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Moroccan rock phosphate solubilization during a thermo-anaerobic grassland waste biodegradation process

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In order to investigate the presence of thermo-tolerant rock phosphate (RP) solubilizing anaerobic microbes during the fermentation process, we used grassland as sole organic substrate to evaluate the RP solubilization process under anaerobic thermophilic conditions. The result shows a significant decrease of pH from 6.5 to 4.8, and solubilizing from 7 to 15.8% of the phosphorus from the RP in the reactors after 90 days of incubation at 45°C. In these conditions, the organic acids produced were qualitatively and quantitatively identified as: acetic, butyric and propionic acids. This biological RP solubilization is due to the presence of a single thermo-tolerant bacterium isolated and identified as *Bacillus subtilis* from the anaerobic reactors. This *B. subtilis* strain was shown to be able to solubilize RP in liquid cultures containing insoluble RP as sole phosphate source. The mechanisms involved in these weathering processes confirmed the production of organic acids which were identified and quantified. This study is expected to lead to the development of novel, non-polluting farming practices by entering in the formulation of novel multi-functional biofertilizer by inoculating this thermo-tolerant phosphate-solubilizing bacterium into agricultural wastes as a practical and environmental strategy.

Key words: Grassland, phosphate, solubilization, *Bacillus subtilis*, thermo-anaerobic conditions.

INTRODUCTION

Phosphorus (P) is an essential element for life. After nitrogen, P is the second major element effecting plant growth and yield. P contributes to the biomass construction of micronutrients, the metabolic process of energy transfer, signal transduction, macromolecular biosynthesis, photosynthesis, respiration chain reactions and physiological chemical process for plant as well as for seed maturation (Shenoy and Kalagudi, 2005). Unfortunately, the concentration of P in soil solution is very low; varying from 0.001 mg/l in very poor soils to 1 mg/l in heavily fertilized soils (Antoun, 2012).

Therefore, phosphatic fertilizers, such as costly chemical fertilizers that contain large amounts of soluble P, have been applied to the agricultural fields to maximize

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production (Shenoy and Kalagudi, 2005). However, the soluble P in phosphatic fertilizers which is easily and rapidly precipitated to insoluble forms may become unavailable to the plant because of mineral phase reprecipitation (Wakelin et al., 2004). It is estimated that because of P-fixation in soil, plants will take up the year of application, only 10 to 15% of the soluble P added as fertilizers or manure (Brady and Weil, 2008). The unmanaged use of phosphatic fertilizers has increased agricultural costs and instigated a variety of environmental problems (Del Campillo et al., 1999). Therefore, the concept of adding phosphate-solubilizing microorganisms (PSMs) to rock phosphate (RP), a finite non-renewable resource and natural fertilizers, as providers of soluble P presents an economically and environmentally promising strategy (Antoun, 2012; Chang and Yang, 2009).

PSMs may play a major role in developing a sustainable use of P resources and in biogeochemical P cycling in natural and agricultural ecosystems. PSMs can transform the insoluble P to soluble forms by acidification, chelation, exchange reactions, and polymeric substances formation (Delvasto et al., 2006).

Therefore, the use of PSMs in agricultural practice would not only offset the high cost of manufacturing phosphatic fertilizers but would also enhance the solubilization of reprecipitated soil P for crop improvement (Shekhar et al., 2000). Many genera and species of bacteria have been described as PSMs (Hamdali et al., 2008; Yu et al., 2012).

However, all PSMs studied and applied to date have been mesophiles that could only be used under mesophilic and aerobic conditions. These types of microorganisms are not appropriate for the preparation of multifunctional biofertilizer at the high temperatures that occur for decomposing complex organic wastes (Yang, 2003).

Since cellulose is mostly present in plant cell walls, which are very difficult to degrade, only a small fraction of all microorganisms that are specialized for plant cell wall degradation can hydrolyze cellulose (Li et al., 2009), probably because it is present in recalcitrant cell walls (Wilson, 2011). To date, a few study described that anaerobic cellulolytic thermophillic bacteria have a very effective plant cell wall degradation system (Blumer-Schuette et al., 2010).

In the present study, we investigated the presence of thermo-tolerant RP-solubilizing bacteria, during the fermentation of grassland wastes as the sole organic substrate. The effects of these bacteria on RP solubilization through the anaerobic process have also been determinate. The solubilization mechanism and the identification of the selected bacteria were achieved.

MATERIALS AND METHODS

The rock phosphate sample

Rock phosphate originating from the Youssoufia phosphate mine (RP^Y, insoluble rock phosphate powder), was ground with pestle and mortar and passed through a 100 µm sieve. Its chemical characteristics were as follows: 31.4 g 100/g P_2O_5, 50.2 g 100/ g CaO and 2 g 100/ g total organic carbon (Khaddor et al., 1997).

Substrate preparation and fermentation experiments

The grassland experiment was conducted in a greenhouse at 24°C under artificial light for 16 h and at 17°C for 8 h in darkness. The experiment was started on 12 June 2010 on a poorly drained Stagno-Gleyic Luvisol (FAO classification) with rather wet and cool weather at the beginning of the investigation. After two weeks, the sward was maintained at a height of about 5 cm. The pasture was dominated by perennial ryegrass (*Lolium perenne* L) and common bent (*Agrostis capillaries* L). The C and N contents of the grassland topsoil (0-0.15 m) were 1.6 and 0.17%, respectively, with soluble organic carbon (extracted with 0.05 M K_2SO_4) of 87.5%. The grassland was oven dried at 70°C and used as organic substrate for the fermentation experiments.

Dried substrate (165 g) was placed in 5 L Erlenmeyer flasks containing 2 g of RP^Y as sole phosphate source and completed to 4 L with sterile distilled water. Cultures were grown in triplicate on a laboratory digester maintained at 45°C for 90 days in completely filled flasks with rubber stoppers and with shaking at 100 rpm in an incubation shaker to minimize aggregation of the bacteria. Anaerobic conditions were achieved through the consumption of residual oxygen. Similar experiments were carried out with no phosphate source incubated under the same conditions. Samples of 20 ml of each culture were collected periodically (every 10 days). After then, the pH value of the medium was determined with a pH meter equipped with a glass electrode. The culture supernatant obtained by centrifugation (10,000 rpm, 15 min) was passed through a 0.45 mm Millipore filter. The inorganic phosphate content of culture filtrate and organic acids analysis were determined by high performance ion chromatography, HPIC, Type DIONEX Dx-120 (AS11-HC column, injection rate: 2.3ml / min, detector: conductimetric cell).

Isolation of rock phosphate solubilizing bacteria under anaerobic process

At different state of treatment, 0.1 ml of the liquid suspension was sampled and plated in triplicate on the surface of nutrient agar (Difco, USA) to isolate the bacteria. The pH was adjusted to 7 and the medium was sterilized at 121°C for 20 min. After plating under the anaerobic conditions in the presence of Methylen blue as indicator, the agar plates were incubated for 3 days at 45°C.

Selection of phosphate solubilizing bacteria was carried out by plating the nutrient agar isolate on the solid NBRIP medium (Nautiyal, 1999) containing (g/l) Glucose : 10; $MgCl_2.6H_2O$: 5 ; $MgSO_4.7H_2O$: 0.25 ; KCl : 0.2 ; $(NH_4)2SO_4$: 0.1; agar : 15; pH : 7.45; and RP^Y : 0.5 as sole phosphate source. After plating, the agar plates were incubated for three days at 45°C in anaerobic atmosphere. Spores of the phosphate solubilizing bacteria (PSB) isolate and able to show the most active growth on NBRIP were stored in 20% (w/v) sterile glycerol at -20°C.

Estimation of the ability of the selected PSB to release soluble phosphate from RP^Y

Three culture replicates were inoculated with 10^6 spores/ml of the PSB isolate and grown for nine days at 45°C on a rotary shaker (180 g/min) in 250 ml Erlenmeyer flasks containing 50 ml of liquid NBRIP medium with 0.5 g/l RP^Y and maintained under anaerobic conditions. Cultures were centrifuged at 10,000 *g* for 10 min and the pH of the supernatant was measured every day. The superna-

tant was analyzed for P_2O_5 content by the HPIC method as described above. Similar measures were carried out in non-inoculated flasks incubated under the same conditions.

Taxonomic study of the selected strain

Morphological and physiological characterization of selected isolate

The isolate was examined for several phenotypic characteristics. Unless otherwise stated, the tests were carried out in tryptic soy agar (TSA) medium at 32 and 45°C incubation temperature. Flagella were stained using the method of Rhodes. Spores were stained according to the Schaeffer-Fulton method with five days culture on TSA medium in aerobic and anaerobic conditions. Catalase and oxidase production, aerobic nitrate and nitrite reduction and acid-production profiles from carbohydrates were obtained with an API 50CH system (bioMérieux) after growth in 50 CHB/E medium, as described by Logan and Berkeley (1984). Antimicrobial susceptibility was tested in TSA medium according to the method of Bauer et al. (1966).

Amplification and sequencing of the 16S rDNA of the selected strain

The strain isolated in this work was grown on Luria-Bertani medium as standard cultures of *Bacillus sp.* The isolate was incubated in completely filled flasks with rubber stoppers and with shaking at 100 rpm. Inoculation was performed aerobically with an aerobically grown overnight culture with an optical density at 578 nm of 0.3. Anaerobic conditions were achieved after a short time through the consumption of residual oxygen by the inoculated bacteria. The cells for preparations of DNA were harvested after 3 h in the midst of the exponential growth phase.

The 16S rRNA gene was amplified by PCR using standard protocols (Saiki et al., 1988) and the forward primers 16F27 (5'-AGAGTTTGATCATGGCTCAG-3') and the reverse primer 16R1488 (5'-CGGTTACCTTGTTAGGACTTCACC-3') (both from Pharmacia). The PCR products were purified using the Microcon Qiaquick spin-gel extraction kit (Qiagen). Direct sequence determinations of PCR-amplified DNAs were made with an ABI PRISM dye-terminator, cycle sequencing ready-reaction kit (Perkin-Elmer) and an ABI PRISM 377 sequencer (Perkin-Elmer) according to the manufacturer's instructions. The sequence obtained was compared to reference 16S rRNA gene sequences available in the GenBank and EMBL databases obtained from the National Centre of Biotechnology Information database using the BLAST search. Phylogenetic analyses were made using MEGA version 2.1 (Kumar et al., 2004) after multiple alignment of the data by CLUSTAL V (Thompson et al., 1997). Distances and clustering were determined using the neighbour-joining and maximum-parsimony algorithms. The stability of the clusters was ascertained by performing a bootstrap analysis (1000 replications).

Statistical analysis

All experiments were carried out in triplicate or more. All data are reported as means ± SD (standard deviation). The Independent-Samples t-test was used to compare means and the variance homogeneity determination (ANOVA) was conducted with the General Linear Model using type II sum of squares and Tukey's Honestly Significant Difference (P = 0.05) using statistical analysis system software (SAS Institute, 2002).

RESULTS AND DISCUSSION

Isolation of bacteria able to use RP as sole phosphate source

After plating and incubation period of liquid samples at the final phase of the fermentation experiments, only one bacterium isolate could grow when plated on the solid nutrient medium. This thermo-tolerant anaerobic isolate could use RP when plated on the solid NBRIP medium containing RP^Y as unique phosphate source. This is unexpectedly low since the cultures were maintained during the fermentation anaerobic process and most of the phosphate in this biotope is in an insoluble form. Up to date, only a few works reported that some thermo-tolerant phosphate solubilizing microbes were isolated aerobically from compost plants and biofertilizers (Chang and Yang, 2009; Xiao et al., 2008).

Abilities of the selected isolate to release soluble phosphate from RP

Fermentation experiments

The soluble phosphorus content increased significantly between 0 and 10 days from 19.6 ± 0.1 to 36.7 ± 0.2 mg/l, then decreased slightly at 20 days and re-increased after, up to 43.3 ± 0.1 mg/l at 90 days (Figure 1A). The microbial activities were amplified more vigorously at early fermentation phase, resulting in the consumption of soluble phosphorus for microbial growth. These results indicate that thermo-tolerant PSMs can increase the soluble phosphorus content and contribute to the solubilization of RP during the fermentation process. The RP solubilization rate ranged from 7 to 15.8% and increased during the thermophilic anaerobic fermentation experiments (Figure 1A). It was reported that *Bacillus smithii* F18 presented the highest soluble phosphorus percentage (5.3 ± 0.6%) of the total phosphorus after 56 days of composting. In comparison, under aerobic conditions, the control (non-inoculated compost) had the lowest percentage of 2.9 ± 0.2% (Chang and Yang, 2009). Similarly, a moderately thermophilic bacterium *Acidithiobacillus caldus* achieved a phosphorus solubilizing rate of 27.6% in shake flasks containing elemental sulfur (S0) as an energy substrate and only 2.19% for the same system without the additional S0 (Xiao et al., 2011).

Growth in flasks in NBRIP liquid medium

The amount of P solubilized increased with time, and at the end of the incubation period (9 day), the values obtained were significantly different from those of the control (1.6 mg/l of soluble P), irrespective of P source

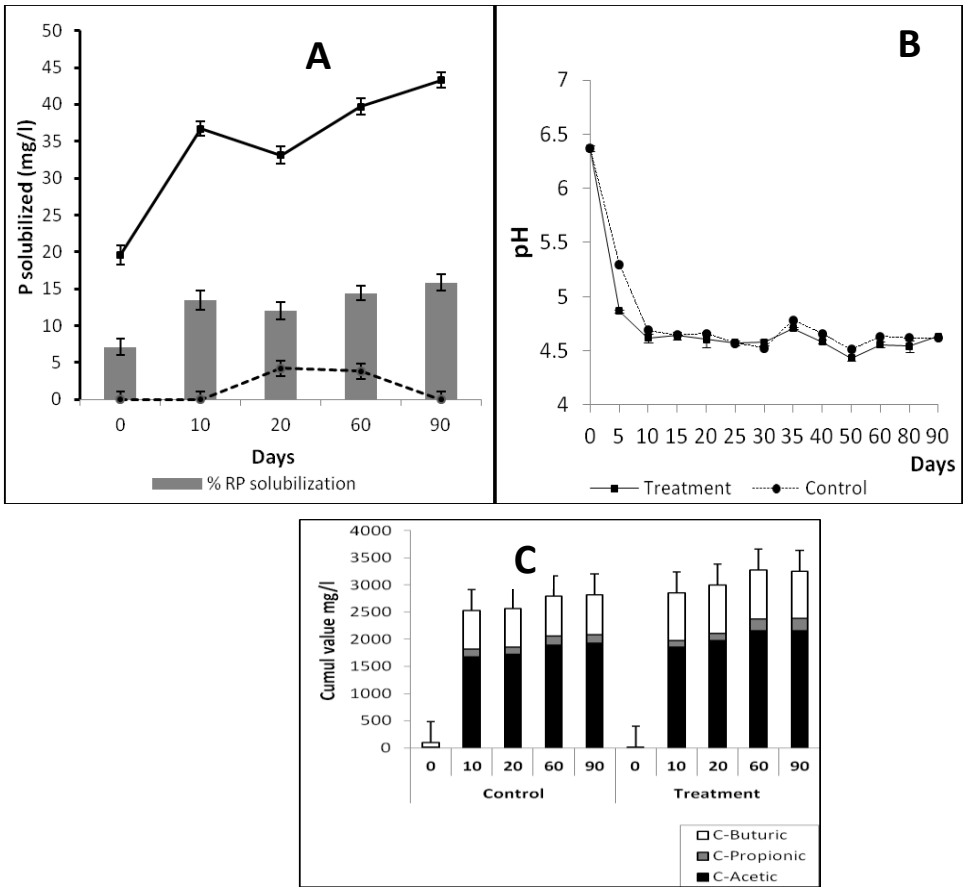

Figure 1. Fermentation experiments in bioreactor with Grassland containing 0.5 g/l RP^Y (Treatment) or without RP^Y (Control) incubated for 90 days under the same conditions. **(A)** Concentration of soluble phosphate released from rock phosphate in the supernatant of cultures. **(B)** Evolution of the pH in the supernatant of cultures. **(C)** Concentration of organic acids produced in the supernatant of cultures. Data points are means and vertical bars are standard deviations (n=3).

(Figure 2A). Phosphate release ranged from 2 to 13 mg/l in the growth medium NBRIP. This showed that the selected anaerobic thermo-tolerant isolate had effectively converted the inorganic insoluble RP into a soluble form. It was indicated that the application of the moderate thermophile of *A. caldus* was an effective method to solubilize phosphorus from RP than that obtained with the mesophile, *Acidithiobacillus thiooxidans* (Xiao et al., 2011). Chang and Yang (2009) reported that an aerobic thermo-tolerant *Bacillus smithii* F18 had the highest RP solubilizing activities with 544.2 ± 30.2 µg/ml of soluble phosphate in Pikovskaya's broth at 50°C.

RP solubilization mechanism by the selected isolate

Fermentation experiments

On Petri dishes, the isolates were surrounded by a clear halo, characterizing microorganisms producing organic acids on the NBRIP media (Nautiyal, 1999). Noticeable acidification of the growth medium was observed during the anaerobic digestion of grassland (Figure 1B) suggesting that the process of RP solubilization involved the excretion of organic acids.

Organic acid production is the main mechanism by which PSMs mobilizes P from sparingly soluble phosphates (Khan et al., 2007). Solubilization of phosphates may result from the drop in pH or from cations chelation by organic acids (Antoun, 2012). In these experiments, pH decreased significantly from 6.5 to 4.8 and remains substantially unchanged until the end of the treatment (90 days) (Figure 1B). Thus, cellulose is a polymer of glucose and it is readily hydrolyzed during acidogenesis (Huang et al., 1986). The pH value of 4.8 indicates microbiological activity and the presence of thermo-cellulolitic acidophilic bacteria. Xiao et al. (2011) showed that the phosphate solubilizing ability of *A. caldus* was the most effective when carried out under pH 2.5 at 45°C. A few studies have reported the presence of cellulolytic bacteria

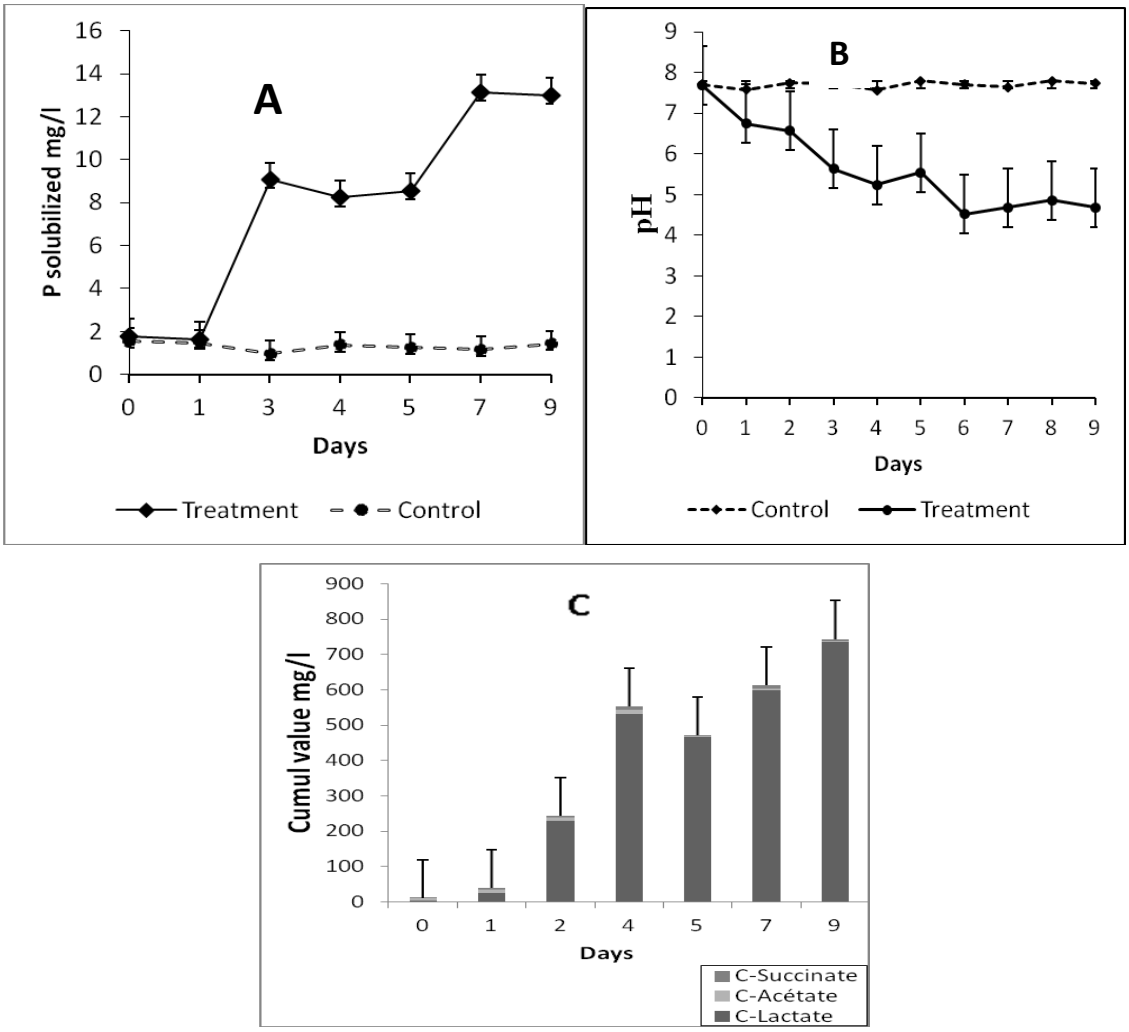

Figure 2. Culture of *Bacillus subtilis* in flasks in NBRIP liquid medium containing 0.5 g/l RP^Y (Treatment) or without RP^Y (Control) incubated for 9 days under the same conditions. **(A)** Concentration of soluble phosphate released from rock phosphate in the supernatant of cultures. **(B)** Evolution of the pH of the culture supernatant. **(C)** Concentration of organic acids produced in the supernatant of cultures. Error bars represent standard deviations of the mean values of the results of three independent culture replicates.

able to grow on native cellulose as only carbon source (Schwarz, 2001). In addition, cellulolysis was considered more effective and faster with thermophilic than mesophilic bacteria (Leschine and Canale-Parola, 1983). It was reported that *Acidothermus celluloticus* was a rare case of cellulolytic species; acidophilic and thermophilic able to grow at optimal pH of 5 and which could grow at pH 3 on cellulose substrate in the medium (Bergquist et al., 1999). In this study, the following acids (acetic, butyric, propionic acids) were found in the culture filtrates during the anaerobic fermentation process, using HPIC chromatography (Figure 1C). These identified organic acid presented after 60 days of anaerobic fermentation the concentration of 3300, 2400 and 2200 mg/l of butyric, propionic and acetic acid, respectively (Figure 1C). It was reported that the acidophilic bacteria can acidify the medium at pH values from 3 to 4 and can transforms sugars into lactic, acetic, propionic, byturic acids, alcohol and CO_2 (Kawagoshi et al., 2005). High concentrations of organic acids and therefore relatively low pH are usually encountered in anaerobic ecosystem environments (Goodwin and Zeikus, 1987).

Growth in flasks in NBRIP liquid medium

Compared with the solubilizing system for RP without bacterium, the pH of the culture with the bacterium was obviously lower during the solubilizing process in the NBRIP liquid medium (Figure 2B). There was a gradual decrease of pH from 7.7 up to 4.8 at day 9 as a result of the consumption of acid by the proton attack on RP. This

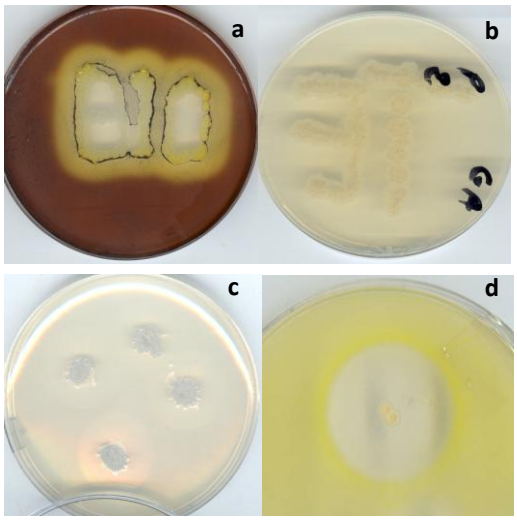

Figure 3. (a) Starch degradation showed by iodine -test of the selected isolate. **(b)** Cream pigmented colonies. **(c)** RP solubilization by the selected isolate on NBRIP medium by acid production mechanism. **(d)** Antibacterial activity against *Micrococcus luteus* ATCC 381.

result confirms the ability of the selected isolate to solubilize RP by producing organic acids. Research of organic acids in the liquid NBRIP culture revealed the presence of acetic, lactic and succinic acids at different concentration (Figure 2C). The presence of formic, propionic, butyric, pyruvic, glutaric, malic, tartric and oxalic acids was signaled at only trace concentration (< 2 mg/l). Lactic acid is the most produced from 2 mg/l to more than 700 mg/l after 9 days of incubation. It was reported that a strong correlation between pH and soluble P concentration, as well as total organic acid production and the P solubilized was observed with the PSB strain under aerobic conditions (Yu et al., 2012). Similarly, it was indicated that a thermophilic aerobic bacterium of *A. caldus* have the best ability to produce organic acids than the mesophilic bacteria (Xiao et al., 2011).

Taxonomic characterization of the selected isolate

Identification of isolated *Bacillus* may follow one of several methods of classification. In this study, the selected strain was tested for taxonomical identification using morphological, cultural, physiological and biochemical criteria as well as other features (Claus and Berkeley, 1986). Cells are rods Gram-positive, cream pigmented, anaero-aerobic, occurring singly or in pairs and occasionally in short chains or filaments. They are motile by peritrichous flagella. In aerobic condition at 30°C, the endospores are mainly ellipsoidal and lie in subterminal positions. Catalase and oxydase are positives. However, when grown on in anaerobic condition at 45°C, the endospores are absents. This result is consistent

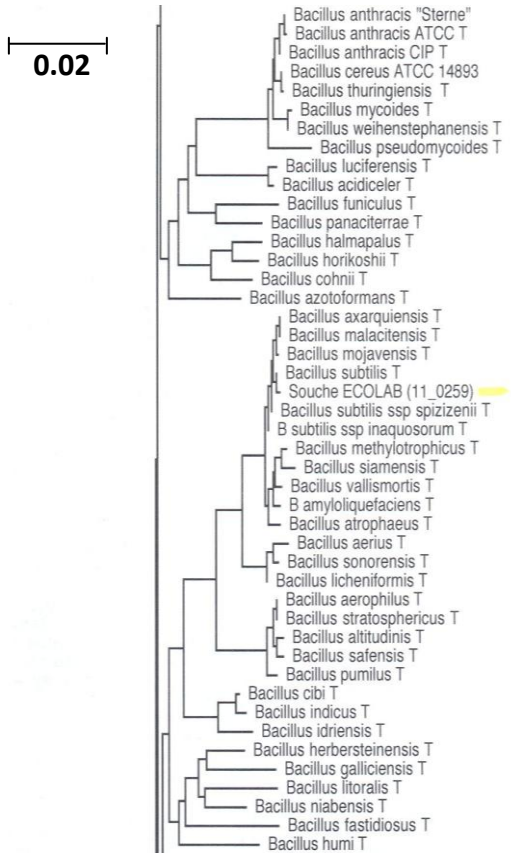

Figure 4. Neighbour-joining tree based on nearly complete 16S rRNA gene sequences of the selected isolate. The significance of each branch is indicated by a bootstrap value calculated for 1000 subsets. Bar, 1 nt substitutions per 100 nt.

with those of Espinosa et al. (2001).

The bacterium grows within a temperature range of 20 to 50°C and pH values comprise between 4.5 and 10. It is halo tolerant, being able to growth in salt concentrations from 0 to 7.5% w/v. The selected strain showed anti-fungal and antibacterial activities. It reduces nitrate aerobic and anaerobically. Starch and cellulose are used. Citrate is used as sole carbon and energy source. Several phenotypic features are shown in Figure 3. The selected isolate was colistin sensible and predicted to belong to the genus *Bacillus*.

The sequencing of the 16S RNA of this strain confirmed this classification. Indeed comparisons of the 16S rRNA gene sequences of the selected strain and those available in the GenBank database indicated that it is phylogenetically closely related to, respectively, *B. subtilis* (98% sequence homology), *Bacillus mojavensis* (97% sequence homology), *Bacillus malacitensis* (97% sequence homology) and *Bacillus axarquiensis* (96% sequence homology) (Figure 4). The phylogenetic tree, constructed using the neighbour-joining method is depicted in Figure 4.

Conclusion

Rock phosphate bio dissolution is due to the presence of a single thermo-tolerant strain of bacteria (*B. subtilis)* isolated by the NBRIP test from the reactors. Tests confirm that *B. subtilis* strain is able to grow anaerobically and can hydrolyse the cellulose substrate. To our knowledge, this study is the first report showing the ability of bacteria to solubilize RP and to degrade cellulosic substrates under thermophilic and anaerobic conditions. The mechanism involved in the RP solubilization is a consequence of proton release from organic acids in the aqueous phase of the fermentation digester.

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