Comparison of Qualitative and Quantitative Methods for Isolation of Phosphate Solubilizing Microorganisms

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Abstract

Phosphate solubilising bacteria possess the ability to solubilise insoluble phosphate to soluble forms enhancing the nutrient status of the soil. This process not only compensates increasing cost of phosphatic fertilisers but also minimises the negative environmental impacts associated with the application of inorganic fertilisers. Phosphate solubilising bacteria (PSB) were screened based on the size of a halo/ clear zone around the colony (NBRIP agar plate assay) and by measuring solubilise phosphorous content (colorimetric method). The aim of this work was to assess the comparative reliability of quantitative and qualitative methods of isolation of phosphate solubilising bacteria. Bacterial strains which showed very poor performance in qualitative method were proven to be good phosphates solubilisers in quantitative method and vice versa. Therefore no positive relationship among the values obtained from qualitative and quantitative methods could be observed. Furthermore qualitative method did not reflect the real ability of the phosphate solubilising bacteria to solubilise insoluble phosphates. From the results of the present study, it can be concluded that isolation of efficient phosphate solubilising bacteria through quantitative method could give better results than that of qualitative method.

Keywords: *clear zone, insoluble phosphates, phosphate solubilising bacteria*

1. Introduction

Phosphorus (P) is the second most important macro-nutrient required by plants (Sharma et al., 2013). Major fractions of soil phosphorus are usually present in the forms which are unavailable to plant as they form complexes with Al or Fe in acid soils or with Ca in calcareous soils (Yin et al., 2015). This leads to wide spread P deficiency in all most all types of soils. There are some diverse groups of soil microorganisms who posses substantial potential to solubilise this insoluble phosphorus to plants available phosphorus especially in soils with limited phosphorus and such microorganisms are generally termed as phosphorus solubilising microorganisms (PSMs) (Xiao et al., 2013; Abbasi et al., 2015; Bakhshandeh et al., 2015). There are some phosphate solubilizing bacteria (PSB), phosphate solubilising fungi (PSF) and actinomycetes that have been reported to be active in conversion of insoluble phosphatic compounds into soluble forms. So far *Pseudomonas, Bacillus* and *Rhizobium* have been reported to be efficient phosphate solubilising bacterial genera. *Aspergillus and Penicillium* are among the most powerful phosphate solubilising fungal strains (Xiao et al., 2011). Organic acid production is identified as an important mechanism responsible for P solubilisation and phosphate solubilising microorganisms are capable of releasing low molecular weight organic acids such as acetate, lactate, oxalate, tartarate, succinate, citrate, gluconate, ketogluconate, glycolate, etc.

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(Wickramatilake et al., 2010) which help in solubilisation of insoluble P in soil. Phosphate solubilising microorganisms form clear zones/halo zones in the surrounding medium through the production of organic acids (Gaur, 1990), thus could be screened by measuring the size of clear zone/halo zone around the colony on the plate (Nautiyal, 1999). Solubilisation index can be measured based on colony diameter and halo zone diameter of such strains. It has been reported that most of the phosphate solubilising microorganisms loss their ability to form halo-zone around the colony on the medium due to repeated sub-culturing (Alam et al., 2002). The reliability of this halo-based technique though widely used for isolation and assessing phosphate solubilising ability of microorganisms is questioned as many isolates which do not produce any visible halo zone on agar plates could still solubilise various types of insoluble inorganic phosphates in liquid medium (Gupta et al., 1994) and as well as under field conditions. Therefore, instead of qualitative techniques for the isolation of phosphate solubilising microorganisms, quantitative methods are increasingly employed as they could quantify the amount of solubilised P released from insoluble substrate as a result of microbial phosphate solubilisation. Under this background, the present study aimed at assessing comparative reliability of qualitative and quantitative methods using calcium phosphate as phosphate source.

2. Materials and Methods

2.1 Sample collection for isolation of phosphate solubilising bacterial strains

Rhizosphere soil samples collected from Agricultural lands in Matara district of Sri Lanka were used in isolating bacterial strains. The soil belongs to Red Yellow Podzolic great soil group and is classified as Hapludults according to the USDA soil taxonomy (Mapa et al., 1999). The climate of the area is tropical monsoonal (Panabokke, 1980), with a warm wet period (April to June) and a relatively dry period (January to March). The area receives an annual rainfall of around 2,500 mm. The distribution of rain is bi-model. Annual mean air temperature of the area is 22-30° C and the relative humidity is about 80%.

Soil samples were collected by gently uprooting the plants. The loosely adhering soil was removed from the roots by gentle shaking. Soil samples were collected to the plastic bag and carried to the laboratory in an icebox. They were kept at 4° C temperature before analyses.

2.2 Isolation of phosphate solubilising bacterial strains

One gram of each field moist soil samples was suspended in 9 mL of sterilized distilled water and mixed vigorously. Serial dilutions were prepared using dilution plate technique (Wollum II, 1982). Each dilution was plated in NBRIP (National Botanical Research Institute Phosphorus) agar plates containing 16 g agar, 10 g glucose, 5 g $Ca_3(PO_4)_2$, 5 g $MgCl_2.6H_2O$, 0.25 g $MgSO_4.7H_2O$, 0.2 g KCl, 0.1 g $(NH_4)_2SO_4$ in 1 L distilled water. The NBRIP medium containing tri calcium phosphate (TCP) act as sole phosphorus source for selectively screening the bacteria which have the ability to release soluble inorganic phosphate (Nautiyal et al., 2000). The pH of the media was adjusted to 7 using HCl. The plates were incubated 4-5 days in an incubator at 30^0C. The colonies with clear halos were considered to be phosphate solubilising colonies and they were then further purified by re-streaking on the fresh NBRIP agar plates at 30^o C. Bacterial strains that exhibited clear zones on the agar plates were selected as phosphate solubilising organisms for further studies. A total 15 phosphate solubilising bacterial strains were isolated and maintained on 30% glycerol stock until use. Phosphate solubilising ability of the isolates was measured qualitatively and quantitatively using NBRIP liquid and solid medium.

2.3 Qualitative assay of phosphate solubilisation

A pin point inoculation of each bacterial strain preserved in glycerol stock was placed in agar plates containing 16 g agar, 10 g glucose, 5 g $Ca_3(PO_4)_2$, 5 g $MgCl_2.6H_2O$, 0.25 g $MgSO_4.7H_2O$, 0.2 g KCl, 0.1 g $(NH_4)_2SO_4$ in 1 L distilled water. The experiment was performed triplicates with three replicates for each bacterial strain. The diameter of halos produced by the strain was measured after inoculation of day 1, 3 and 5 and categorized as low, medium and high on the basis of zone diameter. The strain making a zone of diameter <1 cm was considered as low phosphate solubiliser (0), 1.0-1.5 cm as medium phosphate solubiliser (1) and diameter of halo >1.5 cm as high phosphate solubiliser (2).

2.4 Quantitative assay of phosphate solubilisation

Bacterial strains were grown in sterilized liquid NB (Nutrient Broth) medium (20 mL) at 30° C for two days with continuous shaking at 150 rpm. Aliquots of culture (1 mL) which having 1×10^8 CFU mL^{-1} was then transferred into a 500 mL flask containing 10 g glucose, 5 g $Ca_3(PO_4)_2$, 5 g $MgCl_2.6H_2O$, 0.25 g $MgSO_4.7H_2O$, 0.2 g KCl, 0.1 g $(NH_4)_2SO_4$ in 1 L distilled water. Sterilised uninoculated medium served as a control. A 10 mL sample of each cultured and control were taken into centrifugation tube 1, 3 and 5 days after inoculation and centrifuged in 10 min at 10,000 rpm. The clear supernatant was used in determining phosphorous release into the medium using the phospho-molybdate blue color method (Murphy and Riley, 1962). The experiment was performed triplicates with three replicates for each bacterial strain. Phosphate solubilisation ability of the each strain was categorized into low, medium and high by assigning value 0, 1 and 2 by using Score Indexing technique in each day (Gill et al., 2004).

2.5 Statistical analysis

The data were subjected to analysis of variance (ANOVA) using SAS package (SAS, 1999). The Duncan's Multiple Range Test (DMRT) was applied to test the significance of treatment means at $p\leq0.05$.

3. Results and Discussion

3.1 Isolation of phosphate solubilising bacterial strains

Fifteen bacterial strains (PSB 1 to PSB 15) which exhibited clear zones on the NBRIP agar plates were identified as phosphate solubilising organisms (Figure 1).

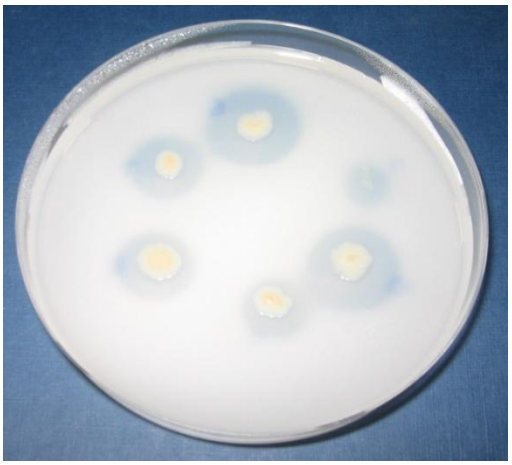

Figure 1. Halo zone produced by isolated strains on agar plates due to Phosphate solubilisation.

3.2 Qualitative and quantitative assay of phosphate solubilisation

All the tested phosphate solubilising bacterial isolates showed positive responses for phosphate solubilisation in agar plates by producing clear zones with different sizes (qualitative) (Table 1). They also could solubilise insoluble phosphate in liquid medium indicating their phosphate solubilising ability (quantitative) (Table 2). However, results of the two methods were found to be significantly different and no sign of similarity in terms of phosphate solubilisation was observed in any of the tested 15 bacterial strains throughout the incubation period.

Table 1: Quantitative phosphate solubilisation of rock phosphate by phosphate solubilising microorganisms after 1, 3 and 5 days after inoculation.

Strain	Day 1		Day 2		Day 3	
	Halo zone diameter (cm)	Rank	Halo zone diameter (cm)	Rank	Halo zone diameter (cm)	Rank
PSB 1	0.94	1	1.65	2	1.51	1
PSB 2	1.05	1	1.53	2	1.52	1
PSB 3	1.13	1	1.12	1	1.23	1
PSB 4	1.17	1	1.87	2	2.11	2
PSB 5	0.85	0	0.89	0	0.96	0
PSB 6	0.78	0	1.01	0	1.07	0
PSB 7	1.27	2	1.75	2	2.31	2
PSB 8	1.40	2	1.44	1	1.46	1
PSB 9	1.40	2	1.47	1	1.52	1
PSB 10	0.61	0	0.97	0	1.07	0
PSB 11	1.17	2	1.14	1	1.18	1
PSB 12	1.15	2	1.32	1	1.34	1
PSB 13	1.25	2	1.31	1	1.35	1
PSB 14	1.31	2	1.23	1	1.33	1
PSB 15	0.66	1	0.67	0	0.74	0

Table 2: Qualitative phosphate solubilisation of rock phosphate by phosphate solubilising microorganisms after 1, 3 and 5 days after inoculation.

Strain	Day 1		Day 2		Day 3	
	Phosphate solubilisation (µg/mL)	Rank	Phosphate solubilisation (µg/mL)	Rank	Phosphate solubilisation (µg/mL)	Rank
PSB 1	98.93	2	379.23	0	916.34	2
PSB 2	82.92	2	279.95	0	456.13	0
PSB 3	101.67	2	410.15	1	1030.27	2
PSB 4	102.19	2	255.41	0	869.14	1
PSB 5	95.09	2	509.68	1	767.57	1
PSB 6	54.40	1	472.29	1	741.89	1
PSB 7	62.39	1	506.76	1	654.96	1
PSB 8	60.41	1	611.04	2	850.91	1
PSB 9	68.11	1	598.65	2	925.22	2
PSB 10	87.39	2	466.22	1	873.87	2
PSB 11	44.01	1	502.48	1	777.93	1
PSB 12	88.87	2	417.57	1	623.42	1
PSB 13	62.97	1	589.41	2	874.32	1
PSB 14	77.52	2	651.82	2	1127.03	2
PSB 15	59.32	1	431.98	1	356.76	0

Based on the results of quantitative method, PSB-6 showed low phosphate solubilising ability, PSB-7, PSB-8, PSB-9, PSB-11, PSB-13 and PSB-15 showed medium phosphate solubilising ability and PSB-1, PSB-2, PSB-3, PSB-4, PSB-5, PSB-10, PSB-12 and PSB-14 showed high phosphate solubilising ability after day 1. Except PSB-6 and PSB-12, all the other strains exhibited different patterns of phosphate solubilisation in solid medium by producing different size of clear zones after day 1. Interestingly, the strains PSB-6 and PSB-12 which produced low and high clear zones respectively around colonies in qualitative method, showed the same trend in quantitative method as well (Table 3).

Table 3: Comparative efficacy of quantitative Vs qualitative method of rock phosphate solubilisation 1st day after inoculation.

Phosphate solubilisation					
Low		Medium		High	
Qualitative	Quantitative	Qualitative	Quantitative	Qualitative	Quantitative
PSB 5	PSB 6	PSB 1	PSB 7	PSB 7	PSB 1
PSB 6		PSB 2	PSB 8	PSB 8	PSB 2
PSB 10		PSB 3	PSB 9	PSB 9	PSB 3
PSB 15		PSB 4	PSB 11	PSB 11	PSB 4
			PSB 13	PSB 12	PSB 5
			PSB 15	PSB 13	PSB 10
				PSB 14	PSB 12
					PSB 14

Similarly after day 3 of inoculation, PSB-1, PSB-2 and PSB-4 showed low phosphate solubilising ability, PSB-3, PSB-5, PSB-6, PSB-7, PSB-10, PSB-11, PSB-12 and PSB-15 showed medium phosphate solubilising ability and PSB-8, PSB-9, PSB-13 and PSB-14 showed high phosphate solubilising ability in quantitative method. However, PSB-5, PSB-6, PSB-10 and PSB-15 showed low phosphate solubilising ability, PSB-3, PSB-8, PSB-9, PSB-11, PSB-12, PSB-13 and PSB-14 showed medium phosphate solubilising ability and PSB-1, PSB-2, PSB-4 and PSB-7 showed high phosphate solubilising ability in qualitative method. PSB-11 and PSB-12 showed medium phosphate solubilising ability in both qualitative and quantitative method (Table 4).

Table 4: Comparative efficacy of quantitative Vs qualitative method of rock phosphate solubilisation 3rd day after inoculation.

Phosphorous solubilisation					
Low		Medium		High	
Qualitative	Quantitative	Qualitative	Quantitative	Qualitative	Quantitative
PSB 5	PSB 1	PSB 8	PSB 5	PSB 1	PSB 8
PSB 6	PSB 2	PSB 9	PSB 6	PSB 2	PSB 9
PSB 10	PSB 4	PSB 11	PSB 7	PSB 4	PSB 13
PSB 15		PSB 12	PSB 10	PSB 7	PSB 14
		PSB 13	PSB 11		
		PSB 14	PSB 12		
			PSB 15		

The strains PSB-2 and PSB-15 showed low phosphate solubilising ability, PSB-4, PSB-5, PSB-6, PSB-7, PSB-8, PSB-11, PSB-12 and PSB-13 showed medium phosphate solubilising ability and PSB-1, PSB-3, PSB-9, PSB-10 and PSB-14 showed high phosphate solubilising ability in

quantitative method after day 5. However, PSB-5, PSB-6, PSB-10 and PSB-15 showed low phosphate solubilising ability, PSB-3, PSB-8, PSB-11, PSB-12, PSB-13 and PSB-14 medium phosphate solubilising ability PSB-1, PSB-2, PSB-4, PSB-7, and PSB-9 showed high phosphate solubilising ability in qualitative method. Interestingly, PSB-15 showed low phosphate solubilising ability, PSb-11, PSB-12 and PSB-13 showed medium phosphate solubilising ability and PSB-1 and PSB-9 showed high phosphate solubilising ability in both qualitative method and quantitative method (Table 5).

Table 5: Comparative efficacy of quantitative Vs qualitative method of rock phosphate solubilisation 5th day after inoculation.

Phosphorous solubilisation					
Low		Medium		High	
Qualitative (PSI)	Quantitative	Qualitative (PSI)	Quantitative	Qualitative (PSI)	Quantitative
PSB 1	PSB 15	PSB 8	PSB 4	PSB 5	PSB 1
PSB 4		PSB 9	PSB 5	PSB 7	PSB 9
PSB 6		PSB 13	PSB 6	PSB 10	PSB 10
		PSB 14	PSB 7	PSB 11	PSB 12
		PSB 15	PSB 8	PSB 12	PSB 13
			PSB 11		
			PSB 12		

Furthermore, no relationship was observed between qualitative and quantitative methods when the values were regressed after day 1, 3 and 5 (Figure 2-4).

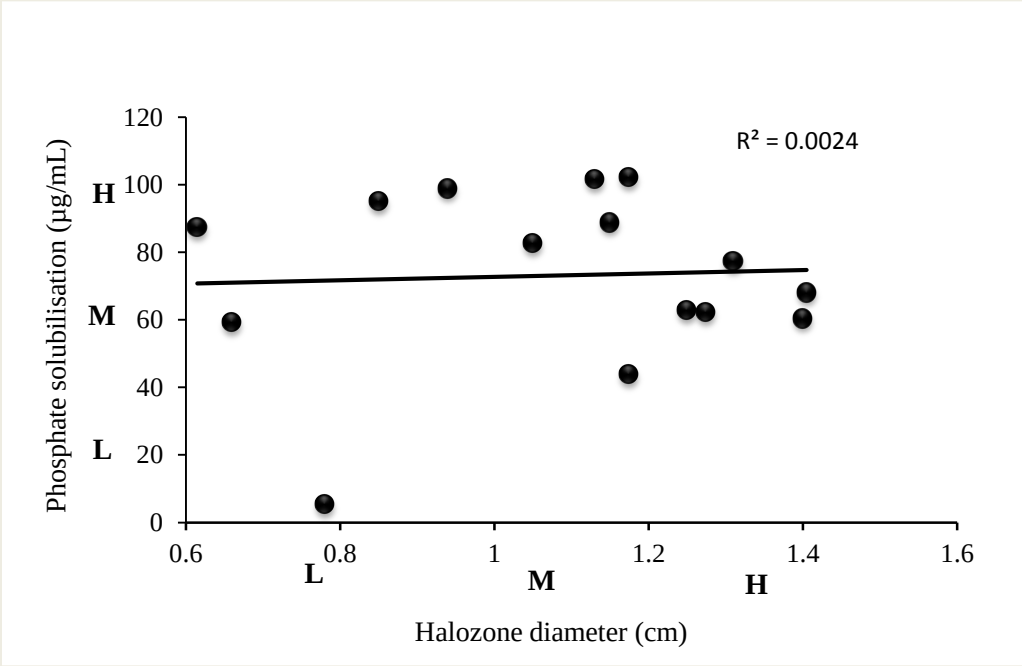

Figure 2. Correlation between qualitative and quantitative methods after day 1 of inoculation.

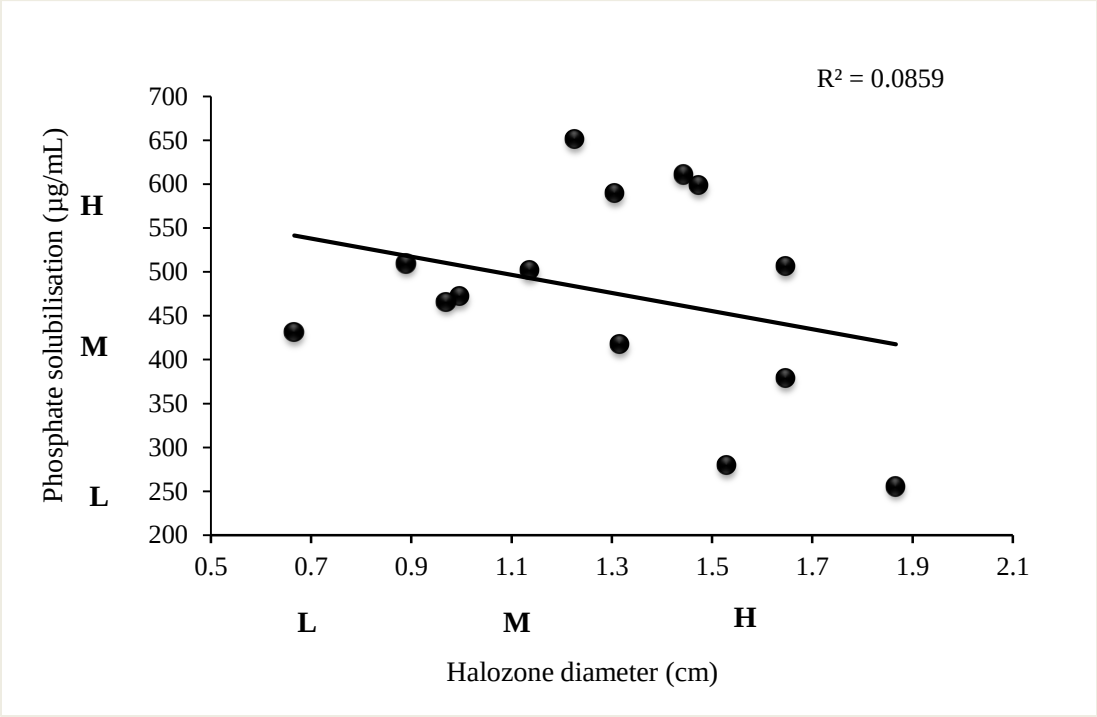

Figure 3. Correlation between qualitative and quantitative methods after day 3 of inoculation.

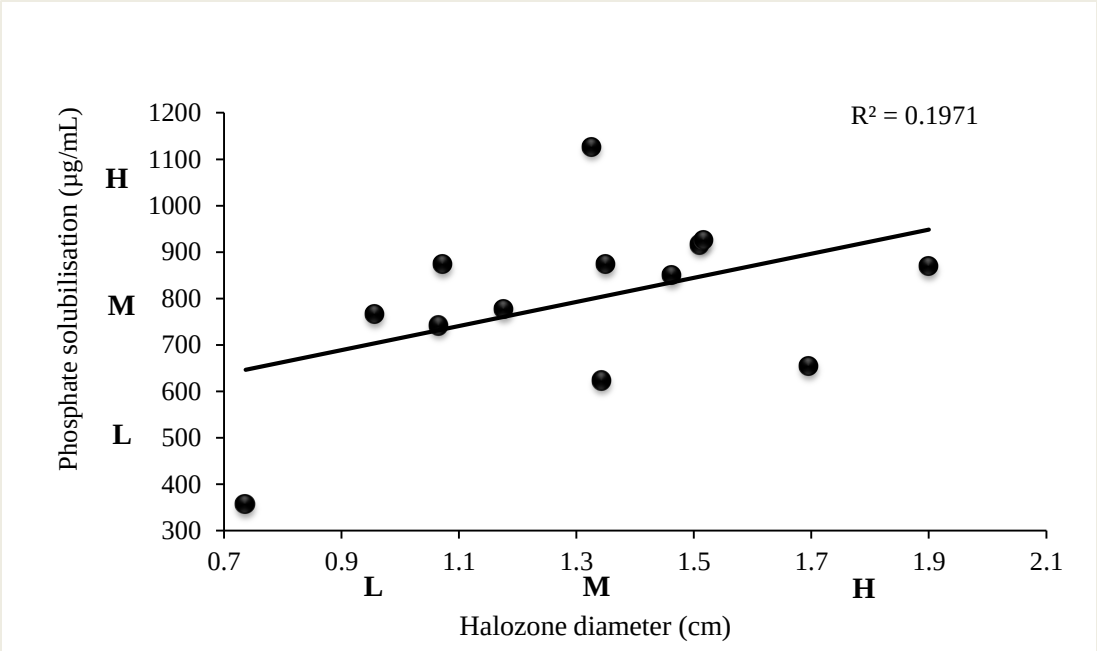

Figure 4. Correlation between qualitative and quantitative methods after day 5 of inoculation.

Present results are in line with the findings of Nautiyal et al. (2000), Baig et al. (2010) and Jain et al. (2017) who also observed different trend in phosphate solubilisation in qualitative and quantitative method. According to Baig et al. (2010) halo/clearing zone produced by the strains RM19 (0.7 cm) and RW27 (0.8 cm) was low in qualitative method, though they showed quite high

phosphate solubilisation activity in quantitative method. Further, they reported that strains which produced low halo/clearing zone in qualitative method, exhibited medium phosphate solubilisation activity in quantitative method. According to Jain et al. (2017) a direct correlation between phosphate solubilisation in solid media and liquid media was observed in 13 strains. In contrast, six strains showed better phosphate solubilisation ability in liquid medium compared with solid medium, and the five strains had better ability on solid medium. The different behavior in solid and liquid medium could be attributed to nutrient availability, growth requirement and varying diffusion rates of different organic acids secreted by phosphate solubilising microorganisms (Jain et al., 2014). Generally phosphate solubilising microorganisms (PSMs) produce organic acids into the surrounding medium which in turn exhibit halo/clear zones. The size of the halo/clear zone varied with the diffusion rates of the organic acids produced by the organism. Therefore the qualitative method fails when the halo produced by PSMs in agar plates inconspicuous or absent (Johnston, 1952). Alam et al. (2002) reported that most of the phosphate solubilising micro-organisms loss their ability to form halo-zone on agar plates due to repeated sub-culturing. Therefore the reliability and accuracy of this qualitative method in isolation of phosphate solubilising microorganisms is questioned because isolates which do not produce any visible halo/clear zone on agar plates (qualitative method) could solubilise various types of insoluble inorganic phosphates in liquid medium (quantitative method) (Gupta et al., 1994). Therefore as revealed by present findings, one should not rely on qualitative method for isolation of phosphate solubilising microorganisms, instead both qualitative and quantitative methods could be performed in parallel to ensure more reliable results.

4. Conclusion

It can be concluded that isolation of efficient phosphate solubilising bacteria through quantitative method could give better results than that of qualitative method.

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