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Isolation, Screening and Optimization for Laccase production by *Scytalidium lignicola* pesante under submerged fermentation

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A B S T R A C T

Twenty Five lignolytic fungi were isolated from decaying wood samples and were screened for the laccase production using guaiacol and ABTS as indicator compounds. The positive isolates were confirmed for the presence of laccase using Azure B and quantitative enzyme assay in presence of catalase using ABTS as substrate. Further laccase producing capacity of fungus was estimated in seven different media where Czapex Dox medium showed highest enzymatic activity of 17.2 U/ml. Effect of carbon and nitrogen sources, initial pH, inoculum size, incubation period, incubation temperature, inducer and inhibitor on laccase production was evaluated. The optimal pH, incubation temperature, incubation period and inoculum size for the laccase production in submerged culture were found to be pH 6, 30°C, 7days and 2disc/100ml medium respectively. Sucrose and sodium nitrate were the most suitable carbon and nitrogen sources for laccase production. Out of the different inducers and inhibitors, the prominent induction was observed with 2mM $CuSO_4$ and 0.1% $MgSO_4$ while sodium azide and formaldehyde inhibited the laccase production almost 94% and 74% respectively. Under the optimal condition the maximum laccase activity was determined to be 28.2U/ml which is supposed to be 1.5 times higher than the unoptimized conditions.

Introduction

Laccases (benzenediol: oxygen oxidoreductase, (EC1.10.3.2) are muti-copper oxidases widely distributed among plants, insects and fungi (Sidhu *et al.*,2014). Laccase reduce molecular oxygen to water and simultaneously perform one-electron oxidation of various substrates such as diphenols, methoxy-substituted monophenols and aromatic and aliphatic amines. (Elsayed *et al.*, 2012). Unlike lignin peroxidases and manganese peroxidase, laccase is capable of catalyzing the oxidation of organic compounds in the absence of H_2O_2 (Ding *et al.*, 2012).The peroxidases have the highest redox potential, being able to catalyze directly the oxidation of non-phenolic compounds. However, the use of oxygen (a non-limiting electron acceptor) by the laccases makes these enzymes more adequate for industrial and environmental applications (Zouari-Mechichi *et al.*, 2006). Due to their low substrate specificity and strong oxidative abilities, laccases have a number of industrial applications including biopulping, prevention of wine decolouration, detoxification of environmental pollutants, textile dye

bleaching, enzymatic conversion of chemical intermediates and the production of valuable compounds from the lignin (Thurston 1994; Patrick *et al.,* 2009).

Microbes that produce laccases have been screened for either on solid media containing coloured indicator compounds that enable the visual detection of the laccase production or with liquid cultivations monitored with activity measurements (Kiiskinen *et al.,* 2004). Usually there are several compounds that have been used as substrates by spectrophotometry methods such as 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonate) (ABTS) (Majolagbe *et al.,* 2012), Syringaldazine (Iyer *et al.,* 2003; Saito *et al.,* 2003), guaiacol, o-dianisidine (Li *et al.,* 2008) and 2,6-dimethoxyphenol (Abdulla *et al.,* 2000). Although many different substrates are available for assaying of laccases but ABTS is considered as a most sensitive among all tested compounds to evaluate the activity of laccase (Li *et al.,* 2008). Discovery of noval laccases with different substrate specificities and improved stabilities is very important for industrial applications, besides developing an effective high yield and economic production medium enhances its utility (Desai *et al.,* 2011).

In this study, we isolated a new fungus from Igatpuri, Nashik, India. The fungus produces large amount of laccase, under the culture conditions. We described here, the confirmatory tests for establishing laccase production and optimization of the parameters to improve the laccase productivity under lab conditions.

Materials and Methods

Isolation of laccase producing microorganisms

Twenty seven samples were collected in sterile tubes from the decaying wood and tree bark scrapings of live trees, from the forests of Igatpuri, Kasara and Bhandardara around Nashik in Maharashtra, India. Sample collection was done during monsoon season in Maharashtra as it was easy to see the growing fungi on the tree bark. Samples were subjected to isolation by standard serial dilution method and inoculated on Potato Dextrose Agar (PDA) medium supplemented with 0.01% W/V peptone, 0.001% W/V yeast extract and 20% W/V glucose (Desai *et al.,* 2011; Alfarra *et al.,* 2013; Singh *et al.,* 2013). The isolated colonies thus obtained were maintained on PDA agar at 4°C for subsequent use as inoculums and subcultured after every 15 days. Twenty five strains were isolated and were designated as DF_1 to DF_{25}.

Screening and validation of laccase production

All the isolated and subcultured fungal strains were inoculated on Petri plates containing PDA medium, amended with 0.02% guaiacol (Fu *et al.,* 2013), 0.1% 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonate) (ABTS) and 0.01% Azure B (Alfarra *et al.,* 2013) each separately. Development of dark brown color around the colonies on guaiacol and of dark green to purple color on ABTS containing medium indicates presence of laccase being produced by the fungus. While absence of clear zone on the Azure B containing plates is confirmation of extracellular laccase production, as it is the substrate of peroxidases.

Laccase activity assay

Czapex Dox medium was used as liquid fermentation media for quantitative estimation of enzyme activities from the selected strains. Two disc of peripheral growing fungal isolates (8mm in diameter) were inoculated aseptically in the broth. The culture was incubated at 25 °C for 7 days. At the end of each growth period, inoculated tubes were collected and centrifuged at 5000

rpm for 5 min. at 4ºC (Sidhu *et al.,* 2014). The filtrate was tested for laccase enzymes activity as follows and enzyme activity was expressed in U/ml.

Catalase (1,000U/ml) was added to the assay solution and incubated for 1h at 37ºC to remove the possible effect of H_2O_2 produced by the fungus (Sheikhi *et al.*, 2012). Laccase activity was determined spectrophoto-metrically at 420nm (ϵ420=36,000 $M^{-1}cm^{-1}$) with 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonate) (ABTS) as a substrate. The assay mixture contained 150µL of 0.5mM substrate (ABTS), 2.7mL of 0.1M sodium acetate buffer of pH 4.5 and 150 µL of culture supernatant and was incubated for 5min. Absorbance was read at 420nm in a spectrophotometer against a suitable enzyme and substrate blank. The activity was defined as the amount of enzyme that oxidized 1umol of ABTS per min and the activities were expressed in U/ml. All spectrophotometric measurements were carried out using a UV-Vis spectrophotometer (Systronics, PC based double beam spectrophotometer 2202). All assays were carried out in triplicate (Bourbonnais *et al.,* 1995; Moore *et al.*, 2011; Zhu *et al.,* 2016).

Screening of different liquid media for laccase production

Different media like malt extract broth, potato dextrose broth, Czapeck Dox, Sabrourd's Medium, Yeast glucose broth, (Amrutha and Abhijit, 2015) Basal liquid Medium (Saparrat *et al.,* 2007; Rajendran *et al.,* 2011), Rose Bengal medium (Christie and Shanmugam, 2012) were screened for laccase production. Medium showing highest enzyme production was further used for optimization experiments.

Optimization of carbon and nitrogen sources for laccase production

Czapeck Dox medium has shown superior results over all the other media screened for laccase production. To investigate the best carbon source, the sucrose in the Czapek Dox media was replaced with other carbon resources including fructose, maltose (Prasher *et al.,* 2015), glucose, galactose, lactose and cellulose (zhu *et al.,* 2016). Effect of different organic and inorganic nitrogen sources like malt extract, urea (Elsayed *et al.,* 2012), peptone (Kenkebashvili *et al.,* 2012), ammonium nitrate and ammonium chloride (Prasher *et al.,* 2015) were evaluated by replacing sodium nitrate from Czapek Dox media. 100ml Media with above carbon and nitrogen sources was prepared in Erlenmeyer flask 250mL and autoclaved. Two disc (8mm in diameter) of a 5 days old culture were inoculated in this media and incubated at ± 25 ºC for 7 days. Control with standard Czapek Dox media was run in parallel.

Optimization of other parameters for laccase production

The initial pH of the medium was adjusted from pH 2 to pH 8 at the interval of 1 unit using 0.1M HCl and 0.1 M NaOH to study its effect on laccase production. Temperature optimization was done by incubating the culture flasks at temperature ranging from 0 to 50ºC (Mahmoud *et al.,* 2013). To study the effect of inoculum size on laccase production, number of discs (8mm in diameter) inoculated were varied form 1-5. 100ml of Czapek Dox medium with above mentioned variations was prepared and autoclaved. Except the experiment conducted to check the effect of inoculum size, to remaining flasks two discs (8mm in diameter) of a 5 days old culture were inoculated and culture flasks were incubated at ± 25ºC for 7 days.

In order to find the optimal time of incubation for the maximum laccase production 100mL Czapek Dox media was prepared and autoclaved. To this two discs (8mm in diameter) of a 5 days old culture were

inoculated and incubated at ± 25°C for 10 days. The culture harvested every day from day 2 to day 10 and was used to determine enzyme activity (Kumar *et al.,* 2016).

Effect of inducers and inhibitors on laccase production

Different inducers were investigated for their capacity to increase laccase production such as ABTS (0.6mM), guaiacol (1mM), CuSO4 (2mM), $MgSO_4$ (0.1%) and tannic acid (0.2mM). Different inhibitors such as sodium azide, EDTA (Ethylene diamine tetraacetic acid) (Mahmoud *et al.*, 2013; Nadeem *et al.*, 2014), formaldehyde and L-cysteine at the concentration of 1mM were added in the medium to evaluate the inhibition of enzyme production. 100ml of Czapek Dox medium devoid of $MgSO_4$ with above mentioned inducers was prepared, as $MgSO_4$ is also considered as one of the inducer. 100ml of Czapek Dox medium consisting of above listed inhibitors was also prepared and autoclaved. To this sterilized medium two discs (8mm in diameter) of a 5 days old culture were inoculated and incubated at ± 25°C for 7 days. Czapek Dox media without the addition of any inducer and inhibitor was run in parallel. Further effect of different concentrations of CuSO4 (0.5mM, 1.0mM, 1.5mM, 2.0mM, 3.0mM, 4.0mM and 5.0mM) and $MgSO_4$ (0.02,0.04,0.06,0.08,0.1,0.12,0.14 and 0.16%) was also evaluated in the similar way.

Enzyme production under optimized conditions

In order to determine the combined effect of the above mentioned parameters and to maximize the laccase production, Czapek Dox medium with all the optimized parameter was prepared. The medium was sterilized and inoculated with two discs (8mm in diameter) of a 5 days old culture and was incubated at ± 25°C for 7 days. The enzyme activity was estimated at the end of the incubation period and was compared with the unoptimized Czapek Dox medium.

Results and Discussion

Isolation and screening of fungal culture for laccase production

Twenty five different fungal isolates were isolated from the decaying wood scrapings which were named as DF_1 to $DF_{25.}$ All the isolates were subcultured and screened for laccase activity on Czapek Dox medium with 0.02 % guaiacol. Laccase enzyme react with guaiacol to give reddish brown color product, out of the above 25 isolates 12 isolates were found to be positive.

All the positive isolates were evaluated quantitatively by performing enzyme assay using ABTS as the substrate of the enzyme. The crude enzyme extract was treated with catalase to degrade any endogenously produced hydrogen peroxide (H_2O_2), this helps to eliminate any false positive results due to the presence of peroxidases.

Out of the 12 positive isolates only two isolates (DF_8 and DF_{12}) showed prominent laccase activity which is 16.2U/ml and 12.61U/ml respectively. The positive isolates were separately grown for secondary screening on Czapek Dox medium containg 0.1% ABTS and 0.01% Azure B. Both the isolates showed characteristic blue color zone surrounding the colonies due to the oxidation of ABTS. ABTS assay is considered to be more efficient assay for screening of laccase producer as far as cost, time and method is concerned (Kumar *et al.,* 2016). None of the two isolates showed zone on the Azure B containing plates. According to Kumar *et al.*, (2016) several fungi which secrete lignin peroxidase and manganese peroxidase have ability to decolorize or degrade Azure B dye.

But laccase enzyme cannot decolorize Azure B. Presence of laccase is sometimes confused with the presence of peroxidase as both are capable of oxidizing variety of phenolic indicators but absence of clearance zone on Azure B containing plate is confirmation of the laccase production.

Out of the Twenty five isolated fungi, isolate no. DF8 and DF12 were confirmed for the production of laccase by both qualitative plate assay and quantitative enzyme assay. Both the isolates were characterized on the basis of morphological characters from Agharkar Research Institute. They were identified as *Scytalidium lignicola* pesante and *Curvularia pallenscens* Boedijn. Further work was carried out on *Scytalidium lignicola* pesante, as it was found to be maximum producer of the laccase enzyme over the other isolate in quantitative enzyme assay.

Optimization of different media

Different media like Malt extract broth, Potato dextrose Broth, Czapeck Dox, Sabrourd's medium, Yeast glucose broth, Basal liquid media, Rose Bengal medium were screened for laccase production, Czapek Dox proved to be the best medium showing 17.2 U/ml of laccase activity at pH 7.3 and temperature ± 25^{o}C.

Janusz *et al.,* (2006) in their studies, got significantly good laccase production using Czapek Dox and Linderberg –Holm medium on 11th day and 8th day of incubation in *Rizoctonia praticola*. Similarly Czapek Dox showed maximum laccase production in *Cochliobous sp*. isolated from plastic dumped soils by Tirupati *et al.,* (2016).

Generally it is believed that Czapek Dox supports the growth of the organisms which are capable to utilize sodium nitrate as the sole source of nitrogen.

Influence of carbon sources on laccase production

Seven carbon sources like fructose, maltose, glucose, galactose, lactose, cellulose and sucrose which are present in control Czapek Dox medium were screen to find the most significant carbon source. Medium containing sucrose exhibited maximum enzymatic activity of laccase *i.e.* 19.3U/ml followed by cellulose *i.e.*, 14.7U/ml. In one of the study on *Pycnoporus Sanguineus* for production of laccase enzyme, the workers Eugenio *et al.,* (2016) demonstrated effect of maltose, fructose, glucose and sucrose from which sucrose exhibited highest production of laccase. Results are also in accordance with a similar study carried out by Jose and Joel *et al.,* (2014) on *Agaricus* sp. where highest yield was obtained in medium containing sucrose. The ability of the organism to give comparatively better result on cellulose demonstrates the possibility of presence of cellulase in the organism which enables organism to utilize cellulose present in the medium.

Influence of nitrogen sources on laccase production

Effect of different organic nitrogen sources like malt extract, urea, peptone and inorganic nitrogen sources like ammonium nitrate, sodium nitrate and ammonium chloride was studied on laccase production. Highest laccase activity of 18.6U/ml was observed with sodium nitrate which is closely followed by ammonium nitrate i.e. 16.2U/ml. These results demonstrate the fact that *Scytalidium lignicola* can effectively utilize inorganic nitrogen sources containing nitrate. Sodium nitrate is also reported to have significant influence on laccase production by Muthukumarasamy *et al.,* (2015). It was also found to be best nitrogen source for laccase production by *Dictyoarthrinium synnematicum* Somrith (Prasher *et al.,* 2015).

Fig.1 Oxidative polymerization of guaiacol to form reddish brown zones in the medium by *Scytalidium lignicola*

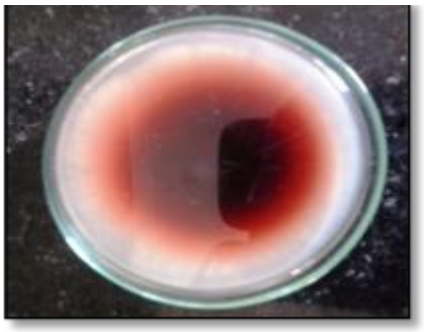


Fig.2 Blue color zones on medium containing ABTS by *Scytalidium lignicola*.

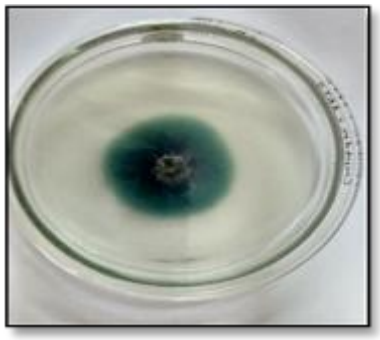
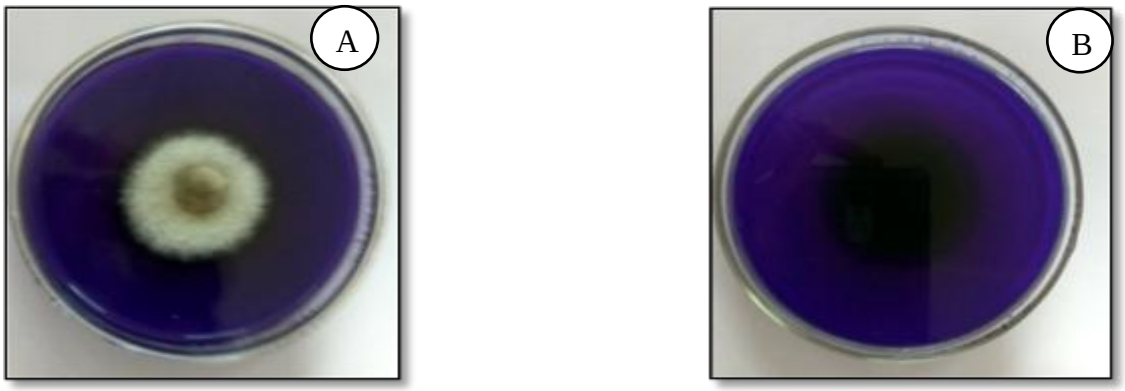
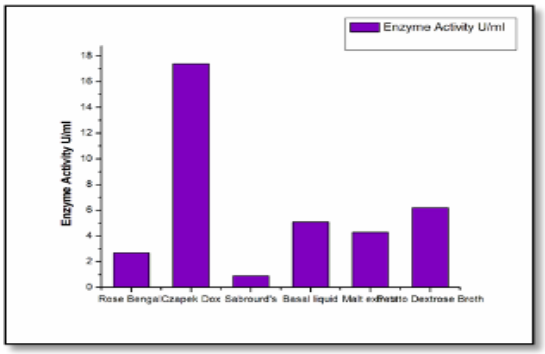


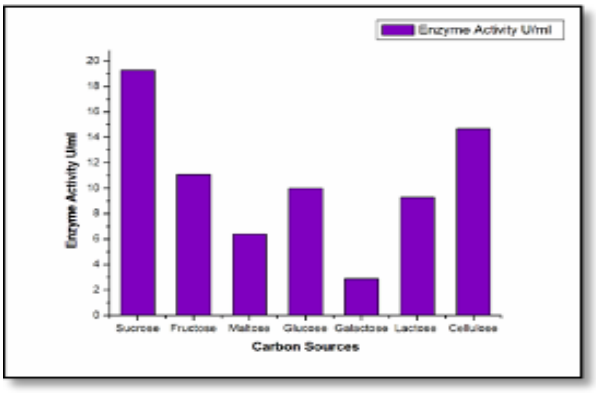
Fig.3 No clearence zone surrounding the colony of *Scytalidium lignicola* on Azure B containing plate. A.Top view of the plate, B.Inverted view of the plate



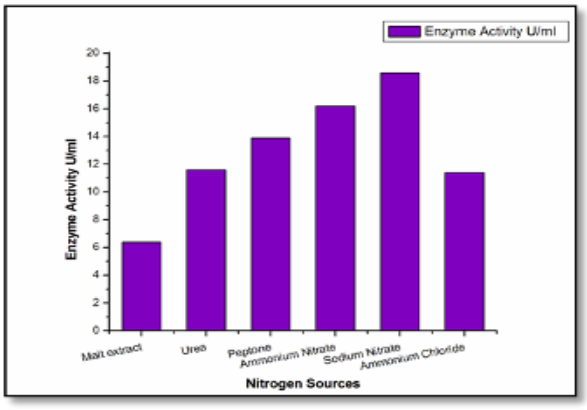
Graph.1 Graph displaying effect of different media on activity of enzyme produced under submerged condition



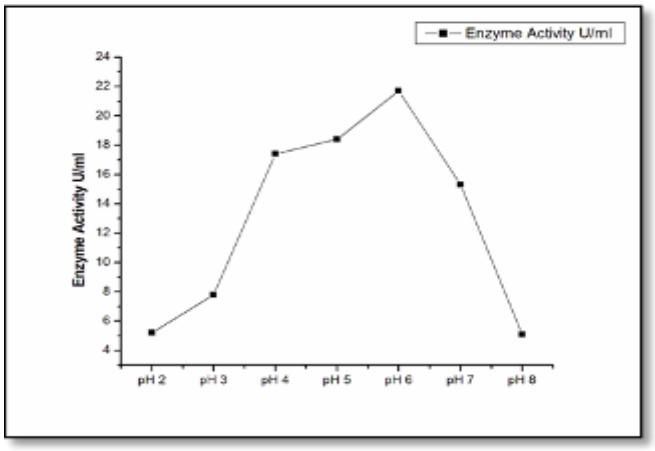
Graph.2 Graph displaying influence of different carbon sources on the activity of enzyme produced under submerged condition



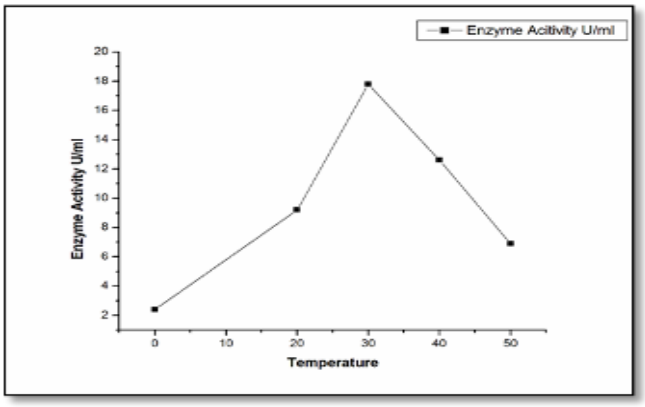
Graph.3 Graph displaying influence of different nitrogen sources on the activity of enzyme produced under submerged condition



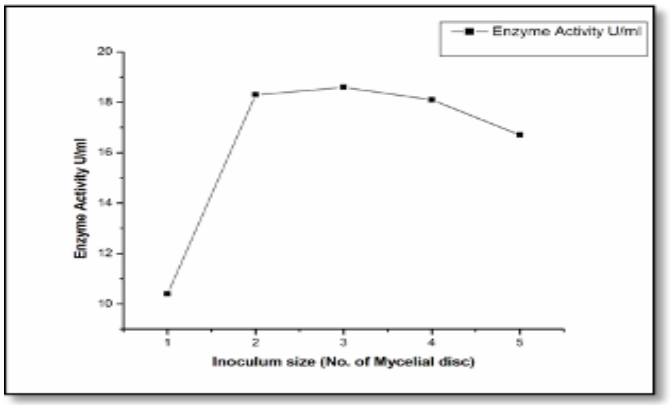
Graph.4 Graph displaying influence of pH on the activity of enzyme produced under submerged condition



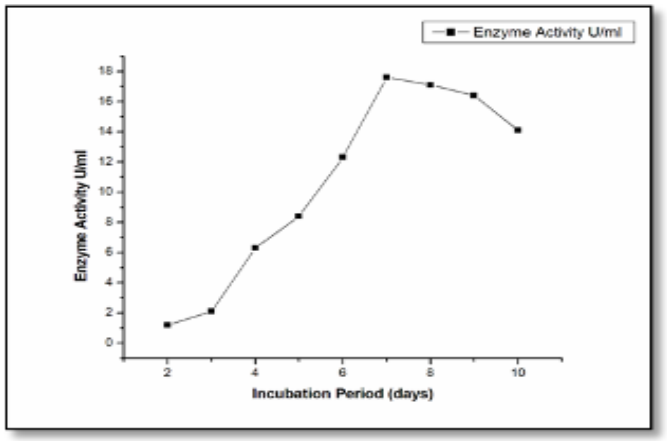
Graph.5 Graph displaying influence of temperature on the activity of enzyme produced under submerged condition



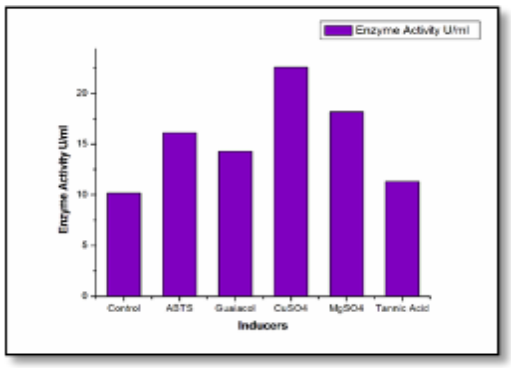
Graph.6 Graph displaying influence of inoculum size on the activity of enzyme produced under submerged condition



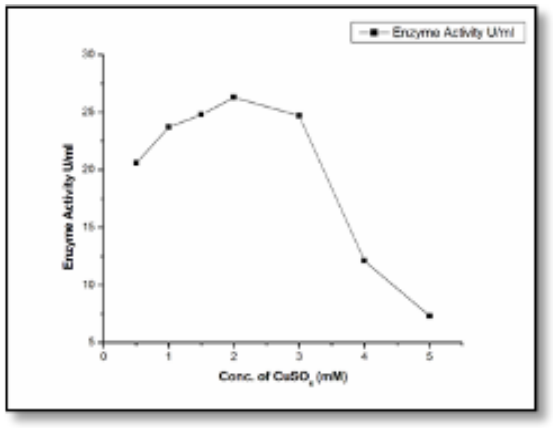
Graph.7 Graph displaying influence of incubation period on the activity of enzyme produced under submerged condition



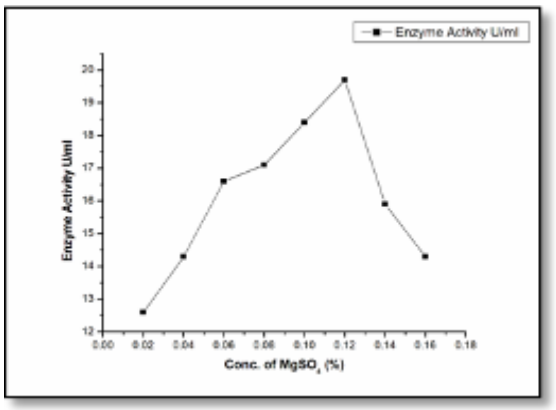
Graph.8 Graph displaying influence of different inducers on the activity of enzyme produced under submerged condition



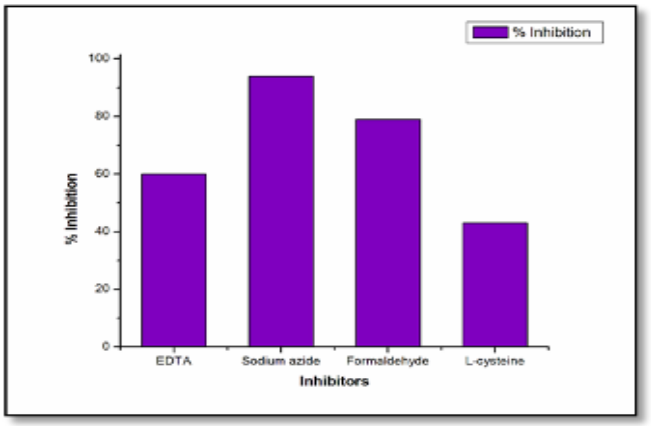
Graph.9 Graph displaying influence of $CuSO_4$ on the activity of enzyme produced under submerged condition



Graph.10 Graph displaying influence $MgSO_4$ on the activity of enzyme produced under submerged condition



Graph.11 Graph displaying influence of different inhibitors on the activity of enzyme produced under submerged condition



Influence of initial pH on laccase production

There is high correlation between laccase production and changes in the medium pH during cultivation. In current study effect of pH ranging from pH 2 to pH 8 was studied on laccase production. It is observed that pH 4, pH 5 and pH 6 are most influential for laccase production by the organism with pH 6 showing maximum activity of the laccase *i.e.* 21.7U/ml. Most fungal laccase reach their maximum activity when the initial pH of the nutrient medium ranges from 4 to 6 (Janusz *et al.,* 2006). The highest activity of laccase was observed at pH between 4.5 and 6.5, in *Pleurotus osteatus* (Nadeem *et al.,* 2014). Also Sivakumar *et al.,* (2010) reported highest laccase production at pH 5 in case of *Ganoderma* sp.

Influence of temperature on laccase production

Study of effect of different temperature reveals that 30°C is the optimum temperatue for the laccase production by *Scytalidium lignicola.* The data obtained is in agreement with most of the other studies carried out to demonstrate the effect of temperature on laccase production. In the work done by Chenthamarakshan *et al.,* (2017) on *Marasmiellus palmivorus* the maximum laccase production was observed at 28°C while 30°C gave better production of laccase in *Pleurotus osteatus* (Nadeem *et al.,*2014). Results obtained are similar to the results obtained by Risdianto *et al.,* (2010) who got maximum laccase production by under solid state fermentation in between 25°C and 31°C by *Marasmium* sp. Normally, the fermentation processes (SSF as well as SmF) develop with mesophilic microbial strains (Risdianto *et al.,* 2010). As *Scytalidium lignicola* also belongs to mesophilic organisms, the growth temperature is in the range of 20-40°C.

Influence of inoculum size on laccase production

Inoculum size plays a significant role in any fermentation. A lower level of inoculum may not be sufficient to initiate the growth, whereas a higher level may cause competitive inhibition (Patel *et al.,* 2009). Therefore determining exact inoculum size is critical step. To demonstrate the effect of inoculum size on laccae production, 100ml of Czapek Dox medium was inoculated with varying

number of mycelial discs (Nos.1-5). Although there is slight increase in laccase activity with increasing inoculum size but the increase is not significant beyond 2 disc/100 medium. So, addition of two mycelial discs is sufficient to achieve the desired enzyme production.

Influence of incubation period on laccase production

Incubation period is a critical factor to be kept in mind for getting maximum productivity of the enzyme in shortest possible time at industrial scale. The broth was harvested everyday from day 2 to day 10 to determine proper incubation period necessary for obtaining maximum enzyme productivity. The production of laccase increased linearly with increasing incubation period up to day 7 where the enzyme activity was found to be 16.9U/ml. From day 7 onwards there was not substantial increase in enzyme activity which proves that incubation up to 7 days is sufficient to obtain maximum enzyme production.

Influence of inducer on laccase production

Interestingly, addition of some of the inducers resulted in strikingly enhanced production of laccase. The inducer employed were ABTS (0.6mM), Guaiacol (1mM), $CuSO_4$ (2mM), $MgSO_4$ (0.1%) and Tannic acid (0.2mM). Although all the inducers applied resulted in enhanced activity of the laccase but the effect of copper was quite prominent when compared with other inducers. Addition of CuSO4 and $MgSO_4$ to growth medium resulted in high production of laccase *i.e.* 22.6U/ml and 18.2 U/ml respectively which is quite high as compared to the control i.e. 10.2U/ml. Copper is an essential micronutrient for the most living organisms, copper requirement by microorganism are usually satisfied in very low concentration, ranging between 1 to 10 mM. Copper present in higher concentration in its free cupric form is extremely toxic to microbial cells. Periasamy *et al.,* (2011) studied the effect of different metal ions on laccase production, out of different metals Hg^{2+}, $Cd^{2+,}$ Zn^{2+}, Cu^{2+}, Mn^{2+}, Mg^{2+} screened, Cu^{2+} was best for laccase production followed by Mg^{2+} in *Pleurotus ostreatus*. Kenkebashvili *et al.,* (2012) reported that *Coriolopsis gallia* grown at 2.0mM Cu^{2+} exhibited high laccase activity as compared to the culture grown at 0.5mM and 1mM Cu^{2+} concentration. In a study carried by Sel *et al., (*2015) $MgSO_4$ at the concentration of 0.05% has shown significant influence on laccase production by *Pleurotus ostreatus*. Keeping in mind the importance of Cu^{2+} and Mg^{2} on laccase production, experiments were also carried out to find out the optimum concentration of both metal ions for the production of laccase. It can be observed from the data that 2mM of $CuSO_4$ and 0.12% of $MgSO_4$ is optimum for the laccase production by *Scytalidium lignicola*.

Influence of inhibitors on laccase production

The laccase production by *Scytalidium lignicola* was inhibited 94% in presence of 1mM sodium azide and 79% by formaldehyde conversely L-cysteine and EDTA had a relatively low inhibitory effect *i.e.,* 43% and 60% respectively. These results are very similar to the previously reported laccase from Basidomycete *Fomitella fraxinea* (Mi *et al.,* 2007). It is formerly reported that binding of sodium azide to the types 2 and 3 copper sites affects internal electron transfer, thus inhibiting the activity of laccase, also formaldehyde being an organic solvent is assumed to alter pH of aqueous solution resulting in reduced enzyme production (Tiwari and Chittora, 2013). EDTA is a metal ion chelating agent and can inhibit metalloprotease activity and L-cysteine is a strong reducing agent of disulphide bond

between protein molecules (Xu *et al.*, 2016). Laccase from *C. thermophilium* was also strongly inhibited by Cu-chelating agents (Chefetz *et al.*, 1998).

Enzyme production under optimized conditions

After optimizing all the above mentioned parameters the laccase enzyme production was carried out in modified Czapek Dox medium incorporating 2mM $CuSO_4$ and 1% $MgSO_4$ as enzyme inducer and adjusted at initial pH 6. The sterilized medium was inoculated with 2 mycelial discs and incubated at 30°C for 7 days. The enzyme activity obtained under optimized conditions was 28.2U/ml which is 1.5 folds of the enzyme activity obtained in control *i.e.* 17.4U/ml.

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