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Research Article

SCREENING THE EFFECT OF NUTRITIONAL PARAMETERS ON BIOMASS AND LACCASE PRODUCTION IN SUBMERGED MEDIUM BY LITTER DECOMPOSING BASIDIOMYCETE *AGARICUS HETEROCYSTIS*

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ABSTRACT

An indigenous litter decomposing edible *Agaricus heterocystis* isolated from IIT Chennai, India was assessed for fungal biomass and laccase production in submerged medium. Nutritional parameters such as medium composition, temperatures, pH, carbon sources and nitrogen sources, and metal ions were optimized. Fungal biomass requirements were determined by the mycelia dry weight method. The optimal medium for laccase activity and biomass production were reported as malt dextrose broth and potato dextrose broth, respectively. The optimal temperature for fungus mycelial biomass yield was recorded as 30°C, whereas the optimal temperature for laccase activity was 25°C. The optimal pH for fungi mycelial biomass yield and laccase activity were 6.5 and 5.5, respectively. Of the five carbon sources tested, fructose supported the highest fungus mycelial biomass yield of 175 ± 1.50 mg/30 cm^3, and the highest laccase enzyme production with 48.17 ± 1.85 U/ml of laccase ($p \leq 0.05$). The most utilized nitrogen sources for fungus mycelial biomass yield and laccase activity were ammonium tartarate and yeast extract, which stimulated the laccase production of 56.89 ± 2.52 U/ml and fungus mycelial biomass yield 197 ± 3.45 mg/30 cm^3. Among the different metal ions tested, only copper sulphate showed highest laccase activity (50.12±3.14 U/ml) and maximum biomass production (192±2.7 mg/30cm^3). The implications of these findings were discussed.

Keywords: Laccase, LDF fungi, *Agaricus heterocystis*, Nutritional parameters

INTRODUCTION

Basidiomycetous inhabitants are classified as wood decomposers, litter decomposers and mycorrihizal symbionts according to the way in which they obtain nutrients. However, most white-rot fungi grow preferentially in compact wood (trunks, logs, branches and stumps). Since these fungi specialize in colonizing compact wood (timber and stumps) and cannot compete in soil for a prolonged time, their actual contribution to the removal of recalcitrant lignin polymer under natural conditions seems to be limited.[1,2] There is, however, a second ecophysiological group of ligninolytic basidiomycetes—the *litter-decomposing fungi*—which have recently been shown to possess a ligninolytic enzyme system similar to that of white-rot fungi[1,3,4]. The lignocellulosic complex in particular includes lignin that is attacked by a number of enzymes including manganese peroxidase (MnP), lignin peroxidases (LnP) and laccase.

Laccase is a copper containing polyphenol oxidase (EC1.10.3.2) first discovered in the Japanese lacquer tree, *Rhus vernicifera*[5] over 100 years ago. It is structurally and evolutionarily related to the large blue copper protein group.[6,7] It is a common enzyme and has been found to be widely distributed in plants[6] and fungi.[8] Laccase is dependent on four copper ions, which are distributed among three different highly conserved binding sites, for its function, with each copper ion appearing to play an important role in the catalytic mechanism.[7,9] It catalyses the four-electron reduction of oxygen to water, and this is typically accompanied by the oxidation of a phenolic substrate, including lignin phenolic units and chlorophenols, anthraquinone dyes and, to a certain extent, some polycyclic aromatic hydrocarbons (PAHs), aromatic amines.[10,11]

In fungi, laccase has been well documented to act as a ligninolytic enzyme.[12] The enzyme has applications in the pulp, paper industry and food industry, where laccase plays a role in tea and coffee fermentations and in vinification, also in bioremediation, biotechnology applications.[11,13] Laccase plays some other function such as sporulation, pigment production and fruit body development.[9] Some LDF are known to produce laccase including the MnP-forming species *Paneolus sphinctrinus*, *Marasmius quercophilus* and *Agaricus bisporus*.[14–16]

With microbial enzymes dominating world markets, more innovation and improvisation is needed to increase the efficiency of production at an economical rate. The understanding of physiological mechanisms regulating enzyme synthesis in lignocellulose bioconversion could be useful for improving the technological process of edible and medicinal mushroom production.[17–18] The ligninolytic machinery in most basidiomycetes is highly regulated by medium composition, nature of carbon source, concentration of carbon source, pH of fermentation broth, fermentation temperature, amount and nature of nitrogen source and presence of inducers (Cu^{2+}, Mn^{2+}, Fe^{3+}, etc).[19–22] Laccase production have been found to be highly dependent on the cultural conditions of the fungus.[15,23] Fungal biomass have also 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[24]; however, the media supporting high biomass yield did not necessarily support high laccase yields.[25]

However, the application of laccase in biotechnological process requires the production of high amounts of enzyme at low cost and hence the current focus on laccase research is oriented towards the search for efficient production systems. Hence, the present attempt was made in LDF fungi to optimize the nutritional parameters on laccase and biomass production.

MATERIALS AND METHODS

Microorganism and inoculum preparation

Fruiting body of the *Agaricus heterocystis* was isolated from south eastern part, IIT Chennai, India, and the culture was maintained on potato dextrose medium at room temperature. Inoculum of *A. heterocystis* was prepared from mycelia grown on the same medium incubated at room temperature for 4–6 days. From the plate, 7-mm diameter mycelial disc was used as the inocula.

Culture conditions

Incubation was carried out statically at 30 ± 1°C in 250 ml Erlenmeyer flask containing 30 ml of the medium inoculated with 7 mm agar plug from 6-day-old mycelia grown on malt-extract agar. Periodic harvesting of the mycelia was performed using the filter paper. An aliquot of supernatant was collected aseptically and culture filtrates were used as enzyme sources.

Screening of nutritional parameters on laccase, biomass production

Optimization of laccase, biomass production by *A. heterocystis* was studied using different medium *viz.*, Potato-dextrose broth, Malt dextrose extract broth, Czapek-dox broth, Modified Melin Norkrans broth, Beetroot-potato broth, Yeast-peptone glucose broth, Sabouraud dextrose broth and Glucose malt extract salt medium. Various carbon sources such as fructose, lactose, sucrose, maltose, starch; nitrogen sources such as ammonium sulphate, ammonium tartarate, beef extract, peptone, yeast extract; metal ions such as copper sulphate, manganous sulphate, magnesium chloride, zinc sulphate, ferric chloride 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.

Estimation of fungal biomass

Fungal biomass yield (mg/30 cm^3) was determined as cell dry weight after centrifugation (12,000 rpm, 10 min) of 30 ml of the culture medium and dried to constant weight at 60°C.

Enzyme assay

Extracellular laccase activity was measured spectrophotometrically by the following method of Wolfenden and Wilson[26] with 2, 2'azinobis (3 ethyl benzothiazoline 6 sulfonate) (ABTS) as substrate. The reaction mixture consisted of 1 ml of 1 mM ABTS in 100 mm sodium acetate buffer pH 5 and 0.1 ml of enzyme. The reaction was monitored by measuring the change in A 436 (ε436 = 29.3 m × *r*–1 con–1) for 30 min. One unit enzyme activity was defined as the amount of enzyme that oxidifies 1 M of ABTS/min at 25°C. The activities were expressed in U/ml. The data represented are means of three replicates (mean ± SD).

RESULTS

There was significant differences in fungus 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 among the eight different nutrient medium tested, only malt dextrose broth showed the highest laccase activity (39.39 ± 2.14 U/ml) (Fig 1a), whereas the potato dextrose broth showed maximum fungal biomass production (143 ± 0.05 mg/30 cm^3) (Fig 1b).

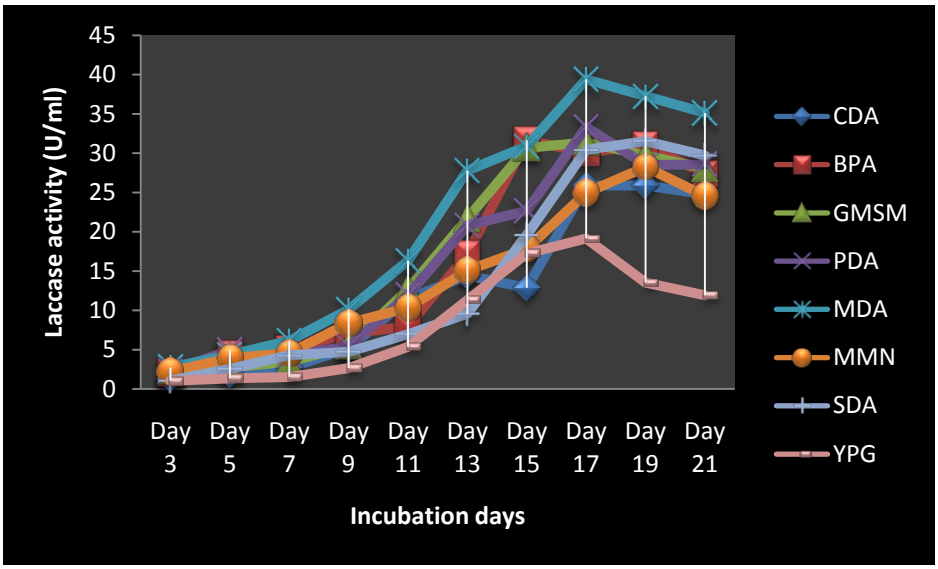

Fig. 1a: Effect of different medium compositions on laccase activity

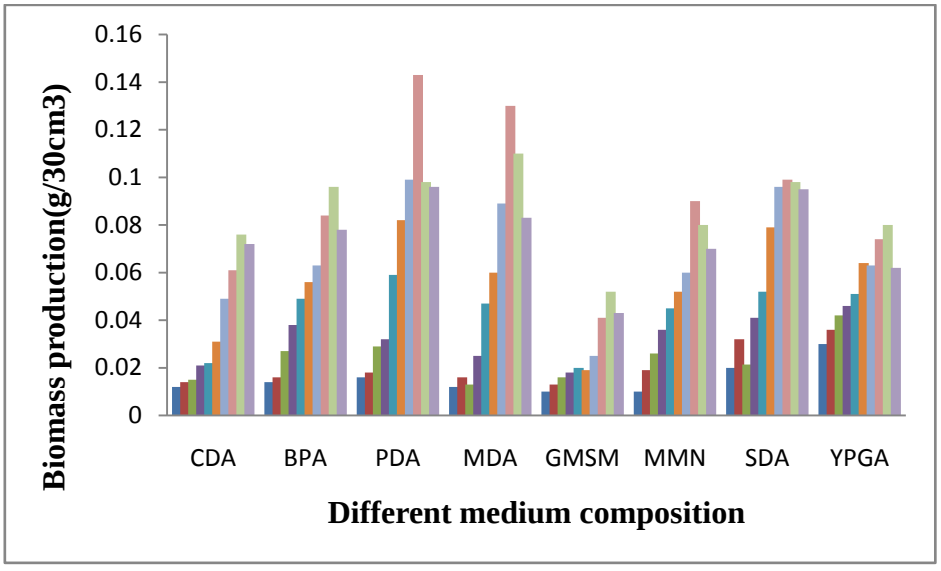

Fig. 1b: Effect of different medium compositions on biomass production

When the incubation temperature increased, fungus mycelial biomass yield and laccase activity decreased simultaneously. Temperature of 25°C was observed to be optimal for laccase activity (46.16 ± 2.12 U/ml) (Fig 2a), whereas 30°C for fungus mycelial biomass yield (154.18 ± 5.20 mg/30 cm³) (Fig 2b).

Increase in fungus mycelial biomass yield and laccase production was observed from pH 4.0 to 6.5, but thereafter fungus mycelial biomass yield and laccase activity decreased. The highest fungus mycelial biomass production was recorded at pH 6.5 (193.25 ± 3.45 mg/30 cm³) (Fig 3a); however, higher laccase activity of 47.33 ± 2.86 U/ml (Fig 3b) was obtained at pH 5.5.

Fructose, lactose, sucrose, maltose and starch were tested as carbon sources for fungus mycelial biomass yield and laccase production. The best fungus mycelial biomass yield (175.32 ± 1.50 mg/cm³) (Fig 4a) and highest laccase activity (48.17 U/ml) were achieved with fructose (Fig 4b).

Similar results were observed with different nitrogen sources such as ammonium sulphate, ammonium tartarate, beef extract, peptone, and yeast extract in submerged medium. All the nitrogen sources used in this study significantly promoted biomass yield and laccase production by the fungus. The best stimulatory nitrogen source for fungus mycelial biomass yield (197.25 ± 3.45 mg/30 cm³) was achieved using yeast extract as nitrogen source (Fig 5a), while the highest laccase activity (48.89 ± 2.52 U/ml) was induced with ammonium tartarate (Fig 5b).

Among the different metal ions tested only copper sulphate showed highest laccase activity (50.12 ± 3.14 U/ml) (Fig 6a) and maximum biomass production (192 ± 2.7 g/30cm³) (Fig 6b).

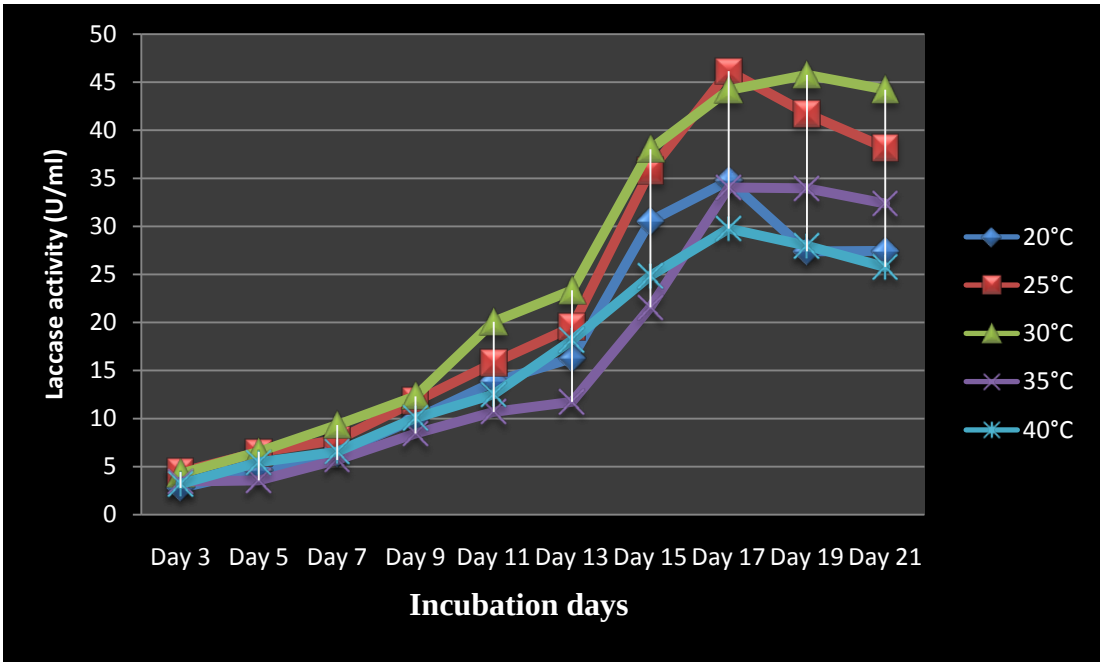

Fig. 2a: Effect of different temperatures on laccase activity

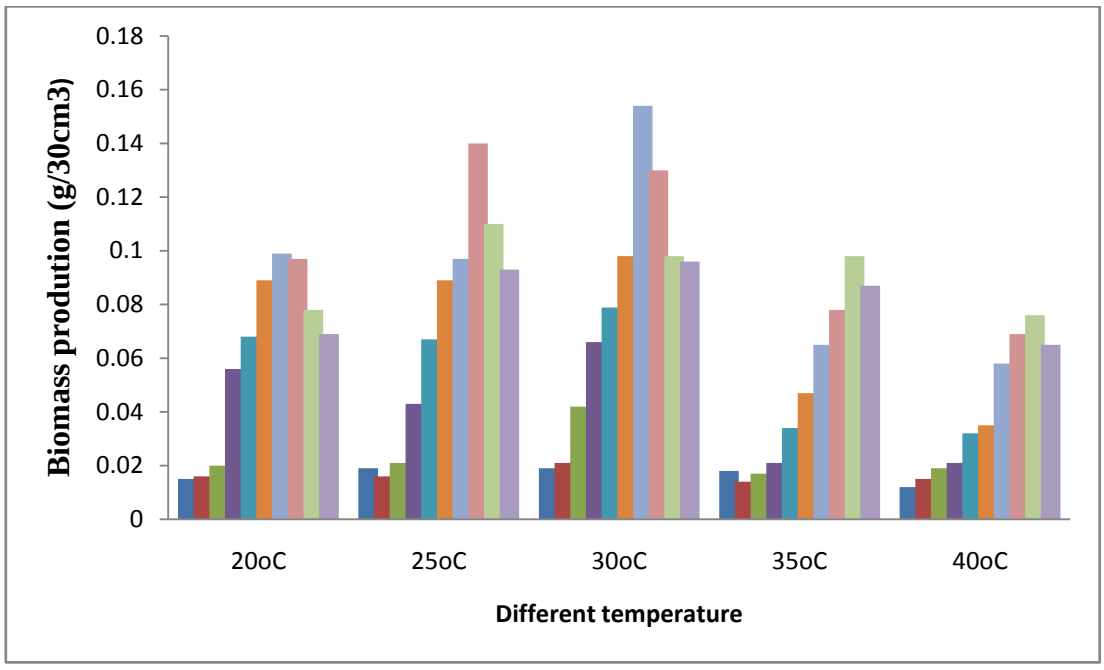

Fig. 2b: Effect of different temperatures on biomass production

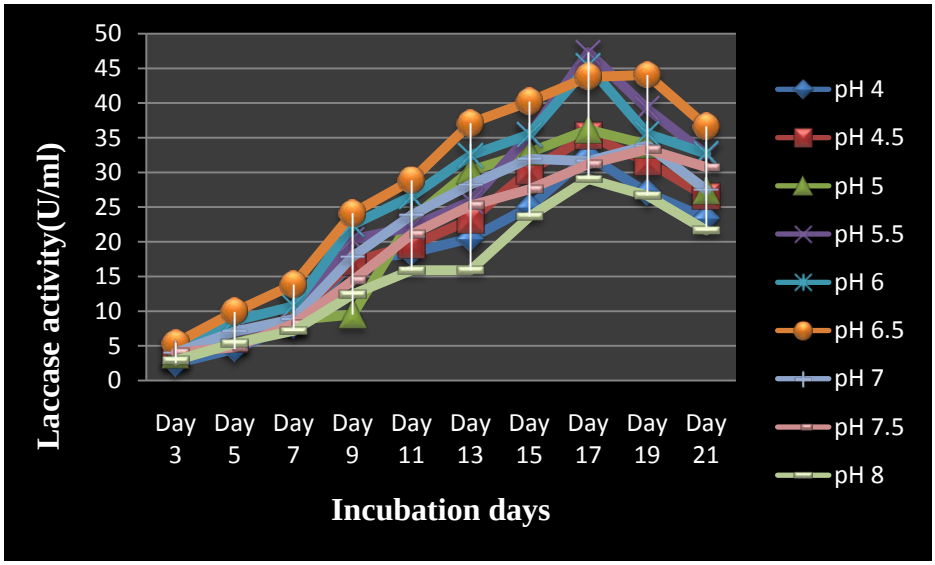

Fig. 3a: Effect of different pH on biomass production

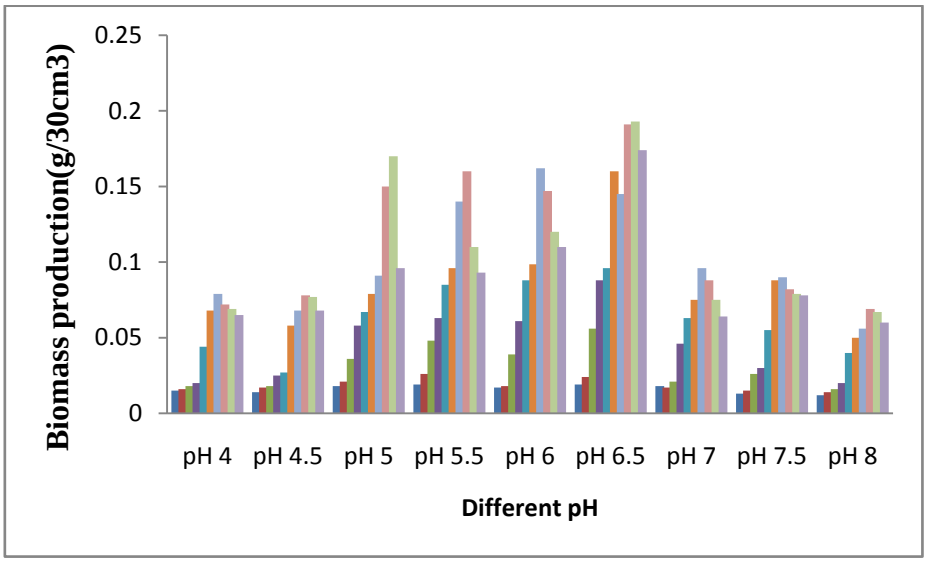

Fig. 3b: Effect of different pH on biomass production

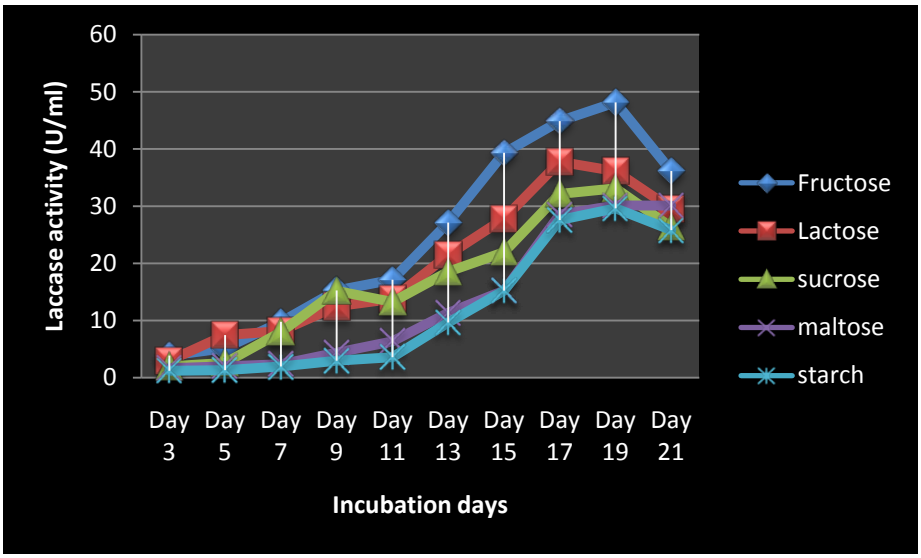

Fig. 4a: Effect of different carbon sources on laccase activity

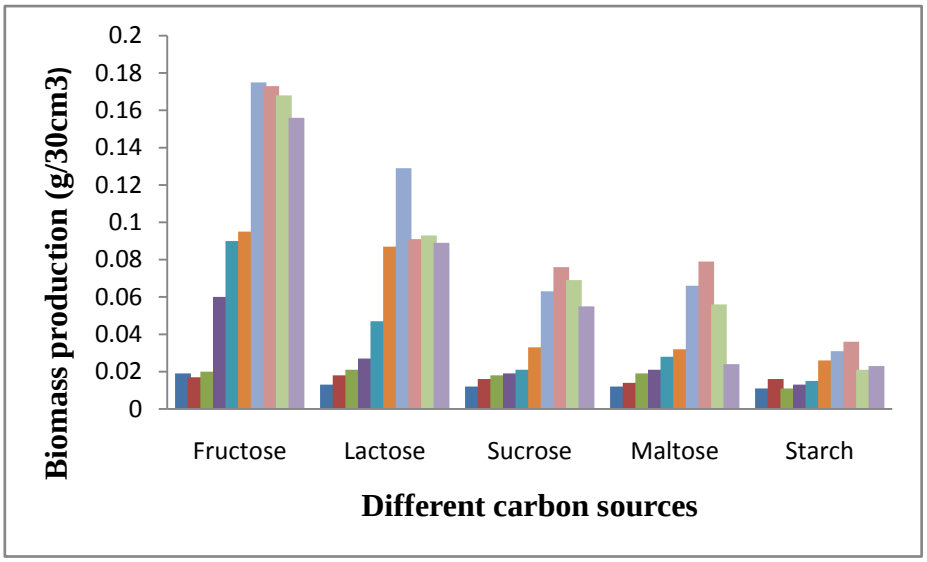

Fig. 4b: Effect of different carbon sources on biomass production

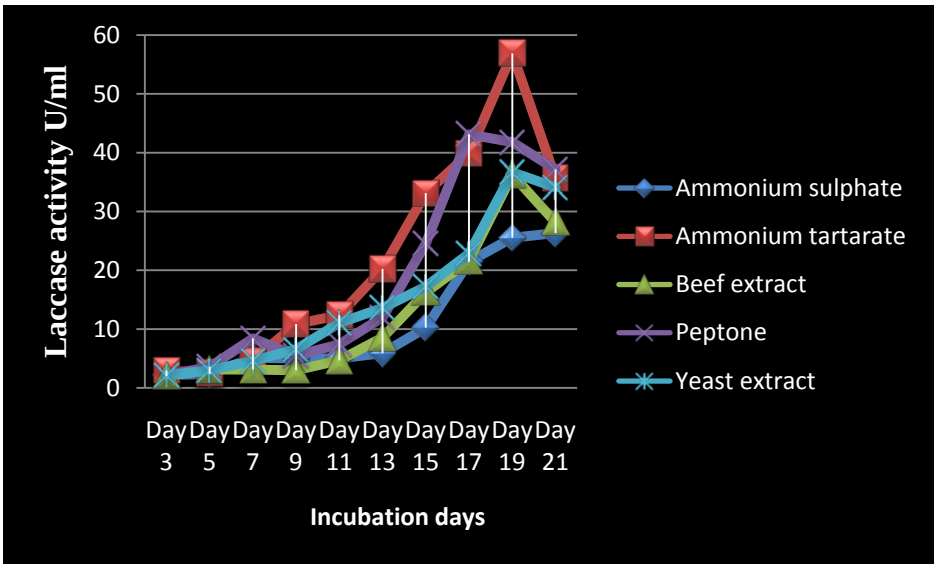

Fig. 5a: Effect of different nitrogen sources on laccase activity

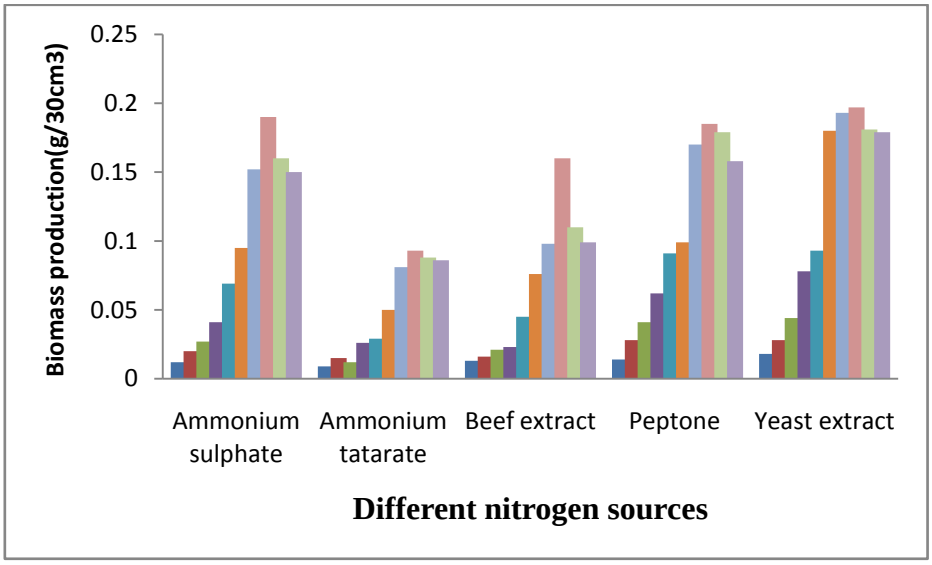

Fig. 5b: Effect of different nitrogen sources on biomass production

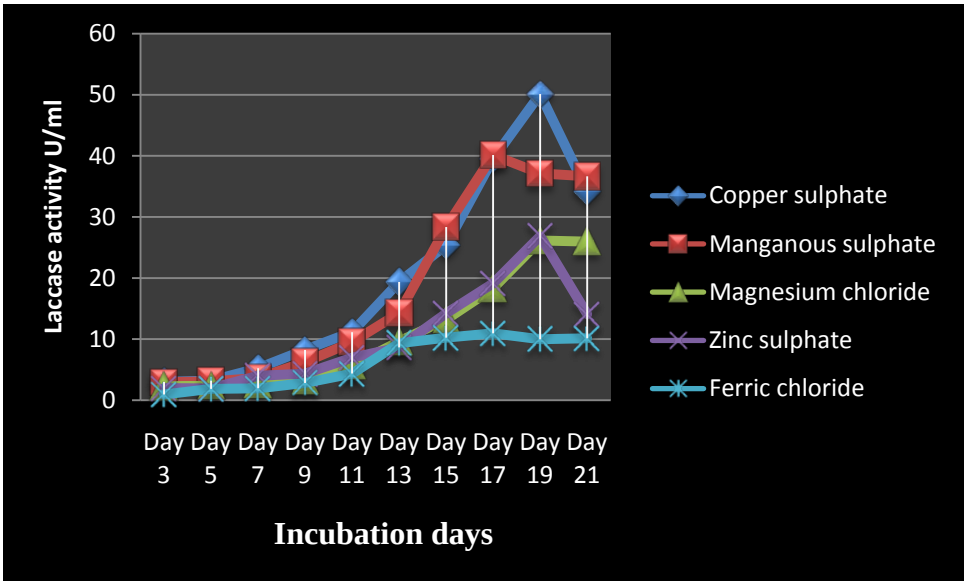

Fig. 6a: Effect of different metal ions on laccase production

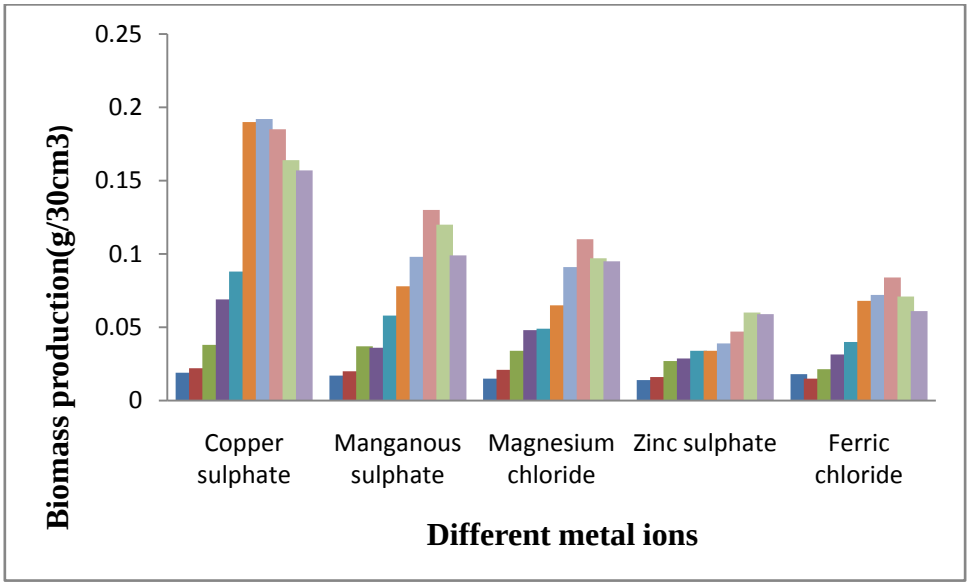

Fig. 6b: Effect of different metal ions on biomass production

DISCUSSION

Fungal biomass yield and activity of laccase were analysed from 3 to 21 days of incubation. The process of submerged cultivation involves the growth of microorganisms in a liquid medium rich in nutrients under aerobic conditions. In order to achieve high production, the studies are first focused on the optimization of nutritional and operational conditions. Laccase production by fungi has been shown to depend markedly on the composition of the culture medium, carbon, nitrogen content and phenolic inducer compounds.[27] Best enzyme production in MEB can be attributed to the fact that it provides the complete pool of amino acids required for enzyme synthesis.[28,29] Moreover, malt extract is rich in the aromatic amino acids tryptophan and tyrosine. Tryptophan is a precursor for the synthesis of a large number of N-substituted aromatic secondary metabolites of fungi.[30] These may then act as inducers for MnP in a way similar to veratryl alcohol and guaiacol, which are substrates as well as inducers for ligninase and laccase. Biomass production and laccase activity was observed to be high at temperature 25–30°C, with temperature 25°C as the optimum for laccase production; and laccase activity of this fungus was neither favoured by low nor high temperatures. Similar observations were made by Jonathan *et al.*[31] on biomass production of *Pleurotus florida*. Also, Iqbal *et al.*[22] found substantial decrease in ligninolytic enzymes of *Trametes versicolor* IBL-04 when cultivated at temperatures higher than 30°C. Results from this study showed that pH values of 6.5 and 5.5 were 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 8.5. This may be attributed to the fact that change in pH may alter the three-dimensional structure of the enzymes.[32] Safari et al.[33] also reported that culture pH is an index of fungi enzyme activity; wherever the pH was low, fungal activity was high.

For these reasons, enzymes are known to be active over a certain pH range. Krishna-Prasad *et al.*[20] observed that the optimum pH for laccases activity of *Pleurotus ostreatus* was 5.5. Among the different carbon sources that were tested for biomass production and laccase production, fructose stimulated the highest laccase and biomass production. 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,[34] whereas Mansure *et al.*[35] showed that the use of fructose instead of glucose resulted in a 100-fold increase in the specific laccase activity of basidiomycetes. Bettin *et al.*[36] showed a similar result that fructose was a good carbon source for laccase production in *Pleurotus sajor-caju*. Fasidi[37] found that glucose and fructose stimulated mycelial biomass production in *Volvariella esculenta.* 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, yeast extract stimulated higher biomass yield and ammonium tartrate stimulated higher production of laccase. These results are in agreement with the findings of Vahidi *et al.*[38] who reported increase in antifungal activity when yeast extract was used as nitrogen source. Collins and Dobson[39] investigated the effect of five ammonium tartarate concentrations (0.5–54.3 mM) increase the laccase activity in *T. versicolor culture*. Among different metal ions tested, copper sulphate showed highest biomass and laccase activity. Since laccase is a copper-containing protein,[40] copper supplementation of the culture medium is reported to enhance enzyme production in many basidiomycetes.[39,40] Copper is a strong inducer of laccase in many fungal species, including *T. versicolor*, *M. quercophilus* and *P. ostreatus.*

This work provides the baseline information on growth parameters optimization for LDF *A. heterocystis* under submerged culture conditions. Future studies will focus towards purifying the enzymes as well as testing them in industrial and environmental biotechnology.

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