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Full Length Research Paper

Dynamics of acid phosphatase production of the ectomycorrhizal mushroom *Cantharellus tropicalis*

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Ectomycorrhizal mushroom *Cantharellus tropicalis* was grown in axenic culture to study the effect of pH, temperature, nitrogen, carbon, phosphorus and heavy metals/trace elements on production of acid phosphatase and mycelial growth. The results of present study showed optimum mycelial growth with pH 4 at 15 and 35 ± 2°C. The ectomycorrhizal mushroom mycelia utilized lactose and yeast extract as best carbon and nitrogen source for biomass production. Ferrous sulphate supported the maximum mycelial growth when different trace and heavy metal were used. Among phosphorus sources, di-Sodium hydrogen phosphate supported maximum growth. The acid phosphatase production did not follow a uniform pattern as inferred from observations in this study. Moreover, results showed that high biomass did not mean more acid phosphatase production. However, pH 5 at temperature 15 and 35 ± 2°C supported high enzyme production. Fries Das medium supplemented with inositol and yeast extract produced maximum acid phosphatase at *in vitro* conditions. Ferric chloride produced considerably higher acid phosphatase among different trace elements tested. The present study demonstrates various factors affecting acid phosphatase production, an important feature for selecting ectomycorrhizal mushrooms for field inoculations.

Key words: *Dendrocalamus, Cantharellus*, acid phosphatase, ectomycorrhizal mushrooms.

INTRODUCTION

Ectomycorrhizal infection can increase the growth of host plant by increasing surface area and absorbing essential nutrients from soil which are otherwise unavailable to host. These fungi are mutualistic symbionts and increase nutrient uptake by production and secretion of surface bound extra cellular enzymes. They solubilize insoluble forms of nutrients not readily available to uninfected plant roots and have a significant role in carbon, nitrogen and phosphorus cycling in forested ecosystems (Cullings et al., 2008). The observed association of ectomycorrhiza with organic matter in forest soils has led to the suggestion that nutrients are obtained enzymatically from organic sources (Reddell and Malajczuk, 1984). Effect of ectomycorrhizal mushrooms on host plant growth or nutrient status depends on enzyme activities *viz.,* phosphatase, laccase, glucuronidase, cellobiohydrolase, *N*- acetyl- glucosamine, leucine aminopeptidase, xylosidase and - glucosidase (Courty et al., 2007; Mosca et al., 2007), which provides an ecophysiological advantage for enhancing nutrient acquisition (Cameron et al., 2006).

Studies on acid and alkaline phosphatase have increased due to practical application of ectomycorrhizal mushrooms in field inoculations. There are several reports of acid phosphatase activity of *Amanita, Hebeloma, Tricholoma* etc (Antibus et al., 1986; Alvarez et al., 2005; Buee et al., 2005, 2007; Courty et al., 2005). These surface phosphatase activity are useful in selecting effective mycorrhizal symbiont for field inoculation of tree seedlings in reforestation of degraded land or mine sites. According to Antibus et al. (1986) and McLachlan (1980) external factors greatly affect production and activity of acid and alkaline phosphatase, thus affecting efficiency of potential ectomycorrhizal fungi. However, still the data on diversity and distribution of enzyme activities in native ectomycorrhizal

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Table 1. Different condition for experiment.

S. no	pH	Conditions				
		Temperation	Carbon source	Nitrogen source	Heavy metal and trace elements	Phosphorus source
1	1	5	Dextrose	Ammonium nitrate	Ferrous sulphate	Potassium di-hydrogen phosphate
2	2	10	Sucrose	Ammonium chloride	Magnesium sulphate	di-Sodium hydrogen phosphate
3	3	15	Maltose	Ammonium acetate	Manganese sulphate	di-Potassium hydrogen phosphate
4	4	20	Citric acid	Sodium nitrate	Cobalt sulphate	di-Ammonium hydrogen phosphate
5	5	25	Oxalic acid	Ammonium phosphate	Ferrous sulphate hepta hydrate	
6	6	30	Fructose	Potassium nitrate	Zinc sulphate	
7	7	35	Lactose	di-Ammonium sulphate	Copper sulphate	
8		40	Mannose	Ammonium oxalate	Iron citrate	
9			Inositol	Yeast extract	Ferric chloride	
10					Nickel sulphate	

communities are inadequate (Courty et al., 2005). *Cantharellus tropicalis* Rahi, Rajak and Pandey is a delicious, edible, basidiomycetous fungus forming ectomycorrhiza with *Dendrocalamus strictus* Nees (Sharma, 2008; Sharma et al., 2008, 2009a,b ; Sharma et al., 2010a,b) in tropical region of Central India and also possesses medicinal properties. *C. tropicalis* uses a broad range of phosphorus sources under *in vitro* studies conducted. When it is grown in defined media, it releases phosphatase [acid phosphatase-(EC 3.1.3.2, orthophosphoric monoester phosphohydrolase)] as media has acidic pH (Sharma, 2008). Activity of acid phosphatase in *C. tropicalis* under controlled conditions has been studied (Baghel et al., 2009). However, there is no study on factors affecting acid phosphatase production by *C. tropicalis*. In present study, we assayed mycelia of *C. tropicalis* at different pH, temperature, nitrogen sources, carbon sources for growth and enzyme production and checked difference in enzyme production in response to various test factors.

MATERIALS AND METHODS

Growth conditions

Modified potato dextrose agar (PDA) plates were made for preparing inoculums [potato extract 200 g, dextrose 20 g (Hi- media, India), agar 20 g (Hi-media, India), malt extract 2 g (SRL, India). *C. tropicalis* culture from stock tube were transferred in 90 mm transparent plastic Petri dish (Hi-Media, India) and incubated for 10 - 15 days at 28 ± 2°C in BOD incubator (Caltron, India). When inoculum was ready, a 9 mm disc of 15 days old *Cantharellus* culture was cut with sterile cork borer and aseptically transferred to 150 ml Erlenmeyer flask with standard Fries Das Medium (FD) consisting- 5.0 g malt extract, 0.5 g KH_2PO_4, 0.5 g $MgSO_4$, 0.5 g NH_4Cl, 100 μm thymine HCL, pH adjusted to 5.6 which is best media for the growth of *C. tropicalis* mycelia in liquid media (Sharma, 2008). We used 50 ml of FD in 150 ml Erlenmeyer flask for growth and enzyme production of acid phosphatase. Mycelia were harvested after 15 days for biomass estimation and production of acid phosphatase. Three replicates were prepared for each experiment.

Experimental design

To examine the effect of pH on mycelial growth and production of acid phosphatase, liquid media was set at different pH levels (1-12 ± 0.2) adjusted with 1-5 M NaOH and 1N HCl and incubated at 28 ± 2°C. For studying the effect of temperature, inoculated flasks were kept at different temperature (5 - 40 ± 2°C) for 15 days before harvesting (Table 1).

To study the effect of different carbon source FD medium was replaced singly by various carbon compounds (Table 1). The quantity of different compounds was adjusted so as to obtain an amount of carbon equivalent to that present in 0.5 g of dextrose in the original FD medium except for starch and cellulose. For studying the effect of nitrogenous compounds, N source of FD medium was substituted by different nitrogen compounds (Table 1).

Different N source were incorporated separately in FD medium at the same nitrogen level as present in 0.5 g of ammonium chloride. A study was conducted to determine the effect of trace elements, heavy metals and phosphorus on growth and acid phosphatase enzyme production in *C. tropicalis* (Table 1).

Measurement of acid phosphatase production

Measurement of biomass and enzyme production was done according to Tibbett et al. (1998a) and Antibus et al. (1986) with slight modification according to the lab

Table 2. Effect of pH on growth and acid phosphatase production of *C. tropicalis*

S. no	Initial culture pH	Number of days (15)		
		Mycelial dry weight, mg (mean ± sem)	Enzyme production, mg p- NP liberated $h^{-1}g$ (mean ± sem)	Final culture pH (mean ± sem)
1	pH-1	-	-	-
2	pH-2	-	-	-
3	pH-3	-	-	-
4	pH-4	283 ± 0.068	2.975 ± 0.002	4.2 ± 0.317
5	pH-5	250 ± 0.015	4.300 ± 0.002	5.6 ± 0.850
6	pH-6	116 ± 3.333	3.537 ± 0.000	4.6 ± 0.100
7	pH-7	100 ± 0.020	4.068 ± 0.020	5.0 ± 0.066

Table 3. Effect of temperature on growth and acid phosphatase production of *C. tropicalis.*

S. no	Incubation Temperature (± 2 C)	Number of days (15)		
		Mycelial dry weight, mg (mean ± sem)	Enzyme production, mg p- NP liberated $h^{-1}g$ (mean ± sem)	Final culture pH[1)] (mean ± sem)
1	5	-	-	-
2	10	-	-	-
3	15	170 ± 0.020	3.512 ± 0.004	3.8 ± 0.00
4	20	160 ± 0.000	3.480 ± 0.010	4.5 ± 0.50
5	25	140 ± 0.035	3.425 ± 0.008	4.9 ± 0.25
6	30	140 ± 0.020	3.512 ± 0.008	4.6 ± 0.85
7	35	170 ± 0.013	3.512 ± 0.002	4.3 ± 0.41
8	40	120 ± 0.011	0.65 ± 0.003	5.0 ± 0.34

[1)] Initial culture pH for all treatments were 5.5.

requirements. Mycelia was separated from culture medium by gentle filtration through pre-weighed Whatman filter paper No.1 (Econ, India). Subsequently, it was washed (2-3 times) in modified universal buffer (MUB) (Skujins et al., 1962) prepared by titrating 120 ml of a stock buffer (7.26g tris- hydroxyl methyl amino methane buffer, 6.96 g maleic acid, 8.4 g citric acid, 3.7 g boric acid, 4 ml 0.5 M NaOH solution made up to 120 ml with d/w).

Mycelia was placed in 30 ml screw cap test tubes (Riviera, India), 4 ml of MUB (pH 5.5 for assay of acid phosphatase) and 2 ml of p-nitrophenol phosphate solution (made in MUB buffer) were added to it. The screw cap vials were closed with cap and incubated at 37 ± 2°C for 2 h in incubator. After incubation 4 ml 0.5 M NaOH was added to screw cap vials, mixed well for a few seconds and supernatant was filtered through Whatman No.1 filter paper. The yellow colour complex of p- nitrophenol (PNP) was measured using 1 cm glass cuvette (Optiglass Ltd, UK) in a spectrophotometer (Scigenics 118, India) at 410 nm. The amount of p-nitrophenol released was calculated by referring to a calibration graph and comparison with standard curve and represented as mg p-NP liberated $h^{-1}g^{-1}$.

Measurement of biomass

The biomass from each mycelial assay was required to calculate cleaved substrates on a mass basis. The assayed mycelia were re-filtered (as previously described) and together with the residual and assayed portion of mycelium were dried overnight at 70 ± 2°C and weighed (± 0.01 mg).

RESULTS

Optimum growth was obtained at pH 4 followed by pH 5 (Table 2). Mycelium of *Cantharellus* did not grow at pH lower than 4. At pH 7 mycelium growth was 35% of that obtained at pH 4. The effect of temperature on mycelial biomass showed that growth at 15 ± 2°C and 35 ± 2°C were considerably higher than other temperatures (Table 3). All carbon sources except oxalic acid (in which *Cantharellus* failed to grow) had a stimulatory effect on fungal growth. Lactose, sucrose, fructose, inositol supported good mycelial growth whereas media supplemented with citric acid and mannose showed least biomass (Table 4). All nine nitrogen sources supplemented in FD medium supported good mycelial biomass production. In general, organic sources supported best growth followed by ammonium (except NH_4Cl) and nitrate sources (Table 5). Media supplemented with trace elements *viz.,* Co, Zn, Cu and Ni showed no growth in flask, whereas ferrous sulphate and ferric chloride

Table 4. Effect of Carbon source on growth and acid phosphatase production of *C. tropicalis.*

S. no	Carbon source	Number of days (15)		
		Mycelial dry weight, mg (mean ± sem)	Enzyme production, mg p- NP liberated $h^{-1}g$ (mean ± sem)	Final culture pH[1)] (mean ± sem)
1	Dextrose	120 ± 0.010	0.468 ± 0.012	4.8 ± 0.033
2	Sucrose	310 ± 0.020	0.275 ± 0.002	3.1 ± 0.057
3	Maltose	260 ± 0.023	0.412 ± 0.028	3.5 ± 0.057
4	Citric acid	110 ± 0.016	2.281 ± 0.054	5.0 ± 0.033
5	Oxalic acid	-	-	-
6	Fructose	320 ± 0.023	0.375 ± 0.002	4.0 ± 0.033
7	Lactose	370 ± 0.051	0.330 ± 0.000	3.3 ± 0.088
8	Mannose	110 ± 0.020	0.55 ± 0.048	4.0 ± 0.033
9	Inositol	330 ± 0.092	0.675 ± 0.002	3.9 ± 0.033

[1)] Initial culture pH for all treatments were 5.5.

Table 5. Effect of nitrogen on growth and acid phosphatase production of *C. tropicalis.*

S. no	Nitrogen Source	Number of days (15)		
		Mycelial dry weight, mg (mean ± sem)	Enzyme production, mg p- NP liberated $h^{-1}g$ (mean ± sem)	Final culture pH[1)] (mean ± sem)
1	NH_4NO_3	340 ± 0.055	0.312 ± 0.011	3.7 ± 0.120
2	NH_4Cl	216 ± 0.019	1.100 ± 0.252	5.5 ± 0.066
3	$C_2H_3O_2NH_4$	278 ± 0.033	0.725 ± 0.008	3.2 ± 0.057
4	$NaNO_3$	235 ± 0.110	0.562 ± 0.023	3.5 ± 0.046
5	$(NH_4)_2HPO_4$	383 ± 0.102	0.588 ± 0.024	3.0 ± 0.120
6	KNO_3	227 ± 0.004	0.825 ± 0.002	6.1 ± 0.088
7	$(NH_4)_2SO_4$	286 ± 0.054	0.594 ± 0.009	2.9 ± 0.078
8	$(COONH_4)_2\ H_2O$	333 ± 0.052	-	4.2 ± 0.115
9	Yeast extract	388 ± 0.044	3.175 ± 0.162	4.4 ± 0.67

[1)] Initial culture pH for all treatments were 5.5.

supported mycelial growth (Table 6). When FD medium was supplemented with different phosphorus sources it supported accumulation of mycelial biomass. However, mycelium growth on di-sodium hydrogen phosphate was significantly higher (370 ± 0.083 mg) (Table 7).

The pH had strong effect on production of wall bound acid phosphatase. Maximum production was observed at pH 5 followed by 7. The final pH

Table 6. Effect of Heavy metals and trace element on growth and acid phosphatase production of *C. tropicalis*.

S. no	Trace elements	Number of days (15)		
		Mycelial dry weight, mg (mean ± sem)	Enzyme production, mg p- NP liberated $h^{-1}g$ (mean ± sem)	Final culture pH[1)] (mean ± sem)
1	$FeSO_4$	236 ± 0.008	1.348 ± 0.042	3.4 ± 0.20
2	$MgSO_4$	135 ± 0.015	1.023 ± 0.085	4.0 ± 0.00
3	$MnSO_4$	186 ± 0.052	1.020 ± 1.415	3.7 ± 0.43
4	$CoSO_4$	-	-	-
5	$FeSO_4$. $7H_2O$	116 ± 0.039	3.60 ± 0.099	3.0 ± 0.11
6	$ZnSO_4$	-	-	-
7	$CuSO_4$	-	-	-
8	$C_6H_5FeO_7$	163 ± 0.020	0.803 ± 0.515	4.1 ± 0.20
9	$FeCl_3$	213 ± 0.012	1.742 ± 0.830	3.2 ± 0.11
10	$NiSO_4$	-	-	-

[1)] Initial culture pH for all treatments were 5.5.

Table 7. Effect of phosphorus on growth and acid phosphatase production of *C. tropicalis*.

S. no	Phosphorus source	Number of days (15)		
		Mycelial dry weight, mg (mean ± sem)	Enzyme production, mg p- NP liberated $h^{-1}g$ (mean ± sem)	Final culture pH[1)] (mean ± sem)
1	KH_2PO_4	280 ± 0.046	5.170 ± 7.513	3.8 ± 0.088
2	Na_2HPO_4	370 ± 0.083	3.400 ± 2.725	3.8 ± 0.218
3	K_2HPO_4	290 ± 0.018	3.950 ± 6.834	3.9 ± 0.185
4	$(NH_4)_2$ HPO_4	290 ± 0.040	5.375 ± 0.750	3.3 ± 0.0885

[1)] Initial culture pH for all treatments were 5.5.

of both treatments was approximately pH 5 which may be an important factor related to enzyme production (Table 2). It seems acid phosphatase production was not affected by various temperatures tested, except at 40 ± 2°C which showed marked reduction in enzyme production (Table 3). Of the nine carbon sources used, citric acid supported highest acid phosphatase production, while sucrose, inositol and lactose supported less enzyme production followed by inositol respectively. All tested nitrogen source showed enzyme production. However; yeast extract produced highest amount of enzyme. Among heavy metals and trace elements, ferric chloride produced highest amount of acid phosphatase (Table 6). All phosphorus sources produced large amount of acid phosphatase. However, potassium di- hydrogen phosphate and di- ammonium hydrogen phosphate produced significantly high enzyme when supplemented as only phosphorus source (Table 7).

DISCUSSION

Similar to many other ectomycorrhizal mushrooms *C. tropicalis* grow slowly (3-4 cm/wk) (Sharma, 2008). However, a fungus tries to adapt itself to conditions prevailing in soil environment. The optimum pH for growth was found to be pH 4 for *C. tropicalis* (Table 2). Temperature studies indicate maximum biomass production at 15 and 35 ± 2°C, which can be an adaptation to wide range of temperature prevailing in Central India during rainy season. However mycelium of *C. tropicalis* did not show any change in colour or growth morphology. Different temperature environments have considerable effect on physiological and ecological consequences of ectomycorrhizal associations (Tibbettt and Cairney, 2007). Hacskaylo et al. (1965) measured biomass after 24 days for *Suillus punctipes, Rhizopogon roseolus, Amanita rubescens,* and *Russula emetica* (all temperate species). Similar findings were reported for *Hebeloma* by Tibbett et al. (1998b). Like some secondary metabolites acid phosphatase production is directly related with mycelial growth of fungus.

Phosphatase production by ectomycorrhizal mushrooms is regulated directly or indirectly by several abiotic and biotic factors. Large increase in acid phosphatase production can be related with increase in mycelial biomass. Culture pH strongly influences extra cellular acid phosphatase production, as metabolic activities are

sensitive to external pH change whether in soil or *in vitro* studies (Baxter and Dighton, 2005). However culture pH affects extra cellular enzyme production by inhibiting enzyme stability after it is secreted into growth medium (North, 1982). In present study, culture pH showed a stimulatory effect on acid phosphatase production by *C. tropicalis* at pH 4 (Table 2). Generally ectomycorrhizal phosphatase has a pH optimum approaching that of native soil (Antibus et al., 1986). Moreover, it has been shown that increased ectomycorrhizal hyphal activity induces soil acidification and promotes greater production of acid phosphatase enzyme (Liu et al., 2005). Temperature 15, 30 and 35 ± 2°C showed highest enzyme production followed by 20 and 25 ± 2°C (Table 3). Although, differences in enzyme production were not pronounced, wall bound p–nitrophenyl phosphatase (p-NPPase) activities were detected for *C. tropicalis* at all growth temperature and tend to be high at higher temperature. The results of present study are different from other ectomycorrhizal fungi, which cannot be strictly compared due to difference in growth condition, species and culture media. *C. tropicalis* showed acid phosphatase production at 15°C which was also observed with arctic fungal strains by Tibbett et al. (1998b). Moreover, according to them, increased enzyme production at 15°C may be caused by cell plasma membrane confrontation and consequent leakage of intracellular p- NPPase. Based on results of present study, there is a need to work on ecological significance of extra cellular p- NPPase production at low temperature. It should be outlined that this is still an preliminary study and requires detail work such as the localization of enzyme in fungal cell as Bae and Barton (1989) reported that alkaline phosphatase are localized in cell wall of *Cenococcum graniforme.*

Citric acid appeared to be best for phosphatase production for *C. tropicalis,* even though it does not produce maximum biomass. Results suggest that, in general other carbon source represses acid phosphatase production in *C. tropicalis.* When different nitrogen sources were tested yeast extract produced highest amount of acid phosphatase. Other ammonium and nitrate sources which produced quite high amount of mycelia did not had any stimulatory effect on phosphatase production as observed by Kieliszewska-Rokicka (1992). However, *C. tropicalis* utilizes ammonium source much efficiently than nitrate sources, producing high mycelia biomass in pure liquid cultures (Sharma, 2008). This is true for other ectomycorrhizal mushrooms as observed with *Paxillus involutus* (Batsch) Fr. where ammonium source stimulated the mycelia growth and acid phosphatase (Kieliszewska-Rokicka, 1992). Trace elements reduce enzyme activity by interacting with enzyme-substrate complex, by denaturing the enzyme protein, or interacting with the protein active group (Nannipieri, 1995). Liquid media supplemented with ferrous sulphate, ferric chloride, magnesium sulphate and manganese sulphate had pronounced effect on regulation of enzyme production. Media supplemented with trace elements *viz.* Co, Zn, Cu and Ni showed no growth in flask, hence, no acid phosphatase enzyme production (Table 6). The change in final pH is shown in Table 6. Trace elements (metal ions) are assumed to inactivate enzymes by reacting with sulfihydril groups, a reaction analogous to the formation of a metal sulfide. It has been generally recognized that copper and cadmium are more toxic than other metals (Hattori, 1992). However, Gibson and Mitchell (2005) studying ericoid endomycorrhizal fungi showed that copper has no effect on wall-bound phosphatase activity up to 5 mM concentration. The production of extracellular p- NPPase appears to be enhanced when grown in a standard P medium (that is, with potassium di-hydrogen phosphate); however, effect of different concentration of phosphorus needs to be tested. Overall, results demonstrate no regulation of p-NPPase synthesis at different sources of P (Table 7) . Dighton (1983) has also stated that, phosphatase production by basidiomycete fungi in liquid culture is independent of P in medium. Although, synthesis of p-NPPase is regulated by concentration of substrate, ability to restrict secretion of enzymes where product is plentiful and to increase production where product is limited suggests an economic regulation of phosphatase production attuned to environmental P concentration (Tibbett et al., 1998b). Baxter and Dighton (2005) found that phosphatase enzymes are differentially expressed under contrasting phosphorus conditions by different ectomycorrhizal fungi that may be due to difference in mineralization of organically bound phosphorus. *Piloderma* has also shown species specific substrate preferences in response to organic and inorganic sources of phosphorus (Rosling and Rosenstock, 2008). Calleja et al. (1980) have also suggested that wall- bound phosphatase are most important in cleavage and acquisition of P as intimate contact with substrate would guarantee uptake of liberated P molecules. However, ECM fungi differ greatly in their capacity to produce acid phosphatase due to differential potentiality to utilize phosphorus (Meyselle et al., 1991) which can also be affected by season and succession. Moreover, Courty et al. (2006) found less seasonal differences in ectomycorrhizal acid phosphatase activity while working with *Lactarius quietus, Cortinarius anomalus* and *Xerocomus chrysenteron.*

Although, the epifluorescent microscopy is an advance method to quantify acid phosphatase activity of ectomycorrhizal mushroom grown in soil or in liquid medium, studies on production is also important for ectomycorrhizal mushroom mycelia. Physiological conditions are an important factor in the ectomycorrhizal development and phosphatase production which forms a key factor of the symbiosis. Thus this study on phosphatase of *C. tropicalis* will help in understanding process of mycorrhization in *Dendrocalamus* and

selection of a potential strain for field inoculation with nursery seedlings. However, further studies are required on intra specific variation of acid phosphatase production would help us to screening potential strain/isolate for inoculation of host plant *Dendrocalamus* for forest plantation programs.

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