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

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Isolates of the ectomycorrhizal fungus *Cantharellus tropicalis* were grown in axenic culture to study the effect of pH, temperature, nitrogen source, carbon source, phosphorus source and heavy metals on the 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 tested. Among the phosphorus sources, di- Sodium hydrogen phosphate supported maximum growth. The acid phosphatase production did not follow a uniform pattern as inferred from the observations in this study. High biomass did not mean high acid phosphatase production. However, pH 5 at 15 and 35±2°C supported high enzyme production. Fries Das medium supplemented with Inositol and yeast extract produced maximum acid phosphatase in *in vitro* studies. Ferric chloride produced considerably higher acid phosphatase among different trace elements tested. The present study demonstrates various factors of acid phosphatase production, an important feature for selecting ectomycorrhizal mushrooms for field inoculations.

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

Introduction

Ectomycorrhizal infection can increase the growth of host plant by increasing the surface area and absorbing essential nutrients from soil which are otherwise unavailable to the host. These fungi are mutualistic symbionts and increase the 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

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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 β-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 intensified due to the 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; Buée *et al*., 2005, 2007 and Courty *et al*., 2005). These surface phosphatase activity are useful in selecting effective mycorrhizal symbiont for field inoculation of tree seedlings in the reforestation of degraded land or mine sites. According to Antibus *et al*. (1986) and McLachlan (1980) external factors greatly affect the production and activity of acid and alkaline phosphatase, thus affecting the efficiency of potential ectomycorrhizal fungi. However, still the data on diversity and distribution of enzyme activities in native ectomycorrhizal 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; Sharma *et al*., 2009a,b) in the tropical region of Central India and benefit the host plant. It also possesses several medicinal properties. *C. tropicalis* uses a broad range of phosphorus sources in *in vitro* studies conducted by Sharma (2008). When it is grown in defined media, it releases phosphatase (probably acid phosphatase) as the media has acidic pH (Sharma, 2008). Activity of acid phosphatase in the ectomycorrhizal fungus *C. tropicalis* under controlled conditions has been studied (Baghel *et al*., 2009). However, so far, there is no effort made to study the factors regulating acid phosphatase production. In the present study, we assayed the mycelia of *C. tropicalis* at different pH, temperature, nitrogen sources, carbon sources for the growth and enzyme production and checked the difference in its response to various factors.

Materials and methods

Growth conditions

Modified Potato Dextrose Agar (PDA) plates were prepared for preparing inoculums (with 2% malt extract). *Cantharellus tropicalis* cultures from stock were transferred in 90 mm transparent plastic petriplate (Himedia, India) and

incubated for 15 days at 26 ± 2°C in BOD incubator (Caltron, India). When inoculum was ready, a 9 mm disc of 15 days old *C. tropicalis* culture was cut with sterile cork borer and aseptically transferred to 150 ml Erlenmeyer flask. Standard Fries Das Agar Medium (FDA) consisting-5.0g malt extract, 0.5g KH_2PO_4, 0.5g $MgSO_4$, 0.5g NH_4Cl, 100µm thymine HCL, pH adjusted to 5.6 is best media for the growth of *C. tropicalis* mycelia in liquid media (Sharma, 2008). We used 50ml of FDA in 150ml Erlenmeyer flask for different experimental setups for growth & enzyme production of acid phosphatase. Mycelia were harvested after 15 days for biomass estimation and production of acid phosphatase. Three replicate were prepared for each experiment.

To examine the effect of pH on mycelial growth and production of acid phosphatases, liquid media was set at different pH levels (1-12) adjusted with 1-5M NaOH and 1N HCl. Inoculated flasks were kept at different temperature (5°C-40°C) for 15 days before harvesting. To study the effect of different carbon source FDA medium was replaced singly by various carbon compounds. The quantity of different compounds was adjusted so as to contain an amount of carbon equivalent to that present in 0.5g of dextrose except for starch and cellulose as its empirical formula was not known. In this case quantity was similar to that of 0.5g of dextrose in the original FDA media. For studying the effect of nitrogenous compounds N source of the FDA medium was substituted by different nitrogen compounds. Different N source were incorporated separately in FDA medium devoid of any nitrogen source at the same nitrogen level as present in 0.5g of ammonium chloride. A study was conducted to determine the effect of trace elements on growth and acid phosphates enzyme production in *C. tropicalis*. Different trace elements used were ferric sulphate, ferrous sulphate, copper sulphate, ferric citrate, ferric chloride, nickel sulphate, magnesium sulphate, cobalt sulphate, zinc sulphate, manganese sulphate.

Measurement of acid phosphatase production

Measurement of biomass and enzyme activity was done according to Tibbett *et al*. (1998) and Antibus *et al*. (1986) with slight modification according to the lab requirements. Mycelium was separated from the culture medium by gentle filtration through pre-weighed Whatman filter paper No.1 (Econ, India). Subsequently, it was washed in modified universal buffer (MUB) (Skujins *et al*., 1962) prepared by titrating 120ml of a stock buffer (7.26g tris-hydroxyl methyl amino methane buffer, 6.96g maleic acid, 8.4g citric acid, 3.7g boric acid, 4ml 0.5M NaOH solution made up to 120ml with d/w).

Mycelia was placed in 30ml screw cap test tubes (Riviera, India), 4ml of modified universal buffer (pH 5.5 for assay of acid phosphate) and 2 ml of p-

nitrophenol phosphate solution (made in the same buffer) were added to it. The screw cap test tubes were stopppered and incubated at 37±2^{o}C in an incubator for 2hr. After incubation 4ml 0.5M NaOH was added to the screw cap test tubes, mixed well for a few seconds and the supernatant was filtered through Whatman No.1 filter paper. The yellow colour complex of p- nitrophenol (PNP) was measured using 1cm glass cuvette (Optiglass Ltd., UK) in a spectrophotometer (Scigenics 118, India) at 410nm. 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.

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^{o}C and described prior to weighing (±0.01mg).

Results

Mycelial growth

Optimum growth was obtained at pH 4 followed by pH 5 (Table 1). Mycelium of *Cantharellus* did not grow at lower pH. 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^{o}C and 35±2^{o}C were considerably higher than that in the medium which were incubated at other temperatures (Table 2). All the tested carbon sources except oxalic acid (in which *Cantharellus* failed to raise) had a stimulatory effect on fungal growth. Lactose, sucrose, fructose, inositol were optimal carbon sources for mycelial growth whereas media supplemented with citric acid and mannose showed least biomass (Table 3). All the nine nitrogen sources supplemented in the FDA medium supported good mycelial biomass production. In general, organic sources supported the best growth followed by ammonium (except NH_4Cl) and nitrate sources (Table 4). Media supplemented with trace elements *viz.,* Co, Zn, Cu and Ni showed no growth in the flask, whereas ferrous sulphate and ferric chloride supported good mycelial growth (Table 5). When the FDA medium was supplemented with different phosphorus sources, all of them supported the accumulation of mycelial biomass. However, mycelium growth on di-sodium hydrogen phosphate was exceptionally higher (370±0.083mg) (Table 6).

Table 1. Effect of pH on growth and acid phosphatase production of *C. tropicalis*.

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±.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 2. Effect of Temperature on growth and acid phosphatase production of *C. tropicalis*.

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

Acid phosphatase production

The pH had strong effect on the production of wall bound acid phosphatase. Maximum production was observed at pH 5 followed by pH 7. The final pH of both treatment was approximately pH 5 which may be an important factor related to the enzyme production (Table 1). It seems acid phosphatase production was not affected by the various temperatures tested, except at 40±2°C which showed marked reduction in enzyme production (Table 2).

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

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

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

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	$(NH_4)_2C_4HO_6$	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

Of the nine carbon sources used, citric acid supported the 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 the highest amount of enzyme. Amongst the heavy metals and trace elements, ferric chloride produced the highest amount of acid phosphatase (Table 5). All the 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 the only phosphorus source (Table 6). Although the difference was quite clear mention the data as well as

unit if you have calculated, more phosphorus sources are needed to be tested before any conclusion is drawn from the present study.

Table 5. Effect of Heavy metals and Trace element on growth and acid phosphatase production of *C. tropicalis*.

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	$NiSO_4$	-	-	-

Table 6. 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	$(NH_4)_2HPO_4$	290±0.040	5.375±0.750	3.3±0.0885

Discussion

Similar to many other ectomycorrhizal mushrooms, *Cantharellus tropicalis* grows slowly (Sharma, 2008). The pH of soil is due to various exudates released by plant and microorganism. Fungi adapts to the condition prevailing in the soil environment. The optimum pH for growth was found to be pH 4 for *C. tropicalis* as shown in Table 1. Temperature studies indicate maximum biomass production at 15 and 35±2°C, which can be hampered to the temperature prevailing in Central India during rainy season. Different temperature environments have considerable effect on the physiological and

ecological consequences of ectomycorrhizal associations (Tibbettt and Cairney, 2007). Hacskaylo *et al*. (1965) measured biomass after 24d for *Suillus punctipes, Rhizopogon roseolus, Amanita rubescens,* and *Russula emetica* (all temperate species) between 24°C and 13°C similar findings were reported for *Hebeloma* by Tibbett *et al*. (1998). However, the mycelium of *C. tropicalis* did not show any change in colour or growth morphology.

Like other secondary metabolites acid phosphatase production is directly related with the 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 may be related with increase in mycelial biomass when mycelia were incubated for 15 days in liquid media at 26±2°C. Culture pH strongly influences extra cellular acid phosphatase production, as the metabolic activities are sensitive to external pH change whether in soil or *in vitro* studies. However culture pH could affect extra cellular enzyme production by inhibiting enzyme stability after it is secreted into the growth medium. In present study, culture pH showed a stimulatory effect on acid phosphatase production by *Cantharellus tropicalis* at pH 4 (Table 1). Generally, ectomycorrhizal phosphatase has a pH optimum approaching that of native soil (Antibus *et al*., 1986). As phosphatase are essential in acquiring phosphorus, they should do so at native soil temperature. This study tested the range of temperature on which it can be produced at maximum and *Cantharellus* of Central India would be accustomed. Temperature 15, 30 and 35±2°C showed highest enzyme production followed by 20 and 25±2°C (Table 2). Although the 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 tended to be greatest 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. Increased enzyme production at 15°C may be caused by cell plasma membrane confrontation and consequent leakage of intracellular p-NPPase as observed for arctic fungal strains by Tibbett *et al*. (1998). However there is a need to work on ecological significance of extra cellular p-NPPase production at low temperature.

Of the various carbon sources, citric acid appeared to be the best for phosphatase production for *C. tropicalis,* even though it does not produced maximum biomass. The results suggest that, in general other carbon source represses the 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.

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. Trace elements reduce enzyme activity by interacting with the 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 the regulation of enzyme production. Media supplemented with trace elements *viz.* Co, Zn, Cu and Ni showed no growth in the flask and hence no acid phosphatase enzyme production (Table 5). The change in final pH is shown in Table 5. 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 the other metals (Hattori, 1992). However, Gibson and Mitchell (2005), while studying on ericoid endomycorrhizal fungi, showed that copper has no effect on the wall-bound phosphatase activity up to 5 mM concentrations. The production of extracellular p-NPPase appears to be enhanced when grown in a standard P medium, with the exception of Na_2HPO_4 whereas KH_2PO_4 and $(NH_4)_2$ HPO_4 when supplemented in medium. However, the effect of different concentration of phosphorus needs to be tested. Overall, the results demonstrate no regulation of p-NPPase synthesis at different sources of P (Table 6). Although, synthesis of p-NPPase is regulated by concentration of substrate, the ability to restrict the secretion of enzymes where the product is plentiful and to increase production where product is limited, suggests an economic regulation of phosphatase production attuned to environmental P concentration. Baxter and Dighton (2005) found that the phosphatase enzymes are differentially expressed under contrasting phosphorus condition 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.*

Thus the preliminary study on phosphatase of *C. tropicalis* will help in understanding the process of mycorrhization in *Dendrocalamus.* Further studies

on intra specific variation of acid phosphatase production would help us in screening potential strain/isolate for inoculation of host plant *Dendrocalamus* for forest plantation programs.

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