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Assessing hydroxyapatite biosolubilization by bacterial strains isolated from EL Halassa Khouribga P deposit.

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Abstract

Natural phosphate represents an important reserve of phosphorus (P_i), essential element for plant nutrition and development. However, direct application of phosphate fertilizers has been proved ineffective for the phosphorus deficit soils remediation due to its low solubility. The aim of this study is to assess the ability of several bacteria species isolated from El Halassa, Khouribga deposit to solubilize inorganic phosphate. This ability has been tested and followed on NBRIP agar and broth medium with hydroxyapatite $Ca_5(PO_4)_3(OH)$ as sole phosphorus source. The results showed that *Pseudomonas spp*., *P. cepacia* and *E. sakazakii* presented the highest P solubilization efficiency (PSE) on NBRIP agar, with values of 79%, 77% and 60% respectively. Meanwhile, *E. sakazakii*, *P. pseudomallei* and *P. cepacia* showed the highest ability to release the soluble phosphate with concentrations of 1728 ± 0.5, 1432.2 ± 1 and 1272.1 ± 0.5 mg P_i L^{-1} respectively in the presence of $Ca_5(PO_4)_3(OH)$ as sole phosphorus source. Monitoring of the orthophosphates release in the medium for each strain, revealed that orthophosphates amounts progressively increase during first days of incubation and at the same time as the pH decreases. This can be explained by the secretion of organic acids and H^+ protons by these microorganisms.

Keywords: *P biosolubilization; NBRIP; hydroxyapatite; Khouribga mine; Enterobacteriaceae*

1. Introduction

Phosphorus (P) is the second major macronutrient required for plant growth and development [1]. Its major role is the storage of energy and its release during cellular metabolism. Although the sol generally contains about 0.04 to 0.12% of phosphate in organic and inorganic forms respectively. However, large quantities are present in insoluble metal complexes (with iron, aluminum, silicon...) in acidic soils [2] or calcium carbonate in alkaline soils [3]. Only 0.1% of phosphorus (P) exists in a soluble form available for plant uptake and nutrition [4]. This P deficiency is one of the most important factors limiting agricultural production in many countries around the world, particularly in Mediterranean area where other abiotic stresses such as drought and salinity are strongly dominant [5-7]. Therefore, the application of large quantities of chemical fertilizers with soluble forms of P is necessary to achieve maximum plant productivity in these area [3]. A large portion of this soluble inorganic phosphate applied to soils as chemical fertilizers quickly transforms into immobilized forms and becomes unavailable to the plants. This P immobilization is due either to its adsorption by calcium ions present on the exchange complex or to its precipitation by the free iron or aluminum ions which are in large quantities in the soils [8]. Besides, traditional methods of phosphate fertilizer production are based on many chemical compounds. It takes place by the treatment of insoluble mineral phosphates, which require intensive treatment with sulfuric acid at high temperature. This process consumes intensive energy and has become ecologically undesirable and economically expensive [9]. Insoluble inorganic phosphates, including phosphate rock, can be transformed into available soluble forms near plant roots by the action of certain soil microorganisms called mineral phosphate soluble microorganisms (MPS), which in turn metabolize organic compounds, mainly sugars and organic acids for their growth [2, 5, 10]. These microorganisms belong also to the Plant Growth Promoting Rhizobacteria (PGPR) due to its action in plant growth stimulation. Several researchers have shown that several microorganisms are able to dissolve phosphorus in the soil and thus improve the plants growth and productivity [11-13]. These microorganisms belong to two groups (i) those which live in the free state not far from the roots and often even on the root without being attached to them and (ii) those who live in symbioses with many plant species [14]. Symbiotic microorganisms have been widely studied and used to increase the production of several crops [15]. In mining environments, microbial population varies from that of the normal soil or aquatic environment due to minerals predominance. There is very little room for water and life by subsurface volume. Some microorganisms are found in pores, fractures and fluid inclusions. Soil desiccation, even if it causes many microorganisms death, but never results in complete soil sterilization [16]. Some strains of Rhizobia, *Pseudomonas* and *Bacillus* species have the ability to solubilize the inorganic phosphate to forms that can be used by plants for their growth and development [17]. Domey and Lippman, [18] reported that by the inoculation with bacteria solubilizing phosphorus, wheat plants increased their shoot biomass by 8% and phosphorus uptake by 17 to 57%. Chabot et al., [19] reported that field inoculation with *Enterobacter* and *Pseudomonas* isolates, obtained, after 60 days of growth, significantly increases (7-9%) corn plants elongation. However, only one *Enterobacter* isolate produced an increase in foliar fresh matter by 23% of maize after 108 days of growth. These results have been confirmed by Wang et al., [20] who have also shown that dry biomass and nutrient uptake become more important in sandy soils. In addition, inoculation of wheat with different combinations of bacteria in the presence of natural phosphates stimulated nitrogenase activity and promoted the N_2-fixation and the solubilization of natural phosphates [21, 22]. However, the selection of high solubilizing bacteria from mining environment is little studied and several results varies from solubilizing media to another. The aim of this work was to determine the capacity of certain strains isolated from the phosphate mine to solubilize hydroxyapatite in NBRIP medium.

2. Materiel and methods

2.1. Bacterial isolation and identification

Bacterial colonies were isolated from the mine soil after series of dilution using distillated water, the suspensions were used to inoculated YSA media and a selection based on the morphological and biochemical properties of the obtained colonies has been adopted [23]. Bacterial isolates were then individually purified, transferred to nutrient agar and stored on broth at -22°C with 15% glycerol until use. Twelve bacterial strains isolated and identified from the EL HALASSA phosphate deposit (32°40'60N, 6°49'60W) were used in this study (Figure 1). *Serratia marcescens*, *Pseudomonas cepacia*, *Aeromonas hydrophila*, *Acinetobacter* sp, *Enterobacter sakazakii*, *Leclercia adecarboxylata*, *Bacillus cereus*, *Pseudomonas pseudomallei*, *Bordetella* sp., *Rahnella aquatilis*, *Pseudomonas spp.* and *Citrobacter freundii*.

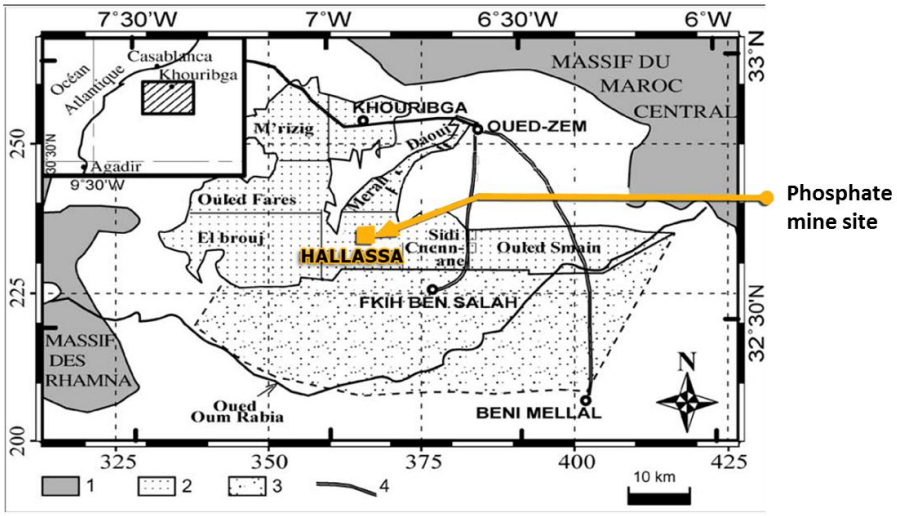


Figure 1. Geographical location of sampling points [23].

2.2. Solubilization test on NBRIP agar

The capacity of these microorganisms to dissolve natural phosphates is thus studied on an agar medium of the National Botanical Research Institute's phosphate (NBRIP) with 10 g Glucose, 0.5 g $MgCl_2$, 0.25 g $MgSO_4$, 0.2 g KCl, 15 g $(NH)_2$ SO_4 per L in which the phosphate in the form of hydroxyapatite ($Ca_5(PO_4)_3OH$) was added separately at a rate of 5 g L^{-1} [24]. The prepared media were sterilized in an autoclave at 121°C. for 30 minutes and then cast in petri dishes. the media were after that separately inoculated by the studied strains and after 10 days of incubation at 30°C. Based on the clearing halo zones diameter, P solubilization index (PSI) and P solubilization efficiency (PSE) were calculated for each bacterial isolate according to Premono et al. [25]:

$$PSI = \frac{Colony\ diameter + Halozone\ diameter}{Colony\ diameter}$$

$$PSE\ (\%) = \frac{Solubilization\ diameter}{Growth\ diameter} \times 100$$

2.3. Solubilization test on NBRIP broth.

A preculture of each of the ten bacterial isolates was prepared beforehand to inoculate Erlenmeyers of 250 mL each containing 100 mL of NBRIP broth medium with inorganic phosphate $Ca_5(PO_4)_3OH$ at the rate of 5 g L^{-1} as sole source of phosphorus. One milliliter of suspension preculture was added to each of the ten Erlenmeyers containing the NBRIP broth medium. The autoclaved and non-inoculated media were used as controls. The inoculated media and the

controls were incubated under continuous stirring at 37°C on a shaker incubator. 2 mL sample was daily taken under aseptic conditions from the incubated Erlenmeyer. The samples were then centrifuged at 2500 × g for 15 min and 250 µL of the supernatants was used for the soluble phosphorus analysis in the orthophosphates form. The pH of the medium was also determined using a glass pH meter. The quantity of the released orthophosphate was determined with the acidic molybdate tartrate method as described and validated by AFNOR [26]. This colorimetric technique consists in the formation of a complex (orthophosphates-molybdate) in the presence of a solution of double antimony and potassium tartrate. This complex is then reduced by ascorbic acid and gives a blue color. The intensity of the staining is proportional to the orthophosphates concentration in the medium. The optical density of the samples was measured using a spectrophotometer at 700 nm. Bacterial growth was determined using the cascade dilutions of 10^{-1} from each Erlenmeyer contained the suspension of each studied bacteria. 0.1 mL aliquots of the dilutions were plated on a solid nutrient medium. Seeded boxes were incubated at 37°C for 48 h. The developed colonies were then counted using a colony counter and the number of colony forming units (CFU) was calculated according to the formula:

$$CFU\,mL^{-1} = (Number\ of\ colonies) \times (Dilution\ factor)$$

2.4. Statistical analyses

Statistical analyses were performed with ANOVA one way using SPSS v21. Means were calculated and presented as mean ± standard errors for all of the studied parameters.

3. Results

3.1. Measurement of the solubilization of inorganic phosphate on NBRIP agar.

According to the results presented in table 1, the solubilizing power may be influenced by the type of the tested microbial strains. *Pseudomonas spp., P. cepacia and E. sakazakii* presented the highest solubilization index (PSI) of 1.79, 1.76 and 1.6 respectively in the presence of hydroxyapatite as the sole source of phosphorus in comparison to the other bacterial strains.

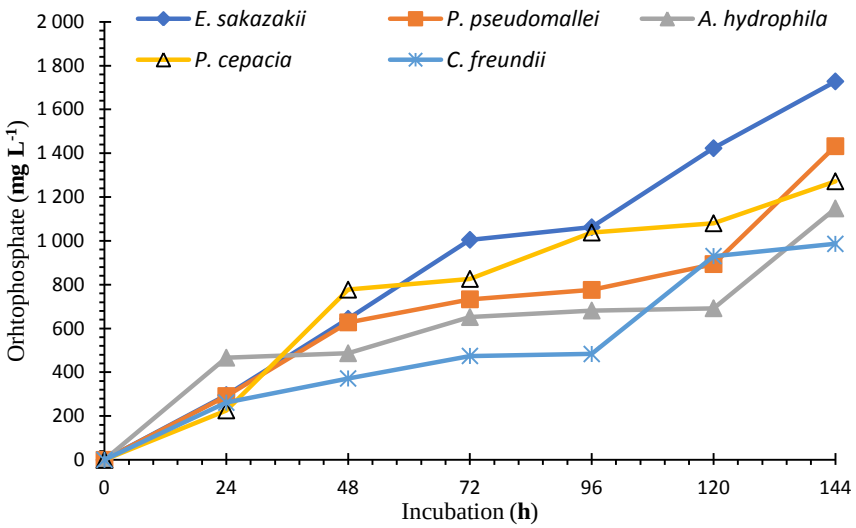
Table 1. Hydroxyapatite solubilization by the studied strains on NBRIP agar.

Species	Halozone diameter (mm)	Colony Diameter (mm)	PSI	PSE
S. marcescens	**23**	4	1.17	17.4
Bordetella sp.	**23**	10	1.43	43.4
P. pseudomallei	13	4	1.30	30.7
B. cereus	No halo	No halo	-	-
L. adecarboxylata	18	8	1.44	44.4
E. sakazakii	**20**	12	**1.6**	**60**
Acinetobacter sp.	No halo	No halo	-	-
A. hydrophila	No halo	No halo	-	-
P .cepacia	**26**	**20**	**1.76**	**76.9**
R. aquatilis	No halo	No halo	-	-
C .freundii	No-halo	No halo	-	-
Pseudomonas spp.	19	15	**1.79**	**78.9**

These strains presented also the highest solubilization efficiency (PSE) values of 78.9%, 76.9% and 60% respectively under these conditions. *S. marcescens* strain presented the lowest PSI and PSE of 1.17 and 17.4% respectively compared to the other strains. Besides, this strain next to *Bordetella* sp., *P. cepacia* and *E. sakazakii* presented the highest halozone diameters of 23, 23, 26 and 20 mm respectively under these conditions. Meanwhile, *B. cereus*, *Acinetobacter sp*., *A. hydrophila*, *R. aquatilis* and *C. freundii* did not present any halozone of solubilization around their colonies in the presence of hydroxyapatite. P. cepacia presented the highest colony diameter of 20 mm, while *S. marcescens* and *P. pseudomallei* presented the lowest value of 4 mm in the medium.

3.2. Solubilization of inorganic phosphate in liquid medium

The result illustrated in figure 2 showed that orthophosphates amounts progressively increase during the first incubation days at the same time as the pH of the media decreases. There were significant (p <0.001, Table 2, 3) variations between the studied strains in these conditions. Indeed, *P. cepacia*, *P. pseudomallei* and *E. sakazakii* released the significantly (p <0.05) highest soluble P concentrations of 777.1 ± 0.8, 627.7 ± 0.3 and 0.644 ± 0.7 mg P_i L^{-1} after 48 h of incubation respectively. Meanwhile, *Acinetobacter sp.* and *S. marcescens* presented the lowest P release of 402.4 ± 0.5 and 392.1 ± 1.2 mg P_i L^{-1} respectively under these conditions. At the end of incubation (144h), *E. sakazakii* and *P. pseudomallei* released the highest P values of 1728 ± 0.5 and 1432.2 ± 1 mg P_i L^{-1} in the medium respectively, while *Bordetella sp*. presented the lowest value of 607.4 ± 1.1 mg P_i L^{-1} form the hydroxyapatite as the sole P source. The results showed that the pH of the NBRIP broth media decreased during the incubation for all of the studied strains (Figure 2). It is noted that the pH of the medium has decreased to 5.09 which is the lowest observed pH value in this test. *C. freundii*, *E. sakazakii* and *P. cepacia* presented the lowest pH values of 5.09, 5.23 and 5.25 respectively in the presents of hydroxyapatite as sole source of P. Meanwhile, *S. marcescens* presented the highest pH value of 5.44 under these conditions



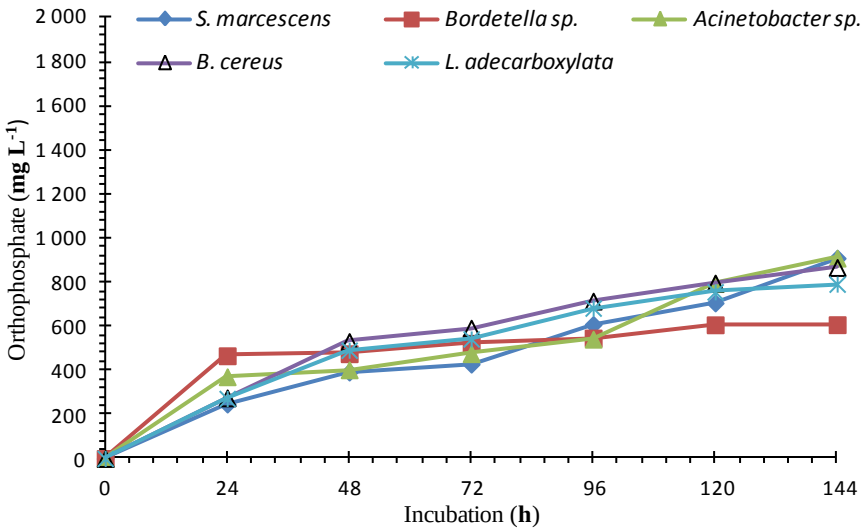


Figure 2. Solubilization of hydroxyapatite by the studied bacterial strains isolated from the mining soil.

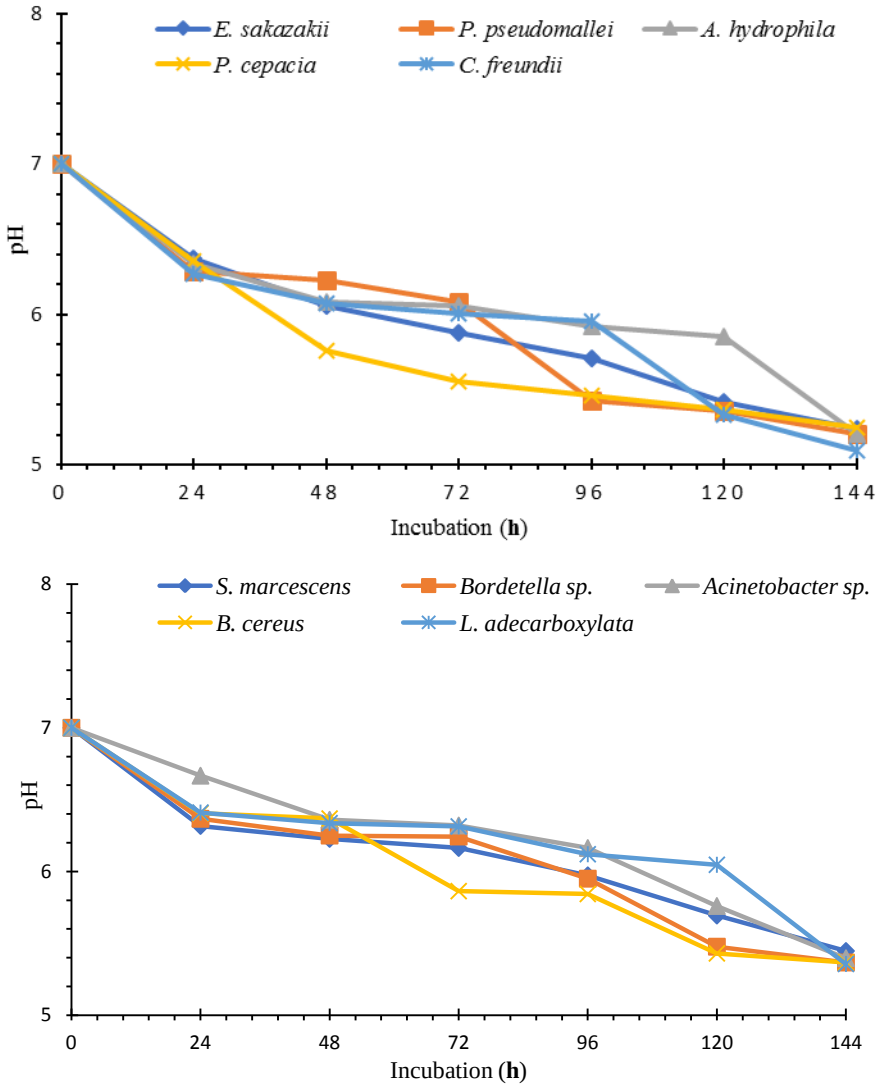


Figure 2. kinetics of the pH of the media of the solubilization by the bacterial strains isolated from the mining soils.

3.3. Bacterial growth

The result showed a strong correlation ($p < 0.01$, $r^2 = 0.145$, Table 3) between the UFC and P release in the media for all of the studied strains with significant variation in their behavior (Figure 3). However negative correlation ($p < 0.001$, $r^2 = 0.209$, Table 3) has been detected between the UFC, P release and pH of the media for all of the studied bacterial strains (Figure 4). *E. sakazakii*, B. cereus and S. marcescens presented the highest CFU values of 735, 492 and 460 $\times 10^3$ UFC mL^{-1} respectively, while Bordetella sp. presented the lowest value of 784 $\times 10^3$ UFC mL^{-1} in the presence of hydroxyapatite as sole P source.

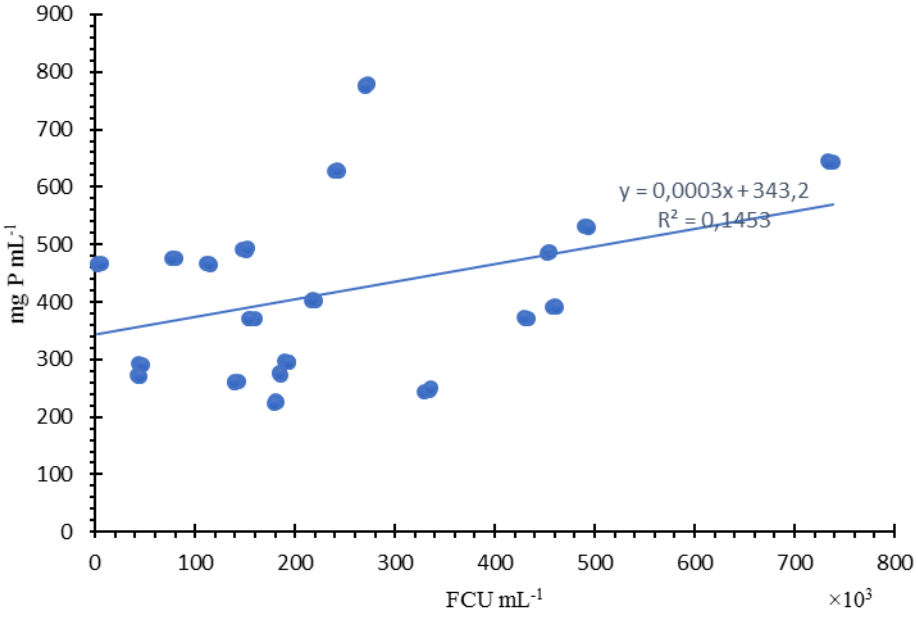


Figure 3. Interaction between bacterial growth (FCU) and P release for the studied strains

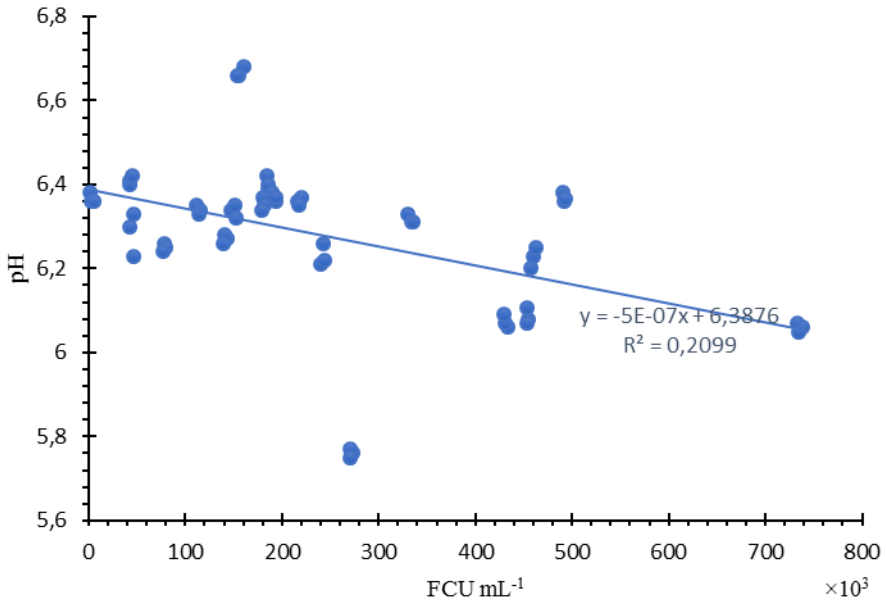


Figure 4. Interaction between bacterial growth (FCU) and pH of the medium for the studied strains.

4. Discussion

In this study ten bacterial strains were isolated form the Khouribga mine and tested for their ability to solubilize inorganic phosphate in the form of hydroxyapatite. the results showed that *S. marcescens* and *P. pseudomallei* presented the highest P solubilizing capacity in NBRIP agar after 10 days of incubation in comparison with the other studied strains. Fernandez et al., [13] have shown that biosolubilization activity is presented as a halo or clear or yellow zone on the plaque, which is used to evaluate this activity of bacterial strains. This is in agreement with several previous studies. Kadmiri et al. [23] and Widawati et al. [27] have isolated *Bacillus* sp., *Enterobacter alvei*, *E. agglomerans*, and *Pseudomonas* sp. and demonstrated their ability to solubilize inorganic forms of P. Rudresh et al., [28] reported that the main phosphate solubilizing bacterial genera is *Pseudomonas*, A strain of *Enterobacter agglomerans* showed significant capacities for the hydroxyapatite solubilization as well as organic phosphorus hydrolysis [29]. In spite of the good obtained results by this method, it has been demonstrated that several microorganisms' incapable of producing clear zones around their colonies can solubilize inorganic phosphates in liquid medium [24, 30]. Consequently, a quantification of the phosphorus released by these strains in liquid medium was then carried out. In this study, the two strains belonging to the *Pseudomonas* genus (*P. pseudomallei* and *P. cepacia*) significantly solubilized the hydroxyapatite in NBRIP agar with solubilizing indexes of 3.25 and 1.30 respectively in comparison with the control. In the broth media, these two strains released 291 ± 0.5 and 225.8 ± 0.8 mg of P_i mL^{-1} respectively. Indeed, they were very efficient at the end of incubation with values of 1432.3 ± 1.08 mg P_i L^{-1} and 1272 ± 1.15 mg P_i L^{-1} respectively. However, *E. sakazakii* presented the highest released P value of 1728 ± 0.5 mg P_i L^{-1} in NBRIP broth after 144 h of incubation. These results are similar to those reported by Kim et al., 1997, who demonstrated that some species of the genus *Pseudomonas* gave 230 mg L^{-1} in the first days of incubation solubilization from hydroxyapatite. In addition, the majority of the studied species were able to release large P_i quantities after 48h of incubation, while *Acinetobacter* sp. and *S. marcescens* did not release significant quantities of orthophosphate during this period of incubation. According to the results of this study, *Acinetobacter* sp. has not shown remarkable growth in the presence of hydroxyapatite, this may be explained by their poor adaptation to the used media. For all of the studied strains, a relatively high incubation time was required to reach the maximum concentrations of soluble phosphorus in the media. Thus after 4 to 6 days of incubation it was observed that *Acinetobacter* sp. released from 541.5 to 909.6 mg P_i L^{-1}. According to our knowledge, some strains such as *L. adecarboxylata* are mentioned for the first time in a report as inorganic phosphate solubilizing bacteria. This strain presented in NBRIP agar an important solubilization halo of 1.8 cm. Nevertheless, this strain showed weak capacities of solubilization of hydroxyapatite in liquid NBRIP broth in comparison with the other strains. Moreover, this species expresses great degradation capacities of polycyclic aromatic hydrocarbons that can be used in the remediation of contaminated sites by this type of pollution [31]. Consequently, this bacterial strain requires further studies in terms of solubilizing different type of complexed forms in different types of media. Moreover, the bacterial population which showed phosphorus solubilization activities was close to 16.9% relative to the total bacterial population isolated from the phosphate deposit. This proportion is lower than those reported by Farhat et al. [12] and Hamdali et al. [32] which are 18.6% and 18.3% respectively. This can be explained by the original biotope of these isolates and by the mechanisms involved in the biosolubilisation process. In this study, the results showed that the maximum concentration of released soluble P from the hydroxyapatite was 1728 mg P_i L^{-1} by *E. sakazakii* after 144 h of incubation, while the minimum pH decrease was 5.09 by *C. freundii* at the end of the incubation time. Strong negative correlations have been noted for all of the studied species between bacterial growth, orthophosphates release and the medium acidity. This is in accordance with the results reported for *S. marcescens* by Farhat et al. [12]. Indeed, the

authors demonstrated that for this species, the decrease of the pH is due to the release of organic acids by the bacteria in the medium. In addition, inorganic phosphate solubilization in some cases is attributed to the production and distribution of acids [33, 34]. These are carboxylic acids synthesized and released by microorganisms in order to solubilize complexed P forms. This is also responsible for the decrease of the pH [11, 35]. A very weak correlation has been found between P solubilization in broth media and the PSI and PSE calculated from the halozone diameters in NBRIP agar. Similar results have been reported by Alam et al. [36] and Manzoor et al. [37]. This could be explained by the fact that many microorganisms' incapable of producing halozone in the agar medium can mobilize soluble Pi in the broth. Indeed, in this study, *B. cereus*, *Acinetobacter* sp., *A. hydrophila* and *C. freundii* did not produce halozones around their colonies and yet were able to release relatively important quantities of P_i in the broth medium. In fact, it has been reported that the liquid media methods are more sensitive for detecting high potential P solubilizing bacteria [24, 38]. In summary, our results suggested that the application of high phosphate solubilizing bacteria such as *E. sakazakii* and *Pseudomonas* with or without rock phosphate may be useful for enhancing P uptake and utilization by plants and constitute a substitute method for biological fertilization instead of costly and environmental undesirable existing methods.

Table 2. One way ANOVA between the studied strains

Variables	df	Square Means	F
Orthophosphate	9	$40{,}8\times10^{4}$	$178076{,}6^{***}$
Acidity	9	0,304	$668{,}1^{***}$
UFC	9	$10{,}2\times10^{11}$	$25537{,}9^{***}$

Table 3. Pearson correlations of the studied parameters

	CFU	P_i	pH
CFU	1	0.381**	-0.458**
P_i		1	-0.610**
pH			1

5. Conclusion

Results of our study indicated that the majority of the isolated strains displayed important capacity to mobilize large soluble P quantities in the NBRIP broth. This ability was related the difficult pedoclimatic conditions of the mining environment in question. The P release was increased with the decrease in the pH of the media as well as bacterial growth for the majority of the studied strains. *E. sakazakii* and bacteria of the *Pseudomonas* genera were the most performing strains in hydroxyapatite solubilization in comparison with the other studied bacteria. *C. freundii* presented important capacity of solubilization in the broth media.

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