# Optimization of nutritional requirements for mycelial growth and production of ligninolytic enzymes for endophytic *Lasiodiplodia hormozganesis* in submerged culture

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***Abstract***
*Ligninolytic enzymes have been obtained from Lasiodiplodia hormozganensis isolated as an endophyte from Ficus krishnae L. plant. In this study effects of different culture media, carbon source, nitrogen source, temperature, pH and days of incubation on mycelial growth and sporulation of Lasiodiplodia hormozganensis were evaluated. Twelve basal media were utilized and Richard's medium stimulated the best growth followed by Czapek's I, Czapek's II and Coon's medium. The optimum temperature that supported the best growth of this fungus was 32º C while the optimum pH was 4.0.*

*Out of various carbon sources tested, sucrose was found superior for growth followed by glucose and fructose. As nitrogen source, potassium nitrate performed best among several inorganic nitrogenous compounds whereas L-Tyrosine was convenient from all amino acids for fungus growth. Qualitative and quantitative analysis of ligninolytic enzyme has also been determined along with variation in their activity due to different carbon and nitrogen sources.*

**Keywords**: Physiology, Endophytic fungi, Enzymes.

## Introduction

Endophytic fungi are those which invade the intercellular regions of plant parts. These fungi have ability to produce wide range of extracellular enzymes and secondary metabolites. One of the most utilized enzymatic systems in various biotechnological processes, pharmacological and agicultural industries, is oxidative ligninolytic system.

It mainly comprised of lignin peroxidase, manganese peroxidase and laccase[21]. These enzymes also play important role in litter degradation in senescent host tissues. The presence of MnPs, LiPs and laccasses in endophytic fungi has not been investigated except for few reports[53].

There is very little published information on the lignin-degrading ability of endophytic fungi worldwide. *Lasiodiplodia hormozganensis*, a coleomycetous fungus, was reported from India as endophyte in *Ficus krishnae*[44]. Earlier it was reported from Iran associated with *Mangifera indica* and *Olea* sp.[1] worldwide. Later on it was isolated from lesions found on mango branches and fruits from non-native environments in Australia[48] and Brazil[34] respectively.

It was also reported from Oman (UAE) as a pathogen in *Citrus aurantifolia* S., *Phoenix dactylifera* and *Mangifera indica*[3,4], in *Carica papaya* from Brazil[38], in *Dodonaea viscose* from Iran[42], *Vitis vinifera* from in the Sao Francisco Valley, Brazil[12].

The growth and development of this fungus in terms of nutritional requirements and environmental factors are yet unknown. The aim of current work is to understand the physiological conditions favourable for growth of fungus and evaluation of ligninolytic enzymes production in liquid medium secreted by endophytic fungi isolated from *Ficus krishnae*.

In this study, effects of different culture media, carbon source, nitrogen source, temperature, pH and days of incubation on mycelial growth and sporulation of *Lasiodiplodia hormozganensis* were evaluated. To evaluate the potential of *Lasiodiplodia hormozganensis* to secreate lignin modifying enzymes, qualitative and quantitative analysis of ligninolytic enzyme was performed along with variation in their activity due to different carbon and nitrogen sources.

## Material and Methods

**The test organism:** The fungus *Lasiodiplodia hormozganensis* was isolated from *Ficus krishnae*. The mycelial culture was maintained on PDA plates.

**Experimental design:** The growth of the test organism was assessed on twelve different basal media (Table 1), five different temperatures ranging from 16ºC- 32ºC (with difference of 4ºC each) on the optimum medium and different pH ranging from 3-9 (with unit difference) for growth of the fungal mycelium for 10 days.

To determine the physiology of particular fungus, there is need to study optimum days required for growth of the fungus. For this, fungus was incubated for a month at an optimum basal medium (Richard's), temperature (32ºC) and pH (4). The mycelia weight was observed after alternate days. The fungus was also tested for carbon and nitrogen requirements. For that ten different carbon sources, nine different inorganic sources and twenty- one different amino acids were utilized.

**Table 1**
**Composition of different basal media per 1 litre**

| Name of Media | Ingredients | Intial pH |
|---|---|---|
| Asthana and Hawker's medium | 5g Glucose, 3.5g Potassium nitrate, 1.75g Potassium dihydrogen phosphate, 0.75g Magnesium sulphate heptahydrate | 5 |
| Brown's I | 2g Glucose, 2g Asparagine, 1.25g Potassium Phosphate, 0.75g Magnesium sulphate heptahydrate | 5.1 |
| Brown's II | 2g Glucose, 10g Potato Starch, 25mg Asparagine, 1.25g Potassium Phosphate, 0.75g Magnesium sulphate heptahydrate | 5.2 |
| Coon's | 3.5g Maltose, 0.25g Asparagine, 1.25g Potassium dihydrogen phosphate, 0.5g Magnesium sulphate heptahydrate | 5.3 |
| Czapek's I | 30g Sucrose, 1g Potassium dihydrogen phosphate, 2g Sodium nitrate, 0.5g Magnesium sulphate heptahydrate, 0.01g Ferrous sulfate heptahydrate, 0.5g Potassium chloride | 5.0 |
| Czapek's II | 50g Sucrose, 1g Potassium dihydrogen phosphate, 3g Sodium nitrate, 0.25g Magnesium sulphate heptahydrate, 0.01g Ferrous sulfate heptahydrate | 4.7 |
| Dox's | 15g Sucrose, 1g Potassium dihydrogen phosphate, 2g Sodium nitrate, 0.05g Magnesium sulphate heptahydrate, 0.01g Ferrous sulfate heptahydrate, 0.05g Potassium chloride | 4.7 |
| Elliot's | 5g Glucose, 1.06g Sodium Carbonate, 1g Asparagine, 1.36g Potassium Dihydrogen phosphate, 0.5g Magnesium sulphate heptahydrate | 6.5 |
| Glucose Nitrate | 20g Glucose, 2.5g Potassium nitrate, 5g Potassium dihydrogen phosphate, 0.25g Magnesium sulphate heptahydrate, 0.005g Ammonium sulphate | 4.5 |
| Glucose Peptone | 10g Glucose, 2g Peptone, 1g Potassium dihydrogen phosphate, 0.5g Magnesium sulphate heptahydrate | 5.0 |
| Raulin's | 70g Sucrose, 4g Tartaric acid, 4g Ammonium nitrate, 0.6g Potassium Carbonate, 0.4g Magnesium sulphate heptahydrate, 0.07g Zinc sulphate heptahydrate, 0.07g Ferrous sulphate heptahydrate, 0.25g Ammonium sulfate 0.6g Ammonium phosphate | 2.5 |
| Richards's | 50g Sucrose, 5g Potassium dihydrogen phosphate, 10g Potassium nitrate, 2.50g Magnesium sulphate, 0.02g Ferric chloride hexahydrate | 4.0 |

**Preparation of medium:** Twenty five ml of liquid medium was poured into each 100 ml flask. The medium was autoclaved at 121ºC for 15 min. After cooling, one disc (10 mm) of the test fungus was cut with the help of sterilized cork borer from the margin of seven days old culture grown on PDA Petri plate and placed in each flask. Incubation was carried out at 28 ± 1º C for 10 days (taken tentatively). Three replicates for each medium were studied.

After the tenth day, the mycelial mat in each conical flask was filtered through a preweighed Whatmann No. 1 filter paper. These were dried in the oven at 80º C for 24 h and weighed. The experiment was conducted in completely randomized design (CRD). Variation was determined in terms of mycelial weight after 10 days (taken tentatively) of the inoculation[39] using an electronic balance (Sartorius Analytical BL 210S). The final pH of the culture filtrate of the individual replicate was checked over digital pH Meter 813.

After optimization of days of incubation, further experimentation were conducted for 16 days (Optimum days standardize for maximum mycelia weight). For C/N experiments, the original carbon source (Sucrose) and nitrogen source (Potassium Nitrate) of the Richard's medium were replaced by equivalent amount of carbon and nitrogen sources under analysis. The medium devoid of any carbon/nitrogen source serves as a control.

**Enzyme activity assays:** Extracellular enzymatic activities were first assayed at 25±1°C for 15 days in Petri dishes with a basal solid medium (1 g/L $KH_2PO_4$; 0.5 g/L $C_4H_{12}N_2O_6$; 0.5 g/L $MgSO_4 \cdot 7H_2O$; 0.01 g/L $CaCl_2 \cdot 2H_2O$; 0.001 g/L $CuSO_4 \cdot 5H_2O$; 0.001 g/L $Fe_2(SO_4)_3$; 0.001 g/L $MnSO_4 \cdot H_2O$; and 0.01 g/L yeast extract) containing glucose 4 g/L, agar 16 g/L and 0.1g/L Azure B. After 10 days, discoloration of blue colored medium was recorded which indicated the activity of lignin modifying enzymes[7].

**Quantitative analysis:** Enzyme activities of the supernatant collected after each optimization step were determined using spectrophotometer. Lignin peroxidase (LiP) activity was determined spectrophotometrically at 310 nm through the oxidation of veratryl alcohol to veratryl aldehyde[51]. The activity of manganese peroxidase (MnP) (EC 1.11.1.13) was measured at 465 nm by using guaiacol as a substrate[8]. Laccase (Lac) enzyme activity was measured by monitoring the increase in absorbance at 420 nm due to the oxidation of guaicol[6,11].

**Analysis of data**: All the experiments and enzyme assays were performed in triplicate in Complete Randomized design (CRD). The results obtained were subjected to One – way Analysis of Variance (ANOVA) while the test of significance was carried out by Tukey's test using Statistical Package for the Social Sciences (SPSS) 16 software. The results have been presented as mean ± S.E.

## Results

**Effect of basal media:** In current study, all basal media supported the growth of fungus. After 10 days of incubation, Richard's medium stimulated the best growth followed by Czapek's I, Czapek's II and Coon's medium whereas least growth was observed in Raulin's medium (Fig. 1). The fungal was olivaceous grey to black in colour forming a thick mycelia mat along with aerial mycelium.

**Effect of temperature:** The growth of mycelium occurred at all tested temperatures. The dry weight of mycelium increased significantly with increase in incubation temperature. Out of five different temperatures, maximum mycelial weight was observed at 32ºC whereas least at 16ºC (Fig. 2).

**Effect of pH**: The growth rate of the mycelium was different at varied H-ion concentrations. The highest dry weight of the fungus was observed at pH 4 whereas it was lowest was at pH3 (Fig. 3).

**Effect of days of incubation:** The continued increase in mycelia dry weight was found until 16$^{th}$ day of culture after which the growth retards up to 24$^{th}$ day and remained constant thereafter by the end of month (Fig. 4). The retardation in growth might be due to quick depletion of the nutrients in the medium.

**Effect of carbon source:** Out of various carbon sources tested, all of them showed moderate growth but sucrose was found superior for growth followed by glucose and fructose (Fig. 5). Sucrose is a good candidate for the carbon source because of its ease-of-use and low cost compared with other carbon sources[16]. The carbon sources associated with highest weight mycelium are as follows: Sucrose> Glucose> D (-) Fructose> D (+) Raffinose> Sorbose> (+) Xylose> Maltose> Starch> D (+) Lactose> Pectin> Control.

**Effect of the carbon source on ligninolytic enzyme production:** Among all carbon sources tested, the highest laccase activity was measured in pectin followed by raffinose whereas no activity was detected in submerged cultivation of *L. hormozganensis* in Richard's medium with various carbon sources. MnP activity was observed in all the sugars tested and it was also found to be maximum in pectin followed by starch. The highest LiP activity was expressed in pectin followed by sucrose and xylose whereas it was found to be nil in medium containing lactose. No activities of these enzymes were recorded in medium without carbon source (Fig. 6).

**Effect of inorganic nitrogen source:** Among nine different nitrogen sources along with control which is free from any nitrogen source, nitrates (potassium and sodium nitrates) yield more mycelium as compared to media supplied with ammoniums (ammonium acetate, ammonium chloride, ammonium phosphate and ammonium sulphate) (Fig.7). Potassium nitrate performed better than sodium nitrate followed by ammonium oxalate and ammonium sulphate. The biomass yield varied from 70 to 786.7 mg/25mL depending on the compound added.

**Effect of the inorganic nitrogen source on ligninolytic enzyme production:** Laccase activity was found to be dominant in expression as compared to MnP and Lac in different inorganic nitrogen compounds. Maximum laccase activity was expressed in medium supplemented with sodium nitrate followed by ammonium acetate.

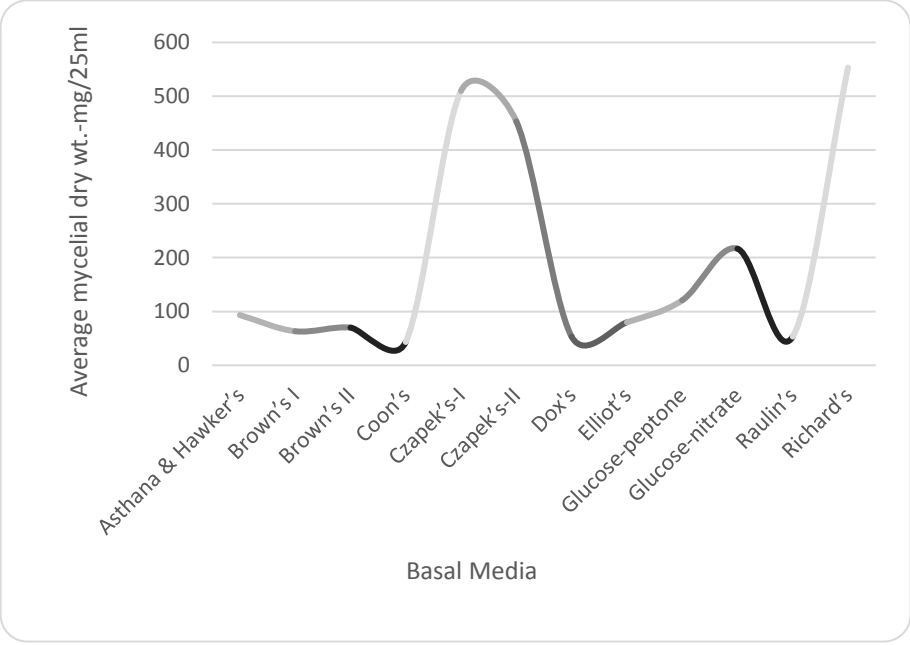


**Fig. 1: Average mycelial dry wt.-mg/25ml of *Lasiodiplodia hormozganensis* with different basal media at 24°C after 10 days of incubation (taken tentatively)**

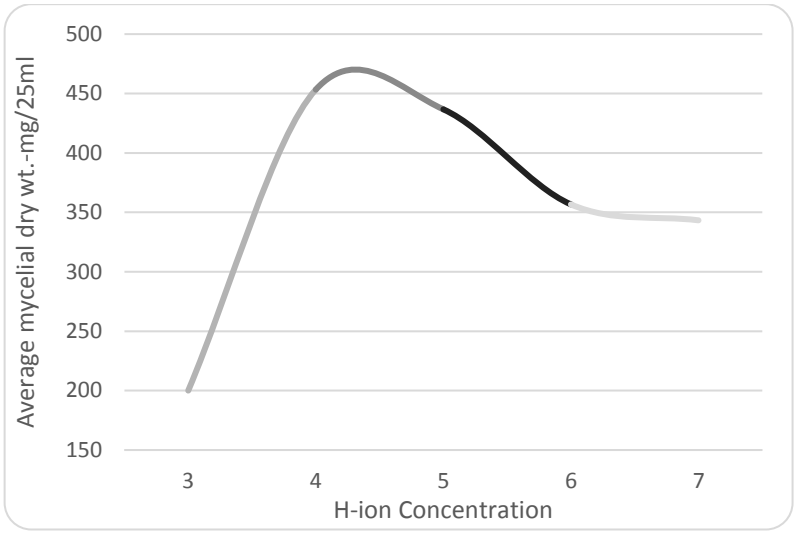


**Fig. 2: Average mycelial dry wt.-mg/25ml of *Lasiodiplodia hormozganensis* with Richard's media at different temperatures after 10 days of incubation (taken tentatively)**

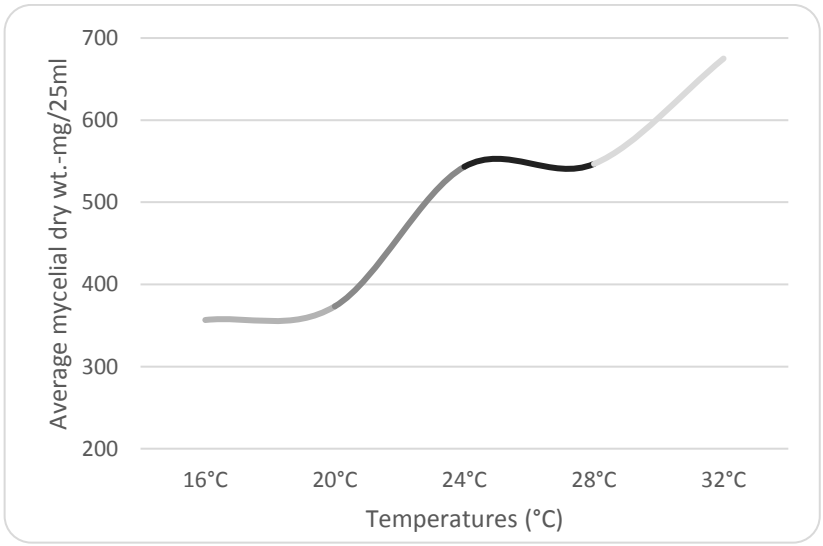


**Fig. 3: Growth (average mycelial dry wt.-mg/25ml) of *Lasiodiplodia hormozganensis* with Richard's media at 32°C at different pH after 10 days of incubation (taken tentatively)**

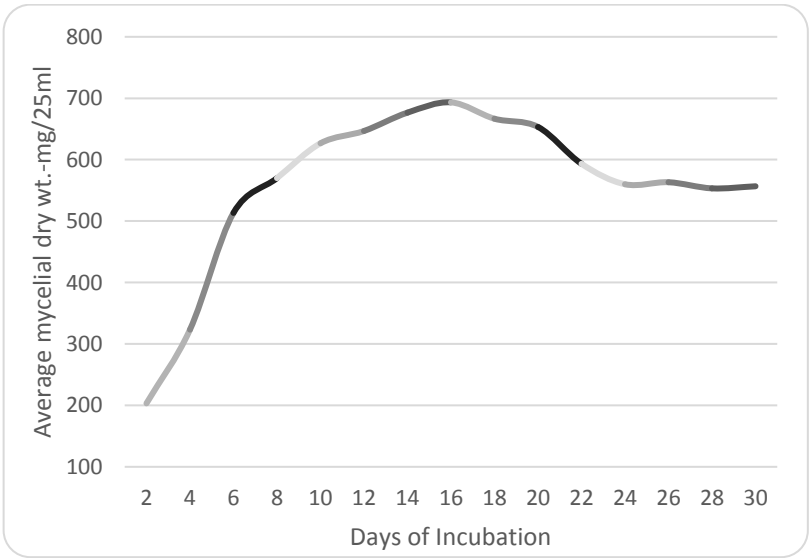


**Fig. 4: Growth rate (average mycelial dry wt.-mg/25ml) up to 30 days of incubation of *Lasiodiplodia hormozganensis* with Richard's media at 32°C at different pH 4.**

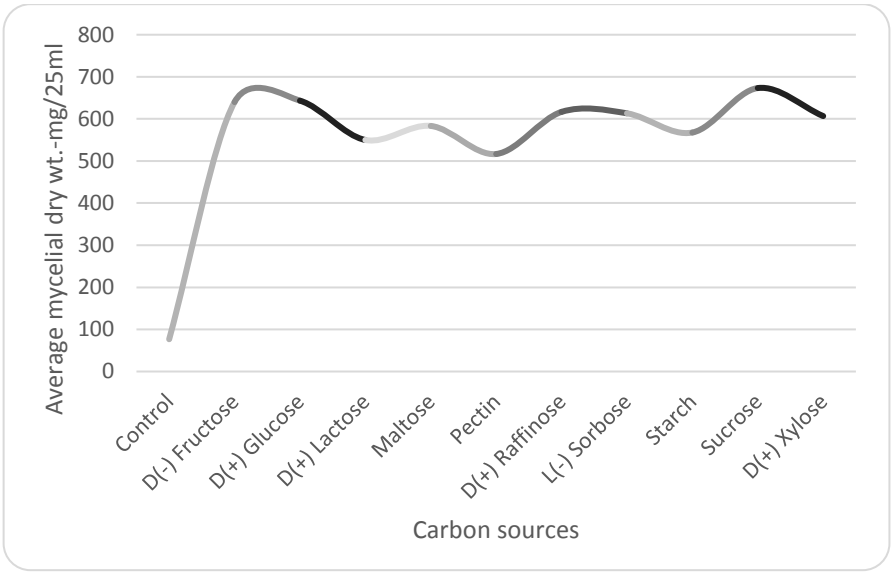


**Fig. 5: Growth rate (average mycelial dry wt.-mg/25ml) of *Lasiodiplodia hormozganensis* in different carbon compounds at 32°C and pH 4.0 after 16 days of incubation**

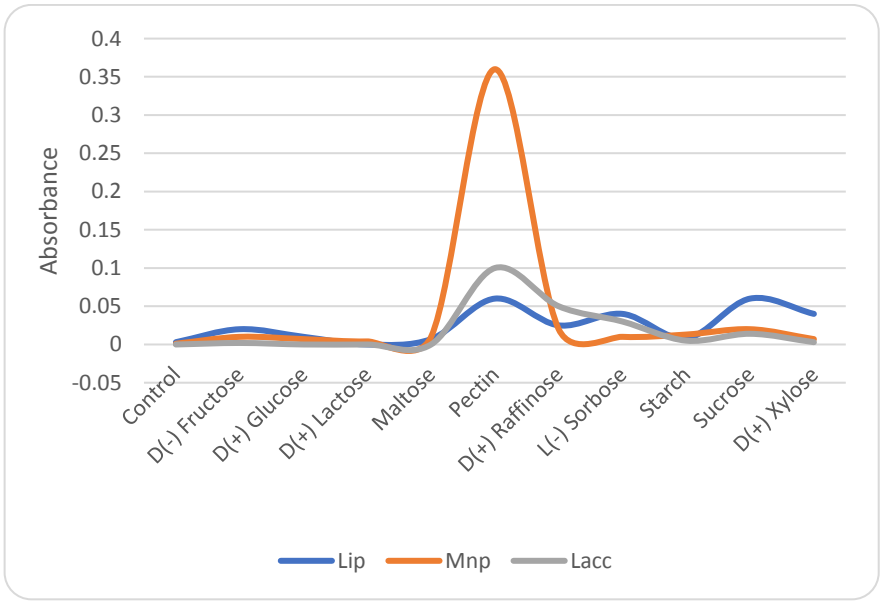


**Fig. 6: Ligninolytic enzyme production of *Lasiodiplodia hormozganensis* in different carbon compounds at 32°C and pH 4.0 after 16 days of incubation**

Laccase production in sodium nitrate was almost two fold of that produced in ammonium acetate. The medium with ammonium acetate exhibited highest MnP activity whereas no activity was observed in ammonium sulphate, potassium nitrate, sodium nitrate and sodium nitrite. Lip activity was observed among ammonium acetate, ammonium chloride, ammonium sulphate and ammonium phosphate whereas it is absent among remaining nitrogen sources (Fig. 8).

**Effect of organic nitrogen source:** Among twenty two different organic nitrogen sources including control, the medium supplemented with sucrose and L-Proline was best suited for mycelia growth. Out of these, single amino acid had negative impact on growth of this fungus as the dry weight obtained from medium supplied with was even less than that in control (without nitrogen source) (Fig. 9). Except Cysteine-HCl, all organic nitrogen source cultures developed superficial and aerial mycelium which was greyish to dark black in color.

The optimum production of biomass in relation to amino acid utilization is as follows in decreasing order: L-proline> L-Tyrosine> DL-alanine> DL-threonine > L-leucine> L-Lysine mono HCl > DL-valine> DL-tryptophan> DL-methionine> L-Asparagine> DL-serine HCl> Glycine> Dihydroxy-phenylalanine> L-ornithine mono HCl> DL-Aspartic acid> L-glutamic acid> L-histidine HCl> L-α amino-n butyric acid> L-arginine HCl> L-Cystine> Control> L-cysteine mono HCl.

Among 22 different organic nitrogen sources, L-Arginine HCl was identified as best enhancer for production of Lip

enzyme whereas L-ornithine mono HCl and L-Lysine mono HCl were best producers of MnP enzyme. The maximum laccase activity was obtained in medium containing L-Asparagine and L-Arginine HCl as nitrogen source. The fungus was reported to produce three of the isoenzymes on all the nitrogen compounds tested except DL- serine and Tyrosine which inhibited the production of MnP (Fig.10).

## Discussion

Different fungi require different nutrients for its growth as all fungi are not able to utilize same substrate for their growth. This study revealed that Richard's medium is best medium for growth of *Lasiodiplodia hormozganensis*. Similar results were obtained for growth of *Microsporum gypseum*[48], *Colletotrichum gloeosporioides*[14], *Aspergillus niger*[11], *Alternaria solani*[26], *Botriodiplodia theobromae*[36] and *Chrysosporium indicum*[22].

A broad range of temperature tolerance for growth and sporulation of *L. hormozganensis* (i.e. 16 to 32ºC) is an interesting feature. The maximum growth was observed at 32ºC. Kalaw et al[23] also reported the maximum growth of *Ganoderma lucidum*, *Lentinus tigrinus* and *Coprinopsis cinerea* strain incubated at 32 + 0.91ºC.

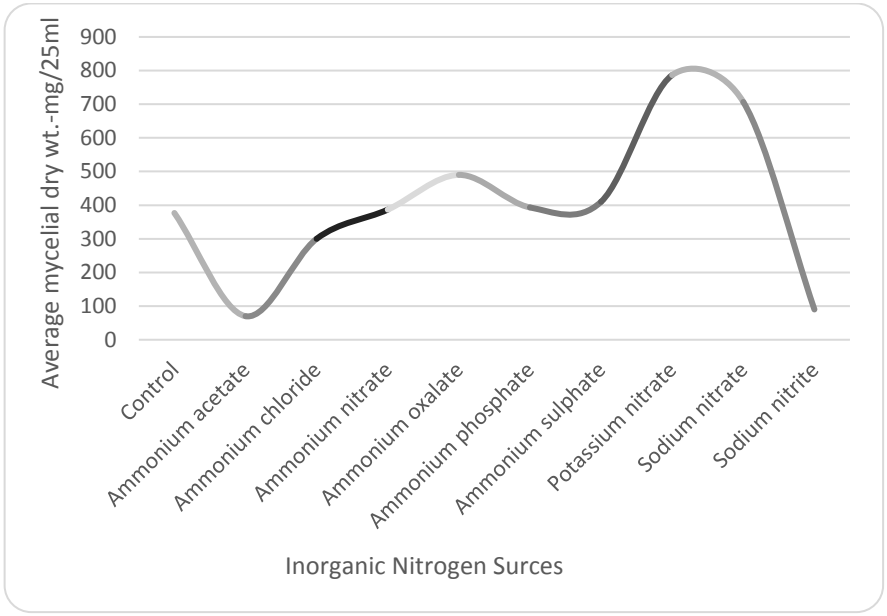


**Fig. 7: Average mycelial dry wt.-mg/25ml) of *Lasiodiplodia hormozganensis* in different inorganic nitrogen compounds at 32°C and pH 4.0 after 16 days of incubation**

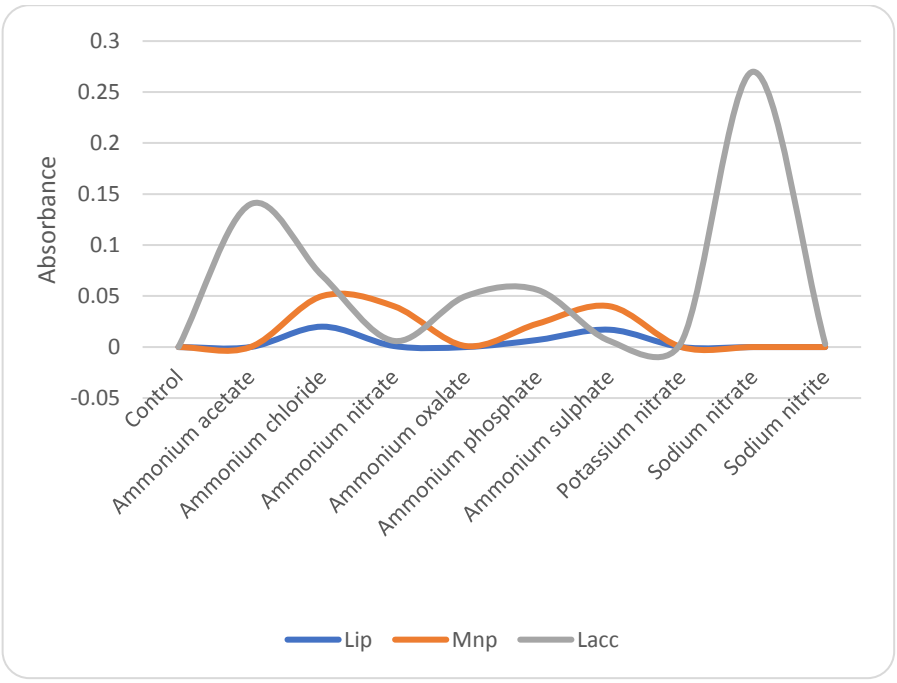


**Fig. 8: Ligninolytic enzyme production by *Lasiodiplodia hormozganensis* in different inorganic nitrogen compounds at 32°C and pH 4.0 after 16 days of incubation.**

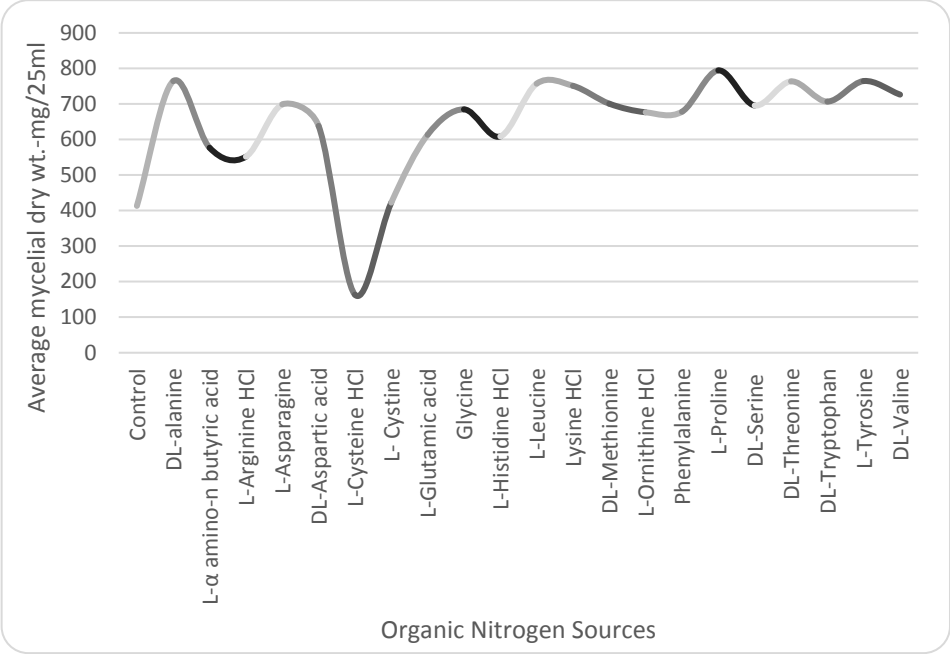


**Fig. 9: Average mycelial dry wt.-mg/25ml of *Lasiodiplodia hormozganensis* in different organic nitrogen compounds at 32°C and pH 4.0 after 16 days of incubation.**

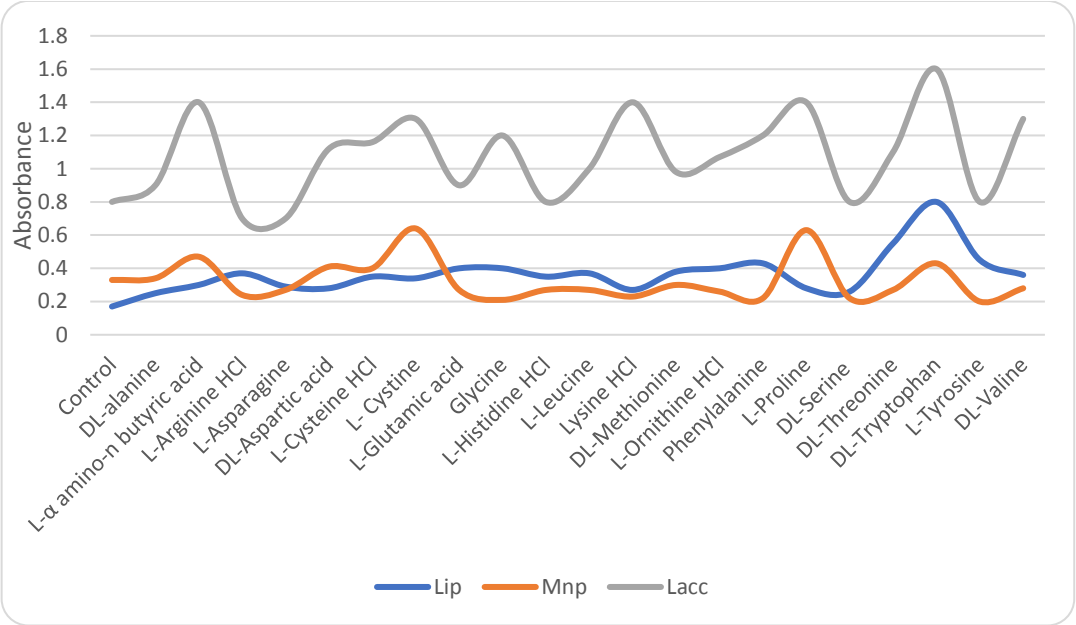


**Fig. 10: Ligninolytic enzyme production by *Lasiodiplodia hormozganensis* in different organic nitrogen compounds at 32°C and pH 4.0 after 16 days of incubation.**

This fungus can grow in wide pH range from 4-9 which makes it adapt to the surroundings. The highest mean of dried mycelial weight (453.3mg) was obtained at 32ºC and pH 4.0 in Richard's medium. The results are in accordance to data reporting maximum mycelia growth of *Trichoderma* spp.[27], *Aspergillus parasiticus*[2] and *Lentinula edodes*[46] at pH 4 whereas least mycelia weight was recorded at pH 3 as obtained in *Pleurotus ostreatus* var. *florida* [40].

Different fungi require varied growth periods to attain maximum growth in particular medium. It is essential to find optimum growth period to understand fungal physiology. The fungus under current study achieves maximum dry weight of mycelium after 16 days of incubation, after which mycelia weight starts decreasing. The decline in fungal dry weight may be due to autolysis of cells or depletion of nutrients in the medium.

Carbon is the most important constituent for the growth of mycelium. It constitutes about half of total dry weight of mycelium. Different fungi utilize different carbon sources for their growth and development. Here the growth of mycelium is observed to be much more in all carbon sources as compared to control (without any carbon) whereas it is

maximium in sucrose. Similar results were also obtained by Zou[59] in *Oudemansiella radicata*, Nagadesi and Arya[37] in *Lenzites sterioides*, Mathan et al[35] in endophytic *Aspergillus terreus* KC 582297, Latha et al[29] in *Lasiodiplodia theobromae*, Rajput et al[47] in *Trichoderma harzianum* and Wiriya et al[56] in *Termitomyces* sp. CMUTM001 and CMUTM002.

Nitrogen comprises 6% of total dry weight of mycelium. The current study revealed that potassium nitrate is found best among different inorganic nitrogen sources for growth of *Lasiodiplodia hormozganensis.* The fungi may have capacity to produces reductase or other associated enzymes which are essential for the metabolism of the nitrate[55]. Potassium nitrate was found to be best by various other researchers including Manjunath et al[33] (*Colletotrichum lindemuthianum*), Lal et al[28] (*Curvularia lunata)*, Taware et al[52] (*Alternaria carthami*) and Gawai and Shinde[20] (*Colletotrichum coccodes* and *Colletotrichum capsici*).

*L. hormozganensis* was able to utilize all amino acids as sole nitrogen source. Out of various organic nitrogen sources, L-Proline was found to yield maximum mycelia weight followed by L-Tyrosine,L- Leucine and Lysine HCl. Similar results were also reported in studies related to different strains of *Hirsutella rhossiliensis*[31], *Trichoderma viride, Flagellospora penicilloides, Pestalotiopsis submerses*[9] and *Arthrinium phaeospermum*[43] whereas these results are in contrary with literature data for *Tetracladium marchalianum, Tetrachaetum elegans*[9] and *Alternaria alternata*[24].

Different nutritional factors as well as culture conditions affect the production of ligninolytic enzymes. It is not mandatory that the media yielding maximum biomass will support high enzymatic yields[58].

Among ten different carbon sources tested, maximum ligninolytic enzyme (all three isoenymes) production was observed in pectin. Similar results have also been reported by various researchers. Alves et al[5] screened glucose, fructose, galactose, galacturonic acid, xylose, lactose, sucrose, mannitol, pectin and inulin for laccase production by *Botryosphaeria* sp.

They reported that there is increase in production of laccase on most of carbon sources studied except inulin and galacturonic acid. *Colletotrichum truncatum* was also reported to produce maximum laccase activity when cultured on a defined medium based on pectin and asparagine as carbon and nitrogen sources respectively[30]. Dhakar et al[15] also found that pectin enhances the production of Lip and MnP in *Aspergillus niger.*

Ligninolytic enzyme production can be regulated by both the nature and concentration of nitrogen source[19]. The current results revealed highest laccase activity with sodium nitrate. Sodium nitrate was also described as the best nitrogen source for laccase production by Elshafei et al[17] in *Penicillium martensii* NRC 345, Prasher et al[45] in *Dictyoarthrinium synnematicum* Somrith and Sidhu et al[50] in *Scytalidium lignicola.* MnP and Lip activities were produced in maximum quantity in media supplemented with ammonium chloride as nitrogen source. Ammonium chloride also resulted in production of applicable amount of Lip in *Phanerochaete chrysosporium* and *Pleurotus ostreatus*[25] and MnP in *Phanerochaete chrysosporium*[54].

These results have been found to be contradictory to those obtained by Prasher et al[45] where *Dictyoarthrinium synnematicum* does not exhibit Lip and MnP activity in ammonium chloride.

The presence of amino acids in the media affects the production of ligninolytic enzymes. A sucrose-asparagine medium is reported to be best for laccase production in *Pycnoporus sanguine*[18] which is in accordance to our results. Asparagine also resultened in maximum laccase production in *Botryosphaeria rhodina*[13] and *Pleurotus ostreatus*[41] along with a combination of inorganic nitrogen source $NH_4NO_3$.

## Conclusion

Optimization of nutritional parameters for growth and development of particular fungus is needed to lower the process cost required for their utilization in biotechnological purposes. An endophytic fungus, *Lasiodiplodia hormozganensis,* is found to survive in wide range of nutritional conditions. Here ligninolytic enzyme production by this endophyte has been explored. The oxidative enzymes produced by these fungi might have helped them for penetrating inside their host whereas the extracellular enzymes will allow them to decompose the debris and litter after host senescence.

These enzymes producing endophytes can be regarded as species with bioremediation potential. There is need to explore the potential of endophytic fungi as enzyme producers which can further be utilized in various industrial processes.

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