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Plant Growth Promoting Traits of Phosphate Solubilizing Bacteria Isolated from Agricultural Lands in Sothern Sri Lanka

B.C. Walpola* and R.H.A.N. Hettiarachchi

ABSTRACT

Inorganic phosphate solubilization by phosphate solubilizing bacteria (PSB) is known to be one of the major mechanisms associated with plant growth promotion. The use of plant growth-promoting bacteria as bio-inoculants/bio-fertilizers is thus considered to be an environmentally friendly approach of soil enrichment. Fifteen PSB from different agricultural lands including terrestrial, aqua and swamp regions of Southern Sri Lanka were isolated using serial dilution plating on NBRIP agar plates and screened for various plant growth-promoting traits. The highest phosphate solubilization (1127 µg/ml) was exhibited in PSB-14 which was identified as *Enterobacter* sp. IAA production was detected by 6 strains and among them, bacterial isolate PSB-12 showed the highest IAA production (24.2 µg/ml) followed by PSB-7 and PSB-1 (21.3 and 20.9 µg/ml respectively). Except for PSB-8 and PSB-13 strains, all the other isolated strains showed a positive response for ammonia production. Except for PSB-2, PSB-7, PSB-10 and PSB-11, all the other studied isolates showed HCN production. PSB-3, PSB-9 and PSB-14 isolates were found to be strong HCN producers. All the strains showed catalase activity implying that they were capable of growing under stress conditions. PSB-1, PSB-12, PSB-14 and PSB-15 strains showed all the tested plant growth promotion traits.

Keywords: IAA production, Plant growth-promoting rhizobacteria, Phosphate solubilizing bacteria, Phosphate solubilization

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INTRODUCTION

Phosphorus (P) is known to be a major macronutrient having a defined role in the growth and development of plants (Awasthi *et al*., 2011). Though the majority of agricultural soils contain a high reserve of P, the concentration of available P in soil is generally very low (0.4-1.2 g/kg) (Fernández *et al*., 2014; Joe *et al*., 2018). Furthermore, a certain portion of the applied phosphorus fertilizers is immobilized into insoluble forms with Al or Fe in acid soils or with Ca in calcareous soils (Mundra *et al*.,

2011). In this context, the role of phosphate solubilizing microorganisms (PSMs) is important as they are effectively involved in the solubilization of insoluble inorganic phosphates (Khan *et al*., 2010). Phosphorus-solubilizing activity is considered to be the most important among the multiple properties of the soil microorganisms that promote plant growth and nutrient absorption.

In addition, they enhance plant growth via synthesis of phytohormones such as auxins (Jeon *et al*., 2003; Egamberdiyeva, 2005), cytokinins (Gracia de Salamone *et al*., 2001), and gibberellins (Gutierrez-Manero *et al*., 2001) or other growth-promoting or protecting substances like siderophores (Wani *et al*., 2007), hydrogen cyanide (Kang *et al*., 2010), enzymes and/or fungicidal compounds such as chitinase, cellulose, protease (Dey *et al*., 2004; Lucy *et al*., 2004; Hamdali *et al*., 2008) which ensure antagonism against phytopathogenic microorganisms. Among plant growth-promoting bacterial strains, rhizobia are involved in the symbiotic fixation of atmospheric nitrogen with legumes (Satyaprakash *et al*., 2017). Therefore, PSMs (Phosphate solubilizing microorganisms) may effectively contribute towards the enhancement of the plant performance while improving the efficiency of chemical fertilizers (Bechtaoui *et al*., 2019). Bacterial species belonging to genera *Pseudomonas, Azospirillum, Azotobacter, Klebsiella, Enterobacter, Alcaligenes, Arthrobacter, Burkholderia, Bacillus* and *Serratia* are well-known phosphate solubilizers (Satyaprakash *et al*., 2017). Chamikara (2019) isolated *Pseudomonas aeruginosa*, *Achromobacter xylosoxidans* and *Pseudomonas fluorescens, Bacillus subtilis* respectively from Aloe vera and mung bean cultivated soils in Sri Lanka.

In Sri Lanka, the available information about the phosphate solubilizing plant growth-promoting rhizobacteria is generally poor. In this context, the objectives of the present study were to isolate phosphate solubilizing bacterial strains and assess their plant growth-promoting characteristics.

MATERIALS AND METHODS

Isolation of Strains and Phosphate Solubilization

Soils used in this study to isolate phosphate solubilizing microorganisms were locally collected from rhizosphere soil samples from different agricultural

lands in Southern Sri Lanka (Latitude: from 6.009460N to 6.163220N and longitude: from 80.473530E to 80.676000E). Each rhizosphere soil sample was collected by carefully uprooting a plant and shaking the soil adhering to the roots into a sterile polythene bag. The soil samples were immediately transported to the laboratory for further study.

A 10 g of field moist soil from each soil sample was weighed and transferred to a 250 ml Erlenmeyer flask containing sterilized 90 ml of 0.85% NaCl solution. The mixture was then shaken for 30 minutes at approximately 150 rpm. Immediately after shaking, a series of tenfold dilutions of the suspension was made by pipetting 1 ml aliquots into sterilized 9 ml of 0.85% NaCl solution. Aliquots of 0.1 ml of the sample from each of these dilutions were spread on to a petri dish with National Botanical Research Institute Phosphorus (NBRIP) medium containing 10 g of glucose, 5 g of $Ca_3(PO_4)_2$, 5 g of $MgCl_2.6H_2O$, 0.25 g of $MgSO_4.7H_2O$, 0.2 g of KCl and 0.1 g of $(NH_4)2SO_4$ in 1 L distilled water (Nautiyal, 1999). The pH of the media was adjusted to 7 using HCl. The plates were incubated for 7 days in an incubator at 30 ^{0}C. The colonies with clear halos are considered to be phosphate solubilizing colonies. Predominant colonies were further purified by re-streaking on the fresh NBRIP agar plates at 30 ^{0}C. Fifteen bacterial strains that exhibited large clear zones on the agar plates were selected as phosphorus solubilizing strains for further study (Figure 1).

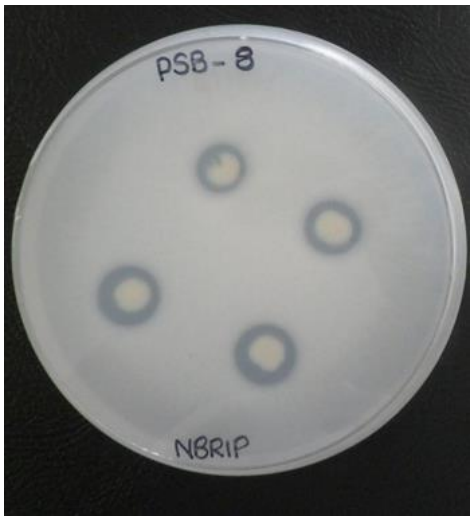


Figure 1. Isolated phosphate solubilizing bacterial strain (PSB-8) showing clear halos produced in NBRIP solid medium.

Isolated bacterial strains were inoculated on agar media and cultured for 48 h at 30 ^{0}C. The bacterial colony shape and colour were observed to study the morphological aspects of the colonies. Gram staining of purified PSB was performed according to the Vincent method (Vincent, 1970) and

was observed under a microscope (VanGuard 1400 series) (Figure 2).

Bacterial strains were grown in sterilized liquid NBRIP medium (20 ml) at 30 ^{0}C for 2 days with continuous shaking at 150 rpm. Aliquots of culture (1 ml) were then transferred to a 500 ml flask (n=3 per strain) containing sterilized liquid NBRIP medium (200 ml) and incubated for 7 days with continuous shaking at 30 ^{0}C. Sterilized un-inoculated medium served as a control. A sample (10 ml) of each cultured and control were taken daily and centrifuged at 8000 rpm for 15 min.

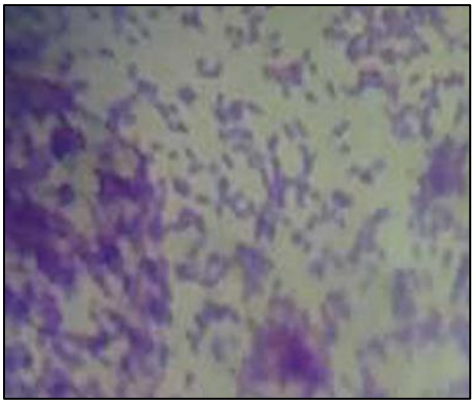


Figure 2. Gram-positive phosphate solubilizing bacterial isolate (PSB-9).

The clear supernatant was used in determining the amount of phosphorous released into the medium according to the phospho-molybdate blue color method (Murphy and Riley, 1962).

Plant Growth-promoting Traits of Isolated PSB

Production of Indole Acetic Acid

Bacterial strains were grown in sterilized 100 ml liquid NBRIP media containing 1 ml of 0.2% tryptophan and incubated for 72 hrs with continuous shaking at 30 ℃. A sterilized uninoculated medium was served as the control. Each was taken into a centrifugation tube every 24 hrs and centrifuged 10 min at 12000 rpm. The clear supernatant was used to determine IAA production as described by Gutierrez *et al*. (2009). Clear supernatant of 1 ml was mixed with 4 ml of the Salkowski's reagent (50 ml of 35% perchloric acid and 1 ml of 0.05 M $FeCl_3$ solution). The mixture was incubated in the dark at 37 ℃ for 30 minutes. Development of pink colour indicated IAA production and optical density was taken at 530 nm using UV spectrophotometer (Shimadzu-UV mini 1240). The concentration of IAA produced by cultures was measured with the help of a standard graph of IAA obtained in the range of 10 to 100 $\mu g\ ml^{-1}$.

Production of Ammonia

The bacterial isolates were tested for the production of ammonia in peptone water. Fresh cultures were inoculated into 10 ml peptone water and incubated for 48 hrs at 30℃. Nessler's reagent (0.5 ml) was added to each bacterial suspension. The development of brown to yellow colour was considered to be a positive response for ammonia production (Cappucino and Sherman, 1992).

Production of Hydrogen Cyanide

HCN production was tested by growing bacteria in the 10% tryptic soy agar (TSA) supplemented with glycine (4.4 g L^{-1}). A filter paper previously soaked in picric acid and Na_2CO_3 (0.5% and 2%, respectively) solution was fixed onto the underside of the lids of plates and incubated for 5 days at 30 ℃. A change in filter paper colour from yellow to orange-brown was considered to be the indication of HCN production (Donate-Correa *et al*., 2004).

Catalase Activity

Isolates were grown on nutrient agar media at 30 ℃ for 24-48 hrs. A loop-full of each culture was mixed with 50 µl of 3% (v/v) hydrogen peroxide (H_2O_2) on a glass slide and incubated at room temperature for 1 min to observe the evolution of oxygen which was recorded as positive for catalase reaction (Chaiharn and Lumyong, 2009).

Each of the above tests was based on three repeat experiments and triplicated per bacterium.

Identification of the Best Bacterial Strain

Partial sequencing of 16S rRNA for the bacterial strain was performed with the help of a DNA sequencing service (Solgent, Daejeon, South Korea) with universal primers, 27F (5′-AGAGTTTGATCCTGGCTCAG -3′), and 1492R (5′-GGTTACCTTGTT ACGACTT-3′) (Weisburg *et al*., 1991). The online program BLAST (NCBI, 2019) was used in identifying the related sequences with known taxonomic information available at the databank of the National Center for Biotechnology Information (NCBI, Bethesda, Maryland, USA). A phylogenetic tree was constructed with the CLUSTAL X program (Thompson *et al*., 1997), which involved sequence alignment by the neighbour-joining

method (Saitou and Nei, 1987) and maximum parsimony with the MEGA4 program (Kumar *et al*., 2001). The grouping of sequences was based on confidence values obtained by bootstrap analysis of 1000 replicates. Gaps were edited in the BioEdit program (Hall, 1999) and evolutionary distances were calculated by Kimura's two-parameter model (Kimura, 1980). Reference sequences were retrieved from GenBank under the accession numbers indicated in the trees.

Statistical Analysis

Data were subjected to ANOVA with the SAS package (SAS Institute, 1999).

RESULTS AND DISCUSSION

Isolation of Strains and Phosphate Solubilization

A total of 15 bacterial isolates (PSB-1 to PSB-15) that exhibited clear zones around the colony after 3 days of incubation were selected as phosphate-solubilizing organisms. All the isolated PSB strains were odourless, had rod and round raised colonies with a smooth and shiny surface. Most of them had white colour colonies except PSB-5, PSB-6, PSB-13 and PSB-15 which had yellow colour colonies. Out of 15 bacterial isolates, 8 isolates (PSB-3, PSB-4, PSB-7, PSB-9, PSB-10, PSB-11, PSB-12, PSB-14) showed positive response for gram staining.

To authenticate the isolated PSB, which formed a clear zone around the colonies, their phosphate solubilization ability was measured using a liquid NBRIP medium. Significantly higher amount of phosphate solubilization was shown in PSB-1 (916 μg/ml), PSB-3 (1030 μg/ml), PSB-9 (925 μg/ml) and PSB=14 (1127 μg/ml) compared to all the other bacterial strains after 5 days of incubation (Table 1). A rapid increase of available phosphorus contents in the liquid medium was observed during the first 5 days of the incubation followed by a gradual decrease towards the end of the incubation. In the case of control, no phosphate solubilization was observed throughout the incubation period. The phosphate solubilization is associated with the production of low molecular weight organic acids such as gluconic acids, oxalic acids, citric acids, succinic acids, etc. (Chaiharn and Lumyong, 2009; Gulati *et al*., 2010). The reduction of available phosphorous content in later stages of the incubation may be due to consumption of the available

phosphorous or depletion of nutrients in the culture medium, in particular, carbon source that is essential for the production of organic acids and microbial activity (Chaiharn and Lumyong, 2009).

However, as reported by Varsha-Narsian *et al*. (1994), the availability of soluble phosphorus in the culture medium might also have an inhibitory effect on further phosphate solubilization. Excretory toxic products may also responsible for such reduction in P-solubilization.

Production of Indole Acetic Acid

Besides phosphate solubilization, the production of phytohormones by phosphate solubilizing microorganisms in the soil can promote plant growth. As stated by previous studies, some strains of PSB can produce a relatively high amount of IAA in a nutrient broth medium supplemented with tryptophan (Nacoon *et al*., 2020; Gusain *et al*., 2015; Walpola and Arunakumara, 2015). Among the phytohormones, the auxin IAA, applied at low concentrations, is known to stimulate

Table 1. Quantity of phosphate solubilization by phosphate solubilizing bacteria at 1, 3, 5 and 7 days after inoculation.

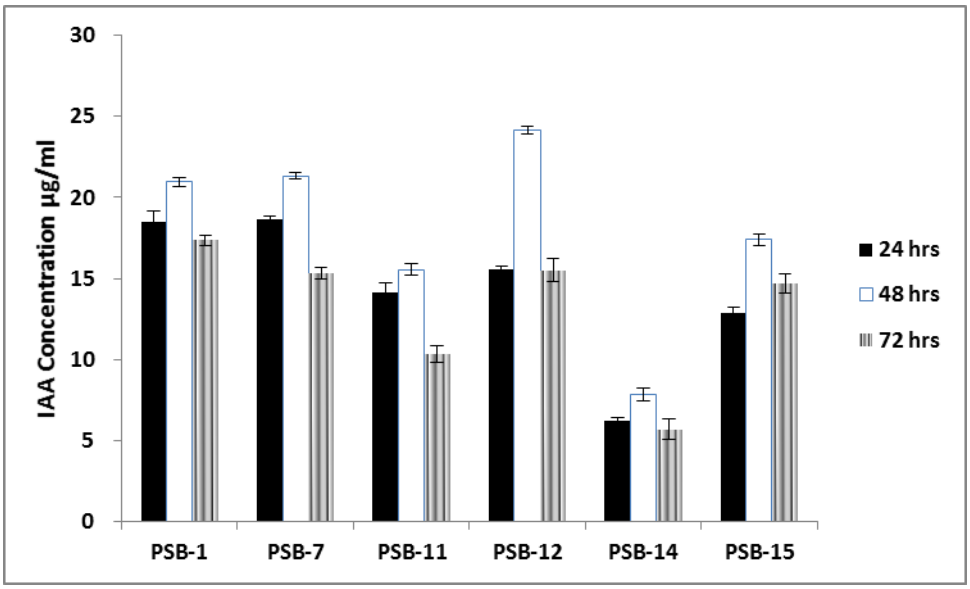
	Phosphate solubilization (µg/ml) Day 1	**Phosphate solubilization (µg/ml) Day 3**	**Phosphate solubilization (µg/ml) Day 5**	**Phosphate solubilization (µg/ml) Day 7**
PSB 1	98.9±13.4	379.2±15.6	916.3±16.4	748.8±15.4
PSB 2	82.9±13.1	279.9±13.9	456.1±14.5	358.5±13.8
PSB 3	101.7±13.5	410.2±14.2	1030.3±11.3	973.5±10.2
PSB 4	102.2±13.8	255.4±13.5	869.1±13.9	749.4±18.5
PSB 5	95.1±12.5	509.7±14.6	767.6±14.1	707.1±14.8
PSB 6	54.4±12.1	472.3±14.1	741.9±15.1	678.5±13.8
PSB 7	62.4±8.9	506.8±15.4	654.9±11.8	586.8±9.5
PSB 8	60.4±9.7	611.0±14.5	850.9±12.4	784.6±9.5
PSB 9	68.1±8.5	598.7±14.6	925.2±9.4	846.6±11.5
PSB 10	87.4±7.5	466.2±13.4	873.9±8.9	811.8±12.6
PSB 11	44.0±5.7	502.5±12.4	777.9±10.4	706.7±8.4
PSB 12	88.9±9.4	417.6±11.4	623.4±11.7	547.8±7.8
PSB 13	62.9±7.8	589.4±15.6	874.3±13.5	794.5±8.9
PSB 14	77.5±8.8	651.8±15.7	1127.0±14.3	1036.7±14.8
PSB 15	59.3±6.7	431.9±14.5	356.8±8.5	316.3±7.8

Values given here are the means (n = 3) ± standard deviation.

both a rapid response (e.g. increased cell elongation) and a long-term response (e.g. cell division and differentiation) in plants (Elhaissoufi *et al*., 2020). The amount of IAA produced by plant growth-promoting bacteria can vary among different species and strains, and it is also influenced by culture condition, growth stage and availability of substrates such as amino acids (Gusain *et al*., 2015). The IAA production by isolated phosphate solubilizing microorganisms is shown in Figure 3. Out of 15 isolates, 6 bacterial isolates were able to produce IAA in a nutrient broth medium supplemented with tryptophan. The bacterial isolate PSB-12 showed the highest IAA production (24.2 µg/ml) followed by PSB-7 and PSB-1 (21.3 and 20.9 µg/ml respectively).

In spite of being good IAA producers, these three bacterial strains (PSB-12, PSB-7 and PSB-1) efficiently solubilized inorganic phosphate in NBRIP culture medium.

All the isolates exhibited the highest IAA production within 24 - 48 hrs followed by reduction as time progressed. Previous studies by Nutaratat *et al*. (2017), Ozdal *et al*. (2016) and Swain *et al*. (2007) also observed the maximum IAA production during the



Values given here are the means (n = 3) ± standard deviation

Figure 3. IAA production of isolated phosphorus solubilizing bacterial strains.

stationary phase of the growth where the production occurred when the growth reached the maximal level, though the duration of the stationary phase depends on the species (Nutaratat *et al*., 2017).

The increase of IAA production during the early stages of the incubation may be attributed to the greater availability of the precursor as reported by Bharucha *et al*. (2013) who also observed increased IAA production up to 96 hours. The reduction in IAA production in later stages might be due to the release of IAA degrading enzymes such as IAA oxidase and peroxidase by the bacteria as reported by Bharucha *et al*. (2013).

Production of Ammonia

Among the plant growth-promoting activities, ammonia production also plays an important role in plant growth by the accumulation of nitrogen and helps in promoting root and shoot growth and biomass production indirectly (Marques *et al*., 2010). Production of this secondary metabolite was found in >85% of the phosphate solubilizing isolates) except PSB-8 and PSB-13 (Table 2). The isolates PSB-5 showed the maximum ammonia production followed by PSB-14, PSB-6 and PSB-4. It was reported that the majority of rhizosphere associated bacteria (Rhizobacteria) possessed ammonia production (Thakur and Parikh, 2018). This observation is consistent with earlier reports, as all *Serratia* strains isolated from maize (*Zea mays* L.) rhizosphere (Agbodjato *et al*., 2015) and all *Bacillus* and *Pseudomonas* strains isolated from chickpea rhizosphere (Yadav *et al*., 2010) had shown positive response for ammonia production.

Production of Hydrogen Cyanide

Except for PSB-2, PSB-7, PSB-10 and PSB-11, all the studied isolates showed HCN production. PSB-3, PSB-9 and PSB-14 isolates were found to be strong HCN producers as they produced a brown colour in filter paper disc during assay for HCN production (Figure 4).
By synthesizing HCN, some bacteria inhibit plant disease development. By synthesizing HCN, some bacteria inhibit plant disease development strengthening the host's disease resistance mechanism (Schippers *et al*., 1990). Thus the ability to produce HCN is considered as a desired quality of plant growth-promoting organisms.

Table 2. Response of phosphorus solubilizing bacteria to qualitative assay of ammonia production.

Strain	Ammonia production
PSB-1	++
PSB-2	+++
PSB-3	+++
PSB-4	++++
PSB-5	+++++
PSB-6	++++
PSB-7	++
PSB-8	-
PSB-9	++
PSB-10	++
PSB-11	++
PSB-12	++
PSB-13	-
PSB-14	++++
PSB-15	+++

(+): positive response [Number of (+) marks express the intensity of activity], (-): Negative response

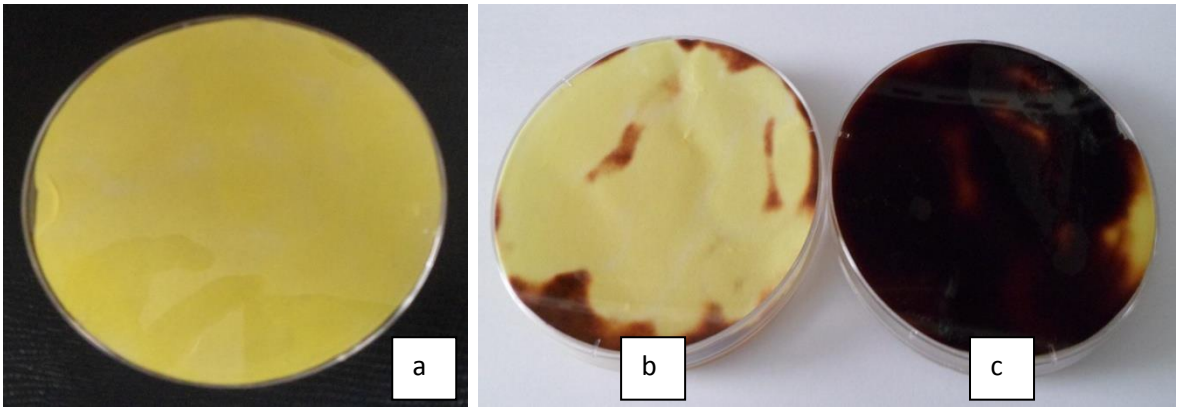


Figure 4. HCN production by the isolates (a) control no color change-negative for HCN production (b) light brown coloration - moderate HCN production (PSB-9) (c) dark brown coloration - strong HCN production (PSB-14).

The presence of HCN in the soil can also act as an efficient biological weed control measure as it inhibits seed germination and seedling vigour (Banerjee *et al*., 2010). It has previously been reported that various bacterial genera capable of producing HCN, including *Alcaligenes, Aeromonas, Bacillus, Pseudomonas*, *Enterobacter* and *Rhizobium* species isolated from various soil niches (Kumar *et al*., 2014).

Catalase Activity

Catalase activity is known to be an important trait of plant growth-promoting rhizobacteria as it could enhance the protection against hydrogen peroxide, a poisonous compound to bacteria and plant roots. Catalase removes the excess H_2O_2 produced in response to salt stress and therefore prevents leakage of H_2O_2 to other cell locations in the plants (Jamil *et al*., 2011). All the isolates in the present study showed a positive response for catalase activity correlating with previous studies (Bartakke *et al*., 2012; Bumunang and Babalola, 2014). Among them, PSB-3, PSB-7, PSB-12 and PSB-14 were found to exhibit the maximum activity.

Phylogenetic Analysis

Among the isolated bacterial strains, PSB-14 showed a remarkable ability in phosphate solubilization, IAA production, ammonia production, HCN production and catalase activity. Sequence alignment and phylogenetic tree drawn based on 16S rDNA gene sequences indicated that the species was *Enterobacter cancerogenus* (Figure 5*)*. The sequence was deposited in the NCBI Genebank under accession numbers KX815170 (Enterobacter cancerogenus LMG 2693).

CONCLUSION

All the phosphate solubilizing bacterial strains have exhibited other plant growth promoting traits including IAA production, ammonia production, HCN production and catalase activity. Catalase activity was detected in all isolates while productions of indole acetic acid, ammonia, hydrogen cyanide were shown in 40%, 86% and 73 % of isolates respectively. Further research with selected strains would be needed to confirm the practical acceptability of the strains for plant growth promotion via greenhouse and field experiments.

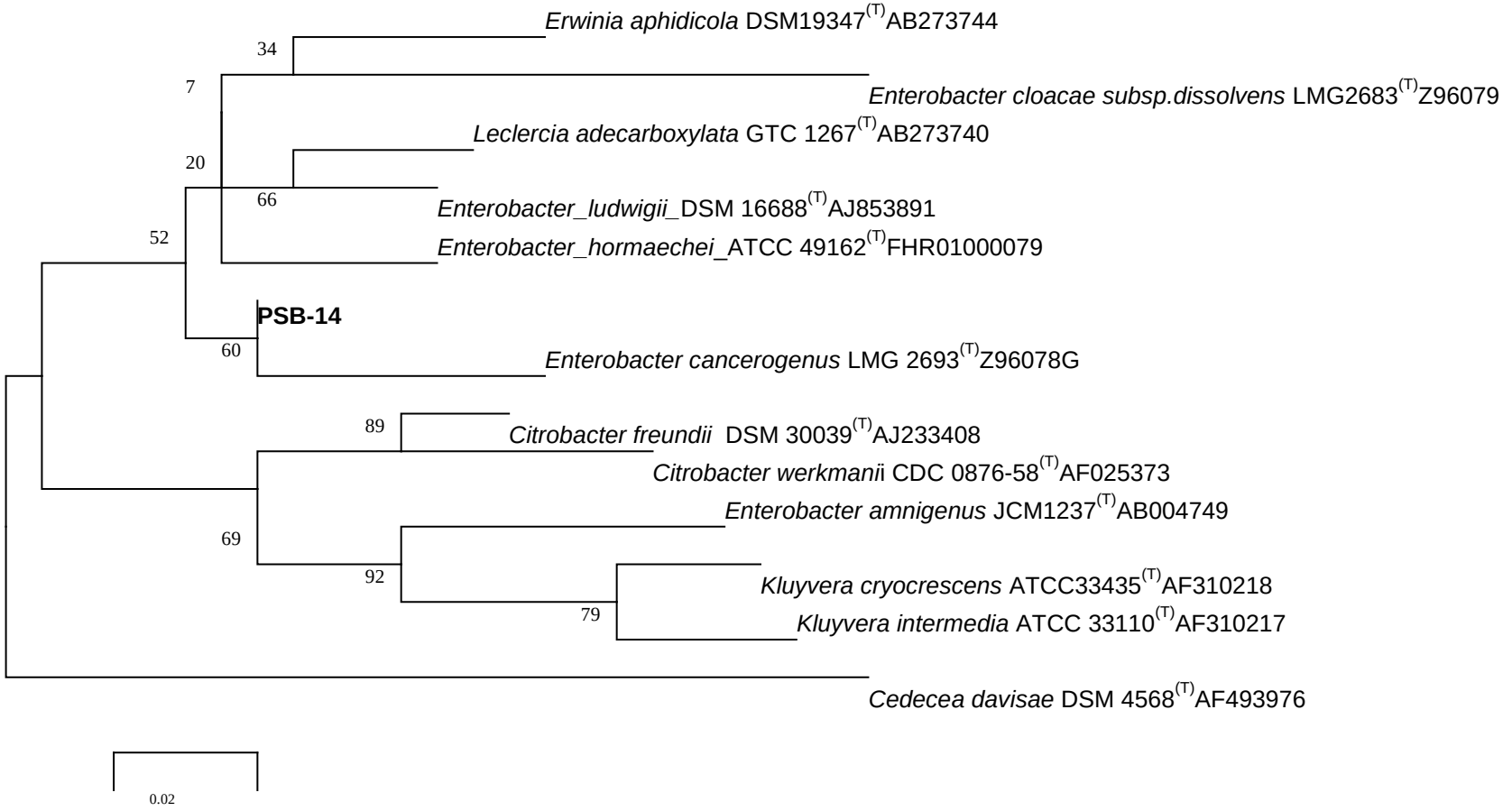


Figure 5. Phylogenetic tree based on 16S rDNA gene sequences, showing the position of PSB-14, *Enterobacter cancerogenus* strain with respect to related species. The scale bar indicates 0.02 substitutions per nucleotide position.

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