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Characterization by high performance liquid chromatography (HPLC) of the solubilization of phosphorus in iron ore by a fungus

Edwin J. Parks[1], Gregory J. Olson[1], Frederick E. Brinckman[1] and Franco Baldi[2]

[1]*Polymers Division, National Institute of Standards and Technology, Gaithersburg, MD, U.S.A., and* [2]*Department of Environmental Biology, University of Siena, Italy*

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SUMMARY

The value of iron ore is adversely affected by phosphorus in concentrations over 0.03% by weight. The present research concerns the use of metabolic products of a *Penicillium*-like fungus to leach insoluble phosphates (hydroxyapatite) from ores. Ion chromatography was used to measure metabolism of glucose into acidic fragments. The rate and products of glucose degradation depended on both the chemical composition of the growth medium (buffered or not) and incubation conditions (shaken or quiescent). The principal products were identified as oxalic acid and isomers of propylene dicarboxylic acid, mainly itaconic acid. Continued, slow metabolism of itaconic acid generates more oxalic acid. Aliphatic acids were not detected. Both iron ore phosphate and calcium phosphate were partially solubilized by either the spent broth or aqueous oxalic acid. Solubilization of ore phosphorus was greatly assisted by hydrochloric acid added to the spent broth in small increments. The data suggest biological alternatives to costly leaching procedures that use only mineral acids.

INTRODUCTION

The solubilization of phosphates from insoluble inorganic phosphate minerals and compounds by microorganisms has been known for many years [1]. Ehrlich [2] summarized microbiological mechanisms of phosphate solubilization which include production of (1) inorganic [1,3] and organic [4–10] acids, (2) chelators [11–13] and (3) hydrogen sulfide, which reacts with iron phosphate [14] to produce phosphoric acid and other products. Organic acids such as 2-ketogluconic acid or oxalic acid may solu-

Correspondence: G.J. Olson, B-320 Polymer Bldg., National Institute of Standards and Technology, Gaithersburg, MD 20899 U.S.A.

bilize some forms of insoluble phosphates by a combination of mechanisms 1 and 2 above [4,7,10,13]. However, the amount of organic acid in the growth medium and the pH are not always directly correlated with the amount of phosphate solubilization [4,15].

Although the importance of microbiological phosphate solubilization is recognized in environmental cycling of phosphorus and in agriculture, there has been little attention given the potential industrial applications of such processes. One application would be the use of microbiological metabolites to provide an inexpensive method for the leaching of phosphorus for iron ore beneficiation. An elevated level of phosphorus in steel results in embrittlement, loss of toughness, reduced ductility, and enhanced stress corrosion behavior [16,17]. Consequently, iron ores and concentrates with phosphorus levels above 0.03% are not competitive. Chemical and physical methods for removal of phosphorus from iron ore concentrates have been investigated but are either ineffective or too costly [W. Hancock, Cleveland Cliffs Iron Co., Ishpenning, MI, private communication]. Full development of domestic iron ore reserves would be assisted by the development of inexpensive methods for iron ore dephosphorization.

In preliminary experiments we found that oxalic acid in water could solubilize calcium phosphate. Oxalic acid and many other chromophoric and aliphatic acids were among the bacterial metabolites of glucose identified by Guerront et al. [18] by means of liquid chromatography. Krausse and Ullman [19] developed a strategy for identifying anaerobic bacteria by comparing the liquid chromatograms of metabolites for characteristic short chain fatty acids. Agnihotri [10] reported the production of citric and oxalic acids during metabolism of glucose by certain fungi. Itaconic acid (2,3-propenedicarboxylic acid) has been produced commercially by the action of fungi on sugars [20]. In the present work we have isolated a fungus which dissolves calcium phosphate, and used a mass-sensitive refractive index HPLC detector to study its glucose consumption, and a UV detector to follow the concurrent generation of chromophoric organic acids, which were identified by comparison with authentic samples. The principal objective was to identify products in the spent culture medium that were capable of solubilizing phosphorus from an iron ore concentrate containing elevated levels of phosphorus.

MATERIALS AND METHODS

Organism isolation and culture conditions

Strains of calcium phosphate-dissolving microorganisms were isolated by spreading an aqueous suspension of garden soil (1% w/v) on modified Babenko agar [5] which contained (in g/l of deionized water): $(NH_4)_2SO_4$, 0.5; NaCl, 0.2; glucose, 10; $Ca_3(PO_4)_2$, 1.0; and microelement solution, 1.0 ml. The microelement solution contained (in g/l of deionized water): H_3BO_3, 5.9; $NaMoO_4$, 5.0; KBr, 0.5; $ZnSO_4 \cdot 7H_2O$, 0.2; $Al_2(SO_4)_3$, 0.15. The pH was adjusted initially to 7.0. Plates were incubated at 28°C for one week. Colonies surrounded by clear zones in the agar, presumably producing phosphorus–dissolving metabolites, were restreaked twice for isolation. The metabolic products of one strain, designated F-1, were further studied by HPLC.

Studies of metabolism

Strain F-1 was grown in 250-ml conical flasks containing 100 ml of liquid Babenko medium [with 0.1 g/l $Ca_3(PO_4)_2$] and incubated in the dark at 28°C under quiescent conditions or on a gyratory shaker at 200 rpm. In some cases the medium was buffered with sodium acetate to a pH of 6.8. In the absence of buffer, the pH of inoculated samples dropped from 7.0 to a final value of 2.5 after 2–3 weeks incubation. To follow the degradation of glucose, 1-ml aliquots were taken at intervals up to 50 days, or longer for quiescent incubation, filtered through a 0.2-μm membrane filter and injected (usually 50 μl originally containing 500 μg of glucose) directly into the liquid chromatograph. Known quantities of organic acids were similarly injected to compare their elution volumes (V_R) and retention coefficients (k') with those of experimental fractions. The retention coefficient k' is defined as

$$k' = \frac{V_o}{V_r - V_o}$$

where V_0, the 'dead volume', corresponds to the volume of eluant required to transport an eluite through the system without retention and V_r is the volume required to transport the analyte through the system.

Liquid chromatography (HPLC)

The column was polystyrene crosslinked with divinylbenzene (PS-DVB), having sulfonate substituents (Shodex ionpack C-811, Alltech Associates, Deerfield, IL), 4.8 mm ID by 50 cm; measured column efficiency 20 000 plates per meter. The detector was a Knauer UV/RI Dual detector (Utopia Instruments Inc., Joliet, IL), UV absorption measured at 254 nm. Other chromatographic parameters were as indicated in legends to figures. The injection valve was from Rheodyne, model 7120 (Berkeley, CA) and the pump was an Altex Model 110A (Altex, Berkeley, CA).

Phosphorus analyses

Phosphorus concentrations in solution were determined either by phosphorus-specific graphite furnace atomic absorption spectroscopy (GFAAS) using a Perkin Elmer model 460 AAs set at a wavelength of 213.8 nm or by colorimetric determination following complexation with phosphomolybdenum blue [21] using a Klett colorimeter with a No. 66 (red) filter. The phosphorus content of iron ore was also determined colorimetrically following sodium peroxide fusion in a zirconium crucible [22]. Phosphorus dissolution from suspensions of minerals in spent culture broth or by authentic organic acids was determined colorimetrically after centrifugation of subsamples at 12 000 × *g* for 3 min. Iron ore concentrate from the Tilden Mine was obtained from W. Hancock of Cleveland Cliffs Iron Co. The company reported that the concentrate contained 0.061% phosphorus, mainly in the form of hydroxyapatite. Our analyses showed 0.058% ± 0.002% phosporus.

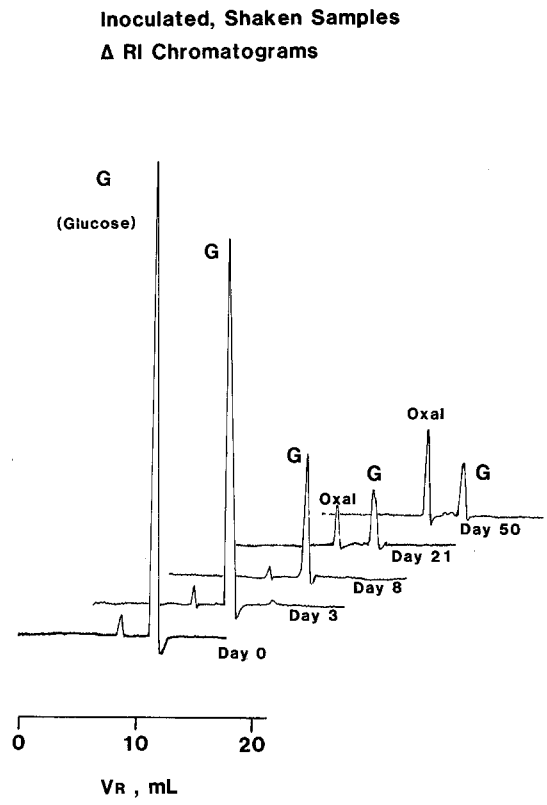

Fig. 1. ΔRI chromatograms of spent growth medium, obtained simultaneously with the UV chromatograms (Fig. 2). At day 0, a small solvent front was followed by the prominent glucose peak which is only slightly evident in the corresponding UV chromatogram, and the glucose peak had nearly disappeared by day 50. It is possible that oxalic acid is indistinguishable from the solvent front at day fifty. ΔRI lacks the sensitivity of the UV detector for itaconic acid, with its conjugated double bonds.

RESULTS AND DISCUSSION

Organism

On Babenko agar medium, strain F-1 produced colonies of cotton-like appearance which become green-colored with time. The green color was due to production of large numbers of conidia which were borne at the tips of conidiophores at the end of the hyphae. The hyphae were septate, and about 3–3.5 μm in width. Conidia measured about 3.5 by 4 μm. Although complete identification of strain F-1, including study of its sexual stage, was not undertaken, the organism bears a strong resemblance to *Penicillium* in that the mycelium produced long, erect

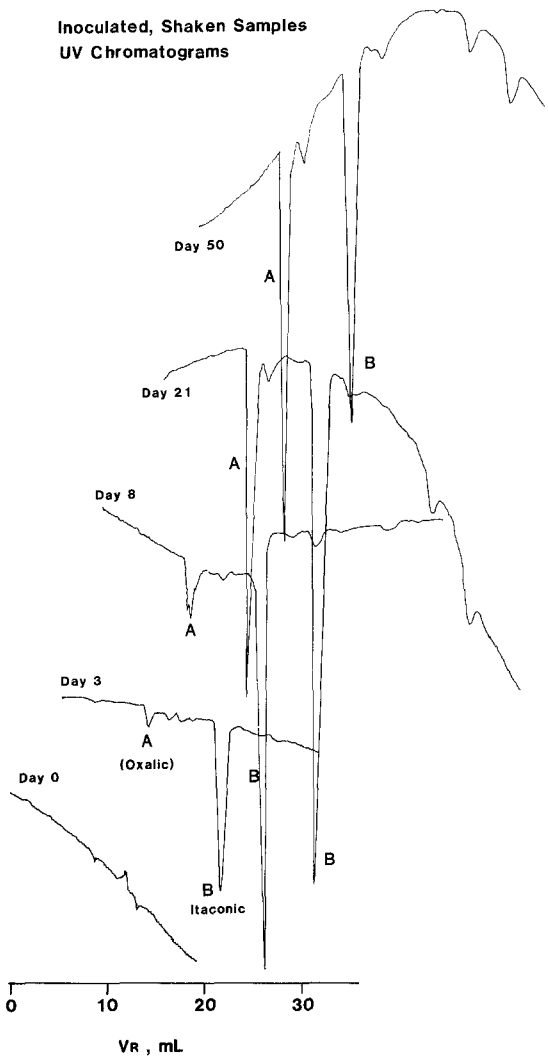

Fig. 2. UV chromatograms of spent growth medium. Injection volume: 50 μl of filtered, spent medium. Eluent: 0.035% (w/v) $HClO_4$ in deionized water. Flow rate: 1.0 ml/min. Major peaks identified (oxalic acid, itaconic acid) by comparison with authentic acids. Note relatively slow early growth of oxalic acid peak compared to itaconic acid, followed, at fifty days, by a relative decrease in the latter.

conidiophores exhibiting multiple branching with long chains of slightly ovoid conidia at the tips [23].

Liquid chromatography of metabolic products

The ΔRI chromatogram of the growth medium before inoculation (Fig. 1) showed a small solvent front (V_R about 8.8 ml) and a glucose peak (V_R = 12.4 ml, k' = 0.409); the latter also contributed a weak absorbance to the otherwise flat UV chromatogram (Fig. 2). During growth of strain F-1, the glucose peak diminished in size (Fig. 1) while in the UV chromatogram at least five peaks appeared (Fig. 2). By comparison with authentic acids the peaks were assigned consecutively to oxalic acid, alpha-ketogluconic acid, and three isomers: glutaconic, itaconic, and mesaconic acids. The major peaks (k' = 0.046, oxalic acid; and k' = 0.865, itaconic acid) continuously increased in size for at least 21 days, the later peak exceeding the earlier one (oxalic acid). But after 50 days, the oxalic acid peak continued to grow while the itaconic acid peak proportionally diminished (Table 1). The generation of acids did not account for all of the change in the mass of glucose since fungal biomass and CO_2 was produced. Sterile controls showed little change in the glucose peak and no appearance of degradation products. The data suggest a metabolic pathway in which glucose first is degraded into the several 5-carbon species, at least one of which, itaconic acid, may further degrade to the two-carbon species, oxalic acid. A similar sample was maintained in the dark for periods up to five months without shaking. The process was slower for the quiescent samples, but the same metabolic products resulted.

Since there were considerable increases in the acidity of the broth, we subjected a sample to buffering, with the pH maintained at 6.5. Both the rate of glucose degradation and the appearance of metabolic products changed; the only prominent product peak was that of oxalic acid.

Solubilization of phosphates

Table 2 indicates that phosphates were partially solubilized by spent culture broth, using either calcium phosphate (9 mg, 20% solubilization), or hydroxyapatite (11 mg, 28% solubilization). By comparison with the chromatograms of samples of known concentrations of oxalic acid (Table 1), the spent broth contained 14.3 mg of the acid in the 10.0-ml aliquot taken from 100 ml of broth. This represents 14.3 percent of the original glucose.

2.5 ml of an aqueous solution containing 97.3 mg of authentic oxalic acid dissolved 80% of phosphorus from 7 mg of calcium phosphate. Direct com-

Table 1

Mass of residual oxalic and itaconic acids calculated from chromatograms

Sample	Retention coefficient (k')	Sensitivity (AUFS)[1]	Acid conc. (g/10 ml)
Oxalic acid[2]	(a) 0.045	0.025	0.0117
Itaconic acid[2]	(b) 0.850	0.25	0.115
Culture medium, day 21	(a) 0.045	0.025	0.0118
	(b) 0.85	0.025	0.0225
Culture medium, day 50	(a) 0.034	0.025	0.0150
	(b) 0.82	0.025	0.0162

[1] Absorbance units, full scale.
[2] authentic compounds.

parisons of the spent broth and the authentic materials were complicated by the presence of itaconic and other acids in the spent broth. However, the efficiency of solubilization appeared to be somewhat higher for the metabolites than oxalic acid alone.

Removal of phosphorus from iron ore

We used Tilden Mine iron ore concentrate to determine the effectiveness of organic and mineral acids and spent culture medium on phosphate removal from ore. Fig. 3 shows results of a time course experiment involving treatment of iron ore concentrate with spent culture broth (1.14 g concentrate in 68 ml). The maximum level of soluble phosphate occurred after 5–10 min. The pH increased from 2.8 to 5.7 over the course of this experiment. The efficiency of dissolution of phosphorus from ore by spent culture medium compared to authentic organic acids was compared after 10 min of reaction (Table 3), using the same concentrations of organic acid, as estimated from chromatograms. The pure organic acid solution, however, had a lower pH (2.1), possibly because of undetected natural buffers in the spent culture. About 20% of the phosphorus was removed by spent culture broth compared to over 50% removal by the more acidic solution containing 12 mM itaconic and oxalic acids.

Further experimentation suggested that the broth and a mineral acid together might synergistically solubilize the phosphorus. HCl was added incrementally to deionized water or spent culture broth (10 ml) containing 100 mg of iron ore concentrate (Fig. 4). Small amounts of HCl (10–25 μl) in the

Table 2

Dissolution of phosphates by spent culture medium[1]

Compound	Solution	PO_4^{2-} in solution (mg/l)	% Dissolved	Final pH
$Ca_3(PO_4)_2$	spent broth	135	20	5.4
	deionized H_2O	4	0.6	7.2
hydroxyapatite	spent broth	150	28	5.4
	deionized H_2O	8	2.2	7.1
none	spent broth	25	–	3.1

[1] 100 ml of a 3-week culture of F-1 grown in Babenko medium was filtered (0.2-μm filter) and 10-ml aliquots added to conical flasks containing 9–11 mg of the above compounds. Flasks were shaken at 200 r.p.m. for 4.5 h at 28°C. The solution was again filtered and analyzed colorimetrically for soluble phosphate [21].

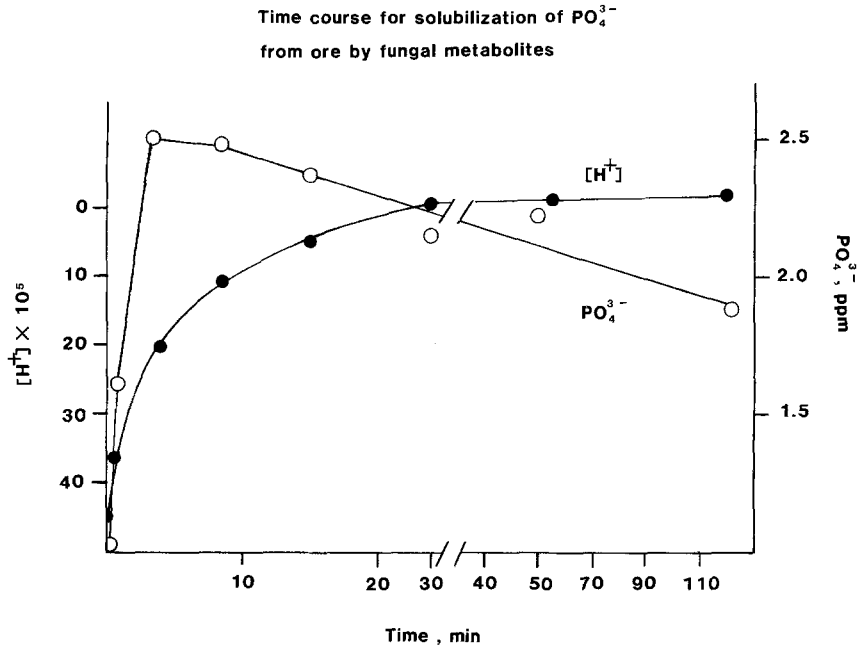

Fig. 3. Solubilization of phosphorus from ore as shown by phosphate in solution after periods of time. The concurrent depletion of acid was nearly finished in 35 min. The decrease in phosphate on extended reaction could indicate its reprecipitation or adsorption to particles of ore or to the walls of the vessel.

broth enhanced phosphate dissolution by a factor of two or three, with smaller increases as the amount of HCl increased up to 100 μl. Following treatment with HCl-emended broth or distilled water, respectively, the iron ore phosphorus levels had declined to 0.029% and 0.025%. The 10-μl spikes of HCl in 10 mL of distilled water leached 33 to 50% of the levels of dissolution effected by 100 μl of HCl. The use of spent culture broth resulted in a 100% increase in phosphorus dissolution over the levels

Table 3

Solubilization of ore phosphorus by spent culture medium and its organic acid constituents[1]

Acids	Acid conc. (mg/10 ml)	pH	P in treated ore (%)
none	–	7.0	0.059
spent broth	32[2]	2.8	0.049
oxalic/itaconic	32	2.1	0.028

[1] Reaction for 10 min (1% pulp density) followed by centrifugation of ore, drying, and analysis for total phosphorus [22].

[2] Estimated from peak heights in UV chromatograms.

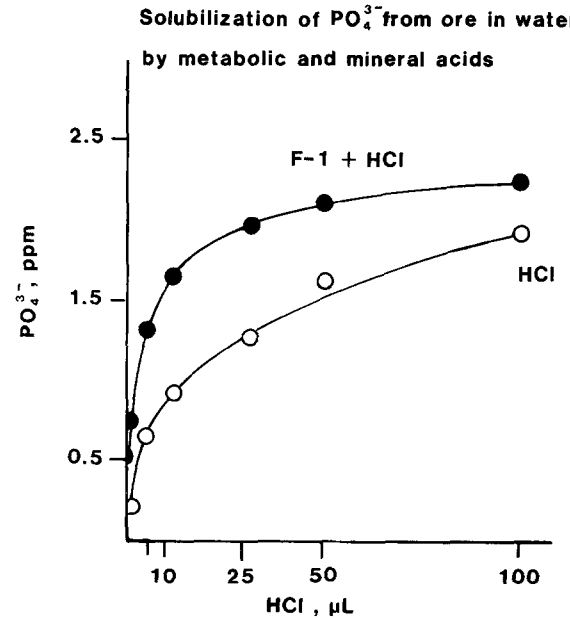

Fig. 4. Solubilization of phosphate from ore in water by metabolic and mineral acids. A synergistic combination of organic and inorganic acids is indicated here with the incremental addition of HCl to broth. The added HCl was most effective in the lowest increments.

achieved with deionized water when both were spiked with 10 μl of HCl per 10 ml.

If a similar process using acid metabolites and low concentrations of mineral acid can be exploited commercially, such high phosphorus ore after treatment would be competitive with iron ores naturally low in phosphorus. Further evaluation of other microorganisms and inexpensive sources of carbon and energy (e.g. cornstarch), is needed to fully evaluate the potential for commercialization of microbial iron ore dephosphorization.

NOTE ADDED IN PROOF

Rogers et al. recently described studies aimed at using microorganisms to solubilize rock phosphate. Cultures of bacteria and fungi solubilized up to 80% of Idaho rock phosphate samples within 9 days (Rogers, R.D., J. Acasi, R.C. Cronn, J.K. Trautman and J.H. Wolfram. 1989. Biosolubilization of phosphate ore. In: Proc. Symp. Biotechnol. in Minerals and Metals Processing, Soc. Mining Engineers, Littleton, CO, in press).

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