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Phosphate Solubilizing Bacteria (PSB) Isolation from Tomato Rhizospheres at Koka District, Ethiopia[#]

Dereje Haile[1,a,*], Bizuayehu Tesfaye[2,b], Fassil Assefa[3,c]

[1]*School of Biology, Hawassa University, Hawassa, Ethiopia*
[2]*School of Plant Science and Horticulture, Hawassa University, Hawassa, Ethiopia*
[3]*Department of Microbial, Cellular and Molecular Biology, Addis Ababa University, Ethiopia*
[*]*Corresponding author*

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ABSTRACT

Phosphorous is an essential macronutrient for plant growth and development. Most of the soil is deficient in plant available phosphorous and due to economic limitations majority of Ethiopian farmers applied inadequate fertilizers. It is essential to stabilize a mechanism to access P for plants with an efficient, cheap, and eco-friendly approach for enhanced crop growth and production. The main objective of this study was to screen efficient phosphate solubilizing bacteria from tomato rhizosphere soil. Using halo zone formation on PVK agar medium, more than 400 PSB isolates were isolated from 13 rhizosphere soil samples. By evaluating SI, texture in the culture-re-culturing process, liquid medium pH change efficiency and growth rate, upmost three promising PSB isolates (K-1-29, K-10-27, and K-10-41) were selected. Incubation of the isolates in PVK broth for five days showed significant pH reduction. For instance, isolate K-10-41 showed significant pH change (4.02) which indicates the organic acid production. Isolation and evaluation of efficient phosphate solubilizing bacteria maintain soil fertility, promote plant growth, induce plant response to pathogens, reduce agrochemical consumption and promote sustainable agriculture. Therefore, these selected PSB isolates need further detailed study for taxonomic identification, plant growth promotion, host range, and phytopathogen response. Local isolation improves environmental adaptation and indigenous competition.

[a] *gaginager93@gmail.com* *https://orcid.org/0000-0001-6555-8038*
[b] *tbizuayehu@gmail.com* *https://orcid.org/0000-0002-2843-1561*
[c] *asefafasil2013@gmail.com* *https://orcid.org/0000-0003-2609-6509*

Introduction

Phosphorous (P) is an essential nutrient (Nesme et al., 2018; Sharma et al., 2013) for plants to synthesize cellular structures and biomolecules (Baliah et al., 2016) including nucleic acids, enzymes/proteins, and phospholipids. It is required for the growth and development (Kour et al., 2021) of roots, leaves, stalks (Sharon et al., 2016), flowers, seeds, and fruits. P accounts about 0.2-0.8% of plant dry weight (Sharma et al., 2013). Both organic and inorganic forms of phosphorus are found in soil (Tian et al., 2021; Yadav and Verma, 2012, Kkan et al., 2009), however, organophos ranges from 30-90% of the total phosphorous. Nevertheless, plants absorb inorganic phosphorous (Naik et al., 2008) that is accessed by solubilizing microbes (Khudhur, 2017) as well as from supplemented chemical fertilizers. It is strongly precipitated (Kour et al., 2021) and switched immediately to unavailable forms for plant absorption (Naik et al., 2008; Kkan et al., 2009). Most of worldwide soils are deficient in plant-available P (Kour et al., 2021). P supplement (Nesme et al., 2018) through synthetic fertilizers application is a very good option for direct plant assimilation (Yadav and Verma, 2012). Yet, it is reported costive, spendthrift (about 75-90% is rapidly fixed or highly reactive to precipitate with Al^{3+} and Fe^{3+} in acidic and Ca^{2+} in alkaline soils) (Baliah et al., 2016; Sharma et al., 2013; Kkan et al., 2009), energy-consuming (Kour et al., 2021), and environmentally unfriend (production, transportation and application is environmentally deleterious). Therefore, it is necessary to establish a mechanism to access P for plants with an efficient, cheap and eco-friendly approach for enhanced plant growth and production.

The majority of Ethiopian arable land needs fertilizer supplement. However, most of the farmers applied fertilizers below the required rate (Simtowe, 2015). The main reasons fall under the economical barrier and lack of awareness. Farmers are unable to purchase fundamental agricultural inputs including fertilizers, high-quality varieties, pesticides (De Putter et al., 2012), herbicides, and other agricultural inputs. Frequently, many of the farmers used previously cultivated seeds, followed traditional hand-based weeding, had no or sub-optimal fertilizers application, and had a very limited amount of pesticide practice. Moreover, fertilizers importation, transportation, and distribution delay affect application timing and the rate.

In Ethiopia, irrigation farming showed a progressive increment, especially in the Rift Valley including Koka, Meki, and Ziway districts. Vegetable production excelled in the area because of the conducive environment, resources, and agricultural input availability. Tomato is one of the main vegetables known for its production and intensive agrochemicals (fertilizers, pesticides, and fungicides) consumption (Haile et al., 2022). The relative productivity, irrigation suitability, manageability for farming, market outlet, and agroecological adaptation promote smallholder farmers to produce tomatoes in these areas. Since tomato is sensitive to nutrient deficiency and other stresses (De Putter et al., 2012) including biotic (fungal, bacterial, viral, nematode, insects, weed, and bird's invasion), and abiotic stress (drought, temperature, heavy rain, humidity, wind, and flood); its production requires intensive protection, inputs supplements and day to day follow-up.

Beneficial microorganisms play a pivotal role (Sharon et al., 2016) in sustainable agricultural production and environmental health (Yadav et al., 2017). It is necessary to enrich beneficial microbes in the soil for improved soil fertility and enhanced plant growth. A wide range of microbial diversity is found in the plants' rhizosphere (Kour et al., 2021; Tian et al., 2021; Baliah et al., 2016) which is the most biologically active soil zone. The region is rich in root exudates that give carbon sources (sugars), amino acids, organic acids, phenolic compounds, and secondary metabolites and create communication signals (attract or repel) to the microbiome. The plant releases plenty of root exudates to give a response for root-root, root-bacteria, root-fungi, root-nematode, and root-insects communication (More et al., 2019).

Multifunctional phosphate solubilizers play determinantal roles (Tian et al., 2021) as biofertilizers, growth promotors, and biocontrol in plant root ecology (Naik et al., 2008). Phosphate solubilizing bacteria (PSB) were applied to maintain soil fertility, promote plant growth, induce plant response to pathogens, reduce chemical fertilizers and promote sustainable agriculture (Kour et al., 2021). They improve soil fertility with minimum cost requirement which is an essential attribute for small-scale farm production.

A huge diversity of soil microbes capable of P-solubilization and other plant growth promotion attributes (Yadav et al., 2017) have been reported so far and remain to be explored. Numerous microorganisms (bacteria, fungi, and algae) are known for their efficient P solubilization (Kour et al., 2021; Baliah et al., 2016; Sharma et al., 2013) and soil maintenance (Naik et al., 2008). They are ubiquitous in number and differ from soil to soil (Yadav and Verma, 2012). They are rich in the rhizosphere though they are found inside the root of the host plant (Yadav et al., 2017). The most familiar efficient P-solubilizers genera are *Bacillus*, *Pseudomonas*, *Rhizobium*, *Penicillium*, *Aspergillus*, *Actinomycetes*, and *Arbuscular Mycorrhizal* (Kour et al., 2021; Kalayu, 2019; Sharon et al., 2016; Singh and Jha 2015). To solubilize insoluble phosphate compounds, microbes used different mechanisms (Sharon et al., 2016; Sharma et al., 2013; Yadav and Verma, 2012; Kkan et al., 2009) such as the synthesis of phosphatase enzymes (alkaline and/or acidic phosphatase) (Khudhur, 2017), produce organic acids and lower the medium pH. P-solubilizing bacteria (Chen and Liu, 2019) play a significant role in plant rhizosphere to access nutrients (Tian et al., 2021; Sharon et al., 2016), promote plants growth (Naik et al., 2008), produce or induce growth hormones (IAA, Auxin, and gibberellin), protect plants from pathogens by producing antimicrobics, chelating iron (Yadav et al., 2017), compute for niche, food and plant exudates. Therefore, these attractive features of PSB make a sound to isolate, screen, characterize, evaluate their efficiency, and agroecology adaptation in order to apply as biological inputs for sustainable farm production. This study paid its effort to isolate competent PSB isolates from tomato rhizospheric soil to develop biofertilizers that possibly impacted agrochemical reduction and enhance tomato production. Local beneficial microbes' isolation, strain identification, host-specific evaluation, and understanding mechanism of interaction is helpful for better farm production, residue reduction, indigenous competition, and sustainable environmental health.

Materials and Methods

Soil samples

13 tomato rhizosphere soil samples were randomly collected from Koka open-field tomato farmlands. Koka is found in the East Shoa zone, Oromia region, Ethiopia, 102km to the south of Addis Ababa along the main road line to Moyale. The area has a good agricultural practice in producing different cereals and vegetables including tomato using irrigation and rainfall. Open farm tomato-producing smallholder farmlands were randomly selected, and soil samples collected. Soil samples were collected at a 30cm radius and 5-20cm depth from the tomato rhizosphere. Half a kilogram of each soil sample was homogenized, packaged in a polyethylene bag, and transported to Hawassa University soil microbiology laboratory for PSB isolation. Samples were preserved in a refrigerator until PSB isolation activity was completed.

PSB isolates screening

All PSB isolation and screening methods were done using Pikovskaya (1948)'s procedure with minor modifications. Collected soil samples grinded, thoroughly mixed, sieved, and serially diluted to 10^{-6}. 100 µl of aliquot solution spread on Pikovskaya's (PVK) agar medium (composition: 0.5 g yeast extract, 10 g glucose, 5 g $Ca_3(PO_4)_2$, 0.5 g $(NH_4)_2SO_4$, 0.2 g KCl, 0.1 g $MgSO_4.7H_2O$, 0.0001 g $MnSO_4.H_2O$, 0.0001 g $FeSO_4.7H_2O$ and 15 g agar per litre of water, pH adjusted to 7) and incubated at 28°C for 8 days. Colony and halo zone diameters were measured in every two days interval to evaluate the solubilization index (SI) of each colony.

Bacterial isolates with higher SI and better morphological structure were stored in the refrigerator (-20°C) for future characterization, plant growth promotion efficiency, and host range evaluation. Bacterial isolates named with a prefix “K” followed by two serial numbers indicating; K for sampling site Koka, first digit stands for the number of soil samples and the last digit indicates the number of bacterial isolates from that specific soil sample. SI calculated as:

$$SI = \frac{\text{Halo zone diameter} + \text{Colony diameter}}{\text{Colony diameter}}$$

Biochemical test

Selected three PSB isolates were subjected for gram staining and biochemical test. Isolates were evaluated for sugar fermentation capability and gas production. Tested sugars include glucose, fructose, raffinose, xylose, mannitol, arabinose, lactose, maltose, sucrose and starch. Six different salt concentrations (1%, 2%, 3%, 5%, 6% and 10%) were added in the broth medium and isolates inoculated to check salt tolerance. They were also tested for Urease production using urea agar, Indole test using kovak’s reagent and tryptophan broth. Each inoculated growing media was incubated at 28°C for five days to check the growth of isolates in the respective treatment.

Results and Discussion

Farmlands in the study area are used for dual crops production that promotes aggressive agrochemicals consumption. Even if it is expected to decline beneficial microbes in such kind of farming systems, it was found that all tomato rhizosphere soil samples comprised bundles of P-solubilizers. In a similar way, Baliah et al. (2016) reported that PSB population was found rich in tomato rhizosphere. This is because the availability of enriched root exudates synchronizes plants and microbial communities in the rhizosphere (More et al., 2019). It is also stated that phosphate solubilizing microbes are abundant (20-40% of the total population) in the rhizosphere to compete for root exudates (Kour et al., 2021). Likewise, Kour et al. (2021) and Yadav and Verma (2012) described among soil P-solubilizing microbes, bacteria constitute 1-50% of the total P-solubilizers with great fold solubilization efficiency.

Halo zone formation on agar PVK medium (Baliah et al., 2016; Naik et al., 2008) used as first line detection of P-solubilization from TCP ($Ca_3(PO_4)_2$) by the candidate strain. Following colony clear zone formation on PVK agar plate, more than 400 phosphate solubilizing bacteria were isolated (data not shown) from 13 tomato rhizosphere soil samples. Isolates were subjected to repeated sub-culturing in order to verify purity and to confirm solubilization efficiency. PSB isolates showed different solubilization index and morphology on the agar medium. Aliyat et al. (2020) described SI and colony morphology used as a primary standard for selection of PSB isolates. During isolation, different microbes (Figure 3) appeared in various colony shape (round, smooth, rough, and branched), size (small, medium and large), and colours (white, yellow, creamy-white, pink, purple, green, blue-green and black). For instance, colourful appearance was observed in some isolates including K-10-1 and K-10-3 (yellow and orange colony respectively). In addition, colony structure and size variation noticed such as branched or root like structure formation (K=9-16), layered and ringed structure (K-7-18 and K-10-3), larger and jelly like structures (K-3-3, K-8-34, and K-10-27). Similarly, Sharon et al. (2016) described the variation of PSB colony structures in size, shape and colour. It was also observed that a few PSB isolates showed antibiotic effects on neighbouring colonies that make a clear zone around the colony possibly indicating self-defence and niche or nutrient competition. Isolates K-10-41 and K-3-3 remove/clean other neighbouring microbes (showed antibiotic impact) as the colony size increased in accordance with incubation days go on. Wang et al. (2017) emphasized the impact of antibiotics production by soil microbes to control colonization dynamics in the rhizosphere.

Kour et al. (2021) stated that clear zone formation is not a sole selection criterion, rather it should be supported by other supplementary screening methods. Using preliminary screening techniques, 10 (Table -1) isolates were selected and furtherly evaluated in PVK broth medium for pH reduction, OD and CFU (Figure 1). These 10 selected PSB isolates scored SI of 3.1 and 1.83 (maximum and minimum respectively) which is higher than Singh and Jha (2015) investigation (seven days incubated PSB isolates scored 1.9 and 1.4 SI) as well as Sharon et.al (2016) report 1.5 and 0.09 SI scored from 7 days incubation.

Table 1. 10 selected PSB isolates colony diameter, hallo-zone diameter and SI

Isolates	Day-2		Day-4		Day-6		Day -8		C Avg.	H Avg.	SI
	C	H	C	H	C	H	C	H			
K-10-1	0.1	0.1	0.2	0.3	0.3	0.5	0.4	0.7	0.25	0.53	3.12
K-10-41			0.2	0.4	0.2	0.4	0.2	0.4	0.2	0.4	3
K-7-2	0.2	0.3	0.7	0.9	0.8	1.1	1.2	1.6	0.73	1.2	2.66
K-3-3	0.2	0.3	0.3	0.4	0.3	0.4	0.3	0.5	0.28	0.45	2.64
K-1-29	0.1	0.2	0.4	0.5	0.6	0.8	0.8	1.1	0.48	0.7	2.47
K-10-3	0.1	0.2	0.3	0.5	0.4	0.6	0.5	0.8	0.33	0.45	2.39
K-8-34	0.1	0.1	0.3	0.5	0.7	1	1	1.4	0.53	0.7	2.33
K-10-27	0.1	0.2	0.3	0.5	0.6	0.8	0.9	1.2	0.48	0.58	2.21
K-9-16	0.2	0.3	0.4	0.6	1	1.3	1.7	2.2	0.83	0.73	1.89
K-7-18	0.1	0.2	1	1.2	1.5	1.8	2	2.5	1.15	0.95	1.83

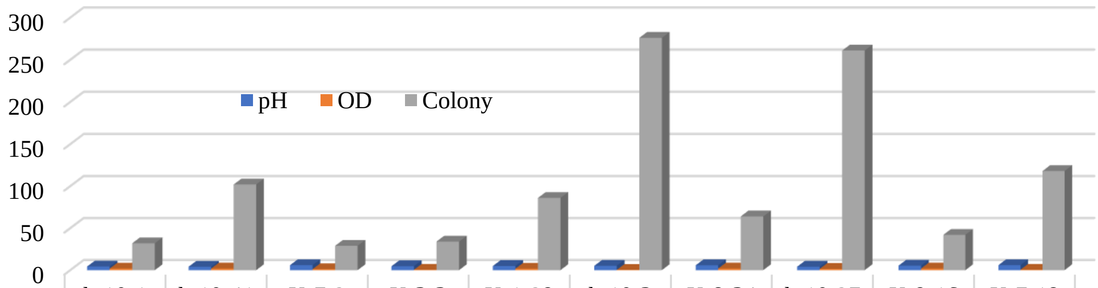
NB: C= colony, H= halo zone. Isolates were labelled as K followed by numbers. K stands for sample site name Koka, first digit stands for sample number and last digits for number of isolates from that specific soil sample.

Table 2. Biochemical characterization of the three selected isolates

Isolate	Glu		Suc		Fruc		Mani		Malt		Xyl		Raf		Star		Lact		Arab	
	F	G	F	G	F	G	F	G	F	G	F	G	F	G	F	G	F	G	F	G
K-1-29	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+
K-10-27	+	+	+	+	+	+	+	+	+	+	-	-	+	-	+	-	+	-	+	+
K-10-41	+	+	+	+	+	+	+	+	+	+	-	-	-	-	+	+	+	-	+	+

Isolate	Urase test	Indole test	Catalase test	1%	2%	3%	5%	6%	10%
K-1-29	+	+	+	+	+	+	+	+	-
K-10-27	+	+	+	+	+	+	+	+	-
K-10-41	+	+	+	+	+	+	-	-	-

NB: Gluc= glucose, Suc= sucrose, Fruc= fructose, Mani= mannitol, Man= mannitol, Xyl= xylose, Raf= raffinose, Star= starch, Lact= lactose, Arab= arabinose, F= sugar fermentation, G= gas, %= salt concentration added per litter.



	k-10-1	k-10-41	K-7-2	K-3-3	K-1-29	k-10-3	K-8-34	k-10-27	K-9-16	K-7-18
pH	4,32	4,02	5,93	4,7	5,01	5,23	5,84	4,12	5,37	5,93
OD	1,947	1,977	1,195	0,469	1,723	0,313	1,986	1,561	1,995	0,498
Colony	32	102	29	34	86	276	64	261	42	118

Figure 1. Selected 10 PSB isolates efficiency in PVK broth medium

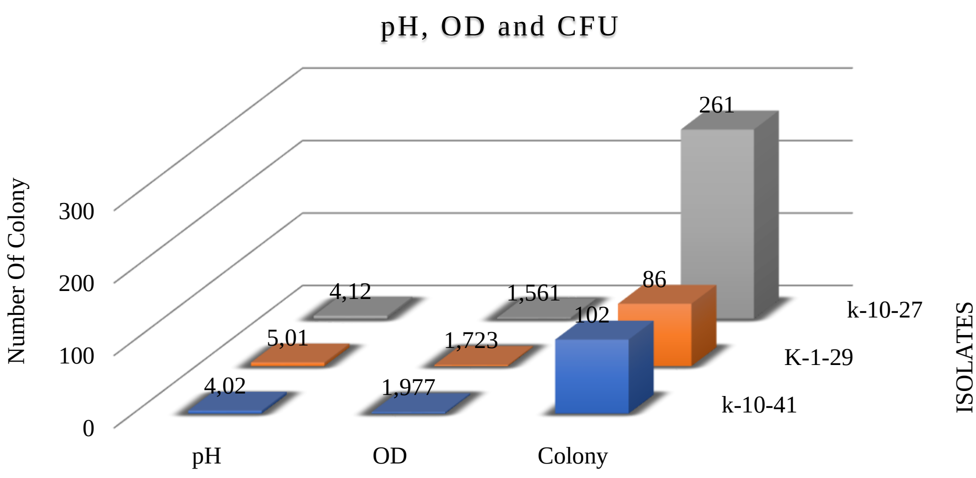


Figure 2. Upmost 3 selected PSB isolates performance in PVK broth (pH change, OD and CFU)

During isolation from the bulk soil solution, some PSB isolates were fast grower and efficient solubilizers whereas others found slow grower and performed differently. Out of 10 screened PSB isolates, 9 of them formed a measurable colony and hallo zone starting on the 2nd days of incubation with a potential increment until 8th days of incubation (Table -1). While isolated, K-10-41 delayed forming a detectable colony and hallo zone after 72 hrs incubation and scored a minimum diameter on the 8th day's incubation. Even though it's expected to increase both in colony and hallo zone diameter along incubation period, K-10-41 growth stays limited/stacked throughout the consecutive incubation days. However, it was observed with antibiotic effect on the neighbouring microbes which possibly give a clue for antagonistic response for plant/tomato pathogens that could be examined in the future with detailed further evaluations. Sub-culturing maintains its growth to medium colony size with a good halo zone formation. It also showed a significant pH reduction (4.02) of liquid PVK medium (Figure 1 and 2) that possibly indicates organic acids production. Different research reports indicate that organic acids production and medium pH reduction signify phosphate solubilization by the candidate PSB strains (Kour et al., 2021; Baliah et al., 2016; Sharon et al., 2016; Kkan et al., 2009). Yadav and Verma (2012) also reviewed that PSMs increased TCP

$(Ca_3(PO_4)_2$ solubilization by secreting organic acid and pH reduction which led to high affinity to calcium and solubilized more phosphorous. Isolate K-7-18 was the largest colony with a significant hallo-zone diameter but it recorded the lowest SI, pH reduction and the 3rd lowest OD reading. SI used to screen competent PSB isolates on PVK agar plate, but other supportive traits help to select efficient strains. There were many PSB isolates which scored SI 3 and above but for some reasons including re-culturing dissatisfaction, impurity with repeated subculturing, poor halo zone formation as well as lack of consistency and reputability, they were not selected. However, none of our PSB isolates SI score compared with Aliyat et al (2020) finding a maximum SI of 4.79$\pm$0.57 recorded after three days incubation.

From 10 selected PSB isolates, K-3-3, K-7-2 and K-9-16 formed 0.2 cm colony and 0.3 cm hallo zone on the 2nd days of incubation (Table -1). The latter two isolates showed static diameter increment along incubation days while K-3-3 did not show significant improvement. Up to 8 days of incubation, isolated K-7-18 showed remarkable diameter increment (0.1 to 2 cm for colony and 0.2 to 2.5 cm halo zone). Significant average colony diameter difference observed among selected PSB isolates (highest was 1.2 cm recorded by k-7-18 and the lowest 0.2 cm by K-10-41). Beside colony diameter, these two isolates showed contrasting efficiency in pH reduction and OD measurements (Figure 1). Similarly, K-7-2 recorded maximum and K-10-41 minimum average of hallo zone diameter (1.2 and 0.4 cm respectively).

Figure 3. PSB isolation from diluted fresh soil sample on PVK agar

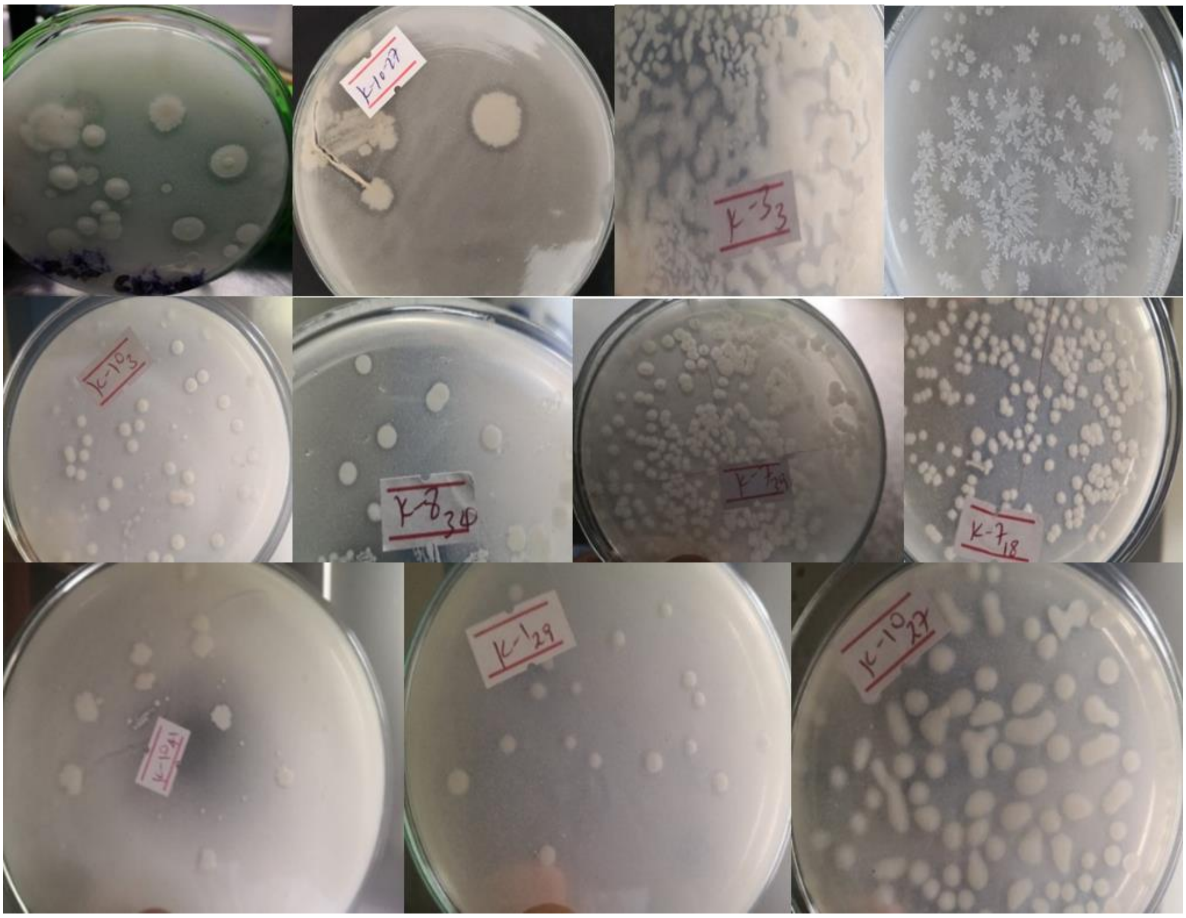


Figure 4. PSB isolates sub-culture and purification on PVK agar

Isolates cultured for five days in PVK broth medium to evaluate pH change and OD. All our isolates reduced growing medium pH. High pH reduction recorded by isolate K-10-41 (4.02) and relatively minimum pH recorded by K-7-18 and K-7-2 (5.93). Supportive findings reported that significant liquid PVK medium pH reduction was observed by Naik et al. (2008) (reduced from 7.4 to 4.8 in 10 days incubation), Sharon et al. (2016) reduced to 5.5-4.5 in 24 hrs incubation. Similarly, Aliyat et al. (2020) reported that PSB strains solubilize TCP in liquid medium complementing with pH reduction. A minimum OD reading (0.313) was recorded by isolate K-10-3 whereas maximum OD (1.995) was recorded by K-9-16. Despite pH and OD reading, colony count (CFU) was also conducted on agar plate (Figure 1). A minimum number of colonies (29) counted by isolate K-7-2 and maximum colony number (276) counted by K-10-3. It is unusual and contradicting to have high OD reading value and low number of colony count, but isolate K-10-3 recorded high OD and less colony count. This might be due to the isolate being a fast grower and if the incubation period extended, the stationary phase passed and entered the death phase. More dead cells accumulated in the broth to raise the OD reading but very few viable/active cells found in the medium. Furthermore, all the three selected PSB isolates were found gram negative, fermented all the given sugars except xylose and raffinose, positive for urease and indole test, grown on 1-3% salt concentration (Table -2).

Generally, by combining the results from growth condition on PVK agar, SI value, culture re-culture consistency, pH change, OD and CFU (Figure 2), upmost three PSB isolates (K-1-29, K-10-27 and K-10-41) selected and tested for different sugar fermentations, gas production, urease, indole and slat tolerance (Table -2) then maintained for future greenhouse and field trials on tomato and other crops following proper taxonomic identification using biochemical and molecular characterization. Isolates K-7-18, K-9-16 and K-7-2 formed larger colonies and halo zone diameter however, their SI was found low. Especially K-7-18 formed the largest colony (2) with wider halo zone (2.5) but unable to confirm during re-culturing and liquid medium growth evaluation. It recorded lowest SI (1.83), OD (0.498) and least pH reduction (5.93). Similarly. K-9-16 measured wider diameter (1.7 and 2.2 diameter for colony and halo zone respectively (Table -1)) and highest OD reading but it's SI (1.89) and minimum pH (5.93) reduction ability hinder the candidate not to be selected. On the other hand, K-10-41 formed the lowest colony and halo zone diameter but it's OD reading (1.977), strong pH reduction (4.02), viable cell counts as well as the additional feature of antagonistic effects on the neighbouring colony ratify the candidate to be selected for further evaluation and possibly to be applied as bioinoculant for tomato farms. Likewise, K-1-29 mostly lies in the midpoint of diameter averages (out of 10 isolates), SI, OD and their consistency in culture-re-culturing process helps the candidate to be selected. K-10-27 also formed medium diameter but recorded the second top viable count and strongly reduced liquid medium pH (4.12) next to K-10-41 (pH 4.02). Since pH reduction is one way indication of TCP-solubilization through organic acids production, it is a good pointer of solubilization efficiency to the candidate isolates.

Many soil microbes are capable of dissolving and transforming insoluble phosphate compounds into plant available phosphate anions. Proper isolation and performance evaluation of PSB isolates help to screen efficient strains for biofertilizer application. Biofertilizers are beneficial microbes that possibly provide substantial benefit in nutrient enrichment and growth promotion of the crops which promote sustainable healthy agricultural production (Yadav et al., 2017). Kour et al. (2021) discussed the benefit of P-solubilizers isolation from different habitats and understanding plant growth promoting attributes. Most P-solubilizers are concentrated and metabolically very active in the rhizosphere soil.

Conclusion

P is essential nutrient for plants but due to its low availability and immediate immobilization, plants use only limited amount of supplemented chemical P-fertilizers. Therefore, isolation of efficient and competent PSB strains from local environment wellnigh help to raise/improve plant available P in the rhizosphere. Tomato rhizosphere soils were found rich with valuable P-solubilizing bacteria. Promising three PSB isolates screened using first hand selection criteria based on halo zone diameter and SI in combination with other supportive filtering methods including broth medium growth performance, pH change efficiency, colony morphology, growth rate, and other important features. Further studies required to characterize isolates, greenhouse and field trial, interaction with host crops and other remaining experimental activities. Properly selected isolates with combined screening methods will help to find efficient broad spectrum and multitasked strains that are capable of nutrient accessing for the plant, induce hormone production, promote stress resistance and sustain production. Moreover, laboratory isolation of efficient strain is not an end by itself. Selected strains should be evaluated at greenhouse and field level for competence, plant growth performance, yield, and host range. Well characterized and evaluated PSB strains improve crop production, reduce agricultural cost, minimize chemical consumption, toxic residues and promote environmental health.

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