Characterization of brushite as a re-crystallization product formed during bacterial solubilization of hydroxyapatite in batch cultures

P. Delvasto\textsuperscript{a}, A. Valverde\textsuperscript{b}, A. Ballestera\textsuperscript{a}, J.M. Igual\textsuperscript{b}, J.A. Muñoz\textsuperscript{a}, F. González\textsuperscript{a}, M.L. Blázquez\textsuperscript{a}, C. García\textsuperscript{c,*}

\textsuperscript{a}Biohydrometallurgy Research Group, Department of Materials Science and Metallurgical Engineering, Universidad Complutense de Madrid, 28040 Madrid, Spain

\textsuperscript{b}Natural Resources and Agrobiology Institute, CSIC, Cordel de Merinas, 40-52, 37008 Salamanca, Spain

\textsuperscript{c}Department of Industrial Technology, Universidad Alfonso X "El Sabio", Av. de la Universidad 1, 28691 Villanueva de la Cañada (Madrid), Spain

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Abstract

Two strains of bacteria (\textit{Burkholderia} sp., strain FeGL01, and \textit{Burkholderia caribiensis}, strain FeGL03) were isolated from a Brazilian high phosphorus iron ore. The capacity of both strains to solubilize hydroxyapatite, Ca\(_5\)(PO\(_4\))\(_3\)(OH), was assessed in plate and batch cultures. In batch cultures, the concentration of solution-P showed two kinetics: an initial one, characterized by a continuously increasing kinetics and a second one, characterized by oscillatory kinetics. To understand the nature of these oscillations, phosphatic residues in the spent broth were collected before, during and after the oscillations, and characterized using scanning electron microscopy (SEM), energy-dispersive X-ray chemical microanalyses (EDX) and X-ray diffraction (XRD). From these studies, it was found that drops in P concentration were related to the formation of an intermediate phosphate in the residues, identified as brushite, CaHPO\(_4\)-2H\(_2\)O. Later increase of available P in the solution was found to be a consequence of re-dissolution of brushite crystals previously formed. Re-crystallization of brushite was also detected in plate cultures after 12–14 days of incubation.

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1. Introduction

Mineral-phosphate solubilization by microorganisms has been studied thoroughly for decades and it is of paramount interest in different fields including soil science, agriculture, bioremediation and biomining. Although there is controversy on the actual mechanism by which P is released from mineral sources, it is generally accepted that microbially mediated acidification, excretion of organic acids (OAs), organic ligands (OLs) and production of extracellular polymeric substances are mechanisms that explain the overall process of P-dissolution by microbes (Illmer and Schinner, 1995; Welch et al., 2002). Apart from solubilization mechanisms, microbial solubilization kinetics of inorganic P-sources deserves special attention. Several works have reported that P released to solution during mineral-phosphate solubilization often shows an oscillatory kinetics. This feature has been observed in cultures of several phosphate-solubilizing microorganisms (PSMs) including \textit{Pseudomonas} sp. (Illmer and Schinner, 1995), \textit{Bacillus} sp. (Kole et al., 1999), \textit{Azospirillum halopraeferans} (Seshadri et al., 2000), \textit{Penicillium} sp. (Kole et al., 1999), \textit{Penicillium radicum} (Whitelaw et al., 1999) and \textit{Aspergillus niger} (Goenadi and Sugiarto, 2000). Illmer and Schinner (1995) suggested as a possible explanation, a process of re-precipitation and secondary solubilization of intermediate phosphate species. These authors concluded that a monitoring of the mineralogical changes undergone by P-substrates along the process may be helpful in understanding why these oscillations of P in solution are produced.
In our present work, we assessed the capacity of two bacterial strains of genus *Burkholderia* to solubilize a mineral source of P, hydroxyapatite, in shake flasks. The mineralogical changes undergone by hydroxyapatite were monitored along the experiment, emphasizing the characterization of newly formed crystalline species by several techniques including scanning electron microscopy (SEM), energy-dispersive X-ray chemical microanalyses (EDX) and X-ray diffraction (XRD). Our aim was to confirm whether or not the observed kinetic oscillations of P in the culture medium were related to a process of re-crystallization and subsequent re-dissolution of intermediate phosphatic phases during the bacterially mediated solubilization of hydroxyapatite.

2. Materials and methods

2.1. Isolation of bacterial strains

The bacterial strains used for phosphate solubilization tests were isolated from a high-phosphorus iron ore from the Jangada mine, located in the Brazilian region of Minas Gerais. XRD and chemical analysis of the ore indicated that hematite and quartz were the main mineral constituents. The phosphorus content of the ore was 0.18%. PSMs were isolated by plating ore extracts in a modified NBRIP medium (Nautiyal, 1999): glucose, 10.0 g/l; MgCl₂-6H₂O, 5.0 g/l; MgSO₄-7H₂O, 0.25 g/l; KCl, 0.20 g/l; (NH₄)₂SO₄, 0.10 g/l, agar-agar, 20 g/l; and 2.5 g/l of hydroxyapatite (noted HAp), Ca₅(PO₄)₃(OH), as insoluble mineral-phosphate source. The pH of the medium was adjusted to 8 before autoclaving. The plates were incubated at 30 °C for 10 days. Colonies that developed clear zones around them, indicating solubilization of hydroxyapatite, were separated and sub-cultured. Up to five strains having solubilization capacity were found; however, only two were chosen for further experiments because these isolates kept unchanged their solubilization ability for more than 18 months of successive sub-culturing (36 generations).

2.2. Identification of the selected strains by 16S rDNA gene sequencing

Total genomic DNA from the bacterial isolates was extracted as described by Rivas et al. (2001). Cells were harvested by centrifugation at 9000 g in a microspin centrifuge for 10 min at room temperature. DNA was extracted with 100 μl of 0.05 M NaOH (DNA-free) by heating at 100 °C for 4 min. Samples were then placed on ice and 900 μl of water was added to each microtube and mixed thoroughly. After an additional centrifugation at 9000g, 700 μl of the supernatant were removed and stored at −20 °C.

Polymerase chain reaction (PCR) was performed using an AmpliTaq reagent kit (Perkin-Elmer Biosystems) following the manufacturer’s instructions (1.5 mM MgCl₂, 200 μM of each dNTP and 2 U of Taq polymerase for 25 μl of final volume of reaction). The PCR amplification of 16S rDNA was carried out using the following primers: 5'-AGAGTTTGATCCTGCGCAG-3' (Escherichia coli positions 8–27) and 5'-AAGAGGTTGATCACCAGCAG-3' (E. coli positions 1502–1522) at a final concentration of 0.2 μM. PCR conditions were as follows: pre-heating at 95 °C for 9 min; 35 cycles of denaturing at 95 °C for 1 min; annealing at 59 °C for 1 min and extension at 72 °C for 2 min, and a final extension at 72 °C for 7 min. PCR products were electrophoresed in a 1% agarose gel at 6 V/cm⁻¹ and visualized by ethidium bromide staining. The band corresponding to the 16S rDNA was purified directly from the gel by centrifugation using Ultrafree®-DA tubes (Millipore) for 10 min at 5000g at room temperature according to the manufacturer’s instructions.

 Sequencing reactions were performed on an ABI377 sequencer (Applied Biosystems) using a BigDye terminator v3.1 cycle sequencing kit as supplied by the manufacturer. The following primers were used: 5'-AACGCTGGCGG-CRKGCTTAA-3', 5'-ACTCTTACGAGGCGACGAG-3', 5'-CTGTCCTGTCTCGTGGAGGT-3', 5'-CGTGCCAGCAGCGGTAA-3', 5'-CAGTATTAGATACCCTTGTTAG-3' and 5'-GAGGAAAGTTGGGATGACG-TC-3', which correspond to *E. coli* small-subunit rDNA sequence positions 32–52, 336–356, 356–336, 512–532, 782–803 and 1173–1194, respectively. The sequence obtained was compared with those from the GenBank using the FASTA program (Pearson and Lipman, 1988). Sequences were aligned using the Clustal W software (Thompson et al., 1997).

2.3. HAp solubilization experiments in batch cultures

The experiments were carried out in 150 ml Erlenmeyer flasks. Each flask received 0.08 g of HAp, and was autoclaved for 20 min at 126 °C. A volume of 30 ml of sterile modified NBRIP broth was added to each flask. The final P concentration in the system was 500 mg/l. Bacteria were inoculated by adding 0.05 ml aliquots (10⁸ cells/ml) of actively growing bacterial cultures to each flask. A series of uninoculated flasks were used as controls. Flasks were kept in orbital shaker at 150 rpm and 30 °C. Triplicate samples were taken at day 0, 2, 3, 7, 10, 12 and 14 for the experiment with strain FeGL01, (*Burkholderia* sp.) and at day 0, 4, 7, 10, 12 and 14 for the experiment with strain FeGL03 (*Burkholderia caribensis*). The pH of the spent broth was measured using a Crison Basic 20 pH meter, with an Ag/AgCl electrode model 5202. Available P in solution was determined by colorimetric methods (AOAC, 1990). Bacterial biomass was monitored by cell-counting with a Thoma-type hemocytometer. All values of pH, P concentration and cell-counting shown are averages of three samples. HAp residues from the flasks were extracted and prepared for analysis as described below.
2.4. Characterization of HAp residues and in-vitro precipitated materials

As a first approach, HAp residues after bacterial solubilization were decanted and dried at 45°C for 48 h. In other cases, residues were washed by centrifuging and decantation with distilled water prior to drying likewise. For XRD analysis, a PHILLIPS XPERT-MPD diffractometer, wavelength Cu-Kα = 1.5406 Å, was employed. Scan duration was 1 h between angles 2θ = 10° and 2θ = 70°. Crystallographic phases present were identified by comparison with standard diffractograms available on International Centre for Diffraction Data (ICDD) database. For electron microscopy and EDX microanalysis, the residues were mounted on a double-sided conductive carbon adhesive tape and then fixed on a brass sample holder. The samples were sputter-coated with gold and examined using a JSM-6400 SEM coupled with EDX at an accelerating voltage of 20 kV.

Both the bacterial strains, grown on modified NBRI agar plates, exhibited the formation of pebble-like materials below and aside the colonies, in regions where HAp was previously located. These re-precipitations occurred after 12–14 days of incubation at 30°C. Agar sections around the pebbles where cut off and dried at 45°C for 48 h. XRD characterization and SEM examination of the re-precipitated material was performed following the same procedure as for flask residues.

3. Results

3.1. Identification of the strains

The complete 16S rDNA sequences of the two isolates used in this study, FeGL01 and FeGL03, were obtained. A comparison with the 16S rDNA sequences available in the GenBank database indicated that both strains are phylogenetically related to the genus Burkholderia. Strain FeGL03 showed a 99.7% of similarity with B. caribiensis MWAP84 (Y17011) and, therefore, it can be considered as belonging to this Burkholderia species. Strain FeGL01 only showed a 97.5% of similarity with B. sacchari (AF263278), its closest relative. Thus, it cannot be assigned to any of the known species of this genus.

3.2. HAp solubilization experiments in batch cultures

The variables chosen for monitoring the HAp biological solubilization process in agitated flasks were biomass generation (expressed as cells/ml) and available P in solution; pH also served as an indirect solubilization parameter. As depicted in Fig. 1(a) for Burkholderia sp. strain FeGL01 and in Fig. 1(b) for B. caribiensis strain FeGL03, biomass growth during incubation period showed two stages, one fast-growth up to day 4th and one steady-growth afterwards. Towards the end of this steady period, biomass values decreased slightly.

As shown in Fig. 1(a) and (b) up to 4th day of incubation, as biomass production increased, pH decreased in the system. It was found that biomass production arrestments for both isolates tested coincide with increases or arrestments in pH evolution. Fig. 1(a) and (b) also show that pH of uninoculated controls did not change significantly throughout the experimental period, staying around the initial value of 6.5. The minimum pH values reached were 4.7 for Burkholderia sp. FeGL01 (3rd day) and 4.0 for B. caribiensis FeGL03 (7th day). In the latter, a steep increase of pH was found after reaching the minimum value, while for Burkholderia sp. FeGL01 pH increase, after attaining the minimum value, was slight and stayed almost constant along the last 4 days of the experiment.

Evolution of available total phosphorus in solution is shown in Fig. 1(c) for Burkholderia sp. FeGL01 and Fig. 1(d) for B. caribiensis FeGL03. P concentration in broth of uninoculated controls stayed almost constant during experiments, showing a variation between 1.4 and 2.4 mg/l. This can be considered as the equilibrium P concentration range in the liquid as a consequence of placing culture medium in contact with HAp. These equilibrium values are exceeded by far in the case of the inoculated experiments. The maximum P solubilization yield found throughout the experimental time was 253 mg/l (50.6%) for B. caribiensis FeGL03 and 220 mg/l (44.0%) for Burkholderia sp. FeGL01. Beyond the 7th day of treatment, P concentration in broth for B. caribiensis FeGL03 showed an oscillating trend. Burkholderia sp. FeGL01 exhibited this behavior between the 10th and 14th day, attaining a minimum on day 12th. For further analyses, reacted solids were collected when these oscillations occurred.

3.3. Characterization of residues from batch culture solubilization experiments

To verify whether there was any connection between the oscillations in the total P concentration in broth and the nature of the reacted material remaining in the flasks after solubilization experiments, SEM examination, EDX microanalysis and XRD analysis were performed. B. caribiensis FeGL03 phosphatic residues were collected at points where oscillations of P concentration in broth occurred, i.e. on 10th day (minimum) and 12th day (maximum). B. caribiensis FeGL03 liquid cultures showed a heavily viscous appearance at the above-mentioned sampling times. As a consequence, phosphatic residues were highly mushy. As a first approach, residues were dried as-collected, i.e. no washing of the residues was performed. This was intended to preserve, as possible, the original characteristics of the residues. As a result, good SEM images were difficult to obtain. Fig. 2(a) shows a SEM image obtained for B. caribiensis FeGL01 phosphatic residues at the 10th day of incubation.

It can be appreciated that HAp agglomerates are covered with a material with high carbon content as indicated by
Fig. 1. Hydroxyapatite solubilization experiments using isolated strains *Burkholderia caribiensis* FeGL03 and *Burkholderia* sp. FeGL01. (a) Biomass generation by isolates as a function of treatment time. (b) Changes in pH during the experiment. (c) Available phosphorus in solution during the experiment. The values are the average ± SD from triplicate experiments.

Fig. 2. Characteristics of unwashed phosphatic residues after 10 days of incubation with *Burkholderia caribiensis* FeGL03. (a) SEM image showing highly etched residues coated by a polymer-like material (arrow). (b) Typical diffractogram of residues compared with standard diffraction maxima for hydroxyapatite, Ca₅(PO₄)₃OH (ICDD file 24-0033) and brushite, CaHPO₄·2H₂O (ICDD file 02-0085).
EDX microanalysis (data not shown). This material was interpreted as microbial exopolymers. *Burkholderia* species have been reported to produce mucoid substances, especially acidic exopolysaccharides (Cérantola et al., 2000; Lagatolla et al., 2002). Moreover, *B. caribiensis* has been described as responsible for lump aggregation in some soils of the Martinique Island, because of its high exopolymer production (Vanhaverbeke et al., 2001). The diffractogram of *B. caribiensis* FeGL03 residues taken at 10th day, Fig. 2(b), showed marked signal noise as well as the presence of a broad peak around 2θ = 20°. This is indicative of low crystallinity in the sample. Since polymeric materials are amorphous, accumulation and attachment of polymeric substances to reacted calcium phosphate surface, as well as the small amount of residues (less than 0.1 g), can explain this observed feature. Nevertheless, at least three diffraction maxima with strong intensity can be easily observed at 2θ angles of 11.6°, 29.2° and 31.6°. In Fig. 2(b) obtained diffractogram is compared with standard diffraction maxima for hydroxyapatite, Ca$_5$(PO$_4$)$_3$(OH) (ICDD file 24-033) and brushite, CaHPO$_4$·2H$_2$O (ICDD file 02-0085). It can be appreciated that these few maxima are coincident with some of the most important maxima of brushite and hydroxyapatite; however, information is not enough to characterize the residues accurately.

Residues of *Burkholderia* sp. FeGL01 were treated differently and results were improved. In this case, residues were sampled on day 7 (steep dissolution period), day 12 (a minimum on P concentration in broth) and day 14 (a maximum on P concentration). These residues underwent a washing process to remove all associated viscous matter. This improved substantially the quality of SEM images and diffractograms, as seen in Figs. 3 and 4.

![Fig. 3. SEM images and EDX microanalysis spectra from residues of solubilization experiments in broth. (a) Typical view of HAp agglomerates in uninoculated flask after 12 days of incubation. (b) and (c) Re-precipitated brushite crystals present in phosphatic residues after 12 days of incubation with *Burkholderia* sp. FeGL01. (d) EDX spectra of original HAp and re-precipitated brushite crystals showing correspondent Ca/P atomic ratios.](image-url)
The general aspect of hydroxyapatite in uninoculated control flasks can be seen in Fig. 3(a). HAp formed aggregates of different sizes. Once HAp has undergone incubation with bacterial cultures, this aggregated arrangement is still preserved; however, it was found that after 12 days of incubation with Burkholderia sp. FeGL01 cultures, new plate-like crystals were among the residues intermixed with the original HAp agglomerates. Fig. 3(b) and (c) show some of the crystals found.

Chemical microanalysis (EDX), performed on new crystals and HAp aggregates, confirmed a chemical difference between the two. EDX spectra of plate-like crystals and HAp aggregates is shown in Fig. 3(d). While HAp aggregates have a Ca/P atomic ratio of 1.67, typical for this kind of calcium phosphate, the plate-like crystals exhibit an atomic Ca/P ratio of 1.04. This latter value is characteristic of several Ca-phosphates including (Vallet-Regi, 2001): dicalcium phosphate (monetite—CaHPO_{4}) and dicalcium phosphate dihydrate (brushite—CaHPO_{4}·2H_{2}O). XRD was performed to fully identify the new phases found in the residues. Diffraction patterns of original HAp, showing the absence of any crystalline change in the residues, i.e. neither new peaks were visible nor previous peaks had disappeared. This diffractogram corresponds to the time interval where the curve of P released to solution showed its steepest slope, as evidenced on Fig. 1(c). This behavior changed, however, at day 12th of treatment. At this point, a decrease of P concentration in broth was detected, as seen on Fig. 1(c). The corresponding diffractogram showed the appearance of a number of new diffraction maxima (peaks) different to those related to the HAp crystalline structure. Comparing those new diffraction maxima with the standard file 02-0085 of ICDD database, the new phase coincides with brushite, CaHPO_{4}·2H_{2}O. Taken together, results shown in Figs. 1, 3 and 4, confirm the precipitation of plate-like crystals of brushite in bacterium-treated experiments when phosphate concentration in broth falls. On day 14th, a new increase on P levels in broth was measured. SEM examination residues at day 14th did not show well formed brushite crystals, and the appearance of the residue was again alike to that of HAp shown in Fig. 3(a). The corresponding diffractogram (Fig. 4) exhibited an intensity decrease of the main diffraction peaks of brushite. This suggests that once brushite was formed and experiment proceeded, a re-dissolution process of this intermediate calcium phosphate occurred. A re-precipitation of phosphatic materials was also found on plate cultures of Burkholderia sp. FeGL01 and B. caribiensis FeGL03 (Section 3.4).

3.4. Characterization of crystals formed in plate cultures

A re-crystallization phenomenon was detected in the plate maintenance cultures after 12–14 days of incubation. Cultures of Burkholderia sp. FeGL01 as well as cultures of B. caribiensis FeGL03 formed clusters of a white precipitate resembling pebbles. These clusters were found in regions below and aside the bacterial colonies, where HAp was previously present. Ripening of the crystals in most cases caused a break of the agar surface. This can be seen in Fig. 5(a), where crystals from the B. caribiensis FeGL03 culture are shown. Some of these clusters were extracted and examined in detail; their morphology is shown in Fig. 5(b). At higher magnification (Fig. 5(c)) the clusters are made up of slab-shaped individual crystallites. These crystallites had an average cross-section of 12 μm² and an average length of 21 μm.

Pebble-like clusters produced in Burkholderia sp. FeGL01 cultures were almost identical in appearance to those shown in Fig. 5(a)–(c). Chemical microanalyses (Fig. 5d) indicated that crystals had a Ca/P atomic ratio of 1.20 and also that magnesium and carbon were present. Although this atomic ratio does not match any of the common calcium phosphate minerals (a complete list can be found in Vallet-Regi, 2001), XRD analyses of precipitates generated by B. caribiensis FeGL03 and...
**Burkholderia** sp. FeGL01 (Fig. 6) indicated that the crystal structure matches that of brushite in both cases.

Diffractograms shown in Fig. 6 do not differ too much from each other. Despite some intensity difference, the position (2θ angle) of all maxima is highly reproducible between both types of precipitates. The slight intensity difference found could be due to several factors including orientation of the crystallites and the presence of foreign ions, i.e. CO$_3^{2-}$ and Mg$^{2+}$, that could be incorporated into the crystalline structure of the brushite during the re-precipitation process. Incorporation of nutrient-medium ions into brushite structure may also explain the unexpectedly high Ca/P atomic ratio found in the precipitates.

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**4. Discussion**

As seen in Fig. 1(a) and (b), initial pH drop in cultures appeared to be related to biomass production. After biomass production reached a steady state in growth, this dropping trend in pH stopped for *Burkholderia* sp. FeGL01 and reversed for *B. caribiensis* FeGL03 cultures. In the same period, the concentration of P in solution remained very low in both cultures, as seen in Fig. 1(c) and (d), indicating a lag between the production of biomass and the total P yield to solution. Since P is a limiting nutrient for bacterial growth and no source of P, other than HAp, is available in the system, bacteria must generate adaptation strategies to scavenge a threshold amount of phosphate.
from the mineral source to sustain a larger population. As far as our results indicate, for the *Burkholderia* species studied, these strategies seems to be the acidification of liquid medium and exopolymer production. Bacterially mediated acidification may have diverse sources, including respiration, NH₄⁺ assimilation and OA release (Illmer and Schinner, 1995). Although no attempts were made to identify or quantify the OAs or OLs released by the isolates, no evidences of re-crystallization of organic complexes with calcium were found within the solubilization residues. As reported in different works (Lapeyrie et al., 1991; Sayer et al., 1997; Welch et al., 2002; Fomina et al., 2005), OA or OL production by microorganisms during solubilization of minerals induce the precipitation of crystals of organic metal-complexes. All these articles report the use of similar experimental conditions and similar characterization techniques as in our work. Consequently, under the conditions tested here, no significant amounts of OAs or OLs seemed to be produced during the process of solubilization of HAp incubated with our *Burkholderia* isolates. Thus, our results suggest that probably only respiration and NH₄⁺ assimilation are the important factors for acidification and subsequent solubilization of HAp. These results are in agreement with Illmer and Schinner (1995), since they reported that a closely related species, *Pseudomonas* sp., incubated with HAp did not produce detectable amounts of OAs.

Another factor that must be pointed out and may be affecting hydroxyapatite dissolution is the presence of microbial extracellular polymers. Extracellular polymers play a key role in the formation of biofilms on minerals or rocks undergoing weathering (Banfield et al., 1999). It has also been reported that bacteria produce surface-bound polymers that coat mineral-phosphate surfaces during solubilization (Welch et al., 2002). These compounds may have different functions, and can often be produced as a response to environmental stresses, such as nutrient limitation (Welch et al., 2002). A mineral dissolution study with microbial extracellular polymers and simple analogs of these compounds shows that acid polysaccharides can dramatically increase the dissolution rate of minerals under acidic conditions or even inhibit it in slightly alkaline conditions (Welch et al., 2002). Moreover, biofilms and surface-bound polymers create microenvironments that enhance mineral dissolution by local acidification (Banfield et al., 1999). In our experiments, a polymer-like material, pasted to highly etched mineral agglomerates, was observed by SEM (Fig. 2a). As previously said, *Burkholderia* species produce high quantities of such compounds. In our experiments, these bacterial polymers could be affecting hydroxyapatite dissolution well. However this effect was not quantified and further experiments will be necessary to determine it.

A phenomenon observed during HAp solubilization experiments was that after a rapid P-dissolution in the first step of the process, P concentration in solution showed oscillatory kinetics characterized by a raise-and-fall pattern. This concurred with a recovery or stabilization of the pH of the medium, as seen on Fig. 1(a) and (b). As previously said, this behavior of the microbial-mediated solubilization of inorganic-P sources has also been detected by other authors (Illmer and Schinner, 1995; Kole et al., 1999; Whitelaw et al., 1999; Seshadri et al., 2000; Goenadi and Sugiarso, 2000). Some authors (Rodriguez and Fraga, 1999) have explained oscillations as a consequence of several factors including: P uptake and release by biomass, precipitation of organic metabolites and/or formation of organo-P compounds. The work of Illmer and Schinner (1995) must be highlighted because it is the only one that focused the explanation of these features on possible mineralogical changes of the phosphate substratum. In the present article, we support a proof that this rise-and-fall pattern is due to re-precipitation processes as Illmer and Schinner (1995) previously hypothesized. X-ray diffractograms, shown in Fig. 4, identified the reacted phosphatic residues at different stages of the HAp solubilization tests. The decrease in solution P concentration on the 12th day, Fig. 1(c), coincided with the appearance of a new Ca-phosphate species, brushite, within the HAp residues. Further re-dissolution of this precipitated brushite, indicated by a drop of intensity of brushite diffraction maxima in Fig. 4, coincided with a new increase of P concentration in the solution (around 14th day). The solubility product, *K*ₚₑ, of these compounds (Erlich, 2002) is in agreement with this observation: The more soluble of both, brushite (*K*ₚₑ = 2.18 × 10⁻⁷), will tend to re-dissolve before hydroxyapatite (*K*ₚₑ = 1.53 × 10⁻¹²).

Brushite is the hydrated form of dicalcium phosphate, CaHPO₄. According to the general mechanism for hydroxyapatite dissolution proposed by Dorozhkin (2002), dicalcium phosphate is formed as an intermediate phase. Dorozhkin’s dissolution mechanism for hydroxyapatite is described by:

\[
\text{Ca}_5(\text{PO}_4)_3\text{OH} + \text{H}_2\text{O} + \text{H}^+ = \text{Ca}_5(\text{PO}_4)_3(\text{H}_2\text{O})^+ + \text{H}_2\text{O},
\]
2Ca₅(PO₄)₂(H₂O)⁺ = 3Ca₃(PO₄)₂ + Ca²⁺ + 2H₂O,  
(2)

Ca₃(PO₄)₂ + 2H⁺ = Ca²⁺ + 2CaHPO₄,  
(3)

CaHPO₄ + H⁺ = Ca²⁺ + H₂PO₄⁻.  
(4)

The generation of brushite in the collected residues is thus supported by Dorozhkin’s dissolution mechanism. These reactions also indicate that mineral dissolution needs an acidic media to proceed and that the rate of dissolution will increase by decrease in culture pH. Hydroxyapatite could be solubilized through bacterial activity by the following steps: (1) Microbial metabolism provides the H⁺ ions necessary to catalyze mineral solubilization. (2) As reaction progresses, H⁺ ions are consumed according to Eqs. (1), (3) and (4). As a result pH will go up in a weaker or stronger way depending upon how retarded the metabolism is with respect to the H⁺ consumption, for each of the strains tested. (3) As the dissolved phosphate concentration increases, the solution may become saturated and new conditions for the re-crystallization of other mineral-phosphate species can occur. In this way, dissolution rates may decrease not only due to a decrease of H⁺ concentration, but also because the solution is approaching the saturation limit (Welch et al., 2002). The latter also could explain why in the first stage of the solubilization process brushite was not detected, but it started to be detected when concentration of P in solution was high enough to stabilize this intermediate phase. Therefore, once brushite becomes stable, it will tend to re-dissolve because metabolic production of H⁺ is sustained by a still large (and stable) number of bacterial cells (Fig. 1(a) and (b)).

A similar reasoning could be applied for the brushite pebbles precipitated on plate cultures shown in Fig. 5. Slow kinetics in plate cultures, due to lower diffusion rates and the constraint imposed by agar gel-structure to crystal formation, will promote the nucleation and ripening of large crystals. Saturation conditions can also be stabilized by the irreversible dehydration undergone by agar medium during incubation. These effects could keep crystals away from further dissolution. As a consequence, size, morphology and composition of obtained brushite crystals differ from those obtained in liquid cultures. Thus, precipitation of brushite after dissolution of HAp is not a kinetics-dependant process, but depends on the establishment of saturation conditions (dynamic in liquid cultures and static in agar plates) that can stabilize, permanently or not, the brushite structure.

As previously stated for liquid culture residues, no evidence of crystallization of organic Ca-complexes was found in plate cultures, although precipitation of such compounds is more likely to occur under these conditions (Lapeyrie et al., 1991; Sayer et al., 1997; Welch et al., 2002; Fomina et al., 2005). This supports the idea that the Burkholderia strains studied in our present work did not produce important amounts of OAs and OLs under conditions tested.

5. A final comment on in-batch experiments

Interpretation of kinetic information about Ca-phosphate solubilization in batch cultures must be taken cautiously. As it has been explained, batch solutions become saturated by reaction products, permitting the re-precipitation of intermediate phosphatic species that would not be stable otherwise. The implementation of any technological approach based on microbial phosphate solubilization should be taken by considering these facts in order to resolve possible interferences on process efficiency. Accordingly, we suggest the use of continuous culture setups when investigating microbial mineral-phosphate solubilization, because they permit medium renewal throughout the process.

6. Conclusions

Two species of genus Burkholderia isolated from a Brazilian high-P iron ore were selected for testing their ability to produce HAp dissolution. As reported by other authors, an oscillatory behavior of P concentration in solution was observed as HAp dissolution progressed and it was found to be related to the re-precipitation and subsequent re-dissolution of an intermediate Ca-phosphate phase, identified as brushite. Such a process was possible because of the accumulation and saturation of the culture medium with different chemical species coming from bacterial metabolism and the reacted mineral itself. This article supports evidences, which may help to clarify this commonly observed event that affects the interpretation of Ca-phosphate solubilization by microbes.

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