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Research Article

INFLUENCE OF TRACE ELEMENTS ON LIGNINOLYTIC ENZYMES ACTIVITY OF *Dictyoarthrinium synnematicum*

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A B S T R A C T

The aim of this study was to evaluate the effect of various trace elements on growth and ligninolytic enzyme production of *Dictyoarthrinium synnematicum* in liquid media and to provide a basis for further physiological study and biotechnological use of the fungus. The effect of 8 different trace elements (i.e. B, Ca, Co, Cu, Fe, Mn, Mo and Zn) with concentrations ranging from 10-6ppm to 400 ppm, on growth and ligninolytic enzymes production has been investigated. These trace elements has significantly influence the mycelial growth and ligninolytic enzymes production of this fungus. The trace elements viz. B, Ca, Mn and Zn were observed to be fungistatic at all concentrations while Co, Cu, Fe and Mo were mostly found to be fungistatic from 1 ppm to 400 ppm concentrations. It did not show Manganese peroxidase activity with any of these trace elements and showed Lignin peroxidase activity with low concentrations of Co, Cu and Mo while laccase activity was observed only with Mo. This is the first report on effect of trace elementon mycelial growth and ligninolytic enzymes production of this species. The study will facilitate research to increase the production of enzymes of the fungus under defined culture conditions.

INTRODUCTION

The major applications of the fungi, particularly those inhabiting dead or living wood causing rot/degradation has been towards commercial production of lignocellulolytic enzymes (Kaushik and Malik, 2009; Maciel *et al*., 2010; Moore *et al*.,2011). As ligninolytic enzymes have a potential role in several industrial, biotechnological (Maciel *et al*., 2010) and bioremediation processes (Li *et al*., 2012; Koyani *et al*., 2013), it is therefore interesting to evaluate these ligninolytic activities in different white rot fungi.Fungal species and strains differ in their sensitivity towards metals and in the protection mechanisms involved and exhibit significant effect of essential heavy metals such as Cu, Cd, Mn or Zn on their growth, reproduction and other metabolic functions (Faveroet *al*., 1990; Rózycki, 1992; Gabriel *et al*., 1996; Chiu *et al*., 1998). Heavy metals regulate extracellular ligninolytic and cellulolytic enzymes at transcriptional level. In general these are potent inhibitors of enzymatic reactions (Vallee and Ulmer, 1972). They may interfere with both the activity of extracellular enzymes involved in the process and fungal colonization. The ability of 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 (Gabriel *et al*.,1995, 1997; Baldrian *et al*.,1999).

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MATERIALS AND METHODS

Microorganisms and culture conditions

Dictyoarthrinium synnematicum was maintained on 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 and nitrogen sources supporting optimum growth of this fungus determined in the previous experiment were incorporated in the selected basal media i.e. Lactose- 10 g, Sodium nitrate- 2.0g, (Prasher and Chauhan, 2015) and other microelements i.e. KH_2PO_4- 1.0 g, $MgSO_4$. $7H_2O$-0.5 g were added per litre of distilled water. Eight different trace elements (B,Ca, Co, Cu, Fe, Mn, Mo and Zn) at different concentrations (10^{-6}-400 ppm) were used to evaluate the effect on mycelial biomass and ligninolytic enzymes production at 24°C, pH-5.0, after 16-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) (Prasher and Rawla, 1988). 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. All these elements were of BDH AnaLar, E. Merk GR or S. Merk GR grade.Twenty five ml of the basal media were apportioned in each 100 ml sterilized Erlenmeyer conical flask aseptically. Extra care was taken to avoid the falling of lint from cotton plugs into basal medium; the plugs were wrapped in muslin cloth used for bandages. An auto-pipette with separate tips was used for each concentration and for each element to avoid the interference of the other elements. The diluted concentrations of the elements were prepared afresh to avoid the fall in concentration due to adsorption of the elements on to the wall of the containers. Each flask was seeded with equal sized mycelial disc having mycelial load of 2.5 mg(by growing the mycelium for 7 days old culture in optimal basal medium under optimum conditions).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.The mycelia were separated by filtration through pre-weighed Whatman filter paper No. 1 and dried to constant weight at 45-50°C, while culture filtrates were used to measure the enzyme activity.

Enzyme assays

Laccase activity*:* It was determined after Coll *et al*.(1993). The reaction mixture was prepared by mixing 0.5 ml of distilled water, 1 ml of 50mM 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 seconds with 30 seconds of time interval.

Manganese peroxidase activity: It was assayed by following the method after 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 (4mM), 600 µl $MnSO_4$ (1mM), 300 µl culture filtrate and 1200 µl distilled water. It was then incubated at 30°C for 2 minutes and the reaction was initiated by addition of 300 µl of H_2O_2 (1mM). The absorbance of the solution due to oxidation of guaiacol was measured at 465 nm on Shimadzu UV visible Spectrophotometer 1800 in 1 minute intervals after addition of hydrogen peroxide.

Lignin peroxidase activity: It was measured after Atalla *et al*.(2010).The reaction mixture contained 600 µl of 0.3M citrate/0.4M phosphate buffer (pH-4.5), 300 µl of 8mM veratryl alcohol, 1890 µl distilled water and 60 µl of culture filtrate. The reaction mixture was then incubated at 30°C for 2 minutes. The reaction was initiated by addition of 150 µl of H_2O_2 (5mM). The absorbance of the solution was measured immediately in 1 minute interval after addition of H_2O_2 at 310 nm on Shimadzu UV Spectrophotometer 1800. One unit of Laccase, Manganese peroxidase and lignin 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

For *Dictyoarthrinium synnematicum* Boron, Calcium, Manganese and Zinc were observed to be completely fungistatic for growth while Cobalt, Copper, Iron and Molybdenum were found to be partially fungistatic, since the higher concentrations of these trace elements i.e. 1-400ppm inhibited the growth and at low concentrations i.e. 10^{-3}-10^{-6} ppm it showed mycelial growth. It exhibited optimum mycelial growth of 10.3±0.05 mg/25ml and maximum Lignin peroxidase activity of 161.2±0.05 IU/ml at 10^{-6} ppm Cobalt concentration (Table 1). With 10^{-6} ppm Copper, it showed mycelial growth of 9.6±0.15 mg/25ml and 107.6±0.05 IU/ml Lignin peroxidase activity (Table 2). It did not show any Manganese peroxidase and Laccase activity with Cobalt and Copper. While with Iron maximum mycelial yield observed was only 7.1±0.20 mg/25ml and 107.6 IU/ml and no activity of Lignin peroxidase; Manganese peroxidase and Laccase enzymes was observed (Table 3). In case of Molybdenum (10^{-6} ppm) the maximum mycelial growth was found to be 7.5±0.05 mg/25ml with Lignin peroxidase activity of 80.6±0.15 IU/ml and Laccase activity of 522.8±0.10 IU/ml, while no Manganese peroxidase activity was recorded (Table 4). Microscopic studies showed that instead of producing much mycelium it showed the production of chlamydospores at different concentrations of these trace elements (Co, Cu, Fe and Mo). The macroscopic deficiency symptoms caused reduction in mycelial dry weight of the fungus and thereby affected production of the ligninolytic enzymes activity. Cobalt, Copper, Iron and Molybdenum were required at very low concentrations (i.e. 10^{-6} ppm) for optimum biomass production of *D. synnematicum* and beyond which, there was very little growth or nil growth with the increase in concentration of these trace elements (Tables 1-4).

DISCUSSION

In requiring B, Ca, Mn and Zn, *D. synnematicum* differs from other fungi like*Fomes annosus* (Koenigs, 1969), *Alternaria burnsii* (Sankhla *et al*., 1970), *Penicillium crustosum* (Chahal and Rawla, 1980) and *Saccharomyces cerevisiae* (Bennett *et al*., 1999) where Boronis found to be beneficial for their growth;*Neurospora crassa* (Schmid and Harold, 1988; Dicker and Turian, 1990), *Saprolegnia ferax* (Jackson and Heath, 1989) and *Aspergillus niger* (Pera and Callieri, 1997), where Ca is required for the growth. Manganese is required for the growth of fungi like *Gonatobotrys simplex*, *Chaetomium*sp., *Hypoxylon punctulatum* and *Neocosmospora vasinfecta* (Barnett and Lilly, 1966).Zincis reported to influence their growth like *Aspergillus flavus* (Cuero *et al*., 2003), *Agrocybe aegerita* (Sharma *et al*., 2004), *Cordycepssinensis* (Dong and Yao, 2005), *Pleurotus ostreatus* (Baldrian *et al*., 2005), *Alternaria alternata* S3S (Ezzouhri *et al*., 2009). 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). Copper is required for the growth of many fungi (Machlis, 1953; Agnihotri, 1966, 1967; Sankhla *et al*., 1970; Dong and Yao, 2005) and is also a known fungicide even in low concentrations. *Dictyoarthrinium synnematicum* resembles other fungi which don't require Cu for their growth e.g. *Saprolegnia* sp. and *Achlya oblongata* var. *oblongata* (Prasher and Rawla, 1988) and *Cercosporagranati* (Chahal and Rawla, 1977). Its stimulatory effect on MnP & LiP

Table 1 Growth (average mycelial dry wt.) and ligninolytic enzymes production of Dictyoarthrinium synnematicum with different concentrations of Cobalt, at 24°C and pH 5.0 after 16 days of incubation.

Cobalt conc. (ppm)	Average mycelial dry wt. (mg/25ml)	Enzyme activity(IU/mL)		
		Lignin peroxidase	Manganese peroxidase	Laccase
Control	7.2±0.10	ND*	ND	ND
0	2.5±0.00	ND	ND	ND
10^{-6}	10.3±0.05	161.2±0.05	ND	ND
10^{-5}	7.8±0.05	107.6±0.15	ND	ND
10^{-4}	6.4±0.05	80.6±0.05	ND	ND
10^{-3}	6.0±0.00	ND	ND	ND
10^{-2}	5.1±0.05	ND	ND	ND
10^{-1}	4.2±0.25	ND	ND	ND
1.0	2.5±0.00	ND	ND	ND
10	2.5±0.00	ND	ND	ND
100	2.5±0.00	ND	ND	ND
400	2.5±0.00	ND	ND	ND

* ND- not determined

Table 2 Growth (average mycelial dry wt.) and ligninolytic enzymes production of Dictyoarthrinium synnematicum with different concentrations of Copper, at 24°C and pH 5.0 after 16 days of incubation.

Copper conc. (ppm)	Average mycelial dry wt. (mg/25ml)	Enzyme activity(IU/mL)		
		Lignin peroxidase	Manganese peroxidase	Laccase
Control	7.2±0.10	ND	ND	ND
0	7.3±0.10	ND	ND	ND
10^{-6}	9.6±0.15	107.6±0.05	ND	ND
10^{-5}	8.9±0.05	107.6±0.15	ND	ND
10^{-4}	8.5±0.05	107.6±0.20	ND	ND
10^{-3}	8.0±0.05	80.6±0.15	ND	ND
10^{-2}	7.8±0.05	80.6±0.15	ND	ND
10^{-1}	2.5±0.00	ND	ND	ND
1.0	2.5±0.00	ND	ND	ND
10	2.5±0.00	ND	ND	ND
100	2.5±0.00	ND	ND	ND
400	2.5±0.00	ND	ND	ND

Table 3 Growth (average mycelial dry wt.) and ligninolytic enzymes production of *Dictyoarthrinium synnematicum* with different concentrations of Iron, at 24°C and pH 5.0 after 16 days of incubation.

Iron conc. (ppm)	Average mycelial dry wt. (mg/25ml)	Enzyme activity(IU/mL)		
		Lignin peroxidase	Manganese peroxidase	Laccase
Control	7.2±0.10	ND	ND	ND
0	2.5±0.00	ND	ND	ND
10^{-6}	7.1±0.20	ND	ND	ND
10^{-5}	6.6±0.10	ND	ND	ND
10^{-4}	6.1±0.05	ND	ND	ND
10^{-3}	5.6±0.05	ND	ND	ND
10^{-2}	5.6±0.05	ND	ND	ND
10^{-1}	5.4±0.10	ND	ND	ND
1.0	5.1±0.15	ND	ND	ND
10	2.5±0.00	ND	ND	ND
100	2.5±0.00	ND	ND	ND
400	2.5±0.00	ND	ND	ND

Table 4 Growth (average mycelial dry wt.) and ligninolytic enzymes production of *Dictyoarthrinium synnematicum* with different concentrations of Molybdenum, at 24°C and pH 5.0 after 16 days of incubation.

Molybdenum conc. (ppm)	Average mycelial dry wt. (mg/25ml)	Enzyme activity(IU/mL)		
		Lignin peroxidase	Manganese peroxidase	Laccase
Control	7.2±0.10	ND	ND	ND
0	6.3±0.10	ND	ND	ND
10^{-6}	7.5±0.05	80.6±0.15	ND	522.8±0.10
10^{-5}	7.2±0.05	53.7±0.05	ND	392.1±0.15
10^{-4}	6.9±0.15	53.7±0.05	ND	392.1±0.20
10^{-3}	2.5±0.00	ND	ND	ND
10^{-2}	2.5±0.00	ND	ND	ND
10^{-1}	2.5±0.00	ND	ND	ND
1.0	2.5±0.00	ND	ND	ND
10	2.5±0.00	ND	ND	ND
100	2.5±0.00	ND	ND	ND
400	2.5±0.00	ND	ND	ND

activity of *Phanerochaete chrysosporium*(Singhal and Rathore, 2001) and on laccase activity of *Pleurotus sajor-caju* (Soden and Dobson, 2001), *Pleurotus ostreatus* (Baldrian and Gabriel, 2002; Hou *et al*., 2004), *Funalia trogii* (Birhanli and Yesilada, 2006), *Pleurotus pulmonarius* (Tychanowicz *et al*., 2006) and *Trametes versicolor* (Lorenzo *et al*., 2006) has also been demonstrated.The result confirms the finding of Stajic *et al*.(2013), who also reported inhibitory effect of Feon Laccase production in *Pleurotus pulmonarius*. Similarly, inhibitory effect of Fe on Laccase activity was observed in *Trametes pubescens* (Galhaup and Haltrich, 2001) and *Ganoderma lucidum* (Murugesan *et al*., 2009). *Dictyoarthrinium synnematicum* resembles other fungi where the mycelial growth decreases in presence of higher concentrations of Mo like *Alternaria* sp., *Fusarium* sp., *Phlebia radiata*, *Pleurotus pulmonarius* and *Physisporinus rivulosus* (Kluczek-Turpeinen *et al*., 2014). Inhibitory effects of Mo on laccase have been observed with *Phlebia radiata*, *Pleurotus pulmonarius* and *Physisporinus rivulosus* (Kluczek-Turpeinen *et al*., 2014). The results obtained in this paper showed that mycelial growth and the production of ligninolytic enzymes by *Dictyoarthrinium synnematicum* to a great extent is influenced by the presence of trace elements in the medium as these trace elements cause significant loss in biomass production of this fungus and so the ligninolytic enzymes activity.

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