ISSN: 0976-3031

International Journal of Recent Scientific Research

Impact factor: 5.114

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Volume: 6 Issue: 10

THE PUBLICATION OF
INTERNATIONAL JOURNAL OF RECENT SCIENTIFIC RESEARCH
http://www.recentscientific.com
E-mail: recentscientific@gmail.com

ISSN: 0976-3031

Available Online at http://www.recentscientific.com

International Journal of Recent Scientific Research
Vol. 6, Issue, 10, pp. 6853-6857, October, 2015

International Journal of Recent Scientific Research

RESEARCH ARTICLE

ISOLATION AND CHARACTERIZATION OF LACCASE PRODUCING FUNGI FROM DIFFERENT ENVIRONMENTAL SAMPLES

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ARTICLE INFO

Article History:

Received 06th July, 2015
Received in revised form 14th August, 2015
Accepted 23rd September, 2015
Published online 28st October, 2015

Key words:

Laccase, Guaiacol, Dye decolourization.

ABSTRACT

The current study includes isolation and characterization of laccase producing fungi from decaying wood region of fallen trees, soil and mushrooms in the area of botanical garden of Karnatak University Campus, Dharwad, Karnataka. In this study, 9 fungal isolates were isolated by serial dilution technique and fruiting bodies of mushroom used for the isolation. They were cultivated on potato dextrose agar (PDA) plate with indicator compound Guaiacol to screen for the laccase production ability. Out of 9 isolates, Only 2 isolates have the ability to produce laccase, one was presumed to be potent and another showed weak laccase production. The most potent strain was used for further studies. Potent fungus was morphologically identified as belong to *Coprinus comatus*. Laccase production was done by Submerged Fermentation (SmF). Various process parameters like different pH, temperature, carbon and nitrogen sources wereon laccase production was investigated in the fermentation process. The laccase activity was highest with sucrose as carbon source and yeast extract as nitrogen source. The highest production of laccase at pH 5 and the temperature for production was recorded at 30°C. Further dye decolourization was performed with the selected fungus.

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INTRODUCTION

Laccases are copper containing enzymes that catalyse the one electron oxidation of various aromatic compounds specifically phenols, anilines and their derivatives while reducing molecular oxygen (O_2) to water (Gianfreda *et al.,* 1999). Its low-substrate specificity and strong oxidative ability has made it to be significantly useful in pulp delignification, textile dye bleaching, effluent detoxification, bioremediation of soils, washing powder components, removal of phenolics from wines (Kiiskinen *et al.,* 2004, Dhouib *et al.,* 2005) enzymatic conversion of chemical intermediates and production of valuable compounds from lignin (Nyanhongo *et al.,* 2002). Laccases (benzenediol: oxygen oxidoreductases; EC 1.10.3.2) exist widely in nature. They are predominantly found in higher plants and fungi (Thurston 1994, Mayer *et al.,* 2004) and recently some bacterial laccases have also been characterized from *Azospirillumlipoferum* (Givaudan *et al.,* 1993), *Bacillus subtilis* (Martins *et al.,* 2003), S*treptomyces lavendulae*(Suzuki *et al.,* 2003) and *S. cyaneus* (Arias *et al.,* 2003). Most of the laccases studied thus far are of fungal origin, especially from white-rot fungi, such as *Phlebiaradiata* (Niku-Paavola *et al.,* 1988), *Pleurotusostreatus* (Palmieri *et al.,* 2000) and *Trametesversicolor* (Bourbonnais *et al.,* 1995).Textile dyes are chemicals of complex aromatic structures designed to resist the impact of detergents, sunshine and temperatures (Nigam *et al.,* 1996). They are chemically and photo chemically stable and are extremely persistent in natural atmospheres. The world-wide annual production of synthetic textile dyestuff has been estimated to be over 1 x 106 ton (Pandey *et al.,* 2007). More than 10,000 different commercially available dyes are used in textile industry for dyeing and printing purposes (Meyer 1981, Govindwar *et al.,* 2011). 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, 2006). Maximizing laccase production can be achieved by optimizing nutritional conditions which includes carbon, nitrogen and inducer sources and physical conditions such as pH, agitation and inoculum size (Yasser *et al.,* 2010, Baldrian 2006].

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Hence, fungi are highly diverse in nature; they have been recognized as a target for screening to find out the appropriate source of enzymes with constructive and novel characteristics (Dong *et al.,* 2005). Plate-test screening based on polymeric dye compounds, guaiacol is an effective method to identify novel laccase producers (Adiveppa *et al.,* 2015). In view of the industrial significance of laccase, in this present study, fungi were isolated from soil and mushrooms and screened for laccase production using the indicator Guaiacol. This investigation led to the confirmation of fungal isolate which is the producer of laccase to introduce a new source of extracellular laccase and the effectiveness of the isolate in dye degradation.

MATERIALS AND METHODS

Collection of Samples

Fungi in the form of fruit bodies were collected from botanical garden Karnatak University, Dharwad, Karnataka. They were placed into plastic bags. Fruit bodies of fungi were cleaned with disinfectants and approximately 3 x 3 mm was placed on PDA medium in petri-dishes. Later on, when the mycelium had grown on the medium in the vicinity of the tissues, the sample was transferred to fresh agar media in tubes. This was repeatedly carried out until pure cultures could be obtained as single cultures or so called fungal isolates. The samples were marked with information such as number, procurement location, growth site and specific characteristics.

Screening of laccase on solid media

Laccase production were carried out by inoculation of mycelium from each strain onto PDA plates containing 0.02% Guaiacol as indicator compound and it was incubated at 30°C for 5 days. The formation of reddish brown halo in Guaiacol supplemented plates indicated a positive laccase secretion.

ANALYTICAL METHODS

Extracellular laccase 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 10min of incubation using UV Spectrophotometer. Enzyme activity is measured in U/ml which is defined as the amount of enzyme catalysing the production of one micromole of coloured product per min per ml (Adiveppa *et al.,* 2015)

$$\text{Volume activity (U/ml)} = \frac{\Delta A470\text{nm/min} \times 4 \times Vt \times \text{dilution factor}}{€ \times Vs}$$

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

Optimization of laccase in submerged fermentation

Culture conditions

Basal medium (per liter): 1.0 g KH_2PO_4, 0.4 g K_2HPO_4, 0.5 g $MgSO_4.7H_2O$, 0.013 g $CaCl_2.2H_2O$, 0.1 g yeast extract, 0.5 g NH_4NO_3, 3.0 g asparagine and 2 ml Tween 80. After autoclaving and cool to room temperature, 2.5 mg/l thiamine and 1 ml/l of a trace-elements solution consisting of (per liter): 4.8 g $FeC_6H_5O_7.5H_2O$, 2.64 g $ZnSO_4.4H_2O$, 2.0 g $MnCl_2.4H_2O$, 0.4 g $CoCl_2.6H_2O$ and 0.4 g $CuSO_4.5H_2O$ was added. Four agar discs (1 cm diameter), cut from the growing edge of a 7-day PDA culture, were used to inoculate each flask (Xiang *et al.,* 2010).

Effect of different pH

The basal media was adjusted to different pH i.e 3, 4, 5, 6, 7 and 8 in separate conical flasks and then inoculated with the spore suspension of the fungus without adding any additional carbon and nitrogen sources. Enzyme assay was done as Guaiacol assay method.

Effect of different temperature

The media flasks were inoculated with the well grown fungusfrom PDA plates and the flasks were incubated at different temperatures viz., 37°C, 40°C, 45°C, 50°C and 55°C and assay was done as Guaiacol assay method.

Effect of carbon sources

The different carbon sources used were starch, sucrose, maltose, lactose, glucose and mannitol. The carbon sources were amended with the 100 ml of basal media at a concentration of 2% and the media was inoculated with 100µl of the spore suspension of the selected fungus. The enzyme assay was performed as Guaiacol assay method.

Effect of nitrogen sources

The different nitrogen sources used in the study were peptone, yeast extract, soya bean meal, beef extract, casein andtryptone which were added to the basal media at 2% concentration and then inoculated with the spore culture of the potent strain. Enzyme assay was performed as Guaiacol assay method.

Decolourization of Dyes

Dye degradation ability of *Coprinuscomatus* was screened in potato dextrose agar amended with dyes at concentration of 100 ppm in the presence of 0.02% agar plate. Mycelia was placed on the centre of dye containing agar plate and incubated at 30°C. Plates were regularly monitored for dye decolourization activities through the change of colour from coloured to colourless for every 24 hour.

RESULTS AND DISCUSSION

Screening of samples for laccase producing fungi

The present study mainly focused on laccase producing fungi from botanical garden of Karnatak University campus, Dharwad, Karnataka, India. A total of 9 fungi colonies were isolated from the different samples. Isolated fungi were maintained in PDA medium. They were screened for potential laccase producing ability using indicator as well as substrate namely Guaiacol. Laccase is a very potent enzyme with ability to act on a number of substrates. All the isolates were inoculated in potato dextrose agar plate which is containing 0.02% Guaiacol as indicator compound and it was incubated at 30°C for 5 days. In the presence of Guaiacol intense reddish brown colour was produced in the medium around the fungal colonies and was taken as the positive reaction for the production of laccase enzyme (Fig. 1) (Adiveppa *et al.,* 2015).

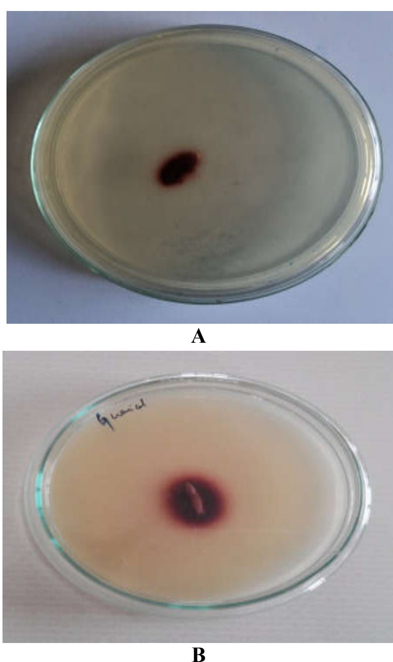


Figure 1 A and B isolates showed positive results for laccase production on Guaiacol indicator

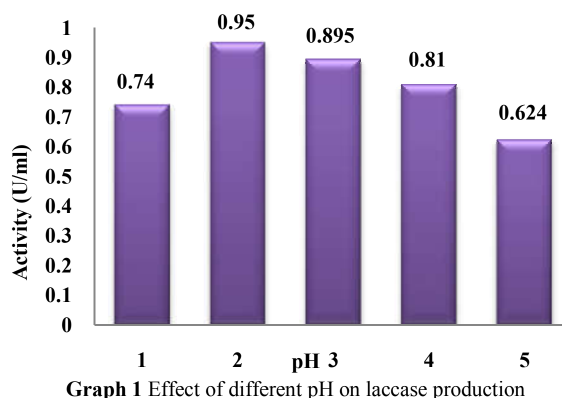
Morphology Identification

Selected laccase producing potent fungus was morphologically identified as *Coprinus comatus* (Fig. 2).

Figure 2 *Coprinus comatus*

Effect of different pH

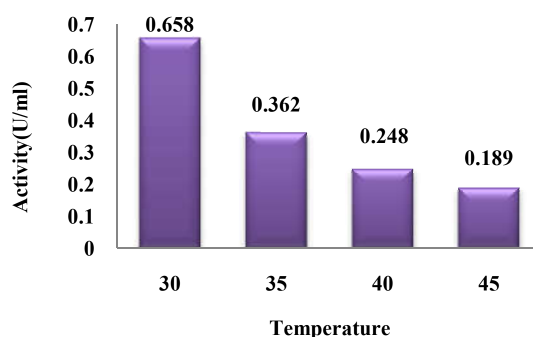
The results showed that the *Coprinus comatus* strain was able to produce maximum laccase activity at pH 5 (0.950 U/ml) followed by pH 6 (0.895 U/ml), pH 7 (0.810 U/ml), pH 4 (0.740 U/ml) and pH 8(0.624 U/ml), (Graph 1) Sivakumar *et al.,* (2010) have reported that *Ganoderma sp.* exhibited optimum laccase production at pH 6.



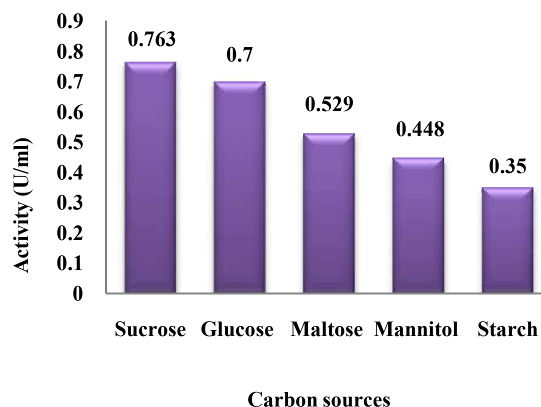
Graph 1 Effect of different pH on laccase production

Effect of different temperature

The results showed that the *Coprinus comatus* strain was able to produce highest laccase activity at temperature at 30°C with high laccase activity (0.658 U/ml) followed by 35°C (0.362 U/ml) and 40°C (0.248 U/ml), 45°C (0.189 U/ml) (Graph 2). Adiveppa *et al.,* (2015) have reported that the *Marasmius sp.* BBKAV79 strain was able to produce temperature is 40°Cwith high laccase activity. Periyasamy *et al.,* (2012) have showed the optimum temperature for laccase production at 50°C in *Pleurotus sp.*



Graph 2 Effect of different temperatures on laccase production



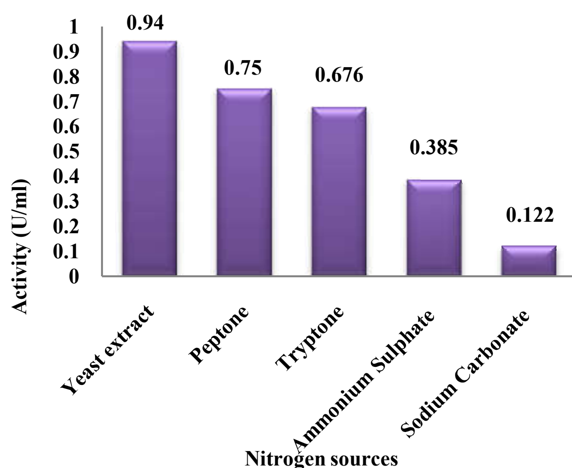
Graph 3 Effect of carbon sources on laccase production

Effect of carbon sources

The results suggested that the Sucrose supported the maximum laccase activity (0.763 U/ml) followed by Glucose (0.700 U/ml), Maltose (0.529 U/ml), Mannitol (0. 448 U/ml) and Starch (0.350 U/ml) (Graph 3). 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.

Effect of nitrogen sources

The results indicate that the Yeast extract supported the maximum laccase activity (0.940U/ml) followed by Peptone (0.750), Tryptone (0.676U/ml), Ammonium Sulphate (0.385 U/ml) and Sodium Carbonate (0.122 U/ml) (Graph 4).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.



Graph 4 Effect of nitrogen sources on laccase production

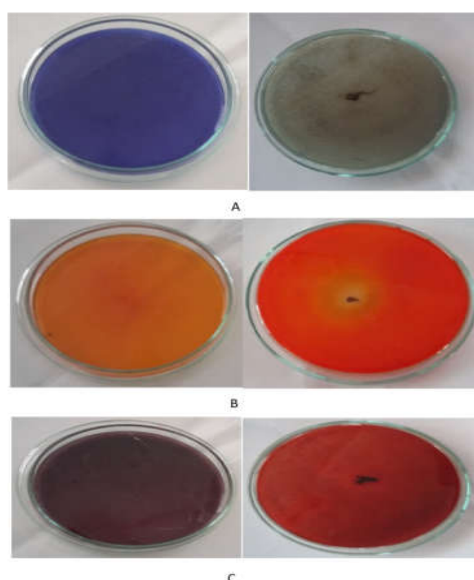


Figure 2 A- Aniline blue, B- Methyl orange, C- Congo red

Dye Decolourization

In the present study, *Coprinus comatus* was investigated for their ability to decolourize 3 synthetic dyes namely Aniline blue, Congo red and Methyl orange by cultivation on a solid media. The decolourization abilities of the fungal strains after 3 to 4 days of incubation are shownin Fig 2. Among the 3 dyes tested in this study, Aniline blue dye was the most rapidly decolourized, followed by Methyl red and Congo red. Similarly, Moorthi *et al.* (2007) tested the white rot fungi *Trametes hirsute* and *P. florida* for their dye decolourizing ability against reactive dyes Blue CA, Black B133 and Corazol violet SR.

CONCLUSION

Screening for laccase producing fungi on plates containing Guaiacol indicator resulted in isolation of 2 fungal strains. One of the strains was identified as potent due to its fast growth rate and grater reactivity towards Guaiacol indicator present in the cultivating medium and morphologically identified as *Coprinus Comatus*. Laccase production by isolated *Coprinus Comatus* strain was carried out in submerged fermentation. *Coprinus comatus* has ability to degrade synthetic dyes. This potent organism can be used for large scale laccase production and its use in treating various industrial effluents.

Acknowledgment

The Authors are thankful to the post-graduate Department of studies in Biotechnology and Microbiology, Karnataka University Dharwad for providing all the necessary facilities.

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How to cite this article:

Adiveppa Bheemappa Vantamuri *et al.*2015, Isolation and Characterization of Laccase Producing Fungi From Different Environmental Samples. *Int J Recent Sci Res* Vol. 6, Issue, 10, pp. 6853-6857.

International Journal of Recent Scientific Research