Characterization of *Pantoea* Sp. Strain MK1D, and *Erwinia* Sp. Strain MK2Y as Tricalcium Phosphate Dissolving Bacteria Isolated from Calcareous Soil

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ABSTRACT

This study aimed to isolate and characterize bacterial strains capable of solubilizing tricalcium phosphate (TCP) as the most commonly and insoluble phosphate in soils. Normal and molecular approaches were used to isolate and characterize phosphate dissolving bacteria (PDB) adapted to arid and semi-arid climate conditions. In this study, two strains were successfully isolated and taxonomically classified as *Pantoea* sp. strain MK1D, and *Erwinia* sp. strain MK2Y based on sequences of their 16S rRNA gene and were given GenBank accession numbers KU358676 and KU358677, respectively. This was confirmed by the formation of large halo zones on both Pikovskaya (PVK) and National Botanical Research Institute's Phosphate (NBRIP) culture media. The significant bacterial growth on liquid media was associated with the reduction of pH from 7.0 to 2.0 which lead to 83% solubilization of added TCP of 0.75 g/L. This study confirmed that both PVK and NBRIP media were reliable and comparable for the isolation of PDB and measuring their efficiency. Further studies are needed to confirm the effectiveness of these strains under pot and field conditions and their potential for commercial biofertilizers production.

Keywords: Tricalcium phosphate, *Pantoea* sp., *Erwinia* sp., solubilization, PVK culture, NBRIP culture

INTRODUCTION

Phosphorus (P) is second to nitrogen in amounts needed for optimal plant growth and yield. It is well known that most soils worldwide are rich in total inorganic and organic phosphorus, but are very poor in soluble and available P forms (McLaughlin et al. 2011). This is, in part, due to its precipitation, surface runoff, and adsorption onto soil minerals especially those rich in calcium and magnesium or iron and aluminum in alkaline and acidic soils, respectively (Richardson et al. 2009; McLaughlin et al. 2011). In general, available P concentration in soil solution range from 0.001 to 0.2 µg/mL representing only about 0.1% of total soil P (Brady and Weil 2002). Therefore, an annual application of chemical fertilizers is used to resupply soils with available P. However, about 90% of this annually added P is usually fixed in soils and becomes unavailable for plants and microbial growth (Sharma et al. 2013; Nash et al. 2014). This requires exploration of more efficient fertilizers and application methods, especially in developing countries where the production of low cost P fertilizers is limited. Long term strategies and solutions are needed from all countries to overcome the serious challenge of phosphate depletion and environmental pollution. The application of biofertilizers of effective phosphate solubilizing microorganisms is a promising biological solution to reeducate the depletion rate of RP reserve and potential environmental pollution during production of other fertilizers.

The use of phosphate dissolving bacteria (PDB) is documented to be an effective, environmentally safe, and economically valuable agricultural practice for improving soil P availability for plants (Subbarao 1988; Sharma et al. 2013). An important function of soil microorganisms is to supply nutrients for soil fertility, plant growth, and optimum crop production. Numerous efforts have been made to develop of specific media to isolate microorganisms capable of dissolving fixed or minimally- soluble forms of P (Pikovskaya 1948; Gupta et al. 1994; Nautiyal 1999). Many of these efforts successfully resulted in the isolation of several microbial species capable of dissolving sparingly soluble P forms such as *Acinetobacter* sp., *Klebsiella* sp., *Enterobacter* sp., *Microbacterium* sp., and *Pseudomonas* sp. (Islam et al. 2007). Ruangsanka (2014) recently reported the isolation of *Buttiauxella izardii*, *Enterobacter cancerogenus*, *Burkholderia ubonensis*, *E. hormaechei*, and *Burkholderia pyrrocinia* as PDB. Many other species were found effective as PDB belong to *Pseudomonas*, *Mesorhizobium*, *Bacillus*, *Acetobacter, Burkholderia, Azotobacter, Klebsiella, Bacillus*, *Rhodococcus*, *Arthrobacter*, *Serratia*, *Chryseobacterium*, *Delftia*, *Gordonia*, and *Phyllobacterium* (Pandey et al. 2006; Shin et al. 2015). Fungi such as *Aspergillus*, *Penicillium*, and *Trichoderma* spp. have also been found effective in phosphate solubilization (Illmer and Schinner 1995).

Calcareous soils are known to have a high content of calcium carbonate and low availability of P and micronutrients. Therefore, the isolation and

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characterization of new PDB recently became crucial toward finding effective and highly adapted PDB strains. This might lead to the production of effective commercial biofertilizers suitable for arid and semiarid climate conditions alternative to the expensive and environment damaging inorganic phosphorus fertilizers. The purpose of this study was to use traditional and molecular-based techniques to: 1) isolate and characterize phosphate-solubilizing bacteria from calcareous soils potential for expansion, 2) determine the ability of isolated strains to dissolve tricalcium phosphate as the most dominant inorganic source of phosphorus, and 3) determine possible mechanisms employed by the identified strains to dissolve TCP using two different culture media.

MATERIALS AND METHODS

Culture media

Two different culture media were used to isolate bacteria from soil with the ability to solubilize insoluble inorganic tricalcium phosphate (TCP). Pikovskaya's (PVK) medium (Pikovskaya 1948) contained 10.0 g glucose, 5.0 g $Ca_3(PO_4)_2$, 0.2 g KCl, 0.1 g $MgSO_4.7H_2O$, 0.5 g $(NH_4)_2SO_4$, 0.5 g yeast extract, 0.002 g $MnSO_4.H_2O$, 0.002 g $FeSO_4.7H_2O$ and 16.0 g agar per liter of distilled water. The second medium is the National Botanical Research Institute's Phosphate growth medium (NBRIP) (Nautiyal 1999) which is devoid of yeast extract and contained 10.0 g glucose, 5.0 g $Ca_3(PO_4)_2$, 5.0m g $MgCl_2.6H_2O$, 0.25 g $MgSO_4.7H_2O$, 0.2 g KCl, 0.1 g $(NH_4)_2SO_4$, and 16.0 g agar per liter of distilled water. The pH of both media was adjusted to 7.0 before autoclaving at 121 ^oC for 20 min. Glucose was filter sterilized by using 0.22 µm PFTE filter membranes and added to media after cooling to 45^oC. Both media were used as solid and as liquid to test the effectiveness of isolates qualitatively and quantitatively. The standard medium LB containing glucose (10.0 g), yeast extract (5.0 g), NaCl (5.0 g), and agar (18.0 g) per liter of distilled water was also used. The LB is a rich medium to provide a faster growth and to support an easier morphological distinction among isolates. All chemicals used in this study were reagent grade or better.

Collection and analysis of soil sample

The soil used for bacterial isolation was collected from the surface layer (0-20 cm) using an acid washed stainless steel hand shovel at the Research Farm of Agriculture College, Fuka, Mersa Matrouh, at the North Western Coast of Egypt. For microbiological use, a fresh sample was transported in icebox to the laboratory and kept at 4 ^oC for 24 h before being used for bacterial isolation. A subsample soil was subjected to air drying, mixing, grinding in a porcelain mortar, and sieving (2 mm). Samples were stored at room temperature in tightly closed plastic container before performing physical and chemical analyses. Composite soil sample was subjected to analysis following standard methods (Olsen et al. 1954; Page et al. 1982; Gee and Bauder 1986).

Isolation of phosphate-solubilizing bacteria by direct plating technique

Direct pour-plating method was used to isolate bacteria dissolving insoluble TCP from soil under investigation. Up to 10^{-5} Ten-fold serial dilutions of soil were made using 0.85% sterile NaCl solution and pour-plated onto PVK medium initially. Plates were inversely incubated at 28 ^oC until large clearing zones were recognized in 3-7 days. Colonies forming halo zones were transferred and streaked onto solid PVK media for phosphate dissolving confirmation and for purification. Isolates were alternatively and successfully streaked onto solid LB and PVK media back and forth to distinguish colonies morphologically. This process was repeated 5-6 times to ensure purity of isolates and to confirm that they sustaining their ability to dissolve insoluble forms of phosphate. Morphological features, including colony size, shape, color, and transparency were used to distinguish potentially different isolates.

Confirmation and solubility index calculation

In addition to PVK, the NBRIP medium was used to confirm the capability of purified isolates to dissolve TCP. Single colonies of purified isolates were transferred by sterilized wood toothpicks and simultaneously stabbed into both solid PVK and NBRIP media. Plates were incubated at 28 ^oC and colonies monitored for the appearance of halo zones. Colony and halo zone diameters of each isolate, in both media, were measured daily and the solubility index was calculated by using the following equation (Edi Premono et al., 1996):

$$PSI = HalZd/Clnd \quad \text{[Eq.1]}$$

where *PSI* is the solubility index, *HalZ*d is the halo zone diameter (mm), and *Cln*d is the colony diameter (mm).

Strains showing a high solubility index were selected for further study. Pure cultures were transferred to LB slants and kept in the refrigerator at 4 ^oC to be used for further studies. Pure cultures were also preserved in 20% sterile glycerol and stored at -70 °C for long term storage. Unique strains were verified to dissolve inorganic precipitated phosphorus (TCP) in liquid NBRIP cultures as described below.

Quantitative measurements for phosphate solubilization and bacterial growth

The efficiency of unique PDB strains isolated from calcareous soil in dissolving precipitated inorganic phosphorus was determined quantitatively by dissolving TCP in liquid broth. Strains were grown in NBRIP liquid medium, with three concentrations of TCP; 0.75, 2.0, and 5.0 g/L. This experiment was conducted in 250-mL Erlenmeyer flasks containing 100 mL of sterilized NBRIP medium and incubated at 28 ^{o}C for 15 d, on a rotary shaker at 150 rpm. Triplicate flasks were sampled nearly every other day under aseptic conditions for measurement of pH, optical density at 600 nm (OD_{600}), and released soluble P. Culture pH was measured in the suspension without centrifugation or filtration. To measure OD_{600}, decanted samples were left to stand for 1 h to allow any insoluble TCP precipitate to settle out of solution and the upper solution was used for measurement of OD_{600}. To measure released P, insoluble TCP and cell debris were pelleted by centrifugation at 6,000 xg for 15 min and the supernatant was used for the determination of soluble P in solution using the colorimetric blue ammonium molybdate method with measurement at 882 nm (Murphy and Riley 1962). The quantities of released P in supernatant were determined using a KH_2PO_4 standard curve with concentrations ranging from 0 to 6 mg P/L. Samples higher than that range were diluted to fit the range. Dilution factor was used to calculate the concentration of P.

Molecular identification of unique PDB isolates

The 16S rRNA gene of morphologically distinct isolates was amplified, purified, and sequenced to identify unique PDB bacterial isolates. A single colony of each isolate was selected, picked with a 1-µl sterile inoculating loop (Fisher Scientific, Pittsburgh, PA), and suspended in 100 µl of sterile solution of 0.05 M NaOH in 1 ml Eppendorf tube. Tubes were boiled (99 to 100 ^{o}C) for 15 minutes for cell lysis. Tubes were centrifuged for 10 min at 6,000 rpm to separate DNA-containing supernatant from cell debris. A 2 µl aliquot of the supernatants were used as DNA templates for PCR reactions for 16S rRNA gene amplification (Weisburg *et al.*1991). The forward primer 27f (5`-AGAGTTTGATCMTGGCTCAG-3`) and reverse primer 1492r (5′-CTACGGCTACCTTGTTACGA-3′) (Integrated DNA Technologies, Coralville, Iowa) were used. PCR mixture (50 µl) contained sterilized Millipore nuclease free water (38.5 µl), 10X PCR buffer (5 µl), dNTPs (2 µl), 27f primer (1 µl), 1492r primer (1 µl), DNA template (2 µl), and Choice *Taq* Polymerase (0.5 µl). PCR was performed using an MJ research PTC 100 (MJ Research, Waltham, Mass., USA) thermocycler. The PCR program was as follow: initial denaturing step for 95 ^{o}C for 10 min, followed by 34 cycles of 95 ^{o}C for 30 sec, 57 ^{o}C for 45 sec, and 72 ^{o}C for 1.5 min with a final extension step for 6 min at 72 ^{o}C in an automated thermal cycler then a storing step at 4 ^{o}C. The PCR products were examined for successful amplification by using gel electrophoresis as described elsewhere (Kandil *et al*. 2015).

Purification and sequencing of 16S rRNA gene

The confirmed 16S rRNA PCR products were purified by using QIAquick PCR purification Kit (Qiagen, Valencia, CA, USA), and the concentration of the purified DNA was determined by Qubit 2.0 fluorometer (Invitrogen, Life technologies, Carlsbad, CA). Samples were submitted to ACGT, Inc (Wheeling, IL) for Sanger sequencing of both DNA strands, using the 27f and 1492r primers. Consensus sequence *contigs* of both strands was generated using *BioEdit* software (Hall 1999) and compared with GenBank database of nucleotides by using the BLASTn algorithm (Altschul *et al.* 1990). A Phylogenetic tree was generated for interrelationships among the strains using MEGA7software (Kumar *et al*. 2016).

RERSULTS AND DISCUSSION

Successful Isolation of PDB from Calcareous Soil

Based on soil analysis, sample was classified as sandy clay loam (61% sand, 24% silt and 15% clay) with a pH 8.28 (2:1, water:soil), electrical conductivity (EC) 2.32 mS/Cm (2:1, water:soil), organic matter 0.17%, 5.7 ppm available P, and 26.7 % total carbonate. Soil of this region is classified as Calcaric Fluvisols and Calcic Yermosols (El-Nahrawy, 2011). Using direct plating methods described above, several halo zones were recognized on PVK plates. Initially, these halo zones contained several mixed bacterial cultures and were transferred to fresh PVK plates for purifications. At the same time, several colonies appeared on PVK but without forming clearing zones and thus were eliminated. For purification, colonies clearing TCP were transferred and streaked back and forth onto fresh PVK and LB plates alternately several times for fast growth and distinct morphological features. Figure 1A shows some halo zones surrounding colonies as they grow on PVK medium (left plate, Fig. 1A). These colonies were streaked later onto NBRIP solid medium and showed a full clearing of TCP precipitate (right plate, Fig.1B).

Selection of highly effective isolates based on solubility index

Based on the diameters of both halo zone and colony, phosphate solubility index (*PSI*) was calculated as described above by Eq.1. Data showed that solubility index varied among isolates, incubation time, and

media. As shown in Fig. 2, although isolates showed higher *PSI* values on PVK medium that that on NBRIP, both media were reliable and effective for the selection and comparison of highly effective PDB strains. Also, data showed that solubility index for each strain was not a fixed value but have changed over time during incubation. Data showed that an incubation period of 4-5 days is reasonable for the selection of potential PDB strains and there was no need to wait for more days to identify effective isolates.

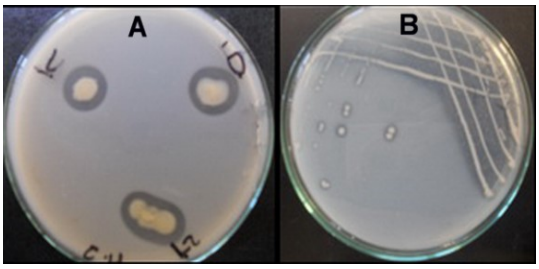

Fig. 1 (A). Halo zones formed by the growth of the isolates on PVK medium supplemented with TCP(5.0g/L) and (B) solubilization of TCP (5.0 g/L) shown as cleared streaked area of NBRIP plate by one of the isolates

Quantitative Measurements for Phosphate Solubilization and Bacterial Growth

Since the solubility index is a qualitative indicator as it varies, based on incubation time, strain, and media, liquid media must be used to quantitatively examine the effectiveness of isolates to solubilize phosphate and determine mechanism(s) by which this is occurring. Results in Figs. 3, 4, and 5 show the growth curves (OD_{600}) of these isolates, their effect on pH of inoculated culture media, and concentration of released soluble P, respectively. Results in Fig. 3 showes that while growth curves had a standard sigmoidal shape, isolates differed in their maximum growth. As shown in Fig.4, growth of isolates was concomitant with a significant reduction of the culture pH from 7 to 2. Ph is the most important factor controlling the solubility and availability of P in cultures and soils. Concurrent with the reduction in pH, there was a significant increase in solublizied P up to 83% comapred to the uninoculated control treatment (Fig. 5). Among isolates tested, strain MK1D showed the greatest growth concurrent with the lowest pH and the greatest amount of solubilized P. Several other isolates were eliminated for further studies due its limited amount of released P.

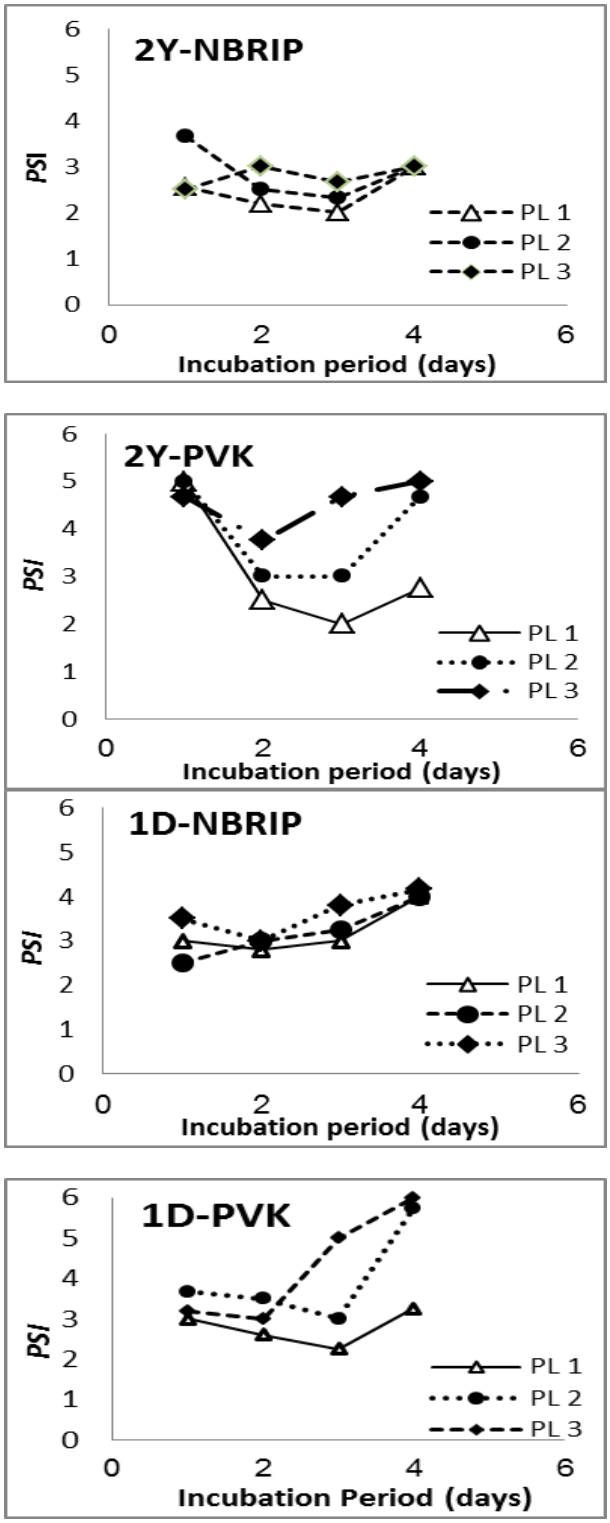

Fig. 2. Phosphate solubility index (*PSI*) for isolates MKID and MK2Y cultured on both PVK and NBRIP as labeled. Diameters of halo zones and colonies were used for *PSI* calculation as *in Eq.1*. Each plate (PL) was treated as a treatment replicate

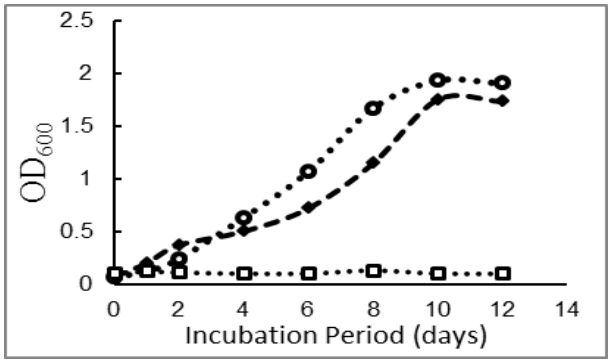

Fig. 3. The growth of strains *Pantoea* sp. strain MKID (♦) and *Erwinia* sp. strain MK2Y (○) on NBRIP liquid medium supplemented with 750 µg/mL TCP against Control (□)

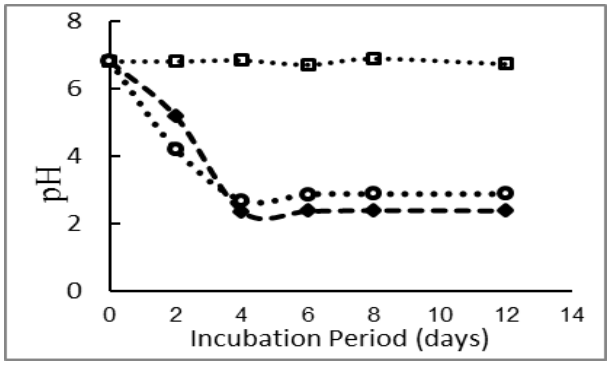

Fig. 4. Changes of culture pH due to the of growth of strains *Pantoea* sp. strain MKID (♦) and *Erwinia* sp. strain MK2Y (○) on NBRIP liquid medium supplemented with 750 µg/mL TCP; Control (□)

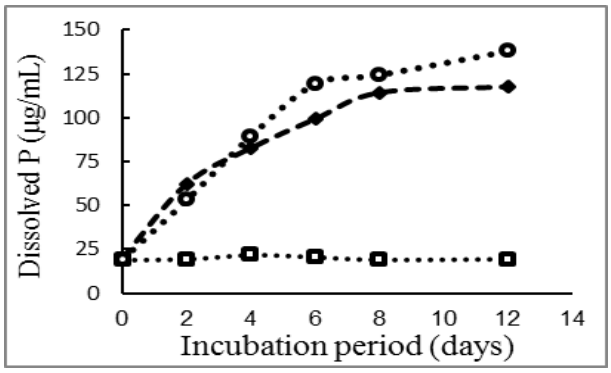

Fig. 5. Solubilization of TCP and amounts of released P during the growth of strains *Pantoea* sp. strain MKID (♦) and *Erwinia* sp. strain MK2Y (○) on NBRIP liquid medium supplemented with 750 µg/mL TCP compared to control (□)

Taxonomic Identification of isolated PDB Strains

After confirming the effectiveness of these isolates in dissolving TCP, the taxonomic identity of the most efficient TCP-solubilizing isolates was determined by the analysis of almost full length sequence of their 16S rRNA gene. Based on BLASTn searches, the sequences of the 16S rRNA genes of the isolates ranged from 97% to 99% identical to several strains in genomic database. Using 16S rRNA sequences of the isolated strains and their closely related species, phylogenetic tree was constructed (Figs. 6 & 7) based on Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method and rooted to an *E coli* strain. The 16S rRNA gene from isolate MK1D was 99% identical to *Pantoea* sp. B21 (JX010971.1), *Pantoea* sp. B28 (KF479585.1), *Pantoea agglomerans* Ast1 (GU204957.1), and *Pantoea agglomerans* strain BJ-Tobacco. As shown in Fig. 7, isolate MK2Y was found to be 99% identical to *Erwinia persicina* WD1608 (EU681952.1), *Erwinia* sp. UIWRF0140 (KR189887.1), and *Erwinia persicina* S9 (KP715898.1). This is one of the few studies reporting that some strains of *Erwinia* spp. are characterized as phosphate dissolving bacteria (Rodríguez et al. 2000; Ivanova et al. 2006). Evolutionary relationships of taxa for *Pantoea* sp. strain MKID (Fig. 6) and for *Erwinia* sp. strain MK2Y (Fig. 7) were inferred using the UPGMA method (Sneath and Sokal 1973). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) is shown next to the branches (Felsenstein 1985). All positions containing gaps and missing data were eliminated. The evolutionary distances were computed using the Jukes-Cantor method (Jukes and Cantor 1969). Evolutionary analyses were conducted in MEGA7 (Kumar et al. 2016). Both trees were rooted to *E coli* (KP789329.1).

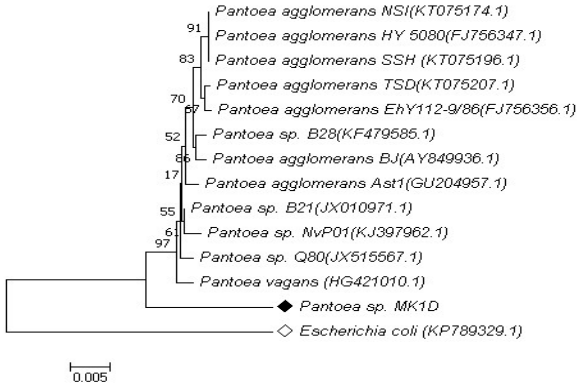

Fig. 6. Evolutionary relationships of taxa for *Pantoea* sp. strain MKID. The evolutionary history was inferred using the UPGMA method. The optimal tree with the sum of branch length = 0.08470423 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The analysis involved 14 nucleotide sequences. There were a total of 1428 positions in the final dataset

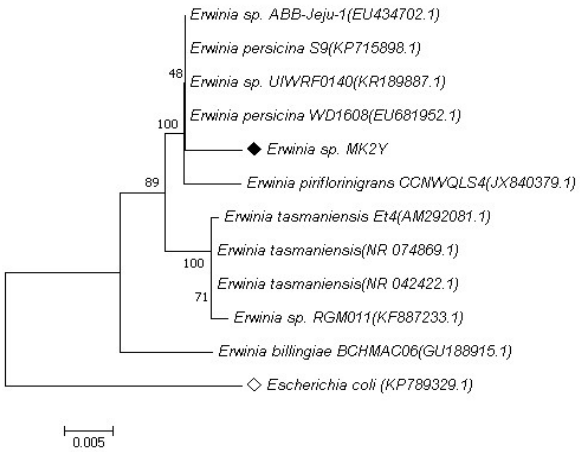

Fig. 7. Evolutionary relationships of taxa for *Erwinia* sp. strain MK2Y. The evolutionary history was inferred using the UPGMA method. The optimal tree with the sum of branch length = 0.07062163 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The analysis involved 12 nucleotide sequences. There were a total of 1207 positions in the final dataset

CONCLUSIONS

In this study, two bacterial strains were isolated from calcareous soil located at Fuka Agricultural Research Farm, Mersa Matruh and were confirmed to be highly effective in solubilizing TCP, the most dominant and insoluble inorganic forms of phosphorus in calcareous soils. Based on nearly full length analysis of 16S rRNA genes, these strains were taxonomically identified as *Pantoea* sp. strain MK1D and *Erwinia* sp. strain MK2Y. This is especially important for soils with poor conditions such as calcareous soils under arid and semi-arid climate conditions where these strains are well adapted. However, for the production of highly effective phosphate biofertilizers, further pot and field studies are needed.

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الملخص العربي

توصيف سلالتي *Pantoea* Sp. Strain MK1D و *Erwinia* Sp. Strain MK2Y المعزولة من التربة الجيرية كبكتيريا مذيبة لفوسفات ثلاثي الكالسيوم

محروس قنديل

تهدف هذه الدراسة إلى عزل وتوصيف السلالات البكتيرية القادرة على إذابة فوسفات ثلاثي الكالسيوم باعتباره أكثر صور الفوسفات شيوعاً وغير القابلة للذوبان في التربة. كما تهدف الدراسة أيضاً مقارنة كفاءة البيئة المغذية الإختيارية والتفريقية الأكثر إستخداماً في هذا المجال (بيئة بيكوفسكايا، PVK) مع بيئة المعهد الوطني للبحوث النباتية بالهند (NBRIP) المستحدثة. وقد أستخدمت الوسائل العادية وكذلك طرق الهندسةالجزيئية لعزل وتوصيف البكتيريا المذيبة للفوسفات والتي تتكيف مع الظروف المناخية للمناطق الجافة وشبه الجافة. هذا وقد تم عزل سلالتين بنجاح وتصنيفها تصنيفاً علمياً سليماً على أساس التسلسل النيوكلوتيدي لجين 16S rRNA الريبوسومي لكلاهما وسميتا *Pantoea* sp. strain MK1D, and *Erwinia* sp. strain MK2Y .كماتم تسجيل هاتين السلالتين في بنك الجينات العالمي GenBank وأعطيتا أرقام الانضمام KU358676 و KU358677، على التوالي. وقد تأكد قدرة السلالتين عل إذابة فوسفات ثلاثي الكالسيوم المترسب في البيئة من خلال تكوين مناطق رائقة كبيرة واضحة حول مستعمراتها عند نموها على كلٍ من بيئة بيكوفسكايا (PVK) وبيئة المعهد الوطني للبحوث النباتية بالهند (NBRIP). وقد اظهرت هاتين السلالتين نمواً كبيراً ومعنوياً على هذه البيئات السائلة. كما صاحب هذا النمو البكتيري الكبير إنخفاض الرقم الهيدروجيني من ٧,٠ إلى ٢,٠ مما أدي إلى إذابة ٨٣٪ من فوسفات ثلاثي الكالسيوم المضاف بتركيز ٠,٧٥ جرام/لتر. وأكدت هذه الدراسة أن كلاً من البيئتين PVK وNBRIP مناسباً وفعالاً لعزل السلالات البكتيرية القادرة على إذابة فوسفات ثلاثي الكالسيوم وكذلك قياس كفاءتها. وبالرغم من نجاح هذه الدراسة كخطوة أولى، فإن هناك حاجةً مستقبليةً إلى مزيدٍ من الدراسات للتأكد من فعالية هذه السلالات تحت ظروف الصوب الزراعية والحقلية وإمكانية إنتاجهاضمن الأسمدة الحيوية التجارية.