Isolation and characterization of phosphate solubilizing bacteria from sugarcane and sugarcane derivatives environments

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ABSTRACT

Phosphorus an essential element for the growth and development of plants, is found in most soils in insoluble form. A large number of microorganisms in the soil have a high capacity for solubilization and mineralization of P. In this sense, the efficiency with which plants are able to access this nutrient through microbial associations could be of considerable economic and environmental benefit. In the present work, phosphate solubilizing bacteria were isolated and characterized from rhizospheric soil, press mud and residual water from biogas production.The isolates were identified as *Pseudomonas luteola* and *Burkholderia cepacia* using the API 20NE system. In the microorganisms studied, their ability to solubilize mineral P and organic P mineralization was determined, showing different solubilization patterns and acid phosphatase activity. The results indicate the strain *Pseudomonas luteola* C3, is the most promising to be used as a microbial inoculant in biofertilizer formulations.
Key words: phosphate solubilization, phosphate mineralization, phosphatases, biofertilization, phosphate solubilizing bacteria.

RESUMEN

El fósforo un elemento esencial para el crecimiento y desarrollo de las plantas, se encuentra en la mayoría de los suelos en forma insoluble. Un gran número de microorganismos en el suelo tiene la capacidad para la solubilización y mineralización de P. En este sentido, la eficiencia con la cual las plantas son capaces de acceder a este nutriente a través de asociaciones microbianas pudiera ser de considerable beneficio económico y ambiental. En el presente trabajo, bacterias solubilizadoras de fosfatos fueron aisladas a partir de suelo rizosférico, fango y agua residual de la producción de biogás y caracterizadas. Los aislados fueron identificados como *Pseudomonas luteola* and *Burkholderia cepacia* usando el sistema API 20NE. En los microorganismos estudiados se determinó su capacidad para solubilizar el P mineral y la mineralización del P orgánico, mostrando diferentes patrones de solubilización y actividad de ácido fosfatasa. Los resultados indican que la cepa de *Pseudomonas luteola* C3 es la más prometedora para ser utilizada como un inoculante microbial en formulaciones de biofertilizantes.
Palabras clave: solubilización de fosfato, mineralización de fosfato, fosfatasas, biofertilización, bacterias solubilizadoras de fosfato.

INTRODUCTION

Phosphorus (P) is an essential nutrient for the growth and development of plants. However, compared to the rest of the macronutrients, it is the element of less mobility and availability in most soils

(1, 2, 3). Although it is abundant in organic and inorganic forms, P is a limiting factor for the growth of plants (4). In contrast to the nitrogen (N) acquisition from the atmosphere through biological fixation, P has to be supplied by the application of phosphorus fertilizers to achieve and maintain high levels of crop productivity (5).

The global reserve of P can be reduced in the next 50-100 years (6), because it is a non-renewable source. Therefore, the efficiency with which plants are able to access soil P through microbial associations could be of considerable economic and environmental consequence. The problem is that the recovery of P by plants from fertilizer is poor; only 10-20 % of the applied P is obtained in the year of application (7, 8). This is because most of the applied P is rapidly "fixed" in the soil in fractions that are poorly available to the plant roots Plant growth promoting bacteria(PGPB) exert a beneficial effect on crops and ecosystems. Within this large group, we found bacteria, capable of solubilizing mineral and organic P present in soil (9, 10, 11), thus playing a fundamental role in the increased availability of this nutrient for plants. The continuous use of these bacteria as natural fertilizers is a strategy for the development of a sustainable agriculture that allows an increased crop yield and an improved soil quality. In the present study, microorganisms with high capacity to solubilize mineral and organic P were isolated and identified.

MATERIALS AND METHODS

Isolation of Phosphate Solubilizing Bacteria

In this study samples were collected in the areas adjacent to the Agro Industrial Complex "Heriberto Duquesne" located in the province of Villa Clara, Cuba. The sampling areas were: oxidation lagoon of the biogas plant, composted sugarcane press mud and rhizospheric cane soil.

For the isolation of bacteria from the oxidation pond, 0.1 ml of residual water was collected and plated on solid NBRIP medium (12). For the isolation from press mud and rhizospheric soil, 10 g of each were resuspended in 10 mL of sterile saline solution (0.85 % NaCl) and shaken vigorously for 45 minutes (min). 1mL of each suspension was plated on NBRIP medium (12). All isolates were incubated at 30 °C for 48 h. After 48 h, colonies which showed translucent solubilization halos around them were selected.All isolates were deposited at the Microbial Culture Collection of the Cuban Research Institute on Sugarcane By-Products (ICIDCA).

Qualitative detection of inorganic phosphate solubilization

The NBRIP medium (Glucose: 10 g, $Ca_3(PO_4)_2$: 5 g, $MgCl_2 * 6H_2O$: 5 g, $MgSO_4 * 7H_2O$: 0.25 g, KCl: 0.2 g and $(NH_4)_2*SO_4$: 0.1 g bacteriological agar 20 g) was used to analyze the solubilization of inorganic phosphorus, indicated by the formation of translucent halos around the colonies (12).

Identification of isolated phosphate solubilizing bacteria

The taxonomic identification of the isolates was performed using the API Test 20NE (bioMerieux). The results were interpreted using API Web StandAlone® software.

Detection of Acid Phosphatase Activity

An indicator medium based on a histochemical detection system was used to detect the acid phosphatase activity on agar plates. LB agar supplemented with 2 mg/ml phenolphthalein diphosphate (PDP, SIGMA), and 0.05 mg/ml methyl green (SIGMA), pH 7.2. The use of the LB medium is a modification introduced to the system developed by Riccio *et al.* (13) using the Tryptose-Phosphate

(TP) medium (Difco Laboratories, Detroit, MI, USA). On this medium, strains expressing acid phosphatase activity ($Pho^{+/-}$phenotype) grow as green stained colonies.

Phosphatase activity from liquid cultures was evaluated in whole cells and supernatant fractions as follows: Cells were collected by centrifugation at 10,000 x g for 5 min and washed with 2 ml of 0.85 % NaCl. The pelleted cells were resuspended in 0.85 % NaCl to a final optical density (OD) of 1.5 (at 600 nm) and pre-incubated at 37 °C for 30 min at 75 rpm (moderate agitation). Then, 50 µl of this suspension was added to the reaction buffer (0.2 M Tris-maleic acid, pH 6.2). The reaction was initiated by the addition of 50 µl of 60 mM para-nitrophenylphosphate (pNPP, sodium salt, SIGMA) and incubated at 37 °C for 30 min. Then the cells were separated by centrifugation and 1.5 ml of 0.33 N NaOH was added to 600 µl of the supernatant to stop the reaction. The liberation of pNP was determined by the change in the absorbance at 420 nm. One unit of activity was defined as the amount of enzyme able to liberate 1 nmol of pNP per ml andmin, under the assay conditions. The activity in the supernatant fractions was determined by the same method as for the intact cells, but without pre-incubation.

For the detection of protein bands with phosphatase activity, the technique described by Thaller *et al.* was used (14, 15). The gel was incubated at 37 °C for 3 h in the renaturation buffer (10 mM Tris-HCL, pH 7.4; 1 % [vol/vol] Triton X-100 and 2 mM $MgCl_2$), with changes at 30 min intervals with the same buffer. Then the gel was equilibrated for 90 min in the equilibration buffer (100 mM NaAc, pH 6.0 and 2 mM $MgCl_2$), with changes every 30 min. Finally, the gel was incubated overnight in a buffer containing 100 mM NaAc (pH 6.0), 4 mM PDP, 2 mM $MgCl_2$ and 0.005 % methyl green. With this detection system the phosphatase activity was visualized by the green colour of the protein bands due to the activity developed by the renaturated polypeptidic chains. The quantity of protein applied in the samples was 36 µg.

Quantification of soluble phosphorus in liquid medium

Soluble phosphorus was evaluated and quantified in the supernatant of the liquid cultures as follows:

Cells were grown in 500 mL flasks with 50 mL of NBRIP liquid medium for 14 days at 30 °C in a rotating shaker at 175 rpm (Lab-Line). After this time, the entire culture was centrifuged (Centrifuge Sigma 3-18K) at 21 390 x *g* for 20 min and cells were discarded. Aliquots of 10 µl of supernatant were diluted in 990 µL of deionized water. From this dilution 50 µL was reacted with 150 µL of the Reagent Phosphate (BioVision). The mixture was manually shaken and incubated 30 min at room temperature. Subsequently the release of the soluble P was detected by the absorbance increase at a wavelength of 650 nm in a Genesys 6 spectrophotometer, with quartz cuvettes of 1 cm light path.

Determinations

Growth was followed by the absorbance at 600 nm. The statistical analysis and regression were carried out with the statistical package Statgraphics Statistical Graphics System version 5.0, with a level of significance of 95 %.

RESULTS AND DISCUSSION

Isolation of Phosphate Solubilizing Bacteria

A total of 10 bacterial isolates were obtained that had the capacity to solubilize tricalcium phosphate, demonstrated by the formation of translucent halos around the colonies (Table 1). All were Gram

negative bacilli, did not form spores or capsules and were actively motile. The use of the NBRIP medium allowed the quick and simple analysis to confirm the phosphate solubilizing phenotype.

Table 1. Bacterial isolates in NBRIP medium at 48 hours

Samples	Isolates
Intermediate wastewater from the oxidation lagoon	RI1
Rhizosphere of sugar cane plants	R5, R7
Sugar cane press mud	C2, C3, C4, C6, C9, C10 (A), C10 (B)

C: sugarcane press mud, R: sugarcane rhizosphere, RI: intermediate residual water.
The number indicates the order of the isolates. A and B: type of colonies.

Qualitative analysis of inorganic phosphorus solubilization

The visual detection and even a semiquantitative estimation of the mineral P solubilization capacity by microorganisms has been possible by detecting clear zones around the colonies when solubilization of ($Ca_3(PO_4)_2$) occurs (12). This method has been successfully used for the detection of phosphate solubilizing bacteria (16).

In this study, all isolates grew on solid NBRIP medium (Figure 1). The formation of translucent halos was measured on days 2, 9 and 14. An increase in the diameter of the solubilization halos was observed. However, in the case of isolates C2, C3, and C6, the halo measurement could not be performed due to the production of a polymer.

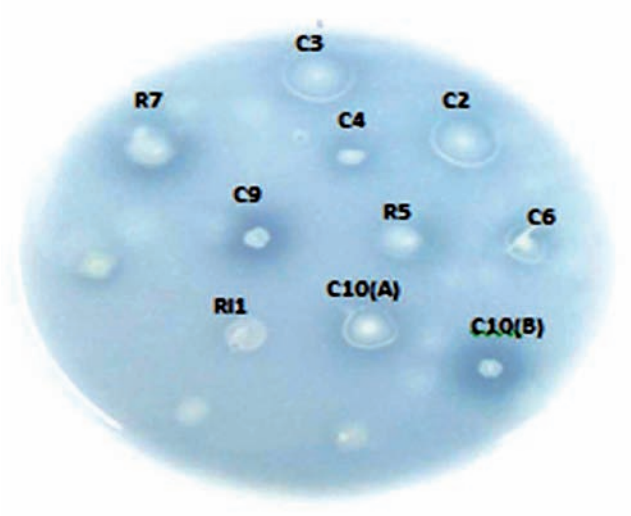

Figure1. Solubilization of tricalcium phosphate on NBRIP solid medium by isolates C2, C3, C4, C6, R7, C9, C10 (A), C10 (B), RI1, R5.

On day 2, isolates C4 and C9 had the largest solubilization halos, showing significant differences between them and the rest of the isolates. The isolates R5, R7, C10(A), C10(B), RI1 showed significant differences between them, although their solubilization halos were smaller than those of C4 and C9 (Figure 2A).

On day 9, isolates C4 and C10(B) showed the largest solubilization halo with respect to the rest of the isolates. Isolates C4 and C10(B) showed no significant differences between them. The isolates R5 and C10(A) also showed a larger solubilization halo than the isolates R7, C9 and RI1 but smaller than the isolates C4 and C10(B) (Figure 2B).

On day 14, isolate C10(B) exhibited the largest diameter solubilization halo compared to the rest of the isolates. Isolates C4, R5 and C10(A), exhibited no significant differences among them (Figure 2C).

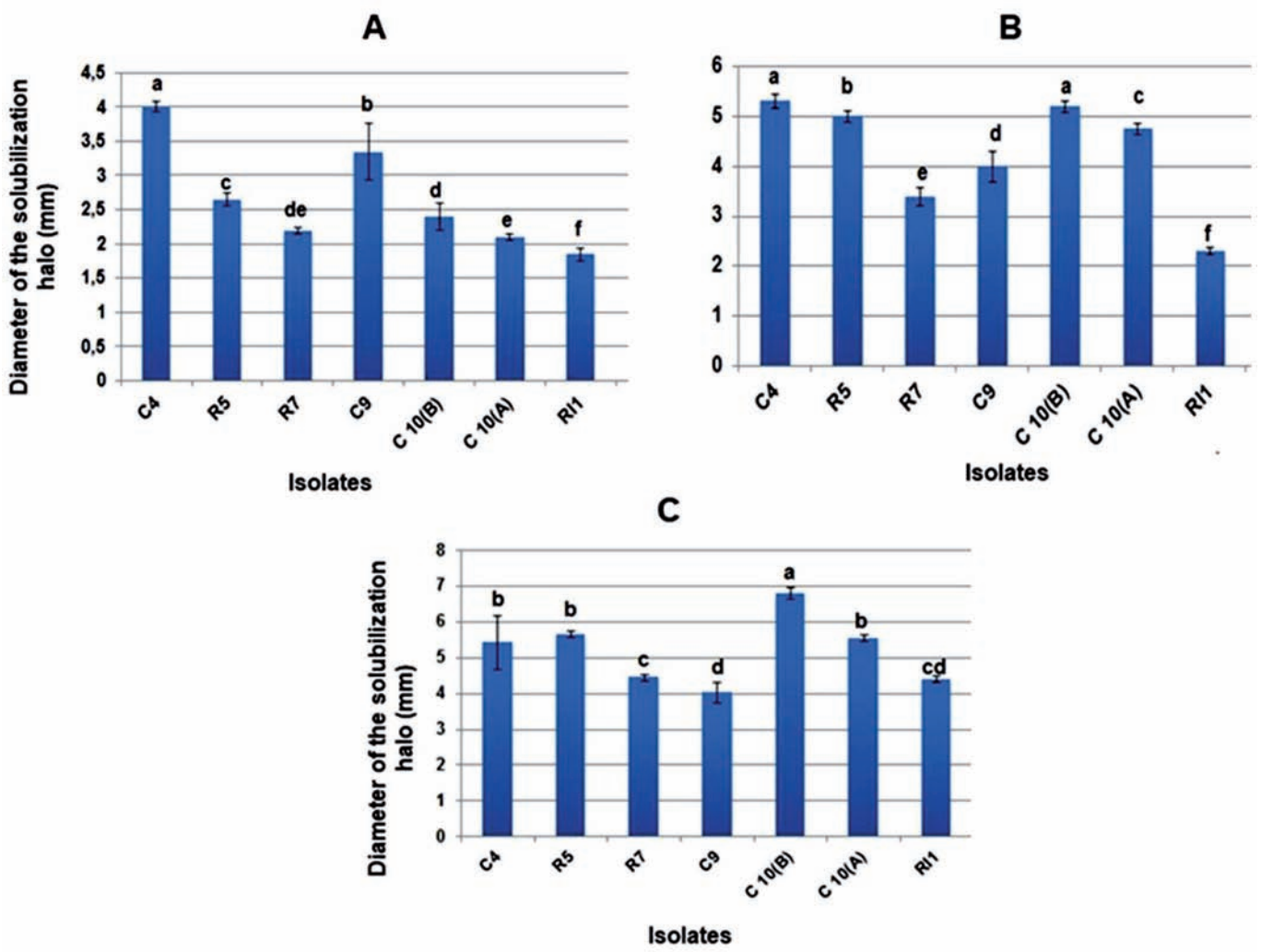

Figure 2. Diameter of the solubilization halo of tricalcium phosphate on NBRIP solid medium **(A)**: Diameter of the halo 2 days after inoculation. **(B)**: Diameter of the halo 9 days after inoculation. **(C)**: Diameter of the halo 14 days after inoculation. Uncommon letters indicate significant differences according to Duncan's test for $p < 0.05$ (I = standard deviation of the mean).

In this study it is demonstrated that the isolates C4, R5, C10(A) and C10(B) are the best phosphate solubilizers because they maintained during the 14 days the largest solubilization halos. However, the C9 isolate that was the one with the highest solubilization halo on day 2 remained below these. The qualitative Petri dish estimation to detect the P solubilization capacity of the isolates coincided with the results of Nautiyal (12), when the isolations made from soil samples showed solubilization halos of up to 7 mm. In addition, the results of the present study were higher than those of Park *et al.* (17), where solubilization halos are reported on NBRIP medium no greater than 4mm.

Identification of the selected isolates

Once the ability to solubilize P on NBRIP medium of all the isolates was verified, they were identified by the API 20NE test (bioMerieux) and the results were read according to the manufacturer's instructions. The isolates selected for their P solubilizing activity were included within the species *Pseudomonas luteola* (18) and *Burkholderia cepacia* (19) (Table 2).

Table 2. Identification of the isolates using the API20NE biochemical test gallery

Isolates	Identification
C2	*Pseudomonas luteola*
C3	*Pseudomonas luteola*
C4	*Pseudomonas luteola*
R5	*Burkholderia cepacia*
C6	*Pseudomonas luteola*
R7	*Burkholderia cepacia*
C9	*Pseudomonas luteola*
C10(A)	*Pseudomonas luteola*
C10(B)	*Pseudomonas luteola*
RI1	*Pseudomonas luteola*

The genus *Pseudomonas* has been associated with the rhizosphere of different plant species such as diazotrophs, and forms part of the rhizosphere of many crops (10, 20). In this study, a prevalence of the species *Pseudomonas luteola* was observed. This could be related to the adaptability that *Pseudomonas luteola* has to numerous ecosystems (21). This species plays an important role in the promotion of plant growth as an excellent P solubilizer, as well as in biological control in crops of economic importance (22, 23). Further, it has the capacity to degrade a large number of xenobiotic compounds, which makes it very effective as a bioremediation agent (24, 25). It is a causative agent of nosocomial infections, so its application in natural environments must proceed with care (26). In this work, *Pseudomonas luteola* was isolated from composted press mud and wastewater from biogas production. This result coincides with the fact that this particular species can be found in many ecosystems due to its capacity to degrade dissimilar organic and inorganic compounds.

The genus *Burkholderia* has been reported as PGPB by numerous authors due to its important role in the promotion and growth of plants. It produces plant growth regulators, performs biological control in several crops of economic importance and stands out for its high efficiency to solubilize and mineralize phosphates (27, 28, 29). The most studied species of this genus is *Burkholderia cepacia*, which has been found associated with the rhizosphere of plants, soil and river waters (30). Particularly, this species possesses antagonistic activity against a wide range of plant pathogens, mainly fungi. For example, root rot is caused by *Aphanomyces euteiches*. It is also considered an excellent bioremediator (31). On the negative side, it is the cause of rot in crops such as onions (19). Also it has been reported as an opportunistic pathogen of man; it has been found in the respiratory tract of patients with pulmonary fibrosis and chronic granuloma (32). Therefore, the necessary safety measures must be taken before commercial application of this microorganism. In this study, *Burkholderia cepacia* was isolated from the rhizosphere of sugarcane. This microorganism has been previously isolated in six varieties of cane, and found to solubilize inorganic phosphorus sources and increase agricultural yield by 25 % (33).

Qualitative detection of phosphatase activity and zymogram of acid phosphatase

The medium TPMG (Tryptose-Phosphate containing PDP and methyl green), was used to select bacteria with phosphatase activity. It is based on the principle that bacteria that do not produce detectable phosphatase activity grow as uncoloured colonies (negative phosphatase phenotype, Pho^-), while bacteria that produce phosphatase activity grow as green colonies (positive phosphatase phenotype, Pho^+) (13). In this study, a modification of the original medium which consisted of LB medium supplemented with PDP and methyl green, was used as a means to qualitatively detect phosphatase activity in the strains studied (Figure 3).

The intense green coloration in strains RI1, C2, C3, R5, C6, R7, C10(A) is due to the fact that they have the Pho^+ phenotype and that they produce at least one active phosphatase enzyme. When this modifies the PDP substrate, it causes the color change of the indicator. In contrast, the uncolored growth of strains C4, C9, C10(B) indicates that they have a Pho^- phenotype under these test conditions.

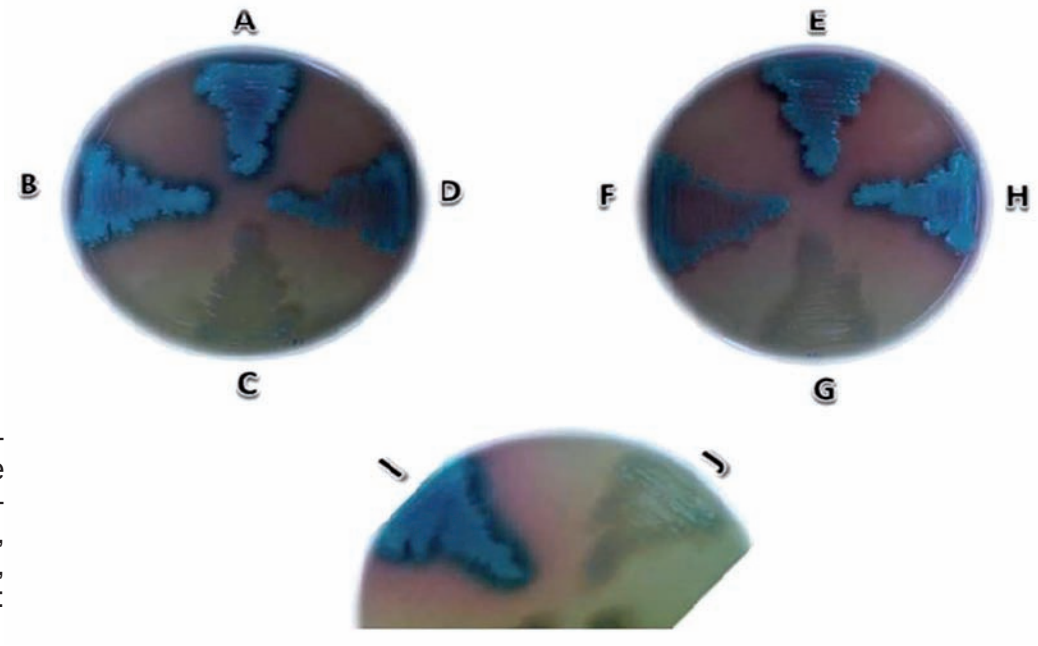

Figure 3. Qualitative histochemical detection of the phosphatase activity produced by the strains: A: C2, B: C3, C: C4, D: R5, E: C6, F: R7, G: C9, H: C10(A), I: RI1(B), J: C10(B).

The result of the zymogram for the cell fractions of the strains under study is shown in Figure 4.

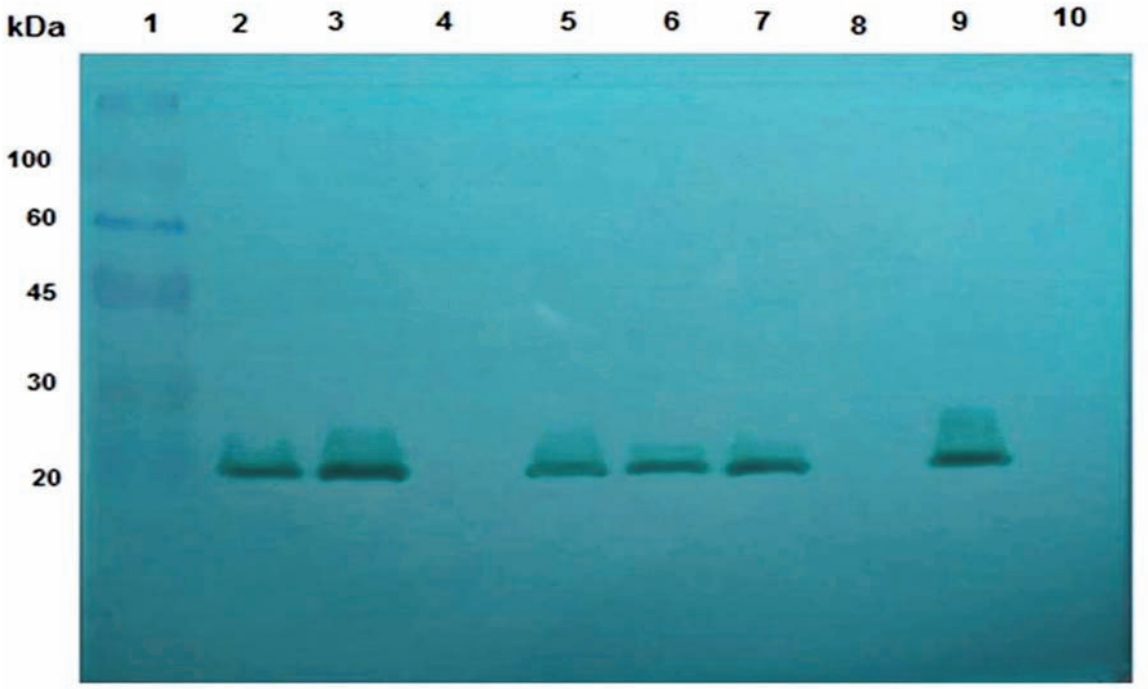

Figure 4. Zymogram for the detection of phosphatase activity in the selected isolates: Molecular weight marker (Color Burst™). 2: C2, 3: C3, 4: C4, 5: R5, 6: C6, 7: R7, 8: C9, 9: C10(A), 10: C10(B).

This technique detects acid phosphatase activity through the formation of a green precipitate, as a result of the activity developed by the renatured acid phosphatase polypeptide chains. In lanes 2, 3, 5-7, 9 (Figure 4) there is a protein band, for which we have estimated a size of approximately 25 kDa. The latter corresponds to the size of non-specific periplasmic acid phosphatases containing low molecular weight polypeptide chains (Lmmp-APs, 25-27 kDa) (15). Phosphatase activity is present in all members of the *Enterobacteriaceae* family. Some species of this family produce high levels of

phosphate-irrepressible acid phosphate activity (HPAP), such as Lmmp-APs, which are responsible for the HPAP phenotype (34, 35).

The results coincide with those of other authors, pointing out that this type of acid phosphatase is widely distributed in the genera *Pseudomonas*, *Enterobacter*, *Burkholderia* (18, 36), and these results confirm the qualitative Petri dish studies (Figure 3).

In the case of lanes 4, 8 and 10 (Figure 4), the 25 kDa band is not observed, indicating that these strains do not show acid phosphatase activity under the conditions tested.

Quantification of acid phosphatase activity in liquid culture

Being soluble in the periplasm of the cell, or bound to the outer surface of the inner membrane, most of the phosphatase activities are detectable using pNPP, which diffuses freely into the periplasmic space of intact cells (37).

In this study, acid phosphatase activity was compared in liquid medium of the cell fractions of all the strains studied (Figure 5). During the 24 hour period analyzed, the RI1 strain showed the highest phosphatase activity compared with the other strains. However, strains C4, C9, C10(B) showed a basal phosphatase activity, not detected in the previous trials. From this we can infer that a negative phenotype in qualitative tests in solid medium does not constitute a criterion of absolute absence of this type of activity, but that it occurs in quantities not detectable by the lower sensitivity of the method or by not presenting the optimal conditions for the production of the enzyme.

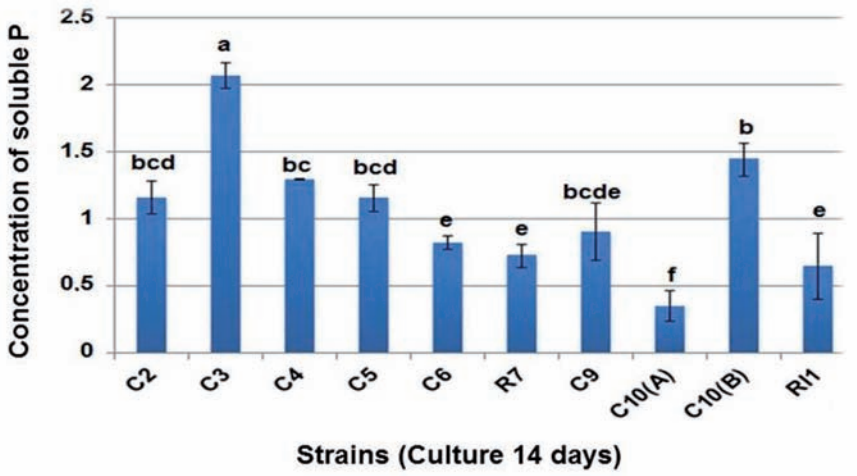

Figure 5. Acid phosphatase activity associated with cells, measured by the production of para-nitrophenol (pNP) nmol / mL by the strains: C2, C3, C4, R5, C6, R7, C9, C10(A), C10(B), CRI1. Uncommon letters indicate significant differences according to Duncan's test for $p < 0.05$ (I = standard deviation of the mean).

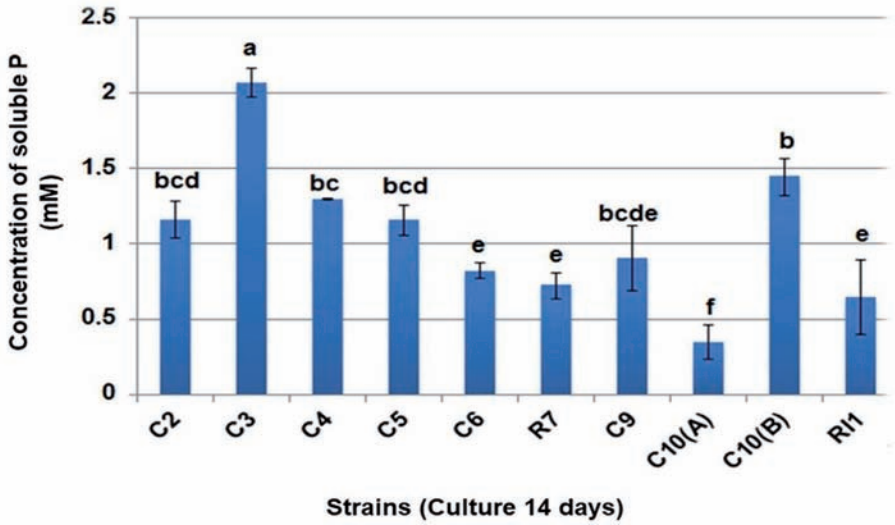

Figure 6. Solubilization of mineral P in liquid NBRIP medium at 14 days of inoculation, by the strains: C2, C3, C4, R5, C6, R7, C9, C10(A), C10(B), CRI1. Uncommon letters indicate significant differences according to Duncan's test for $p < 0.05$ (I = standard deviation of the mean).

Quantification of phosphate solubilization in liquid culture

The determination of soluble P in liquid NBRIP medium to detect the solubilizing activity of P mineral was carried out by means of a colorimetric assay (BioVision). In Figure 6, most strains showed the ability to solubilize tricalcium phosphate by releasing soluble P to the culture medium.

Strain C3 released more soluble P to the medium, showing a significant difference from the rest of the strains. Strains C10(B), C2, C4, R5 showed a moderate concentration of free P in the medium, with significant differences between them and with the rest of the strains, although the concentration of free P with respect to strain C3 was lower. These results coincide in a general way with those reported for *Pseudomonas* sp. by Nautiyal (12), using test conditions similar to those of this study. Similar results have also been reported by Lara *et al.* (38) in strains of *Burkholderia cepacia* and *Pseudomonas luteola*, shown to be excellent P solubilizers according to the authors.

It is important to note that the C3 strain can be considered as a stimulator of plant growth and development because it shows a high solubilization of mineral P.

CONCLUSIONS

Ten bacterial isolates were obtained from rhizospheric soil of sugarcane, press mud and residual waters, with P solubilizing capacity. There were 8 isolates identified as *Pseudomonas luteola* and 2 isolates identified as*Burkholderia cepacia*. All strains had acid phosphatase activity, with *Pseudomonas luteola* RI1 expressing the highest level of activity. All strains had the capacity to solubilize P mineral, with *Pseudomonas luteola* C3 solubilizing the largest amount. *Pseudomonas luteola* C3 is the most promising to be used as a biofertilizer.

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