Influence of the carbon, nitrogen and phosphorus source on the solubilization of insoluble metal compounds by *Aspergillus niger*

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The effects of varying carbon (glucose), nitrogen ($(NH_4)_2SO_4$, KNO_3) and phosphate (KH_2PO_4) source on solubilization of insoluble $Co_3(PO_4)_2 . 8H_2O$, $Zn_3(PO_4)_2 . 2H_2O$ and ZnO by the soil fungus *Aspergillus niger* were assessed. Solubilization activity was quantified by measuring the clear zones produced around colonies of *A. niger* growing on solidified mineral salts medium amended with the insoluble metal compounds. Effects of nutrient variation on solubilizing properties were compared using ratios of colony growth rate on the metal compounds (R_m) to control growth rate (R_c) and the rate of extension of the zone of solubilization (R_s) compared to the colony growth rate on the metal compound (R_m), i.e. $R_m : R_c$ and $R_s : R_m$. Ratios of solubilization rate to growth rate ($R_s : R_m$) on all the compounds decreased with decreasing glucose concentration; there was no solubilization of ZnO below 60 mM glucose and no solubilization of the metal phosphates below 6 mM glucose. Reducing the concentration of ammonium sulphate in the growth medium decreased $R_s : R_m$ but these values were increased when the nitrogen source was nitrate. Reducing the phosphate concentration increased solubilization of $Co_3(PO_4)_2$ but reduced solubilization of $Zn_3(PO_4)_2$. These findings demonstrate that manipulation of carbon, nitrogen and phosphate sources in the growth medium, and variation of the form of the nutrient source, can be used to alter the solubilizing ability of *A. niger*. Whilst, in the natural environment, this response to different nutrient sources allows optimal exploitation of resources, the potential to manipulate nutrients for maximum solubilizing ability may prove beneficial for the optimization of the solubilization of metal compounds with respect to the bioremediation of metal-contaminated wastes and polluted ecosystems. It could also prove useful in other biotechnological applications such as metal recycling and extraction of metals from low-grade ores.

The ability to solubilize insoluble phosphates is widespread among microorganisms. As well as its common occurrence in heterotrophic and autotrophic bacterial species, the conversion of insoluble metal phosphates into soluble forms has been reported in a number of fungal species (Asea, Kucey & Stewart, 1988; Jones *et al*., 1991; Chabot, Antoun & Cescas, 1993; Burgstaller & Schinner, 1993; Sayer, Raggett & Gadd, 1995). Such solubilization is of importance in nutrient cycling as it releases phosphates as well as metal ions into biogeochemical cycles. It plays an important role in phosphate acquisition by microbial and plant species, especially in soils where phosphates can readily become immobile by interactions with soil components such as calcium in alkaline soils (Thompson & Troeh, 1975; Jones *et al*., 1991; Lapeyrie, Ranger & Vairelles, 1991; Illmer & Schinner, 1992).

Solubilization can be accomplished by a range of mechanisms, which include the excretion of metabolites such as organic acids, proton extrusion or production of chelating agents (Hughes & Poole, 1989; Ford & Mitchell, 1992; Burgstaller & Schinner, 1993; Gadd, 1993; Sayer *et al*., 1995; Karamushka, Sayer & Gadd, 1996; Sayer & Gadd, 1997). Phosphatase production by fungi is usually associated with the breakdown of organic phosphorus compounds, such as inositol phosphates, particularly by mycorrhizal fungi (Williamson & Alexander, 1975; Mitchell & Read, 1981; Antibus, Sinsabaugh & Linkins, 1992; Jayachandran, Schwab & Hetrick, 1992). Acidic metabolite production appears to be the major mechanism of solubilization exhibited by filamentous fungi, and the production of citric and oxalic acids by fungi such as *Aspergillus niger*, *Penicillium bilaii* and *P. simplicissimum* is well documented (Franz, Burgstaller & Schinner, 1991; Cunningham & Kuiack, 1992; Strasser, Burgstaller & Schinner, 1994; Sayer & Gadd, 1997).

The importance of fungi in metal biotechnology is becoming increasingly apparent, in relation to the bioremediation of metal-contaminated wastes (Gadd, 1993; Gadd & White, 1993). *A niger* has been shown to produce high concentrations (> 400 mM) of oxalic acid, when grown on sucrose and lactose permeates at pH 6·0 in a 2 l fed batch reactor, which acts as a leaching agent for a variety of metals that form soluble oxalates, e.g. Fe and Li (Strasser *et al*., 1994). *P. simplicissimum*, capable of leaching insoluble metal oxides

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from industrial filter dust (mainly ZnO), develops the ability to produce large amounts of citric acid (> 100 mM after 9 d) in the presence of the filter dust, the trigger for citric acid production possibly being alterations in plasma membrane H^+-ATPase activity (Franz *et al.*, 1991, 1993). Such characteristics mean that fungal solubilization of metal compounds may be of potential in the recycling of metals from industrial wastes and by-products, or obtaining metals from low-grade ores (Burgstaller & Schinner, 1993).

This study looks at the effect of altering nutrient concentration and source on metal solubilization by *A. niger*, a species which has previously demonstrated excellent solubilizing activity (Sayer *et al.*, 1995). In the natural environment, fungi can utilize a wide variety of different nutrient sources, allowing optimal growth in nutritionally demanding and heterogeneous environments (Ritz, 1995). A corollary of this is that by the manipulation of laboratory growth conditions, the solubilizing ability of certain fungal strains could be maximized, which may be of potential with respect to bioremediation and other biotechnological applications.

MATERIALS AND METHODS

Organism, medium and culture conditions

Aspergillus niger (from our own culture collection) was maintained on malt extract agar (MEA, Lab M) at 25 °C. All screening experiments were carried out using a solidified mineral salts medium (MSM) comprising, g l^{-1} distilled deionized water (ddH_2O): $(NH_4)_2SO_4$, 5; KH_2PO_4, 0·5; $MgSO_4.7H_2O$, 0·2; $CaCl_2.6H_2O$, 0·05; NaCl, 0·1; $FeCl_3.6H_2O$, 0·0025; $ZnSO_4.7H_2O$, 0·004; $MnSO_4.4H_2O$, 0·004; $CuSO_4.5H_2O$, 0·0004; D-glucose, 20; agar (Lab M, no. 2), 12. The initial pH of this medium was 5·2. Proportions of carbon, nitrogen and phosphate in MSM were varied by altering concentrations of glucose, ammonium sulphate, potassium nitrate and potassium dihydrogen orthophosphate respectively and independently of the other ingredients. Each of these components was reduced down to 0·01% (w/v) of the normal medium concentration.

Growth and solubilization ability

Commercial preparations of the insoluble metal phosphates $Co_3(PO_4)_2.8H_2O$ and $Zn_3(PO_4)_2.2H_2O$ (Alfa, Johnson Matthey, Royston, Herts., U.K.) and ZnO (BDH, Poole, U.K.) were used to test for the solubilizing activity of *A. niger* under the different nutrient conditions when incorporated in MSM. Both phosphate compounds were used at a concentration of 5 mM and ZnO was used at a concentration of 15 mM, i.e. a 15 mM metal ion concentration in each case. All these compounds have been used successfully in previous solubilization studies with *A. niger* due to the distinct clear zones formed around colonies and the ease of dispersion of the phosphates and oxide within the medium (Sayer *et al.*, 1995). Petri dishes (90 mm) containing 20 cm^3 of MSM were centrally inoculated with 4 mm diam. discs cut from the growing edge of colonies which had been maintained on MEA at 25° for 48 h. *A. niger* was inoculated (six replicates) onto each of the three metal compounds as well as unamended controls on a range of 15 different C:N:P concentrations. Daily measurements were made of the size of the colonies and of any clear zones present. Measurements were discontinued when the colonies had grown to the edge of the Petri dish, or when the clear zones of solubilization had reached the edge of the dish. Daily measurements were used to plot graphs of colony radius and radius of the solubilization zone versus time. From these, extension rates of the colony and solubilization zone were calculated, using least squares regression, over the linear portion of the graph.

RESULTS

Colony extension rate and extension of the zone of solubilization in the presence and absence of the metal phosphates, ZnO and on varying nutrient concentrations were compared by expressing the values as ratios. These were

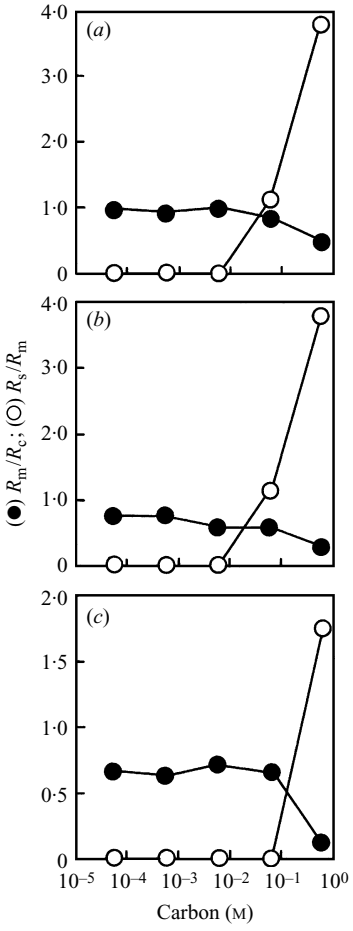

Fig. 1. Growth on and solubilization of (*a*) 5 mM $Co_3(PO_4)_2.8H_2O$, (*b*) 5 mM $Zn_3(PO_4)_2.2H_2O$ and (*c*) 15 mM ZnO by *A. niger* in the presence of varying concentrations of glucose. Results are presented as ratios of the rate of colony extension on metal-amended agar (R_m) in relation to colony extension rate on unamended agar (R_c) (●) and the rate of extension of the solubilization zone (R_s) in relation to colony extension rate on metal-amended agar (R_m) (○). In these, and subsequent data, standard deviations of the rates used to calculate the ratios were all less than 5% (10% for the $Zn_3(PO_4)_2$ solubilization ratios) of the average value (six replicates). Control colony growth rate = 3·02 mm d^{-1}.

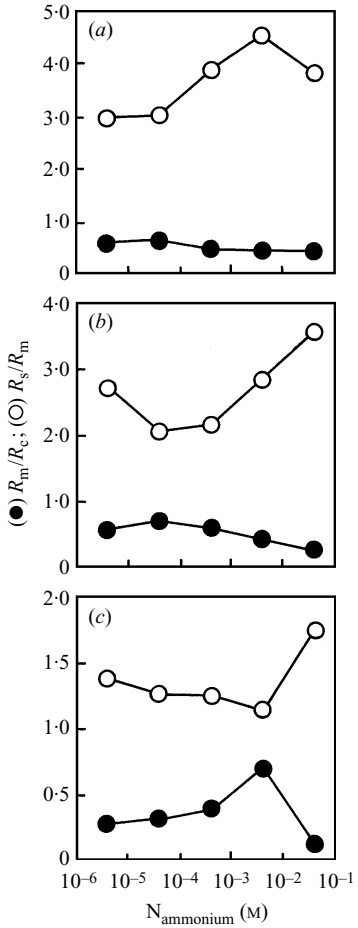

Fig. 2. Growth on and solubilization of (*a*) 5 mM $Co_3(PO_4)_2 . 8H_2O$, (*b*) 5 mM $Zn_3(PO_4)_2 . 2H_2O$ and (*c*) 15 mM ZnO by *A. niger* in the presence of varying concentrations of $(NH_4)_2SO_4$. Results are presented as described in the legend to Fig. 1.

colony extension rate in the presence of a metal compound (R_m) in relation to colony extension rate on control medium (R_c) and rate of extension of the clear zone of solubilization (R_s) in relation to growth rate on metal-amended media (R_m). A ratio of 1·0 means that the control colony growth rate is the same as that in the presence of the metal compound/nutrient treatment whereas, if the ratio is greater than 1·0, it indicates that the metal compound/nutrient treatment is stimulating colony growth rate or the rate of metal solubilization: a ratio of $< 1{\cdot}0$ indicates the reverse of this. In these experiments, solubilization was evident as a clear halo in the agar around the colonies and reprecipitation of the insoluble metal compounds was not observed over the period of incubation.

Influence of altered carbon on growth and solubilization

Reducing the amount of carbon, in the form of glucose, in the growth medium from 600 mM (the maximum concentration tested) to 0·06 mM, i.e. decreasing the C:N ratio, had little effect on the growth of *A. niger* in the presence and absence of $Co_3(PO_4)_2$, $Zn_3(PO_4)_2$ or ZnO (Fig. 1), except that colony growth rate was reduced at the higher concentrations, particularly noticeable on MSM amended with ZnO (Fig. 1*c*). Solubilization ratios on $Co_3(PO_4)_2$, $Zn_3(PO_4)_2$ and ZnO all decreased with decreasing carbon availability and solubilization ceased altogether at 6 mM glucose on the metal phosphates (Fig. 1*a*, *b*) and below 60 mM on ZnO (Fig. 1*c*).

Influence of altered nitrogen on growth and solubilization

When the proportion of nitrogen in the form of ammonium sulphate ($N_{ammonium}$) was altered from a maximum of 40 mM to a minimum of 0·004 mM in MSM, i.e. increasing the C:N ratio, growth on the metal-amended agar was markedly affected on all three metal compounds (Fig. 2). The growth rate of colonies increased with decreasing $N_{ammonium}$ concentration in the presence of both insoluble metal phosphates (Fig. 2*a*, *b*). Conversely, the rate of increase of the solubilization zone decreased with decreasing $N_{ammonium}$ concentration.

Increasing the proportion of nitrate nitrogen ($N_{nitrate}$) in MSM increased the colony growth rate on $Co_3(PO_4)_2$, and $Zn_3(PO_4)_2$ (Fig. 3*a*, *b*) and to a lesser extent on ZnO (Fig. 3*c*). On all three metal compounds, optimum growth occurred at 4·5 mM $N_{nitrate}$, decreasing at the higher concentrations examined. Solubilization ratio values increased with decreasing $N_{nitrate}$ concentrations, although they showed a rather erratic trend on $Co_3(PO_4)_2$ (Fig. 3*a*).

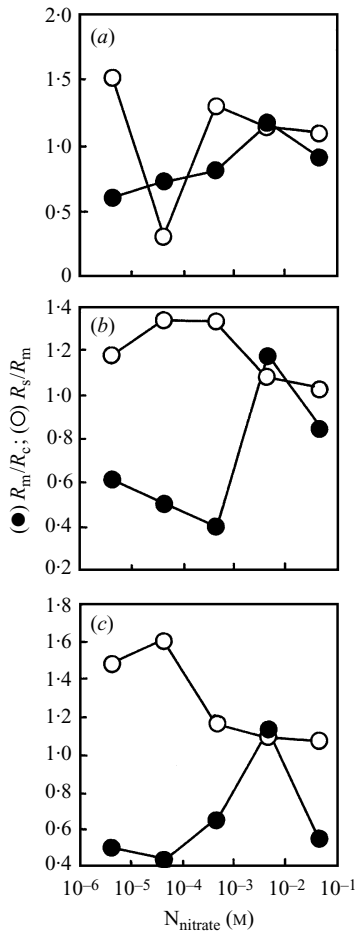

Fig. 3. Growth on and solubilization of (*a*) 5 mM $Co_3(PO_4)_2 . 8H_2O$, (*b*) 5 mM $Zn_3(PO_4)_2 . 2H_2O$ and (*c*) 15 mM ZnO by *A. niger* in the presence of varying concentrations of KNO_3. Results are presented as described in the legend to Fig. 1.

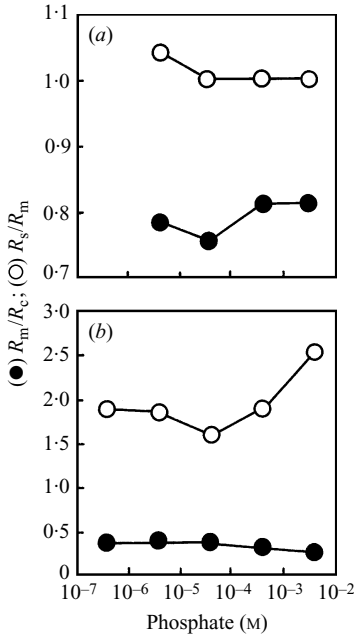

Fig. 4. Growth on and solubilization of (*a*) 5 mM $Co_3(PO_4)_2 . 8H_2O$ and (*b*) 5 mM $Zn_3(PO_4)_2 . 2H_2O$ by *A. niger* in the presence of varying concentrations of KH_2PO_4. Results are presented as described in the legend to Fig. 1.

Influence of altered phosphate on growth and solubilization

Of the three macronutrients investigated, varying the phosphate concentration had the least effect on solubilization by *A. niger* (Fig. 4). Colony growth rates increased with reduced phosphate on $Zn_3(PO_4)_2$ (Fig. 4*b*) but decreased with reduced phosphate on $Co_3(PO_4)_2$ (Fig. 4*a*). Solubilization activity differed on the two metal phosphates: solubilization ratios decreased with decreasing phosphate availability on $Zn_3(PO_4)_2$ (Fig. 4*b*) and increased with decreasing phosphate availability on $Co_3(PO_4)_2$ (Fig. 4*a*).

DISCUSSION

In *A. niger*, as in many fungi, the mechanism of solubilization of insoluble metal compounds is primarily due to excretion of metabolites such as organic acids (Burgstaller & Schinner, 1993; Strasser *et al.*, 1994). Typical published values for organic acid production by *A. niger* are 600 mM citric acid (Mattey, 1992) and 427 mM oxalic acid (Strasser *et al.*, 1994). This process is important in the natural environment because of the solubilization of important nutrients such as essential metals and anionic nutrients, e.g. phosphate, and any factor affecting production of metabolites involved in solubilization will therefore effect solubilization. Nutrient effects on metabolite production by microorganisms have been studied for many years, for example, the effects of substrate composition on calcium phosphate solubilization. Cunningham & Kuiack (1992) showed that citric acid production was stimulated under nitrogen-limiting conditions, while oxalic acid production was stimulated under carbon-limiting conditions. Although organic acid production was not measured directly, our results show that the rate of solubilization of the three insoluble metal compounds decreased with decreasing carbon and decreasing nitrogen (as ammonium) concentration, and increased with decreasing nitrogen (as nitrate) concentration.

The nature and amount of organic acids excreted by fungi are mainly influenced by medium pH and buffering capacity, carbon source and the balance of nitrogen and phosphate (Mattey, 1992). In this study, the glucose level in the medium was shown to be an important factor for the solubilization of both $Zn_3(PO_4)_2$ and $Co_3(PO_4)_2$. Similarly, oxalic acid production in the brown rot fungus *Postia placenta* was stimulated by the addition of glucose and certain glucose polymers (Micales, 1994). Increasing sugar concentrations can increase the activity of glycolytic enzymes and pyruvate carboxylase which can in turn increase citric acid synthesis (Kubicek & Röhr, 1986). Such phenomena may not occur with all fungi though; e.g. oxalic acid production has been found to be promoted under carbon-limited conditions in *Penicillium bilaii* (Cunningham & Kuiack, 1992) and a relatively low sugar concentration (approx. 4 g l^{-1}) was optimal for phosphate solubilization by *Penicillium* spp. isolated from a forest soil (Illmer & Schinner, 1992). This was explained by the fact that suboptimal growth conditions are often necessary for the production of secondary metabolites (Illmer & Schinner, 1992).

N limitation can cause a high conversion of carbohydrate to citric acid in some fungi (Kristiansen *et al.*, 1982), for example, *P. bilaii* (Cunningham & Kuiack, 1992). The form of nitrogen source is of importance, e.g. nitrogen in the ammonium form was necessary for increased phosphate solubilization of rock phosphate (Asea *et al.*, 1988). For a number of ectomycorrhizal fungi, including *Paxillus involutus*, nitrogen source had a significant effect on phosphate solubilization, some strains only being able to solubilize phosphate in the presence of ammonium as the nitrogen source (Lapeyrie *et al.*, 1991). Illmer & Schinner (1992) found that phosphate solubilization by *Penicillium* spp. was minimal around 4 mg ml^{-1} nitrogen and both increasing and decreasing the N-concentration around this figure resulted in higher P-yields which the authors attributed to either inhibition of phosphate uptake by unfavourable C:N ratios or substitution for K^+ by NH_4^+ in phosphate uptake: K^+ is important in P uptake in keeping the cell charge constant. Many fungi are able to utilize both ammonium and nitrate as nitrogen sources and *A. niger* was able to solubilize all the insoluble metal compounds on both N-sources. The reasons why the different nitrogen sources had different effects on solubilization were not investigated in this study, but it is possible that the differential pH changes that occur on the medium with different inorganic nitrogen sources may affect the solubilization processs: a marked fall in pH generally results during growth on ammonium as sole N source for example (Carlile & Watkinson, 1994).

Phosphate is a key nutrient of regulatory importance in microbial secondary metabolite production and limitation of phosphate can improve (or even be considered a prerequisite for) citric acid production (Franz *et al.*, 1991). This 'phosphate effect' is believed to act at an enzymic level rather than at gene expression (Kubicek & Röhr, 1986). The solubilization

rate of $Co_3(PO_4)_2$ was greatest at the lowest phosphate concentration. A stimulatory effect of phosphate limitation was not, however, observed on $Zn_3(PO_4)_2$ in this study.

In conclusion, nutrient effects on the solubilization of insoluble metal phosphates and zinc oxide apparently vary greatly according to nutrient source. In *A. niger*, manipulation of the concentrations of carbon, nitrogen and phosphate, as well as nitrogen source, of the growth medium clearly altered the rate of solubilization of insoluble metal phosphates of cobalt and zinc, and zinc oxide. While the ability of *A. niger* to alter the rate of solubilization in response to changing nutrients is clearly potentially important in the natural environment, manipulation of laboratory media could be useful to optimize the production of organic acids, and this may have potential for biotechnological applications, such as recycling of metals from industrial waste and low-grade by-products, and possibly in the bioremediation of polluted soils.

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