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PHOSPHORUS SOLUBILIZING AND IAA PRODUCTION ACTIVITIES IN PLANT GROWTH PROMOTING RHIZOBACTERIA FROM BRAZILIAN SOILS UNDER SUGARCANE CULTIVATION

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ABSTRACT

Plant Growth Promoting Rhizobacteria (PGPR) has been used as a biofertilizer, bringing benefits to agriculture as Phosphorus Solubilizing Bacteria (PSB), indole-acetic acid (IAA) producers, and with other activites. The goal of this report was the identification of PGPR from soils under sugarcane crops by *16S rRNA* sequencing, and the evaluation of the ability of phosphorus solubilizing and IAA production by biological assays. The isolates of this work were obtained from three areas of sugarcane crop from São Paulo State, Brazil. All isolates came from rhizosphere soil, and in a total of 60 isolates just 10 have showed high ability in phosphorus solubilizing. The selection of PSB may be done by phenotypic and/or genotypic characterization. Among ten isolates *Enterobacter* sp. (FJ890899), *Entrobacter homaechei* subsp. *verschuerennii* (FJ890998), *Burkholderia* sp. (FJ890895), and *Labrys portucalensis* (FJ890891) were able to IAA production.

Keywords: phosphorus solubilization, *16S rRNA*, IAA production, plant growth promoting rhizobacteria.

INTRODUCTION

Sugarcane crop (*Saccharum* spp.) has a great importance in Brazil's economy, mainly due to sugar and ethanol production, for either domestic consumption or exportation, which generates a considerable income for the country. Moreover, sugarcane can be used as alternative forage for ruminant feeding in periods of drought. Considering the high biomass production and low consumption of nitrogen fertilizer, which means low energy balance based on the ratio between the total energy contained in the biofuel and the total fossil energy needed by its obtention, sugarcane emerges as a powerful crop for biofuel production (Urquiaga *et al*., 2005). Currently, there is a strong trend for expansion of sugarcane area, motivated by the increasing interest in obtaining alternative sources of energy that can help reduce the high CO_2 emissions, characteristic of the traditional fossil energy source. Sugarcane ethanol is considered to be an alternative fuel for gasoline (Urquiaga *et al*., 2005).

Phosphorus (P) is considered the second most essential macronutrient following nitrogen, so it may limit plant growth, as it plays an important role in growth of structure, its function, and transfer of energy. Agricultural soils contain large reserves of phosphorus due to its regular application. A large proportion of inorganic phosphorus in soluble form is rapidly fixed to its insoluble form after the application of phosphate fertilizers. In turn, this important nutrient becomes unavailable to the plants (Igual *et al*., 2001; Rodriguez and Fraga, 1999).

The process of phosphorus fixation has been estimated to around 75% of the applied content. Some microorganisms in soil, such as bacteria and fungi, have a central role on the natural P cycle, being responsible for hydrolysis of phosphorus to its inorganic form, making it available to the plants. These processes are mediated by phosphatase enzymes (Sobral, 2003), so that phosphorus solubilizing bacteria act on the insoluble phosphorus, using mainly acidic phosphatase, to produce organic and inorganic acids that reduce the soil pH, thus making the phosphate available for the plants (Nautiyal, 1999; Sobral, 2003).

The phosphorus solubilizing bacteria, when used as soil inoculant, may offer many advantages for plant productivity. For this reason, several molecular techniques have being developed for detection and isolation of efficient phosphorus solubilizing strains. The bacterial genera known for this ability are *Pseudomonas*, *Burkholderia*, *Rhizobium*, *Agrobacterium*, *Azotobacter*, and *Erwinia* (Verma *et al*., 2001). Among the bacteria that act in solubilization of phosphorus, the *Burkholderia* is present in a large number of environments of ecological importance. Many bacteria of this genus have been isolated from soils (Coenye and Vandame, 2003). Igual *et al*. (2001) analyzed different methods to characterize phosphate solubilizing bacteria and concluded that *16S rRNA* sequencing is used as the most important phylogenetic marker for microbial ecology. Because of its high conservation, it allows an estimation of phylogenetic distances among strains.

Besides phosphorus solubilizing, production of hormones by microorganisms in the soil can promote plant growth. Among the phytohormones produced, the auxin IAA, applied at low concentrations, is known for its role in root development and cell division stimulation. This hormone is commonly produced by bacteria such as *Aeromonas verona*, *Agrobacterium* sp., *Azospirillum brasilense*, *Bradyrhizobium* sp., *Rhizobium* sp., and *Enterobacter* sp., among others (Vessey, 2003). Mirza *et al.* (2001) studied the potential of *Enterobacter* isolates as growth promoters in seedlings of sugarcane micropropagated and showed an increase of 55% in root biomass.

In this context, the purpose of this study was to isolate, and characterize by complete *16S rRNA* gene sequencing, the isolates obtained and evaluated through activities of phosphorus solubilizing bacteria and IAA production in relation to phosphate rock from Araxá-MG and Catalão-GO, Brazil.

MATERIALS AND METHODS

Standard strains and phosphorus source

The standard strains used as reference for phosphate solubilizing bacteria were *Burkholderia ferrariae* FeGl01 and *Burkholderia cepacia* ATCC 25416 obtained from Colección Española de Cultivos Tipo (CECT) in Universidad de Valencia and from Brazilian EMBRAPA Agrobiologia, respectively. The phosphorus source used was Ca_5 (OH) $(PO_4)_3$, characterized by phosphate rock, from Catalão/Goiás (28, 1% P) and Araxá/MG (24% P). These sources were obtained from Adubos Catalão and Bunge Fertilizantes industries, respectively.

Soil samples and sample preparation

Soil samples were collected in three sugarcane production area in São Paulo State, Brazil: Pontal (21°02'51.20 "S 48° 06'31 .81" W), Pitangueiras (20° 59'43 .13"S 48º 09'52 .26"W), and Jaboticabal (21° 18'20 .99"S 48º 11'31 .42"W). Rhizosphere samples were collected from 0 to 20 cm depth, following the procedures from Embrapa (2007). Abiotic characteristic of soil samples were obtained by standard methods, and the results are shown in Table-1. All samples were collected between May and June 2007. The soil samples collected were sieved to remove roots, leaves, and other materials. Bacterial strains were isolated by homogeneous serial dilution of 10g of the soil samples in 90 mL of saline solution (NaCl 0.85%), maintained at 25°C for 30 min, at 250 rpm. The 10^{-5} to 10^{-8} serial dilutions were plated in solid NBRIP medium (Nautiyal, 1999) and were incubated for 15 days at 28ºC. The pure isolated colonies obtained were transferred to DYGS culture medium (Rodriguez Neto *et al*., 1989), and then stored in glycerol at -80°C.

Table-1. Chemical analysis of the sampled soils.

Areas	City	Total number of isolates per area	pH	O.M*	P	K	Ca	Mg	Al	H+Al	CEC	V %
			$CaCl_2$	g dm^{-3}	mg dm^{-3}			Mmolc dm^{-3}				
A6	Pitangueiras	3	6,3	35	111	5,4	82	23	0	19	129,4	85,3
A7/A8/A9		10	5,8	38	75	5,8	86	28	0	26	145,8	82,2
A5/A4	Pontal	32	6,3	31	49	5,1	66	16	0	17	104,1	83,7
USC	Jaboticabal	2	5,9	19	96	9,7	70	17	0	20	116,7	82,9
A1/A2	Pontal	13	5,4	23	21	7,1	25	13	0	30	75	60
A3		1	5,2	23	9	5,4	28	14	0	33	80	58,9

*OM: organic matter, **CEC (cmolc kg^{-1}): cation exchange capacity, and ***V: base saturation.

Bacterial grown and DNA extraction

Bacterial isolates were grown in DYGS medium for 24 h at 28ºC, under constant agitation of 160 rpm. After this period, the bacterial suspension was centrifuged at 15, 294 xg for 15 min at room temperature. The precipitate was used for DNA extraction in accordance with Sambrook and Russel (2001). Furthermore, the quality and integrity of DNA was checked by eletrophoretic analysis in 0.8% agarose gel.

PCR amplification and sequencing of 16S RRNA

The *16S rRNA* was amplified by polymerase chain reaction (PCR) using the universal primers fD1 and rD1 (Weisburg *et al*., 1991). PCR reactions contained 10 mM of dNTPs, 5 pmol of each primer, 2 mM $MgCl_2$, 1X PCR buffer, 1.25 U of Taq DNA polymerase (Fermentas®) and 40 ng of genomic DNA. The PCRs were carried out in Gene Amp PCR System 9700 thermocycler (Applied Biosystems®) using an initial step of denaturation at 94ºC for 5 min, followed by 35 cycles of denaturation at 94º C for 30 sec, annealing at 56ºC for 40 min, extension at 72°C for 1.5 min, with a final extension step at 72°C for 7 min. The PCR product of each isolated was purified using GENECLEAN Kit ® (Bio 101). For sequencing reactions 80 ng purified PCR product was mixed with 1.0 µL of DYEnamic ET (GE®), 2.5 X buffer (400 mM Tris-HCl, 10 mM $MgCl_2$), and 5 pmol of each primer, fD1 and rD1. In order to obtain complete sequence of 16S rRNA gene, four reactions were carried out with 5 pmol of each primer, fD1 and rD1, and 3 pmol of each of the internal primers: 362f (for region 339-362), 786f (for region 746-786), and 1203f (for region 1179-1203) (Menna *et al*., 2006). The thermal cycler conditions for the internal primers were carried out as described by Menna *et al*. (2006). The resulting sequencing reactions were purified by ethanol precipitation and analyzed on an ABI 3700 Sequencer (Applied Biosystems®).

Phylogeny analysis based on *16S RRNA* sequences

The high-quality DNA sequences obtained for each strain were analyzed and assembled into contigs using Phred/Phrap/Consed programs being subsequently

submitted to nucleotide similarity comparison with sequences placed in GenBank/NCBI database (Menna *et al*., 2006) using the tool BLAST (Basic Local Alignment Search Tools) (Alstchul *et al*., 1997). These sequences were also compared with others that were placed in Ribosomal Database Project II using the Classifier 1.0 software with 95% confidence (Wang *et al*., 2007). The RDPII uses the naive Bayesian classifier and hierarchical taxonomy based on the Bergey's Manual of Systematic Bacteriology (Garrity *et al*., 2007). The CLUSTALX 2.0 program was used for the multiple sequential alignment of 16S rDNA sequences obtained in this study and other sequences selected in NCBI GenBank (Larkin *et al*., 2007). Starting from the aligned sequences, a phylogenetic tree was generated by the Neighbor-joining distance method (Saitou and Nei, 1987) and the Jukes-Cantor algorithm (Jukes and Cantor, 1969), with 1000 bootstrap replications, using MEGA 4.0 software (Tamura *et al*., 2007).

Evaluation of solubilization on NBRIP medium

Phosphate (5 g L^{-1}) solubilization on NBRIP solid medium was evaluated through the visualization of a clear halo around the colonies until the fifteenth day after inoculation. On the fifteenth day the solubilization efficiency (E) was calculated by the ratio between the halo diameter and the colony diameter (Berraqueiro *et al*., 1976). According to Silva Filho and Vidor (2000), the solubilization process can be classified into low solubilization ($E < 2$), average solubilization ($2 < E < 3$) or high solubilization ($E > 3$). The selected isolates were submitted to a battery of tests in different phosphorus source (Xiao *et al*., 2008) in NBRIP liquid media. The factorial randomized experiments (two phosphorus source x 12 inoculations) were performed in triplicate. Due to the presence of insoluble particles of phosphorus in the supernatant, the samples were sedimented by centrifugation at 10, 000 x g, for 15 min, at room temperature. The supernatant was recovered for assessment of soluble phosphorus by molybdate-vanadate method (Malavolta *et al*., 1989). Statistical analysis was performed using the software Agro Estat (Barbosa and Junior Maldonado, 2008).

In vitro auxin production

The production of IAA was evaluated by the colorimetric method described by Gordon and Weber (1951) with some modifications. These assays were performed in DYGS medium at 30°C by collecting 2 ml of the broth after each 12 h for OD (600 nm) determination. These samples were centrifuged for 10 min at 10,000 x g at 10°C and auxin production was determined as indole 3-acetic acid (IAA) equivalent by mixing 1 ml of supernatant and 2ml of Salkolwski reagent ($FeCl_3.6H_2O$ 2% and HCl_3 37%). The tubes were kept in the dark for 30 min (Hartmann *et al*., 1983). The presence of auxin was visualized by the reddish color and quantified by reading the absorbance at 530 nm. All measurements were performed in triplicate. As a negative control, we used only the culture medium DYGS. To estimate the production of auxin, the standard curve was constructed following concentrations: 0, 1, 3, 5, 7, 10, 25, 50, 100 and 150µg mL^{-1}

RESULTS AND DISCUSSIONS

Analysis of bacterial isolates by *16S rRNA* sequencing

The 16S rRNA sequences from isolates showed high levels of similarity between 91 to 99% with bacterial sequences from GenBank (Table-2). The particular sequence of the A5I46 isolate showed low similarity with the sequences stored in the GenBank, but was similar to the microalgae *Chlorella protothecoides*, whose phosphorus solubilization ability was reported by Grupta *et al*. (1998).

The results obtained by isolates A3I14, A5I55, A6I8, and A9IG showed similarity with genus *Burkholderia*, which has been studied and reported as potential phosphorus solubilizing bacteria. The isolate A3I14 showed high sequence similarity with *Burkholderia caribensis* (GenBank AY586519) identified as a diazotrophic endophytic bacteria in sugarcane crop. Moreover, isolates USC7 and USC8 showed similarity with *Enterobacteriaceae,* which is another group that is able to solubilize phosphorus (Kampfer, 2003).

The isolate A5I42 showed similarity with a genus of the Rhizobiaceae family which presented some evidences on phosphate solubilizing activity (Rodriguez and Fraga, 1999). In spite of these isolates showing similarities with reported phosphate solubilizing microorganisms, A5I53 show similarity to *Flexibacter sancti.* This genus was not described such as PSB yet, so this isolate could be considered a potential PSB for posterior studies.

Based on 95% confidence in Ribosomal Database Project II (RDPII), some sequences were not classified in terms of genus, as seen for A5I46 and USC8 isolates identified as "unclassified chloroplast" and "unclassified Enterobacteriaceae", respectively (Table-2). The isolate A9IG showed contrasting similarities as *Candidatus Burkholderia verschuerenii* in GenBank and as *Citrobacter* in RDPII. The genus *Citrobacter* belongs to the family Enterobacteriaceae that presents the ability of phosphate solubilization (Kampfer, 2003).

Enterobacteriaceae members such as *Pantoea, Citrobacter,* and *Klebsiella* are also known for the ability to fix nitrogen anaerobically (Verma *et al*., 2001). *Candidatus B. verschuerenii* was found to be associated with *Psychotria verschuerenii,* a plant of the Rubiaceae family (Robbrecht, 1988). The presence of this symbiosis is a requirement for the plant normal development.

The results of the identification of A2I61, A3I14, A5I42, A5I46, A5I55, A6I8, USC7, USC8, and the standard bacteria were coincident in both databases searched (Table-2).

Table-2. *16S rRNA* sequences similarities based on Gen bank and RDPII databases.

Isolate	Gen bank accession number	pB**	Gen bank blast	% Id*	Classifier RDPII
A2I61	FJ890893	1502	*Arthrobacter* sp.	99	*Arthrobacter*
A3I14	FJ890892	1525	*Burkholderia* sp. TJ 182	99	*Burkholderia*
A5I42	FJ890891	1358	*Labrys portucalensis* strain	98	*Labrys*
A5I46	FJ890890	1507	*C. protothecoides*	91	unclassified cloroplast
A5I53	FJ890894	1523	*Flexibacter sancti*	97	*Chitinophaga*
A5I55	FJ890895	1530	*Burkholderia* sp. Ak-5	97	*Burkholderia*
A6I8	FJ890896	1528	*Burkholderia gladioli*	97	*Burkhloderia*
A9IG	FJ890897	1536	*Candidatus B. verschuerenii*	97	*Citrobacter*
USC7	FJ890898	1521	*E. hormaechei* subsp.*steigerwaltii*	98	*Enterobacter*
USC8	FJ890899	1184	*Enterobacter* sp. J11	98	unclassified *Enterobacter*
Standard strain	AB252073.1	1491	*Burkholderia cepacia*	99	*Burkholderia*
Standard strain	DQ514537.1	1528	*Burkholderia ferrariae*	99	*Burkholderia*

*Identification percentage
** Fragment in pair bases sequenced

Phylogeny of bacterial isolates based on *16S rRNA*

The cluster analysis based on 16S rRNA sequences allowed a better coherent grouping among all genera distributed in the phylogenetic tree (Figure-1). The B branch grouped most of isolates showing high similarity to the genus *Burkholderia* (Table-2), except the isolate A9IG. The *B. cepacia* and *B. ferrariae* standards bacteria used in phosphate solubilization experiments were also placed in the B branch (Figure-1). On the other hand, A9IG was located in the C group close to *Citrobacter amalonaticus* and *Citrobacter farmeri*, evidencing the importance of RDP II database which allowed a more precise identification of this isolate in genus *Citrobacter*.

The isolate A3I14 (Table-2) showed high similarities with *B. caribensis*, a diazotrophic endophytic of sugarcane, *B. hopsita*, and *Burkholderia* sp. TJ 182. The isolate A5I55 was placed in the same group but in a different branch of *Burkholderia* sp. genus, showing greater similarity to the sequences of *Candidatus Burkholderia verschuereni*. The isolates USC7 and USC8 showed high similarity between them being placed in E group belonging to *Enterobacter*. The isolates *C.protothecoides* (A5I46), *Arthrobacter* sp. (A2I61), *Flexibacter sancti* (A5I53), and *Labrys portucalens*is (A5I42) showed low phylogenetic distance with sequences deposited in the database regarding to species of the same genus, respectively belonging to CP, A, F, and L groups (Figure-1).

In this study, the results presented in Figure-1 show that the sequencing of *16S rRNA* gene allowed the separation of close species at the genus level, coinciding with the results obtained by Coenye *et al.* (2005). The results obtained in this work and by Menna *et al*. (2006) showed that the use of internal primers was very efficient, and allowed a complete sequencing of the *16S rRNA* gene to be obtained for almost all isolates.

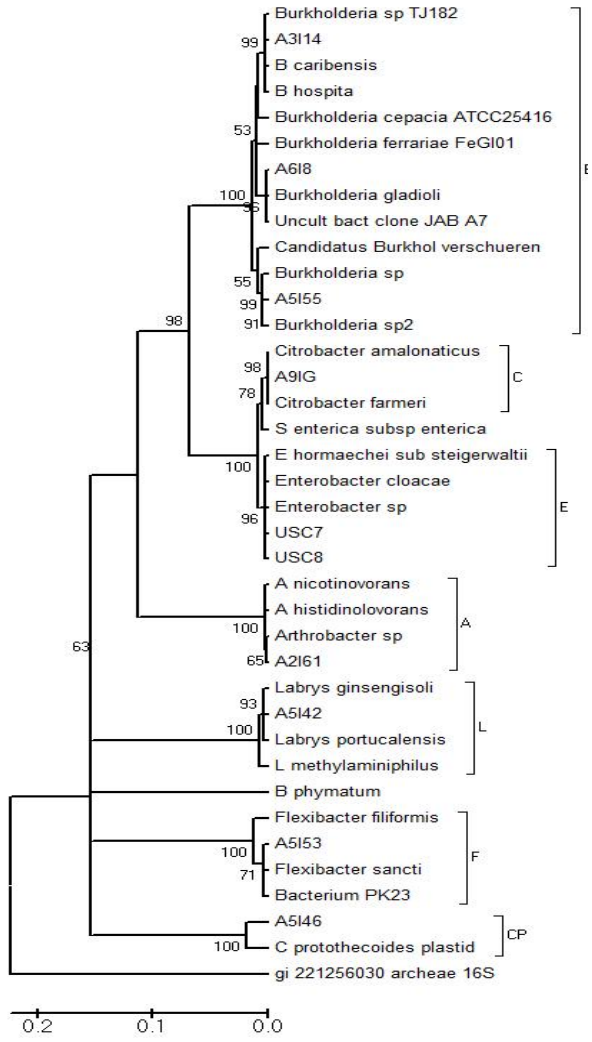

Figure-1. Phylogenetic tree based on *16S rRNA* sequences, showing the taxonomic positions of bacterial isolates related to sequences from databases. The specie studied was identified as an isolate and the standard strain code. The internal numbers represent the bootstrap values. The division keys (B, C, E, L, F, A, and CP) were used for grouping the genera.

Evaluation of phosphorus solubilization on solid NBRIP medium

From sixty isolates, only ten showed high solubilization rates fifteen days after inoculation. The average E values for these were greater than three (E>3) (Table-3). These rates of solubilization and the proportion in which they were found were similar to those previously reported (Silva Filho and Vidor, 2000; Massenssini, 2007), but superior to those found to *Pseudomonas* and *Bacillus* isolates (Nautiyal, 1999). Isolates *C. B. verschuerenii* and *Burkholderia* sp. (FJ890892) had E values of 7.33 and 6.47, respectively; higher than the results obtained by the standard strains. The other evaluated isolates showed E values between 3.17 and 5.4; close to those obtained from *B. cepacia* and *B. ferrariae.*

Table 3. Solubilization ability evaluated on NBRIP medium.

Isolates	**E=(Ø halo/Ø colony) mm****
Arthrobacter sp.(FJ 890893)	3,92
Burkholderia sp.(FJ890892)	6,47
L. portucalensis(FJ890891)	4,91
C.protothecoides(FJ890890)	3,17
F. sancti (FJ890894)	4,35
Burkholderia sp.(FJ890895)	3,75
B. gladioli (FJ890896)	4,25
C. B.verschuerenii (FJ890897)	7,33
E.homaechei subsp. *Steirgerwaltii* (FJ890898)	4,08
Enterobacter sp.(FJ890899)	5,4
*B. cepacia**	4,31
*B. ferrariae**	5,31

* Standard strains.
**Efficiency of phosphorus solubilization up to 15 days after inoculation.

Analysis of phosphorus solubilizing activity by isolates

The phosphorus solubilizing activity was evaluated with two source of phosphorus (Table-4). Some *Arthrobacter* sp. (FJ890893), *C. protothecoides* (FJ890890), and *F. sancti* (FJ890894) isolates showed the lowest values (Table-4) despite showing E> 3 on NBRIP solid medium (Table-3). This may be due to the loss of phosphorus solubilization activity during the storage period (Souchier *et al*., 2007) and the immobilization of phosphorus by microorganisms. For rock phosphate of Catalão, *B. cepacia* and *B. ferrariae* showed the highest values, but did not show difference in the solubilization rate. *Burkholderia* sp. (FJ890892) and *C. B. verchuerenii* (FJ890897) showed similar results, nearly 50% of the values in regard to standards strains. Isolates *L. portucalensis* (FJ890891) and *Burkhlderia* sp. (FJ890895) showed no differences between them, and presented a solubilization efficiency approximately 30% of that obtained by the standards. *B. gladioli* (FJ890896) was the isolate that showed the highest efficiency for rock phosphate solubilization (Table-4), but it is cited by Gonzalez *et al*. (2007) as a pathogen particularly to ornamental plants of the genus *Gladiolous.* This could be limiting to its use in sugar cane, since both are monocots belonging to the same Poaceae family.

B. cepacia and *B. ferrariae* showed the best activities of solubilization for all source evaluated. The results obtained by *B. ferrariae* confirm that from Valverde *et al*. (2006), who isolated this organism from

rock phosphate mines and considered it a great potential solubilizer. *Arthrobacter* sp. (FJ890893), *C.protothecoides* (FJ890890), and *F.sancti* (FJ890894) (Table-4) remained lowest rate of phosphorus solubilization. A minority of isolates had a better rate of phosphorus solubilization for rock phosphate of Araxá, as obtained by Massenssini (2007), with their soil isolates under eucalyptus cultivation. Only *Arthrobacter* sp. (FJ890893), *C. protothecoides* (FL890890), *B.gladioli* (FJ890896), and *B.cepacia* showed better solubilization for Araxá rock phosphate (Table-4).

The values of efficiency of solubilization and pH (Table-4) of the standard *B.cepacia* were similar to those obtained for *B.cepacia* DA23 (Song *et al*., 2008). One of the mechanisms used by microorganisms to perform the solubilization occurs by releasing acids that act directly on phosphate mineral acidifying the environment (Silva Filho *et al*., 2002). Besides this work, others have detected the presence of acid phosphatase in bacteria with optimal pH different for that of *Escherichia coli* (pH 2.5) (Touati and Danchin, 1987), *Mycobacterium tuberculosis* (pH 6.5) (Saleh and Belisle, 2000), and *Streptococcus equisimilis* (pH 5.0) (Malke, 1998).

Table-4. Phosphorus solubility quantification and pH values.

Isolates	Phosphorus solubilizing (µg ml^{-1})			pH	
	Catalão	Araxá	Teste F	Catalão	Araxá
Arthrobacter sp.(FJ 890893)	18,802 Bg	22,790 Ai	283,22**	5,23	5,25
Burkholderia sp.(FJ890892)	46,169 Ac	45,443 Bd	9,41**	4,2	4,23
L. portucalensis(FJ890891)	33,081 Af	29,361 Bh	246,42**	4,95	4,9
C.protothecoides(FJ890890)	17,066 Bh	21,395 Aj	333,85**	4,74	4,34
F. sancti (FJ890894)	14,474 Ai	13,691 Bk	10,93**	4,24	4,14
Burkholderia sp.(FJ890895)	32,616 Af	30,986 Bg	47,30**	4,63	4,67
B.gladioli (FJ890896)	58,043Bb	59,703 Ac	49,12**	4,16	4,12
C. B.verschuerenii (FJ890897)	45,955 Ac	45,092 Bd	13,26**	4,39	4,38
E.homaechei subsp. *steirgerwaltii* (FJ890898)	42,215 Ad	40,652 Be	43,47**	4,5	4,61
Enterobacter sp.(FJ890899)	36,609 Ae	35,740 Bf	13,45**	4,3	4,29
B.cepacia[*]	90,876 Ba	97,694 Aa	827,85**	3,67	3,76
B.ferrariae[*]	90,454 Aa	88,273 Bb	63,06**	3,87	3,61
Test F/inital pH	22897,85**	24073,53**	-	6,5	6,4

[*]Standard strains. Same letter coding values in the column (lowercase) and in the lines (uppercase) did not differ significantly at *P* = 0, 05 (Tukey Test).

analysis of in vitro auxin production

From ten selected isolates, only *Enterobacter* sp. (FJ890899), *E. homaechei* subsp. *steigerwaltii* (FJ890898), *Labrys portucalensis* (FJ890891), and *Burkholderia* sp. (FJ890895) showed significant results for the production of auxin (Figure-2). Bacteria from rhizosphere producing auxin may play a key role in plant growth promotion, particularly in the early stages of development and in the process of rooting. It is known that this stimulus is dependent on the doses of the hormone, because an excess can retard, or even inhibit, the growth of the plant (Broek *et al*., 1999). The isolates that showed the best values for auxin production were *E.homaechei* subsp.*verschuerenii* (FJ890898) and *Enterobacter* sp. (FJ890899) (Figures 2A and 2B), obtained from sugarcane crop, with about one year of cultivation, indicating that the hormone may act at different stages of plant development such as tillering and stalk growth.

Indole acetic acid is obtained from the bacterial stationary phase because it is a secondary metabolite (Ceriglioli, 2005), but the duration of the stationary phase depends on each species. Thus, it is necessary to know the behavior of each isolate to take a reading of the synthesis of auxin at different times of bacterial growth, allowing the determination of the period of maximum hormone synthesis. The isolates in this study had a higher IAA at around 3.70 OD for all isolates. However, this growth rate was obtained at different times for each isolate. The isolated *E. homaechei* subsp. *verschuerenii* (FJ890898) produced 152.63 g ml^{-1}IAA at 60h (Figure-2A); *Enterobacter* sp. (FJ890899) accumulated 136.91 mg ml^{-1} IAA at 48 h (Figure-2B); *Labrys portucalense* (FJ890891) produced 17.63 g mL^{-1} IAA at 96 h (Figure-2C), and *Burkholderia* sp. (FJ890895) showed 58.34 g mL^{-1} IAA at 72h (Figure-2D).

L-tryptophan works as physiological precursor for the biosynthesis of auxin in plants and microorganisms

(Khalid *et al.*, 2004). Root exudates are naturally a source of tryptophan (Dakora and Phillips, 2002) for microorganisms, increasing the biosynthesis of auxin in the rhizosphere. Chagas Junior (2007) evaluated the production of IAA in culture medium containing 0, 10, 25, 50, 100 and 150 mg L^{1} of tryptophan to rhizobia population, and concluded that the largest production of IAA was obtained with 150 mg mL^{-1}. Perin (2007) evaluated the production of IAA by bacteria of the genus *Burkholderia*, but none of those isolates produced the plant hormone, indicating that this ability is rare in diazotrophic species of this genus.

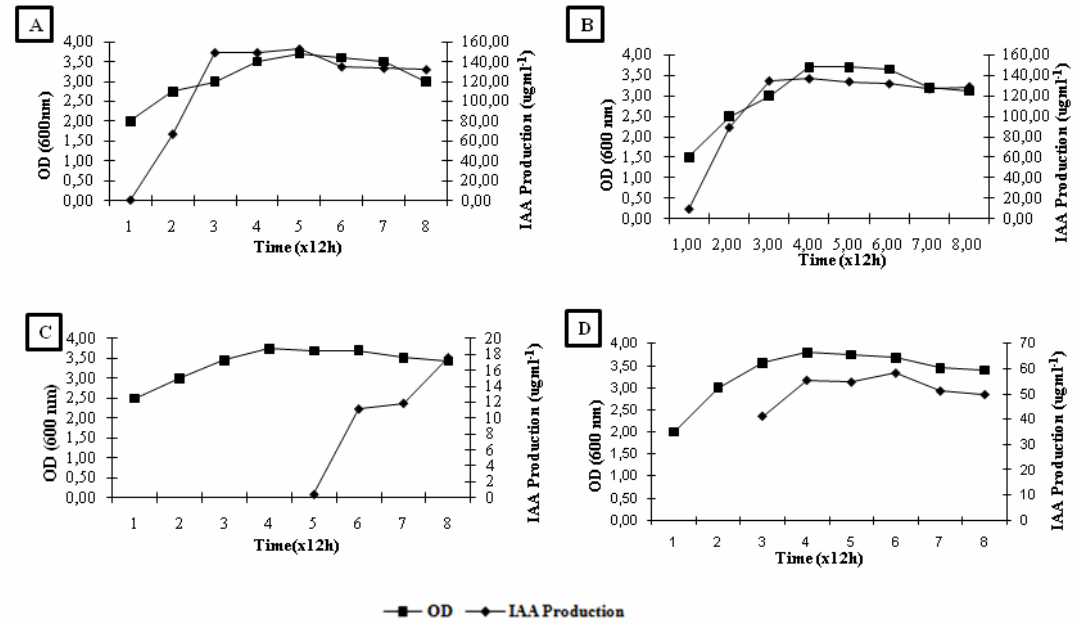

Figure-2. IAA production rate according to growth phase. (A) *E. homaechei* subsp. *verschuerenii* (FJ890898); (B) *Enterobacter* sp. (FJ890899); (C) *Labrys portucalense* (FJ890891) and (D) *Burkholderia* sp. (FJ890895).

CONCLUSIONS

Evaluating phosphorus solubilizing activity, IAA production, and DNA sequencing of *16S rRNA* gene, allowed the detection and identification of the best isolates for plant growth promoting bacteria: *Candidatus Burkholderia verschuerenni* (FJ890897), *Burkholderia* sp. (FJ890892), and *Burkholderia* sp. (FJ890895), without problems with food and environmental contamination.

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