (0.740 U/ml), 14 mm of 4 fungal discs (0.455 U/ml), 14 mm of 3 fungal discs (0.344 U/ml), 14 mm of 2 fungal discs (0.290 U/ml) and 14 mm of 1 fungal disc (0.240 U/ml) (Graph 5).



Graph 5. Effect of inoculum sizefor laccase production

Incubation period

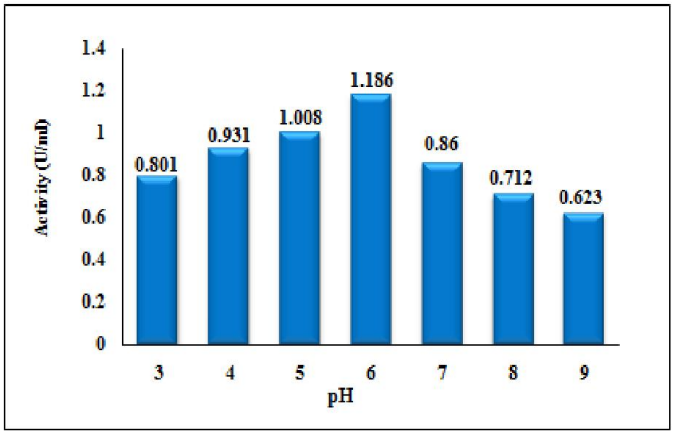
The results indicate that the 9th day of incubation showed excellent laccase production (0.977 U/ml). After which it decreased slowly 10th day (0.920), 8th day (0.840 U/ml), 7th (0.530 U/ml) and 6th day (0.370 U/ml) (Graph 6).



Graph 6. Effect of incubation period for laccase production

pH

The results showed that the *Marasmius sp.* BBKAV79 strain was able to produce maximum optimum pH 6.0 (1.186 U/ml) followed by pH 5 (1.008 U/ml), pH 4 (0.931 U/ml), pH 7 (0.860 U/ml), pH 3 (0.801 U/ml), pH 8 (0.712 U/ml) and pH 9 (0.623 U/ml) (Graph 7).



Graph 7. Effect of pH for laccase production

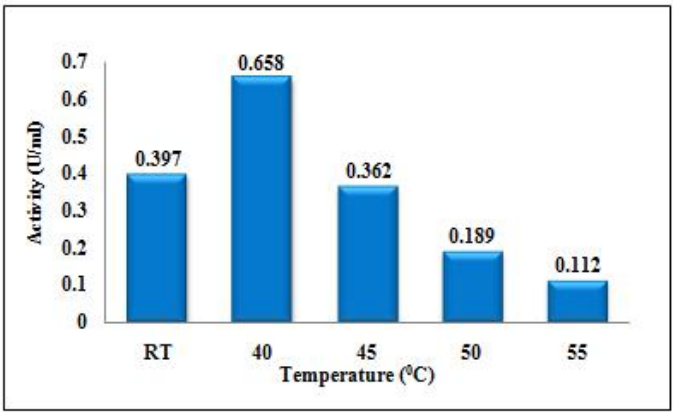
Temperature

The results showed that the *Marasmius sp.* BBKAV79 strain was able to produce temperature at 40 ^{0}C with high laccase activity (0.658 U/ml) followed by 45 ^{0}C (0.362 U/ml), Room temperature (RT) (0.397 U/ml), 50 ^{0}C (0.189 U/ml) and 55 ^{0}C (0.112 U/ml) (Graph 8).

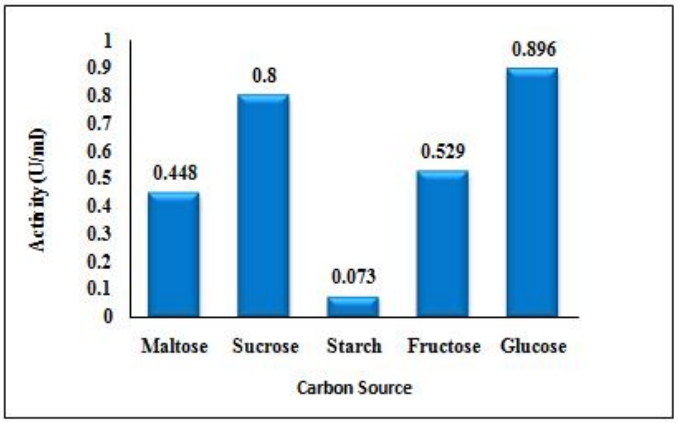
Influence of Nutritional Parameters on laccase production

Carbon sources

The results suggested that the Glucose supported the maximum laccase activity (0.0896 U/ml) followed by Sucrose (0.800 U/ml), Maltose (0.448 U/ml), Fructose (0.529 U/ml) and Starch (0.073 U/ml) (Graph 9).



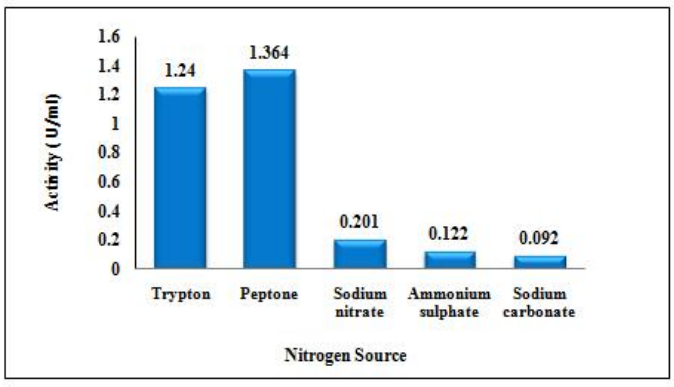
Graph 8. Effect of temperature for laccase production



Graph 9. Effect of carbon sources on laccase production

Nitrogen sources

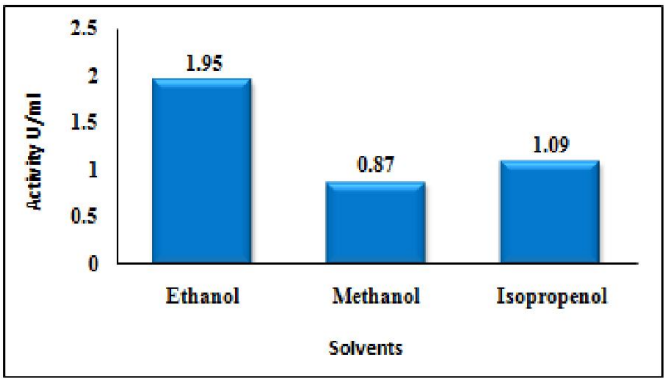
The results indicate that the Peptone supported the maximum laccase activity (1.364 U/ml) followed by Trypton (1.24 U/ml), Sodium Nitrate (0.201 U/ml), Ammonium Sulphate (0.122 U/ml) and Sodium Carbonate (0.092 U/ml) (Graph 10).



Graph 10. Effect of nitrogen sources on laccase production

Solvents

The results showed that the Ethanol supported the maximum laccase activity (1.95 U/ml) followed by Isopropenol (1.09 U/ml) and Methanol (0.870 U/ml) (Graph 11).



Graph 11. Effect of solvents on laccase production

DISCUSSION

Enzyme production is greatly influenced by media components, especially carbon, nitrogen sources, and physical factors such as pH, temperature, inoculum size, incubation time. It is important to produce the enzyme in large scale in inexpensive manner. Hence the influence of various physico-chemical parameters such as inoculum size, incubation periods, pH, temperature, carbon, and nitrogen sources were studied on laccase production. Laccase producing fungi are generally selected based on the primary screening in agar plate containing a suitable substrate. Laccase producing organism showed an intense reddish brown coloured zone around the colonyon the PDA plate amended with Guaiacol, Syrindalzine and Tannic acid. Organism showed purple coloured zone around the colonyon PDA plate amended with ABTS. Sathiyavathi *et al.* (2011) and Gnanadoss *et al.* (2014) also used guaiacol as an indicator for the confirmation of the laccase positive isolates.

The production of laccases under submerged fermentation was evaluated using five different media and enzyme activity was observed to be different in each of the media used indicating the importance of the nutrient composition in enhancing the enzyme production by specific organisms. Similarly, Viswanath *et al.* (2008) have evaluated five different media for the production of laccase by *Stereum ostrea and Phanerochaete chrysosporium*. A fungus has its own requirements for production of laccase under submerged fermentation and hence, has to be standardized. In this study, inorganic and organic nitrogen substances were incorporated in the production medium and their effect on the production of the enzyme was studied. The study also showed that the copper sulphate favoured maximum laccase production by *Marasmius sp.* BBKAV79. Palmieri *et al.* (2000) have showed that the addition of 150 µM copper sulphate to the culture medium resulted in a fifty-fold increase in laccase production compared to the original medium. Collins *et al.* (1997) have reported that copper to be a strong laccase inducer in several species, among them, *T. versicolor* and Dittmer *et al.,* (1997) have showed that *P. chrysosporium* and the same results wereobtained in this study. The influence of optimum pH on laccase activity was studied by recording the absorbance of enzyme catalysed reaction at optimum temperature, using guaiacol as substrate dissolved in different buffers of different pH (pH 4-9).

The present results indicate that the Sodium Acetate buffer (pH 5.5) showed the excellent laccase activity. The *Trametes sp.* showed highest activity at optimum pH 4.5- 5.5 (Kuntal *et al.,* 2013). The *Aspergillus Flavus* showed highest activity at pH at pH 5.5 (Kartikeya *et al.,* 2013). Effect of optimum temperature can be studied by incubating the enzyme mixture containing enzyme, guaiacol and sodium-acetate buffer at different temperatures. The laccase is able to produce optimum temperature at 40 ^{0}C with high laccase activity. The *Trametes sp.*showed highest activity at temperature 45°-50°C (Kuntal *et al.,* 2013). The present study is an attempt to find out the optimum incubation time (time course) for the production of laccase. The results showed that optimum incubation time for laccase activity was determined at 30th hour. Similarly, Kuddus *et al.,* (2013) have showed optimum incubation period for laccase activity by *Pseudomonas putida* was obtained at 108th hours. The results showed that the highest laccase activity at14 mm of 6 fungal discs. Similarly, Elshafei *et al.,*(2012) have showed the maximum laccase activity by *Penicillium martensii* NRC 345 at 14 mm of 5 fungal discs. Further it has been showed that the optimum incubation period for laccase activity was determined at 9th day. Elshafei *et al.,*(2012) have showed that optimum incubation period for laccase activity at 26th day in *Penicillium martensii* NRC 345.

The present results also showed that an initial pH of 6 favoured maximum laccase production by *Marasmius* sp. BBKAV79. Most fungal cultures prefer a slight acidic pH in the medium for growth and enzyme production Sivakumar *et al.* (2010) have reported that *Ganoderma sp*. exhibited optimum laccase production at pH 6.El-zayat *et al.* (2008) have reported that the pH of 5-9 showed a significant influence on the production of extracellular laccase in the *Pleurotus Sp.* Maximum laccase production of 94.3 and 94.0 U/ml was recorded at pH 5.5 and 7.5 respectively, and the moderate activity was observed at pH 9 and then declined. The optimal pH for laccase production was found to be at pH 6.5 in submerged culture of *Chaetomiumglobosum*.

The pH of the culture media strongly affects many enzymatic reactions and transport of compounds a cross the cell membrane as they are sensitive to the concentration of hydrogen ions present in the medium. However, Thurston (2004) has concluded that the effect of pH is limited in case of laccase enzyme production. Temperature has significant effect on the growth and production capacity of laccase enzyme in fungus, the *Marasmius sp*. BBKAV79 was subjected to different temperature conditions on YPD-Cu medium. 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 on microbial growth and enzyme production. The *Marasmius sp.* BBKAV79 strain was able to produce optimum temperature is 40 ^{0}C with high laccase activity. The increase in the temperature resulted in the reduction of the enzyme activity. This is probably due to the fact that increasing the temperature could have inhibited the fungal growth and hence, low/decreased enzyme activities. Periyasamy *et al.,* (2012) have showed the optimum temperature for laccase production at 50 °C in *Pleurotus sp.*
In this study using *Marasmius sp.* BBKAV79, the carbon source of glucose enhanced maximum laccase production. Similarly, Periyasamy *et al.,* (2012) have reported glucose was found to be best carbon source for laccase production. Piscitelli *et al.* (2011) have considered glucose is the best carbon source for production of enzyme. Nitrogen sources are necessary for the proper growth and metabolism of microorganisms. The use of economical nitrogen sources is important for production of laccase as these can significantly reduce the cost. Among the tested nitrogen source peptone enhanced maximum laccase production. Similarly, Gogna *et al.,*(1992) have reported the most widely used nitrogen sources for fungal lignolytic enzyme production are ammonium salts such as tartrate or chloride. Coll *et al.,* (1993) have reported that Ammonium nitrate favoured high laccase production in many white rot fungi, namely, Basidiomycete PM1. Periyasamy *et al.,* (2012) have reported that the peptone was found to be the best nitrogen source for laccase production in *Pleurotus sp*.

The present study results indicating that the ethanol supported the maximum laccase production. Lee *et al.,* (2002) suggested the positive effect of methanol on laccase activity in liquid culture of *Trametesversicolor*. Moreover, ethanol is an abundant agro-industrial by-product, cheaper and environmentally safer. Sivakumar *et al.,* (2010) have showed the positive effect of isopropanol on laccase activity. The findings of the present study clearly indicate that *Marasmius sp.* BBKAV79 is an efficient producer of laccase under submerged fermentation condition. The conventional one factor at a time method of optimization of various parameters for the production of laccase are governed by parameters such as pH, temperature, inoculum size, incubation time, carbon source, nitrogen source and solvents. The optimized medium components under submerged fermentation will help in maximizing laccase production by *Marasmius sp.* BBKAV79 under large scale processes.

Conclusion

During this study, an inexpensive, repeatable and rapid screening technique was developed to evaluate *Marasmius sp.* BBKAV79 for laccase production. The present study was achieved by higher production of laccase under optimized fermentation and nutritional conditions. Yeast extract peptone dextrose-Copper sulphate (YPD-Cu) medium composition enhanced laccase production. Optimum pH and temperature for laccase production was 5.5 and 40 ^{0}C.Time course study for laccase production at 30th hour. Inoculum size for laccase production at 14 mm of 6 fungal discs. 9th day of incubation showed excellent laccase production. pH and temperature for laccase production was 6 and 40 ^{0}C. Glucose and peptone were the most suitable carbon and nitrogen source for laccase production and Solvent for laccase production was Ethanol.

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