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Control of Acid Phosphatases Expression from *Aspergillus niger* by Soil Characteristics

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ABSTRACT

This work studied the acid phosphatase (APase) activity from culture medium (extracellular, eAPase) and mycelial extract (intracellular, iAPase) of Aspergillus niger *F111. The influence of fungus growth and phosphate concentration of the media on the synthesis and secretion of phosphatase was demonstrated. The effects of pH, substrate concentration and inorganic and organic compounds added to the reaction mixture on APase activity were also studied. Both enzymes were repressed by high concentrations of phosphate. Overexpression of iAPase in relation to eAPase was detected; iAPase activity was 46.1 times higher than eAPase. The maximal activity of eAPase was after 24h of fungus growth and for iAPase was after 96h. Optimal pH and substrate concentrations were 4.5 and 8.0 mM, respectively. Michaelis–Menten constant (Km) for the hydrolysis of p-nitrophenyl phosphate was 0.57 mM with* V_{max} *= 14,285.71 U* mg^{-1} *mycelium for the iAPase and 0.31 mM with* V_{max} *= 147.06 U* mg^{-1} *mycelium for eAPase. Organic substances had little effect on acid phosphatases when compared with the salts. Both the APases were inhibited by 10 mM* KH_2PO_4 *and 5 mM* $(NH_4)_2MoO_4$*; eAPase was also inhibited by 1 mM* $CoCl_2$.

Key words: Enzyme inhibitors, molybdenum, overexpression, phosphate, soil chemical characteristics

INTRODUCTION

Phosphorus is one of the major macronutrients needed by the plants after nitrogen, and is indispensable to many biological processes. The most visible effect of its omission or reduction is the substantial decrease in plant growth, as has been reported in potato crops (Balemi et al. 2009). Approximately 95-99% of phosphorus is present in the insoluble form, either organic or inorganic, which is unavailable for plant growth. The amount of phosphorus available is very low in many soils and requires the application of fertilisers (Shen et al. 2011). However, soil microorganisms perform the mineralisation of organic phosphorus compounds and the solubilisation of insoluble mineral compounds, making them available to the plants. This mechanism depends on the environmental conditions and biological processes taking place in the soil (Malboobi et al. 2012).

Organic phosphorus content ranges from 30 to 50% of total soil P (Mullen 2005). Several hydrolytic enzymes play a major role in the mineralization processes of organic P substrates (Annaheim et al. 2010). Organic compounds, which appear as phosphate esters, are hydrolysed by phosphatases to produce phosphate, which can then be assimilated by the plants (Hidayat et al. 2006; Nannipieri et al. 2011). These enzymes are secreted by the plants and microorganisms when the content of soluble phosphate in the soil is low (Samuel et al. 2010). When the phosphorus content is high, these enzymes are repressed. Derepression occurs when the levels of phosphate

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become limiting. It can be inferred that in the soil, which is a complex system, the activity of phosphatases can also be controlled by other factors such as the concentration of substrates of the enzymes, the presence or absence of metals and others. Many elements are important for plant growth (Kevresan et al. 2001) and biological nitrogen fixation (Chagas et al. 2010) but amounts in excess can inhibit phosphatase acid activity (Huang and Shindo 2000; Tsekova et al. 2002, Quiquampoix and Mousain 2005). For example, acid phosphatase from *Ustilago* sp. was inhibited by Ca and Al (Onthong et al. 2007). Addition of Ni II ions to the culture medium caused a substantial decrease in *Rhizopus delemar* growth and acid phosphatase activity (Açikel and Ersan 2010).

Studies of phosphatases characteristics and control have been examined in *A. niger*. Four fungal strains of this fungus produced both acid phosphatase and alkaline phosphatase activities (Nopparat et al. 2007). Both intracellular and extracellular phosphatase activity was detected in *A. niger*, but the extracellular phosphatase activity of other species of fungi was repressed by the insoluble phosphorus (Rinu and Pandey 2010). Activity of phosphatase enzymes is affected by the chemical and physical characteristics of soil (Nannipieri et al. 2010; Fitriatin et al. 2011). Although there has been considerable research on the acid phosphatases in fungi, there is relatively little information about the effect of soil chemical characteristics on these enzymes. Further characterization of phosphatases is required to understand the mechanisms underlying the fungus response to soil Pi starvation. Also, the soil characteristics that affect the process of hydrolysis in P-organic compounds need to be known.

Aspergillus niger, a soil isolate showed a high solubilisation activity of insoluble phosphates, such as Ca, Fe and Al phosphate (Barroso and Nahas 2005). A preliminary study showed that this fungus possessed high phosphatase activity, which was important for the release of soluble phosphate from organic phosphate in the soil. Until now, no other study reported the overexpression of acid phosphatase in fungi. The purpose of this work was to describe the biochemical characteristics of both the extracellular and intracellular acid phosphatases from *A. niger* under the influence by chemical soil characteristics.

MATERIAL AND METHODS

Growth studies

The *A. niger* F111 strain was isolated from the soil in São Paulo, Brazil (Barroso and Nahas 2005). The fungus was grown on Sabouraud agar slants for seven days and was subsequently maintained at 4°C. It was grown in Czapek-Dox broth that contained (g/L): 2.0 $NaNO_3$, 0.5 KCl, 0.5 $MgSO_4.7H_2O$, 0.01 $FeSO_4$, 0.01 and 30.0 sucrose. The pH was adjusted to 6.0. K_2HPO_4 was added at a concentration of 50 µM. When the effect of phosphate was studied, K_2HPO_4 was added at 50, 100, 200, 500, 1000 and 5000 µM. Spores were collected from the agar slants and counted in a Neubauer chamber. Then 0.5 mL of a spore suspension containing 22.3 x 10^6 spores mL^{-1} was inoculated into 50 mL of the culture medium contained in Petri dishes. Plates were incubated at 30ºC for up to 96 h. Cultures were harvested by filtration at 24 h intervals in the time-course assay up to 96 h. Mycelial growth was determined after the mycelium was washed with 50 mL deionised water and dried in the oven at 105°C for 24 h.

Enzyme activity determination

The cell-free filtrates and mycelial extract were used as enzyme source. Mycelia extract was obtained from the washed mycelia with 50 mL deionised water after being ground in a pre-cooled mortar with washed sand and 50 mM-sodium acetate buffer (pH 5.4). Measurement of enzyme activity was done using 4 mM p-nitrophenyl phosphate (p-NPP) in 0.1 M sodium acetate buffer, pH 5.4 (acid phosphatase), or in 0.3 M glycine buffer, pH 9.0 (alkaline phosphatase) as the enzyme substrate. The reaction mixture contained 1.8 and 0.2 mL of cell-free filtrate, or 1.9 mL of substrate and 0.1 mL of mycelium extract. The mixture was incubated at 37°C in water bath for 5 (mycelia extract) or 30 min (cell-free filtrates). After incubation, 1.0 mL 1.0 M NaOH was added to the test tubes. The yellow colour complex of p-nitrophenol (pNP) was measured using a spectrophotometer at 405 nm. The amount of p-nitrophenol (pNP) released was calculated by referring to a standard curve. A unit of activity (U) was defined as the amount of enzyme required to liberate 1.0 µmol of pNP per minute under the assay conditions. Specific activities (U mg^{-1} mycelium) were expressed as units mL^{-1} $hour^{-1}$ mg^{-1} mycelium dry weight.

Acid phosphatases properties

A set of assays was performed to examine the properties of acid phosphatase from cell-free filtrates and mycelia extract on the reaction mixture.

Effect of pH: The effect of pH was determined using 0.5 M buffer: citrate (pH 2.5-3.0), citrate-acetate (pH 3.5), acetate (pH 4.0–5.0), citrate (pH 5.5-6.0) and Tris-maleate (pH 6.5-7.0).

Effect of substrate concentration: The influence of substrate concentration was determined using p-NPP at 0.5, 1, 2, 4, 8 and 16 mM. The Michaelis constant (K_m) and maximum velocity (V_{max}) values were determined from a Lineweaver–Burk plot.

Effect of inorganic and organic compounds: Various compounds were added to the reaction mixture. The relation of these substances and the concentration are shown in Table 1.

Effect of molybdate: Enhancing concentrations of $(NH_4)_2MoO_4$ ranging from 0 to 250 mM were added in the reaction mixture.

Statistical analysis

All the results are sum means of three replicates. Data were subjected to regression analysis using the SAS statistical package for ANOVA Maximum and minimum points were calculated using Maple 8 software.

RESULTS AND DISCUSSION

Effect of inorganic phosphate (Pi)

According to Bünemann (2008), up to 60% of the total organic P can be hydrolyzed by phosphatases. The present results showed that the activity of phosphatases also depended on the content of the available soil P. In general, the P content of Brazilian soils is very low, ranging from 23 to 58 µg P mL^{-1} soil (Marcelo et al. 2009) and from 15 to 79 µg P mL^{-1} soil (Garcia and Nahas 2012). According to results (Fig. 1), the concentration of P of these soils was still sufficient to inhibit the activity of acid phosphatases from *A. niger*. However, depending on the concentrations of C and N, these amounts of P are consumed by microorganisms in the soil; the scavenge for new sources of P stimulates soil microorganisms in the production and secretion of phosphatases for P organic hydrolysis.

Figure 1 shows the active growth *of A. niger* on the medium with increasing concentrations of P*i* ranging from 50 to 5000 µM and decreasing activity of acid phosphatase (APase) from mycelial extract (intracellular, *i*APase) and culture medium (extracellular, *e*APase). *A. niger* grew up to P*i* 200 µM and growth stopped at higher concentrations. The results clearly demonstrated that *A. niger* contained an intra and extracellular acid phosphatase, but not an alkaline phosphatase (data not included). The effect of the P*i* concentration on the growth and phosphatase activity have been shown on *A. niger* (Gargova and Sariyska 2003; Hidayat et al. 2006). Various fungi exhibit both intracellular and extracellular APases; for example, *A. fumigatus* (Bernard et al. 2002) and *A. niger, A. awamori, Emmericella nidulans, E. rugulosa, Penicillium simplicissimum* and *P. rubrum* (Yadav and Tarafdar 2003). The present results suggested that both APase from *A. niger* were repressed by high concentrations of P*i*. *e*APase activity decreased by 86% and *i*APase by 99% when P*i* concentration was enhanced from 50 µM to 5000 µM. The activity of *i*APase decreased more rapidly than that of *e*APase, suggesting that the *i*APase activity was more repressed than the secreted enzyme. Repression of PhoAp activity by the presence of P*i* in the culture medium was found in *A. fumigatus* (Bernard et al. 2002), *A. niger* 307 (Gargova and Sariyska 2003) and *A. niger* N402A (Hydayat et al. 2005). The results obtained in this study indicated that the APases seemed to be different molecular species; however, this needed to be confirmed.

*i*APase activity was 12,128.17 and *e*APase 263.09 U mg^{-1} mycelium at a P*i* concentration of 50 µM, which was 46.1 times higher. Higher activity of intracellular acid phosphatase in relation to extracellular was also found in *A. niger*, *A. terreus* and *A. rugulosus*, but in the ratio 10.1-10.9 (Tarafdar et al. 2001). *A. niger* is a fungus that is recognised by the high solubilising activity of insoluble phosphates, using a mechanism that involves the production of organic acids (Richardson and Simpson 2011; Braz and Nahas 2012). Thus, overexpression of *i*APase seems to contribute in a major way to the metabolic pathway involved in the synthesis of organic acids from C sources. However, the overexpression of *i*APase by *A. niger* seemed to be an intriguing result and needed to be clarified. Yadav and Tarafdar (2003) reported that the extracellular/intracellular acid phosphatase ratio from seven species of fungi, including *A. niger*, ranged from 0.39 to 0.86. The ratio found in this study was very low, ranging from 0.02 (P*i* 50 µM)

to 0.15 (P*i* 5000 µM), due to the low *e*APase activity in relation to *i*APase (Fig. 1). In contrast to these findings, various authors found a higher activity of extracellular APase in relation to the intracellular form from *N. crassa* (Han and Rossi 1989) and from *Humicola lutea (*Aleksieva et al. 2002).

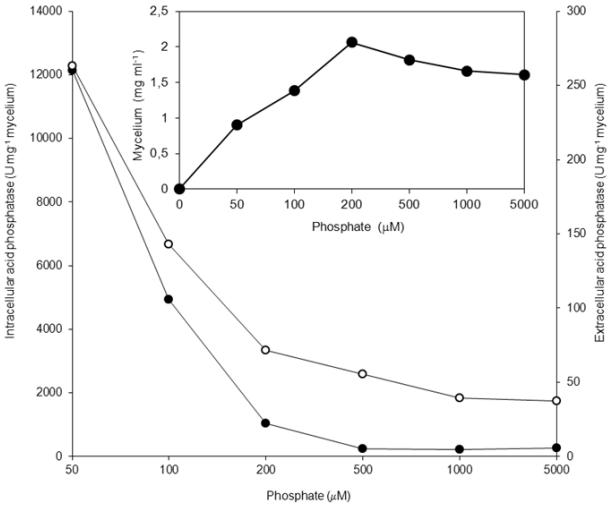

Figure 1 - Effect of phosphate concentration on *Aspergillus niger* growth and acid phosphatases activities. (-●-) intracellular acid phosphatase; mycelium. (-○-) extracellular acid phosphatase.

Time-course of the cultivation

At a *P*i concentration of 50 µM, *A. niger* grew according to a 2nd degree polynomial curve ($y = -0.0199x^2 + 0.3162x - 0.0005$; $R^2 = 0.98$) and the pH of culture media (Fig. 2) decreased rapidly up to 3.0 according to a 3rd degree polynomial curve ($y = -0.035x^3 + 0.4912x^2 - 2.1784x + 5.8974$; $R^2 = 0.98$). The maximal theoretical values were obtained after 7.95 days of incubation for mycelium growth and after 5.74 days for pH decreases. Maximal activity of *i*APase was found after 96h incubation, but *e*APase activity decreased after 24 h (Fig. 2). In contrast, Tsekova et al. (2002) reported that the maximal activity of intra and intracellular enzyme was found after 12h of *A. niger* growth. In addition, the extracellular phosphatase activity continued to increase even when the growth of *A. niger* 307 had stabilised after three days of culture incubation in the stationary phase (Gargova and Sariyska 2003).

The decrease of *e*APase activity during later fungus growth could be due to the repression of P*i* production in the environment. Phosphate is necessary for fungal growth and the APase secretion in P*i*-limited concentration is essential for scavenging organic phosphorus for P*i* uptake by the fungus. Intracellular acid phosphatases have multiple functions involved in the utilisation of P*i* (Pawar and Thaker 2009) in the metabolic process of fungus growth. The synthesis of these enzymes is controlled by the concentration of P*i* and is regulated by the interactions of regulatory genes in *N. crassa* (Davis 2002; Nozawa et al. 2002). It was concluded that the variation of *Pi* concentration in the soil could affect both the fungus growth and, in consequence, the production and secretion of phosphatases.

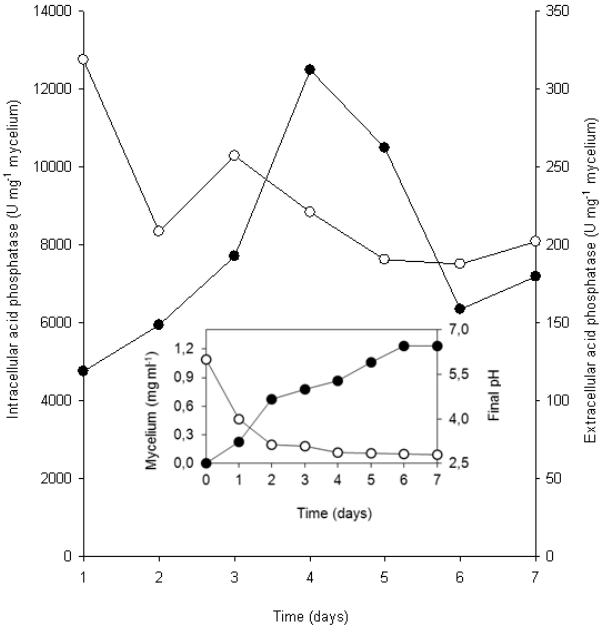

Figure 2 - Time-course of *Aspergillus niger* growth and acid phosphatase activities. (-●-) intracellular acid phosphatase, mycelium; (-○-) extracellular acid phosphatase, final pH.

Effect of pH

Both the intracellular and extracellular APases had pH optimum 4.5 (Fig. 3). The *e*APase activity decreased by 92% at pH 7.0 and the *i*APase by 98% in relation to optimum pH 4.5. However, these results disagreed with those reported by Gargova and Sariyska (2003), who found the optimum pH to be 2.1 for acid phosphatase from *A. niger* 307. At 0-10 cm soil depth, the pH ranged from 5.4 to 6.5 in four crop sequences (Marcelo et al. 2009) and from 4.4 to 6.2 in agricultural and agricultural–pastoral systems (Garcia and Nahas 2012). It was concluded that secreted acid

phosphatase activity from *A. niger* could be severely inhibited in these soils.

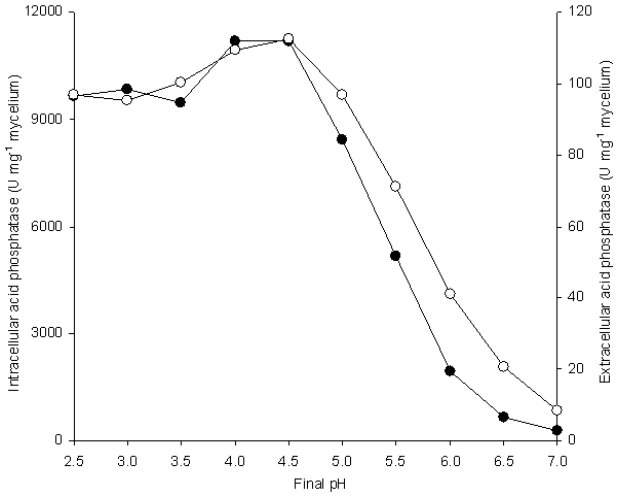

Figure 3 - Effect of pH in the reaction mixture on the acid phosphatases activities. (-●-) intracellular acid phosphatase; (-○-) extracellular acid phosphatase.

Effect of substrate concentration

The activity of both APases increased when p-nitrophenyl phosphate was used at enhancing concentrations from 0.5 to 16.0 mM (Fig. 4). The optimum concentration corresponded to 8.0 mM p-nitrophenyl phosphate. Both phosphatases had Michaelis–Menten type behaviour. The Michaelis–Menten constant (*K*m) was 0.31 mM with V_{max} = 147.06 U mg^{-1} mycelium for the *e*APase, showing a relatively high specificity to the substrate when compared with *e*APase 0.57 mM with V_{max} = 14,285.71 U mg^{-1} mycelium. Similar values of Km for the extracellular acid phosphatases as 0.38-0.53 mM were found for *A. nidulans* (Nozawa et al. 1998). However, higher values of *K*m as 0.95 and 1.3mM were calculated for the acid phosphatases of *A. niger* (Gargova et al. 2006) and *Humicola lutea* (Micheva-Viteva *et al.* 2000), respectively. Also, *K*m values of 0.029 and 0.325mM were reported for the intracellular enzymes of *A. caespitosus* (Guimarães et al. 2003) and *R. delemar* (Tsekova and Galabova 2003), which were low in comparison with the value found in the present work, suggesting that these enzymes had more affinity to the substrate than *i*APase. Orthophosphate monoesters was the dominated fraction of organic P compounds found in the soil (Turner et al. 2003) and could be used as a source of P for soil organisms after phosphate release by phosphatases enzymes. The higher specificity for substrate presented by *e*APase from *A. niger* would allow using the P compounds more efficiently than *i*APase.

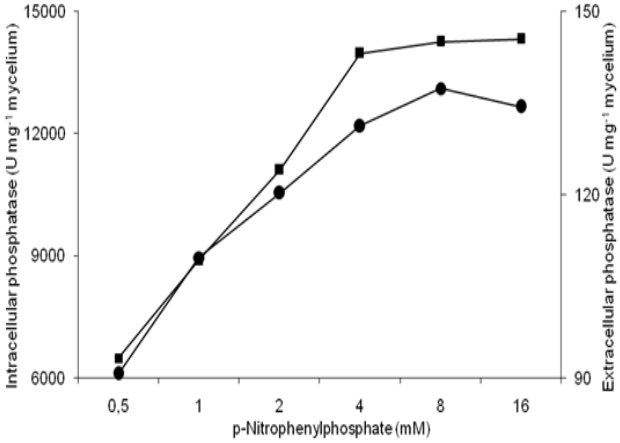

Figure 4 - Effect of substrate concentration in the reaction mixture on the acid phosphatases activities. A) (-○-) extracellular acid phosphatase; B) (-●-) intracellular acid phosphatase.

Effect of inorganic and organic compounds

Organic and inorganic compounds were added to the reaction mixture (see Table 1). Organic substances had little effect on APases when compared to the influence of the salts. The activity of eAPase ranged from 70 to 111% in relation to the control. Tris 1 mM (70%), glucose (74%) and 1 mM Na acetate (79%) were the organic substances that decreased eAPase activity. The activity of iAPase ranged from 91 to 119% in relation to the control and the largest activity was found following the addition of 1 mM Na acetate to the reaction mixture. From the inorganic compounds, the residual activity of eAPase and iAPase ranged from 28 to 99% and 4 to 108% in relation to the control, respectively. The activity of eAPase and iAPase decreased (54 and 51%) following the addition of 10 mM KH_2PO_4 and $CaCl_2$ (86 and 72%, respectively). The residual activity of extra- and intracellular acid phosphatases from *A. caespitosus* were inhibited by 500 mM phosphate, resulting in residual activity of 20 and 31%, respectively (Guimarães et al. 2004). Thus, these phosphatases were less inhibited by phosphate concentrations than found in this study. Salts such as sodium orthovanadate, sodium molybdate, sodium fluoride, and inorganic

phosphate were reported to be inhibitors of acid phosphatase from *Cryptococcus neoformans* (Collopy-Junior et al. 2006) and *Candida parapsilosis* (Kiffer-Moreira et al. 2007). Cobalt is known to induce the activity of phosphatases in bacteria (Gong et al. 2005; Palacios et al. 2005; Wang et al. 2005) and fungi (Venkateswerlu and Sivarama Sastry 1982). The present results showed that 1 mM $CoCl_2$ severely inhibited the activity of eAPase but not iAPase. This could be a negative response because cobalt influenced the absorption of nitrogen by symbiosis (Mengel and Kirkby 2001).

Table 1 - Effect of organic and inorganic compounds on the activity of extracellular (*e*APase) and intracellular (*i*APase) acid phosphatases from *Aspergillus niger.*

Compound	**Concentration**	***e*APase**	***i*APase**
	(mM)	**Residual activity (%)**	
Control	-	100.00	100.00
$(NH_4)_2MoO_4$	5	43.84	3.88
$CaCl_2$	1	86.49	72.32
Na_2SO_4	5	94.28	98.57
$MgCl_2$	1	99.12	101.96
$ZnSO_4$	1	86.96	106.56
KH_2PO_4	0.05	92.05	102.93
KH_2PO_4	0.1	92.41	103.49
KH_2PO_4	1	83.56	94.76
KH_2PO_4	10	53.54	51.26
$(NH_4)_2SO_4$	1	90.02	107.85
$CoCl_2$	1	28.04	100.14
Na and K tartarate	10	93.45	101.26
Na citrate	1	106.06	105.18
EDTA	0.1	97.85	110.26
EDTA	1	89.90	108.53
EDTA	10	98.15	108.68
Glucose	1	74.36	93.41
Tris*	1	69.73	92.72
Fenol	1	89.93	91.95
Fenol	10	98.05	90.57
Na acetate	0.1	79.18	103.07
Na acetate	1	91.94	118.82
Na acetate	10	102.03	91.03
Acetic acid	0.1	100.75	93.78
Acetic acid	1	110.83	97.85
Acetic acid	10	97.06	108.40

*Tris (hydroxymethyl) aminomethane

Effect of Mo

Molybdenum from soil is used by several enzymes from eukaryotes and prokaryotes in reduction and oxidation reactions (Tomatsu et al. 2007). However, various authors have reported that acid phosphatase activity decreased due to the presence of molybdate. This effect was reported in *A. niger*, where the activity was inhibited 44% by 8 mM Na molybdate (Shimada et al. 1977) and corn roots that were also inhibited by 0.01-1.0 mM molybdate (Gallagher and Leonard 1982). Acid phosphatases from *A. fumigatus* were inhibited by NaF, molybdate and vanadate (Bernard et al. 2002). The activity of both APases was severely decreased by 5 mM ammonium molybdate (Table 1). Based on these results, ammonium molybdate was added to the reaction mixture in increasing concentrations of 0-250 mM. Strong inhibition of the *i*APase activity was still observed, even at concentration up to 1 mM, with the residual activity of 18%, and decreased up to 5% at 250 mM (Fig. 5). *e*APase was less inhibited by molybdate, with residual activities of 81 and 19%, respectively (Fig. 6). The importance of these results was related to the content of Mo in the soil (0.1-7 mg kg^{-1}) and that required by the plants

(0.1-2.4 mg kg^{-1}) (Kabata-Pendias and Mukherjee 2007). This suggested that the activity of APase from *A. niger* and other microorganisms could be inhibited by the Mo from the environment.

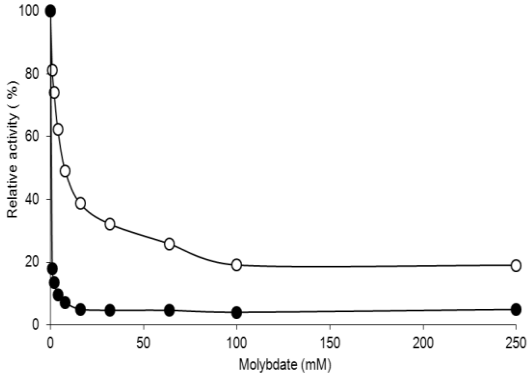

Figure 5 - Effect of molybdate concentration in the reaction mixture on the acid phosphatase activities. (-●-) intracellular acid phosphatase; (-○-) extracellular acid phosphatase.

CONCLUSIONS

A. niger plays a very significant role in the mineralisation of P-organic compounds in the soil, after which free phosphates are released by the action of acid phosphatases (APases). This mechanism occurred in a *P*i-limited manner, which was shown by the fact that both extracellular (*e*APase) and intracellular (*i*APase) APase were repressed at increasing concentrations of phosphate. From the results, it could be concluded that the necessity of *P*i by *A. niger* was not severely limited to up 200 µM, with growth only stopping at higher concentrations. The time-course of fungus growth in 50 µM *P*i showed that the pH of the media decreased up to 3.0 according to 3rd degree equations and the maximal activity of *i*APase and *e*APase was found after 96h and 24h, respectively. The decrease of APase activity in the environment could be interpreted as a mechanism of self-repression by the *P*i available from the organic phosphate sources. The optimum pH and substrate concentration (pNP) for both APases activities were 4.5 and 8.0 mM. The other biochemical properties showed that these enzymes had different characteristics, suggesting that they were not similar proteins. *i*APase activity was overexpressed and had less affinity to the substrate pNP when compared to *e*APase. Both the enzymes were inhibited by *P*i and ammonium molybdate; however, only *e*APase was severely inhibited by $CoCl_2$ and Tris (hydroxymethyl) aminomethane. Finally, these results suggested that *A. niger* growth in the soil was dependent on various factors that influenced the synthesis and secretion of acid phosphatases.

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