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RESEARCH ARTICLES

Nitrogen sources and trace elements influence Laccase and peroxidase enzymes activity of *Grammothele fuligo*

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Abstract

The effect of different organic, inorganic nitrogen sources and trace elements on growth and ligninolytic enzymes production by *Grammothele fuligo* has been investigated. Amongst all the nitrogen compounds used, the most favourable for growth was ammonium oxalate. It showed maximum Lignin Peroxidase activity (80.6 IU/mL) with ammonium chloride. The optimum Manganese Peroxidase activity (4.13 IU/mL) was observed with ammonium acetate. DL-alanine served as the best organic nitrogen source for the growth. The highest MnP (36.7 IU/mL) and laccase (3921.5 IU/mL) activities were revealed in medium supplemented by DL-tryptophan. However, their positive effects on enzyme accumulation were due to a higher biomass production. The higher concentrations of trace elements were found to be fungistatic for its growth viz. B, Co, Cu, Fe (400 ppm) and Co (100 ppm). It exhibited maximum LiP activity (456.9 IU/mL) with 10^{-3} ppm Fe and MnP activity (3.30 IU/mL) with 10^{-6} ppm B and 10^{-3} ppm Ca. The maximum laccase activity (653.5 IU/mL) was observed with 10^{-6} ppm Cu. This is the first report on nitrogen sources and trace elements effect on ligninolytic enzymes production of *Grammothele*. The results will facilitate research to understand the nature of the fungus and to increase its enzymes production under controlled conditions.

Keywords Ligninolytic enzymes · Nitrogen sources · Trace elements

Introduction

Many studies have proved that the nature and composition of culture medium regulate ligninolytic enzymes production by white rot fungi (Abdel-Azeem and Salem 2012). It has been concluded that the differential enzyme production is highly dependent on conditions or strains used (Tekere et al. 2001). Many white rot fungi produce an extracellular laccase under commonly adopted ligninolytic conditions. Studies have been conducted to select new species of white rot fungi for their overproduction to be used at large scale for industrial use such as in detergents, food, feed, pharmaceutical and biofuel (Bonugli-Santosa et al. 2010; Mtui 2012). Nitrogen sources appear to be another powerful factor affecting ligninolytic enzyme production of white rot fungi (Kenkebashvili et al. 2012; Mikiashvili et al. 2005).

Many white-rot fungi exhibit significant effect of essential heavy metals such as Cu, Cd, Mn or Zn on their growth, reproduction and other metabolic functions (Chiu et al. 1998; Gabriel et al. 1996). The metals necessary for fungal growth include copper, iron, manganese, molybdenum, zinc, and nickel. Non-essential metals commonly include chromium, cadmium, lead, mercury and silver (Gadd 1993). The essential metals are relatively less toxic than heavy metals and increase the growth rate of fungi when present at low concentrations (Falih 1997, 1998). It seems that low concentrations of essential heavy metals are necessary for the development of the ligninolytic enzyme system (Périé and Gold 1991; Perez and Jeffries 1992; Singhal and Rathore 2001). The ability of many fungi to accumulate metals can be used for biotechnological applications in removal of heavy metal ions from polluted water, degradation of xenobiotic compounds (Baldrian 2003), bio-monitoring of atmosphere pollution (Baldrian et al. 1999; Gabriel et al. 1996). *Grammothele fuligo* (Berk. & Broome) Ryvarden (the test fungus) is a wood rotting resupinate Agaricomycete growing on the petioles of *Livistona chinensis* (Jacquin) R. Brown. Since

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this fungus is cosmopolitan, however, still it has not been exploited for its ligninolytic enzymes applications in various industries e.g. pulp industry, paper industry etc. therefore it has been selected for the present studies. The aim of this study was to evaluate the significant effect of various nitrogen sources and trace elements for oxidative enzyme production by this fungus since such studies are a pre-requisite for commercial exploitation of these fungi for lignocellulolytic enzymes and organic acid production and no such studies have been conducted on it.

Materials and methods

Microorganism and culture conditions

Grammothele fuligo was maintained on 2% malt extract agar slants at 4 °C in the culture collection of Mycology and Plant Pathology Laboratory, Panjab University, Chandigarh. Glucose-peptone medium was used as the basal medium for the growth of the fungus. The carbon compound supporting optimum growth of this fungus determined in the previous experiment was incorporated in the selected basal media i.e. 10 g D(+) Glucose (Prasher and Chauhan 2013) and other microelements (per liter) i.e. 1.0 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$ were added. 25 ml of the basal media were apportioned in each 100 ml sterilized Erlenmeyer conical flask aseptically. Each flask was seeded with inoculum having mycelial load of 2.5 mg (by growing the mycelium for 4 days old culture in optimal basal medium under optimum conditions) and incubated at optimum temperatures of 24 °C. After 12 days of incubation, found to be optimum days for its growth (Prasher and Chauhan 2013), the mycelia were separated by filtration and dried to constant weight at 45–50 °C, while culture filtrates were used to measure the enzyme activity. Three replicates were kept in each variable in each experiment along with the control. At the end of each experiment, the mycelia were harvested through pre-weighed Whatman filter paper No. 1 and dried at 45 °C in a hot air oven and their dry weights were measured using an electronic balance (Sartorius Analytical BL 210S).

Effect of inorganic and organic nitrogen sources

The different inorganic and organic nitrogen compounds were added in amounts equivalent to that of original nitrogen compound (2 g/l of peptone in Glucose peptone medium, as peptone is a complex nitrogen compound) in the basal medium. The medium was adjusted to pH 7.0 before sterilization. The basal medium without nitrogen source was used as control.

Effect of trace elements

The effects of different trace elements (B, Ca, Co, Cu, Fe, Mn, Mo and Zn) at different concentrations (10^{-6}–400 ppm) were evaluated on mycelial biomass and ligninolytic enzymes production at 24 °C, pH-7.0 with glucose used as carbon source and ammonium chloride acid as nitrogen source, after 12-days of incubation. However, prior to that the trace element contaminants from all the glassware were removed by chelation with disodium salt of EDTA (0.1% w/v aq. solution). The salts used for trace elements were: $FeSO_4.7H_2O$, $ZnSO_4.7H_2O$, $MnCl_2.7H_2O$, $CaCl_2.2H_2O$, $CoCl_2.6H_2O$, $CuSO_4.5H_2O$, $(NH_4)_6Mo_7O_{24}.4H_2O$ and H_3BO_3. The basal medium without added trace elements was served as control. The basal media supplemented with 7 trace elements and omitting one of the elements at a time were designated by zero concentration of the element.

Enzyme assays

Laccase activity: It was determined using the method of Coll et al. (1993). The reaction mixture was prepared by mixing 0.5 ml of distilled water, 1 ml of 50 mM sodium acetate buffer (pH 4.5), 0.5 ml of 46 mM guaiacol and 0.5 ml of culture filtrate. The activity of the enzyme was measured by taking the optical density of the reaction mixture at 440 nm on Shimadzu UV visible Spectrophotometer 1800 up to 90 s with 30 s of time interval.

Manganese peroxidase activity: It was assayed by following the method of Atalla et al. (2010), whereby guaiacol was used as a substrate. The reaction mixture contained 300 µl of 0.5 M sodium succinate buffer (pH-4.5), 300 µl guaiacol (4 mM), 600 µl $MnSO_4$ (1 mM), 300 µl culture filtrate and 1200 µl distilled water. It was then incubated at 30 °C for 2 min and the reaction was initiated by addition of 300 µl of H_2O_2 (1 mM). The absorbance of the solution due to oxidation of guaiacol ($\varepsilon_{465} = 12{,}100\ M^{-1}cm^{-1}$) was measured at 465 nm on Shimadzu UV visible Spectrophotometer 1800 in 1 min intervals after addition of hydrogen peroxide.

Lignin peroxidase activity: It was measured using the method of Atalla et al. (2010). The reaction mixture contained 600 µl of 0.3 M citrate/0.4 M phosphate buffer (pH-4.5), 300 µl of 8 mM veratryl alcohol, 1890 µl distilled water and 60 µl of culture filtrate. The reaction mixture was then incubated at 30 °C for 2 min. The reaction was initiated by addition of 150 µl of H_2O_2 (5 mM). The absorbance of the solution was measured immediately in 1 min interval after addition of H_2O_2 at 310 nm on Shimadzu UV Spectrophotometer 1800. One unit of Lac, MnP, and peroxidase activity was defined as an amount of enzyme that transformed 1 µmol substrate per minute.

Statistical analyses

All the experiments were performed in triplicates. The means of three replicate values for all data in the experiments obtained were tested in a one way ANOVA at $P=0.05$ using PASW Statistics 18 software and Tukey's test was used to evaluate differences between treatments.

Results and discussion

Effect of inorganic and organic nitrogen sources

Grammothele fuligo showed maximum average mycelial dry weight with ammonium oxalate (65.0 ± 1.68 mg/25 ml) followed by ammonium chloride, ammonium phosphate and ammonium sulphate whereas it exhibited least growth with potassium nitrate (32.1 ± 0.49 mg/25 ml) and sodium nitrate (26.5 ± 1.81 mg/25 ml) (Table 1). In case of organic nitrogen sources, it attained moderate growth with DL-alanine (28.8 ± 0.30 mg/25 ml) followed by L-glutamic acid, DL-threonine, DL-serine HCl and L-asparagine. It showed nil growth with L-arginine HCl, di-hydroxy phenyl alanine, hydroxy-proline and L-tyrosine.

It showed poor growth with L-cysteine HCl (10.7 ± 0.05 mg/25 ml), L-cystine (10.7 ± 0.20 mg/25 ml) and L-proline (7.0 ± 0.15 mg/25 ml) (Table 2). It exhibited activity of LiP in medium containing ammonium chloride, ammonium sulphate (80.6 IU/mL) and it did not show LiP activity with any free amino acid. The maximum Laccase and MnP activity was observed to be 3921.5 IU/mL and 36.7 IU/mL, respectively, with DL-tryptophan. The highest MnP activity was observed with ammonium acetate, DL-tryptophan followed by L-cysteine HCl i.e. 4.13 IU/mL, 36.7 IU/mL and 10.3 IU/mL, respectively.

Table 1 Growth (average mycelial dry wt.) and ligninolytic enzymes production of *G. fuligo* with different inorganic nitrogen compounds at 24 °C and pH 7.0 after 12 days of incubation

Inorganic nitrogen source	Average mycelial dry wt. (mg/25 ml)	Enzyme activity (IU/mL)	
		LiP	MnP
Control	47.5 ± 0.94	107.5	5.37
Ammonium acetate	49.1 ± 0.65	ND	4.13
Ammonium chloride	61.7 ± 0.72	80.6	ND
Ammonium nitrate	48.9 ± 0.69	ND	ND
Ammonium oxalate	65.0 ± 1.68	ND	ND
Ammonium phosphate	58.4 ± 1.26	ND	ND
Ammonium sulphate	58.1 ± 1.08	80.6	ND
Potassium nitrate	32.1 ± 0.49	ND	ND
Sodium nitrate	26.5 ± 1.81	ND	ND
Sodium nitrite	2.5 ± 0.00	ND	ND

ND not detected

Table 2 Growth (average mycelial dry wt.) and ligninolytic enzymes production of *G. fuligo* with different organic nitrogen compounds at 24 °C and pH 7.0 after 12 days of incubation

Organic nitrogen source	Average mycelial dry wt. (mg/25 ml)	Enzyme activity (IU/mL)	
		MnP	Lacc.
Control	7.7 ± 0.20	2.06	ND
L-α amino-n butyric acid	8.8 ± 0.20	ND	ND
DL-Alanine	28.8 ± 0.30	ND	ND
L-Arginine HCl	2.5 ± 0.00	ND	ND
L-Asparagine	17.0 ± 0.15	ND	ND
DL-Aspartic acid	13.9 ± 0.05	ND	ND
L-Cysteine HCl	10.7 ± 0.05	10.3	ND
L-Cystine	10.7 ± 0.20	ND	1176.4
Dihydroxy phenylalanine	2.5 ± 0.00	ND	ND
L-Glutamic acid	20.5 ± 0.45	ND	1176.4
Glycine	10.8 ± 0.20	1.65	784.3
L-Histidine HCl	10.0 ± 0.25	ND	ND
Hydroxy-proline	2.5 ± 0.00	ND	ND
L-Leucine	6.4 ± 0.10	4.95	ND
Lysine HCl	3.4 ± 0.10	ND	ND
DL-Methionine	4.0 ± 0.10	ND	ND
L-Ornithine HCl	6.1 ± 0.15	2.47	ND
Phenyl alanine	5.8 ± 0.25	ND	ND
L-Proline	7.0 ± 0.15	ND	ND
DL-Serine HCl	19.2 ± 0.10	1.65	ND
DL-Threonine	20.2 ± 0.40	1.65	ND
DL-Tryptophan	14.9 ± 0.15	36.7	3921.5
L-Tyrosine	2.5 ± 0.00	ND	ND
DL-Valine	10.8 ± 0.05	ND	ND

Many fungal isolates exhibited good to moderate growth with ammonium salts and nitrate salts as have been reported in various reports e.g. *Panellus stipticus* (Prasher et al. 2014) and *Dictyoarthrinium synnematicum* (Prasher and Chauhan 2015). In utilizing nitrate sources of nitrogen the fungus resembles other fungi like *Fusarium oxysporum*, *Phoma nebulosa* and *Botryodiplodia theobromae* (Dandge 2012), *Trichoderma viride* and *Beauveria bassiana* (Mehta et al. 2012). The findings are in conformity with the studies conducted by various workers where L-proline was reported as poor nitrogen source for growth of various fungi viz. *Tetracladium marchalianum* and *Tetrachaetum elegans* (Bisht 2013). However, the present findings are contrary to earlier reports where L-cysteine HCl promoted good to moderate growth of some fungi e.g. *Flagellospora penicilloides* and *Pestalotiopsis submerses* (Bisht 2013) and L-cystine in *Fomitiporia* sp. F6 and Fp strains (Terashima 2013).

L-tyrosine was found to inhibit the growth of the fungus which is contrary to the earlier reports where moderate growth has been reported with L-tyrosine e.g. *Curvularia senegalensis*, *Curvularia prasadii* and *Phoma vulgaris* (Dandge 2012). Stajic et al. (2006) reported the highest Laccase activity with ammonium sulphate as nitrogen supplement and least activity with potassium nitrate in *Pleurotus eryngii* and *Pleurotus ostreatus* strain 493 while *Pleurotus ostreatus* strain 494 exhibited highest enzyme activity with peptone and ammonium chloride. In *Cerrena unicolor* IBB 62 (Elisashvili et al. 2001), the highest Laccase activity was observed in medium supplemented with ammonium sulphate whereas, in *Pleurotus ostreatus* (Stajic et al. 2006), *Trametes gallica* (Levin et al. 2010), *Coriolopsis gallica* (Kenkebashvili et al. 2012), *Fibrodontia* sp. RCK783S (Vaithanomsat et al. 2013) and *Neolentinus kauffmanii* (Johnsy and Kaviyarasan 2014), it was found to be maximum with peptone. However, in case of *G. fuligo*, Laccase activity was not observed with any inorganic nitrogen source. Kenkebashvili et al. (2012) observed maximum MnP accumulation by *Coriolopsis gallica* in medium containing peptone as efficient nitrogen source. Similar results were also observed with *Cerrena unicolor* IBB 62 (Elisashvili et al. 2001) and *Pleurotus eryngii*, *Pleurotus pulmonarius* and *Pleurotus sajor-caju* (Martínez et al. 1996). Glutamic acid (free amino acid) is reported to be the best nitrogen source for the production of Laccase and MnP by fungi like *Coriolus versicolor* and *Trametes trogii* (Levin et al. 2010).

Contradictory results have been reported for the effect of concentration and source of nitrogen on ligninolytic enzymes (Galhaup et al. 2002). It has been reported that the production of ligninolytic enzymes i.e. Laccase, MnP and LiP depends not only on the species of fungi but also on the culture conditions, carbon and nitrogen sources and their concentrations (Mikiashvili et al. 2005; Stajic et al. 2006).

Effect of trace elements

Optimum concentrations of trace elements required for optimum biomass production of *G. fuligo* were found to be: B, Cu, Fe (10^{-3} ppm); Ca (100 ppm); Co, Mn (0–10^{-6} ppm); Mo (10^{-5} ppm) and Zn (10^{-4} ppm). Among these trace elements, it showed maximum biomass production i.e. 12.8 ± 0.74 mg/25 ml with Fe at 10^{-3} ppm.

However, the higher concentrations of these trace elements were found to be fungistatic for the growth of *G. fuligo* viz. 400 ppm of B, Co, Cu, Fe and 100 ppm of Co. It did not show any Laccase activity with Ca, Mn and Zn; and Manganese peroxidase activity with Co and Fe. It showed variability in ligninolytic enzyme activities with different concentrations of different trace elements (Table 3, 4, 5, 6, 7, 8, 9 and 10). It exhibited maximum LiP activity i.e. 456.9 IU/mL with 10^{-3} ppm conc. of Fe and MnP activity up to 3.30 IU/mL with 10^{-6} ppm B and 10^{-3} ppm Ca. The maximum activity of laccase was observed with 10^{-6} ppm Cu i.e. 653.5 IU/mL. It showed all the three ligninolytic enzymes activity at different concentration of Boron (Table 3), Copper (Table 6) and Molybdenum (Table 9); only LiP and MnP activity at different concentrations of Calcium (Table 4), Manganese (Table 8) and Zinc (Table 10) whereas only LiP and Laccase activity were shown with different concentrations of Cobalt (Table 5) and Iron (Table 7).

In requiring Boron, *G. fuligo* (Table 3) resembles other fungi like *Alternaria burnsii* (Sankhla et al. 1970),

Table 3 Growth (average mycelial dry wt.) and ligninolytic enzymes production of *G. fuligo* with different concentrations of Boron, at 24 °C and pH 7.0 after 12 days of incubation

Boron conc. (ppm)	Average mycelial dry wt. (mg/25 ml)	Enzyme activity (IU/mL)		
		LiP	MnP	Lacc.
Control	9.03 ± 0.03	241.9	2.47	522.8
0	8.20 ± 0.08	241.9	3.30	522.8
10^{-6}	9.16 ± 0.16	295.6	3.30	392.1
10^{-5}	9.83 ± 0.03	241.9	2.89	261.4
10^{-4}	10.63 ± 0.03	268.8	2.47	261.4
10^{-3}	12.56 ± 0.26	215.0	2.06	653.5
10^{-2}	10.96 ± 0.29	215.0	2.06	261.4
10^{-1}	9.56 ± 0.03	188.1	2.89	784.3
1.0	9.13 ± 0.08	188.1	2.06	653.5
10	8.46 ± 0.16	161.2	1.65	653.5
100	6.70 ± 0.03	161.2	2.06	915.0
400	2.5 ± 0.00	ND	ND	ND

Table 4 Growth (average mycelial dry wt.) and ligninolytic enzymes production of *G. fuligo* with different concentrations of Calcium, at 24 °C and pH 7.0 after 12 days of incubation

Calcium conc. (ppm)	Average mycelial dry wt. (mg/25 ml)	Enzyme activity (IU/mL)	
		LiP	MnP
Control	9.03 ± 0.03	241.9	2.47
0	4.23 ± 0.08	53.7	1.23
10^{-6}	7.16 ± 0.03	80.6	0.82
10^{-5}	8.43 ± 0.03	80.6	0.82
10^{-4}	8.73 ± 0.08	107.5	2.06
10^{-3}	9.36 ± 0.06	26.8	3.30
10^{-2}	9.56 ± 0.08	26.8	1.65
10^{-1}	9.83 ± 0.03	26.8	1.23
1.0	9.93 ± 0.03	26.8	1.23
10	10.53 ± 0.06	53.7	0.82
100	10.76 ± 0.08	53.7	1.23
400	10.23 ± 0.06	53.7	1.23

Table 5 Growth (average mycelial dry wt.) and ligninolytic enzymes production of *G. fuligo* with different concentrations of Cobalt, at 24 °C and pH 7.0 after 12 days of incubation

Cobalt conc. (ppm)	Average mycelial dry wt. (mg/25 ml)	Enzyme activity (IU/mL)	
		LiP	Lacc.
Control	9.03 ± 0.03	241.9	522.8
0	14.03 ± 0.06	268.8	653.5
10^{-6}	10.56 ± 0.03	215.0	392.1
10^{-5}	9.70 ± 0.05	241.9	392.1
10^{-4}	9.23 ± 0.03	241.9	522.8
10^{-3}	9.03 ± 0.03	215.0	261.4
10^{-2}	9.03 ± 0.03	188.1	261.4
10^{-1}	8.83 ± 0.03	134.4	130.7
1.0	8.53 ± 0.08	107.5	ND
10	5.56 ± 0.12	107.5	ND
100	2.5 ± 0.00	ND	ND
400	2.5 ± 0.00	ND	ND

Table 6 Growth (average mycelial dry wt.) and ligninolytic enzymes production of *G. fuligo* with different concentrations of Copper, at 24 °C and pH 7.0 after 12 days of incubation

Copper conc. (ppm)	Average mycelial dry wt. (mg/25 ml)	Enzyme activity (IU/mL)		
		LiP	MnP	Lacc.
Control	9.03 ± 0.03	241.9	2.47	522.8
0	7.40 ± 0.17	161.2	2.47	522.8
10^{-6}	8.10 ± 0.05	107.5	1.65	653.5
10^{-5}	8.46 ± 0.03	80.6	1.23	261.4
10^{-4}	8.86 ± 0.03	134.4	0.82	261.4
10^{-3}	10.9 ± 0.17	107.5	2.47	392.1
10^{-2}	10.1 ± 0.03	134.4	2.06	261.4
10^{-1}	8.83 ± 0.03	134.4	2.06	522.8
1.0	8.40 ± 0.05	107.5	0.82	392.1
10	7.26 ± 0.03	107.5	ND	ND
100	5.76 ± 0.03	107.5	ND	ND
400	2.5 ± 0.00	ND	ND	ND

Table 7 Growth (average mycelial dry wt.) and ligninolytic enzymes production of *G. fuligo* with different concentrations of Iron, at 24 °C and pH 7.0 after 12 days of incubation

Iron conc. (ppm)	Average mycelial dry wt. (mg/25 ml)	Enzyme activity (IU/mL)	
		LiP	Lacc.
Control	9.03 ± 0.03	241.9	522.8
0	6.73 ± 0.17	161.2	130.7
10^{-6}	7.30 ± 0.15	161.2	130.7
10^{-5}	9.90 ± 0.05	268.8	261.4
10^{-4}	10.1 ± 0.03	376.3	392.1
10^{-3}	12.8 ± 0.74	456.9	392.1
10^{-2}	9.16 ± 0.08	268.8	261.4
10^{-1}	8.63 ± 0.06	241.9	261.4
1.0	8.13 ± 0.06	188.1	261.4
10	7.76 ± 0.08	ND	ND
100	6.36 ± 0.27	ND	ND
400	2.5 ± 0.00	ND	ND

Table 8 Growth (average mycelial dry wt.) and ligninolytic enzymes production of *G. fuligo* with different concentrations of Manganese, at 24 °C and pH 7.0 after 12 days of incubation

Manganese conc. (ppm)	Average mycelial dry wt. (mg/25 ml)	Enzyme activity (IU/mL)	
		LiP	MnP
Control	9.03 ± 0.03	241.9	2.47
0	12.20 ± 0.14	80.6	1.23
10^{-6}	11.66 ± 0.06	80.6	2.06
10^{-5}	11.03 ± 0.08	26.8	1.23
10^{-4}	10.80 ± 0.03	134.4	0.82
10^{-3}	10.30 ± 0.05	161.2	ND
10^{-2}	10.10 ± 0.05	188.1	ND
10^{-1}	9.90 ± 0.05	107.5	ND
1.0	9.63 ± 0.12	80.6	ND
10	9.00 ± 0.05	26.8	ND
100	8.26 ± 0.26	ND	ND
400	5.93 ± 0.12	ND	ND

Saccharomyces cerevisiae (Bennett et al. 1999), where Boron is found to be beneficial for the growth. However, Bowen and Gauch (1966) reported the non-essentiality of boron for the growth of *Saccharomyces cerevisiae*, *Aspergillus niger*, *Neurospora crassa* and *Penicillium chrysogenum*.

In utilizing Ca for growth, *G. fuligo* (Table 4) resembles other fungi. The results are in accordance with other workers like Davis et al. (1928) who reported that Ca has stimulatory effect on fungal growth. Copper is required for the growth of many fungi (Dong and Yao 2005) and is also a known fungicide even in low concentrations. However, *Grammothele fuligo* showed optimum growth at very low concentrations (10^{-3} ppm).

The higher concentration of Cu was found to be fungistatic for the growth of *G. fuligo*. It showed variability in ligninolytic enzyme activities with different concentrations of Cu.

The positive effect of Copper on the production of laccase has been exhibited in *Pleurotus ostreatus* (Baldrian and Gabriel 2002, Hou et al. 2004), *Pleurotus pulmonarius* (Tychanowicz et al. 2006) and *Trametes versicolor* (Lorenzo et al. 2006).

Table 9 Growth (average mycelial dry wt.) and ligninolytic enzymes production of *G. fuligo* with different concentrations of Molybdenum, at 24 °C and pH 7.0 after 12 days of incubation

Molybdenum conc. (ppm)	Average mycelial dry wt. (mg/25 ml)	Enzyme activity (IU/mL)		
		LiP	MnP	Lacc.
Control	9.03 ± 0.03	241.9	2.47	522.8
0	9.43 ± 0.08	134.4	0.82	261.4
10^{-6}	11.20 ± 0.10	188.1	0.82	522.8
10^{-5}	13.83 ± 0.49	134.4	1.23	261.4
10^{-4}	12.26 ± 0.03	107.5	1.65	261.4
10^{-3}	12.06 ± 0.06	107.5	1.23	261.4
10^{-2}	11.30 ± 0.05	134.4	1.23	130.7
10^{-1}	11.13 ± 0.03	80.6	0.82	130.7
1.0	10.43 ± 0.18	80.6	0.82	ND
10	10.10 ± 0.15	ND	ND	ND
100	7.90 ± 0.10	ND	ND	ND
400	7.60 ± 0.11	ND	ND	ND

Table 10 Growth (average mycelial dry wt.) and ligninolytic enzymes production of *G. fuligo* with different concentrations of Zinc, at 24 °C and pH 7.0 after 12 days of incubation

Zinc conc. (ppm)	Average mycelial dry wt. (mg/25 ml)	Enzyme activity (IU/mL)	
		LiP	MnP
Control	9.03 ± 0.03	241.9	2.47
0	9.50 ± 0.10	80.6	1.23
10^{-6}	10.70 ± 0.11	80.6	0.82
10^{-5}	11.21 ± 0.03	53.7	1.65
10^{-4}	11.93 ± 0.23	80.6	1.65
10^{-3}	11.36 ± 0.03	80.6	1.23
10^{-2}	11.10 ± 0.10	107.5	1.23
10^{-1}	10.43 ± 0.03	134.4	0.82
1.0	10.33 ± 0.03	134.4	0.82
10	10.20 ± 0.05	295.6	0.82
100	9.03 ± 0.03	241.9	2.47
400	9.50 ± 0.10	80.6	1.23

There are few reports in the literature which supports the requirement of Co for the growth of fungi e.g. *Alternaria chartarum* and *Alternaria solani* (Madan and Thind 1979) whereas *G. fuligo* showed little growth with Co at very low concentration. *Grammothele fuligo* showed optimum growth and production of LiP and Laccase activities at optimum concentration of Fe. The results confirm the finding of many workers where it is shown to be required for growth and sporulation by many fungi like *Aspergillus flavus* (Cuero et al. 2003) and *Yarrowia lipolytica* (Anastassiadis et al. 2007). It has stimulatory effect on Laccase activity in *Pleurotus ostreatus* (Stajic et al. 2013), however, it is found to be inhibitory for Laccase production in case of *Ganoderma lucidum* (Murugesan et al. 2009) and *Pleurotus pulmonarius* (Stajic et al. 2013). *Grammothele fuligo* has also utilized Mn for producing ligninolytic enzymes and mycelial growth. In some fungi, Mn is shown to be required for synthesis of primary and secondary fungal metabolites e.g. *Agrocybe aegerita* (Sharma et al. 2004) and *Cordyceps sinensis* (Dong and Yao 2005). The positive effect of Mn on Laccase production has been exhibited in *Agrocybe praecox* and *Stropharia coronilla* (Steffen et al. 2002) and *Trametes versicolor* (Lorenzo et al. 2006), whereas *Grammothele fuligo* did not exhibit any Laccase activity with Mn. *Grammothele fuligo* exhibited MnP activity at low concentrations (10^{-6} to 10^{-4} ppm).

The growth of many fungi decreases in presence of Mo like, *Phlebia radiata*, *Pleurotus pulmonarius* and *Physisporinus rivulosus* (Kluczek-Turpeinen et al. 2014) while some species like *Alternaria alternata*, *Aspergillus flavus* and *Cladosporium herbarum* are tolerant to Mo (Hashem 1997). However, *G. fuligo* showed mycelial growth at all concentrations of Mo used in the experiment. Laccase activity has been reported to decrease in presence of Mo in *G. fuligo*, similar effects have been observed with *Phlebia radiata*, *Pleurotus pulmonarius* and *Physisporinus rivulosus* (Kluczek-Turpeinen et al. 2014). *Grammothele fuligo* resembles other fungi where zinc is reported to influence their growth like *Cordyceps sinensis* (Dong and Yao 2005), *Pleurotus ostreatus* (Baldrian et al. 2005) and *Alternaria alternata* S3S (Ezzouhri et al. 2009). However, there is a fungus which showed contrary results with Zinc where it inhibits the growth e.g. *Dictyoarthrinium synnematicum* (Prasher and Chauhan 2017). *Grammothele fuligo* did not show any Laccase activity with Zn at any concentration similar inhibitory effects have been observed in case of *Pleurotus pulmonarius* (Stajic et al. 2013), *Ganoderma lucidum* (Murugesan et al. 2009) and *Dictyoarthrinium synnematicum* (Prasher and Chauhan 2017). However, the stimulatory effect of Zn on Laccase activity has been exhibited in *Pleurotus ostreatus* (Stajic et al. 2013). Since, the tested fungus showed variability in production of laccase, Lip and MnP in relation to different concentrations of trace elements, it becomes necessary to know its ability to produce ligninolytic enzymes in different synthetic media. The results will facilitate research to understand the nature of the fungus and to increase its enzymes production under controlled conditions.

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