Isolation and Characterisation of Phosphate-solubilising Bacteria for the Production of Bio-fertiliser

G. V. G. Priyadarshani*[1], G. Chandrasena[2] and S. M. T. A. Maralanda[1]

[1]*Sugarcane Research Institute, Uda Walawe, Sri Lanka*

[2]*Uva Wellassa University, Badulla, Sri Lanka*

**Corresponding Author: gayanip87@gmail.com*

ABSTRACT

A study was conducted to isolate bacteria capable of solubilising phosphate from the rhizospheres of sugarcane and different grasses aiming at producing a bio-fertiliser using filter-mud and distillery effluent as the carrier medium. Twenty seven phosphate-solubilising bacteria strains were isolated from soil samples collected from roots and rhizospheres of sugarcane, maize and finger millet plants in sugarcane-growing areas in Sri Lanka. They were named from B1 to B27 and identified based on their morphology and bio-chemical features. The phosphate-solubilising ability was studied by determining the phosphate-solubilising index (PSI). The viability of the bacteria with the highest PSI was tested in the carrier media formulations prepared using sugarcane filter mud and distillery effluent .The results revealed that the isolate B22 grown in PVK media had the highest PSI (3.00) followed by the isolates B13 (2.817), B8 (2.792), B18 (2.714), B23 (2.714) and B15 (2.700).The bacterial isolates survived 90 days in filter-mud and 60 days in the distillery effluent.

Keywords: Bio-fertiliser, Filter-mud, Phosphate-solubilising bacteria, Phosphate-solubilising index, Spent-wash, Sri Lanka, Sugarcane

INTRODUCTION

Phosphorus (P) is the second key mineral nutrient for plant growth and development making up about 0.2 % of plant dry weight. Its functions cannot be performed by any other nutrient. It is classified as a major nutrient required by crops in relatively large amounts for optimum growth and reproduction. It plays an important role in many physiological activities of plant such as cell division, photosynthesis, and development of good root system and utilisation of carbohydrate. The total P concentration in agricultural crops generally varies from 0.1 to 0.5 per cent (Gyaneshwar, *et al.*, 2002; Fernandez, *et al.*, 2007).

Plants acquire P from soil solution as phosphate anions. However, phosphate anions are extremely reactive and immobilised through precipitation with cations such as Ca^{2+}, Mg^{2+}, Fe^{3+} and Al^{3+}, depending on the particular properties of a soil. In these forms, P is highly insoluble and unavailable to plants. As a result, the amount available to plant is usually in a small proportion. A greater part of soil phosphorus, approximately 95–99%, is present in the form of insoluble phosphates, and hence, cannot be utilised by plants.

To increase the availability of phosphorus to plants, large amounts of fertiliser are used regularly. But after application, a large proportion of fertiliser phosphorus is quickly transformed into insoluble form. Therefore, very little percentage of the applied phosphorus is used, making continuous application necessary (Abd Alla, 1994).

The principal mechanism for mineral phosphate solubilisation is the production of organic acids. Acid phosphatase plays a major role in the mineralisation of organic phosphorus in soil. The transformation of insoluble phosphate into soluble form is carried out by a number of microbes present in the soil.

Introduction of phosphate-solubilising bacteria (PSB) has been considered as one of the possible alternatives for inorganic phosphate fertilisers for promoting plant growth and yield (De- Freitas *et al.*, 1997; Rodrı´guez and Fraga, 1999; Vessey, 2003; Thakuria *et al.*, 2004). Seed or soil inoculation with PSB is known to improve the solubilisation of the fixed soil P and applied phosphates, resulting in higher crop yield (Yahya and Al-Azawi, 1989; Abd-Alla, 1994; Mehta and Nautiyal, 2001). In fact, PSB render more phosphates into the soluble form than required for their growth and metabolism by secreting organic acids and/or enzymes (*e. g.* phosphatases) (Vessey, 2003). PSB plays an important role in supplementing P to plants, allowing a sustainable use of phosphate fertilisers (Gyaneshwar *et al.*, 2002). Hence, application of efficient PSB strains as bio-fertiliser (bio-inoculant) components into cropped fields has been reported to improve the crop growth and development and increase crop yields (Jones *et al.*, 1997).

Although a small country, the diversity of soils and vegetation in Sri Lanka is high. Under these conditions, there is a prospect of using PSB inocula in crop production systems. A significant reduction in the use of phosphate fertiliser could be achieved if solubilisation of soil-insoluble P is made available to crop plants (Rodrı´guez and Fraga, 1999; Vessey, 2003; Thakuria *et al.*, 2004). This will not only reduce the cost on inorganic fertiliser, but also their adverse environmental impacts.

A large quantity of filter-mud, a by-product of sugar manufacturing is generated during sugarcane juice clarification. Even though, filter-mud is rich in nutrients, in Sri Lanka, it is not utilised. Further more, the disposal of spent-wash generated in sugarcane distilleries has also been a problem for sugar industries due to its high levels of environmental pollutants. Though it can be used in making organic fertiliser, the Sri Lanka still wastes this valuable by-product.

The objective of this study was to isolate and characterise phosphate-solubilising bacteria from the natural environment and study their phosphate-solubilising ability for the production of bio-fertiliser using the above-mentioned sugar factory and distillery by-products as the carrier medium to be used in sugarcane farming to increase the productivity of sugarcane lands while reducing the cost on inorganic fertiliser and minimising adverse environmental impacts.

MATERIAL AND METHODS

Collection of samples

Soil samples were collected from Uda Walawe, Pelwatte, Sevanagala, Badulla, Hingurana and Kilinochchi areas in Sri Lanka where sugarcane is grown. The samples were collected from roots and rhizospheres of sugarcane, maize and finger millet plants.

Isolation of phosphate-solubilising bacteria

PSB were isolated from each sample by serial dilution and spread plate method. One gram (1g) of soil sample was dispersed in 9 ml of autoclaved, distilled water and was thoroughly shaken. One ml of the above solution was again transferred to 9ml of sterilised distilled water to form 10^{-2}dilution. Similarly 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8}serials were made for each soil sample and 0.1ml of each dilution was spread on Pikovskaya's agar medium (PVK) containing insoluble Tricalcium phosphate and incubated at 27 – 30°C for 3 days.

After 3-days of incubation, PSB developed clear zones around bacteria colonies. The colonies with clear zones were further purified

by repeated culturing on PVK agar medium for studying colony morphology. The bacteria isolated were named as B1, B2, and so on.

Morphological characterisation

The bacterial isolates from overnight-grown cultures were spread on the PVK agar medium. The morphological characteristics of bacteria colonies such as configuration, elevation, margins, and colour were observed after 24 hours.

Biochemical identification

The bacterial isolates were identified through biochemical tests, namely, Gram staining, Catalase test and Starch hydrolysis test.

Gram staining: Slides of the isolated bacterial cultures were prepared for observation under light microscope for Gram staining by the Vincent method (1970).

Catalase test: A drop of 3% hydrogen peroxide was added to 48-hour-old bacterial colony on a clean glassslide. This test was performed to study the presence of catalase enzyme in PSB.

Starch hydrolysis test: The isolated PSB was streaked on Nutrient Agar medium supplemented with starch 0.5% and incubated at 37°C for 2-3 days. Iodine solution was added on to the bacteria growing plates to visualise the zone of clearing surrounding the bacteria colonies.

Determination of phosphate solubilisation ability

The phosphate solubilisation ability of the isolated bacteria was studied by spotting the bacteria cultures on the top of PVK agar medium containing insoluble Tricalcium phosphate (Ca_3PO_4). The bacterium that possesses the ability to solubilising phosphate forms a clear zone around the bacterial colonies after inoculation. The clear zone diameter and colony diameter were measured 2, 4, 6, 8, 10, 12 and 14 days after the incubation of the plates at 30°C. The solubilisation index of these isolates was calculated according to Edi-Premono *et al.* (1996).

$$\text{Phosphate Solubilisation Index} = \frac{\text{Total diameter (colony + clear zone)}}{\text{Diameter of colony}}$$

Survival of PSB in carrier media

The survival of PSB in the carrier medium at room temperature was estimated after inoculating the bacteria in to sterilised filter-mud and spent-wash after 90 days of incubation. The population of the bacteria was estimated by counting the Colony Forming Units (CFU).

Survival in filter-mud

The filter-mud obtained from the Sevanagala sugar factory was ground, sieved with 0.5 cm mesh screen and dried in an oven at 60°C for two days. It was then autoclaved at 121°C at for 20 minutes. The selected superior PSB isolate was inoculated into the prepared filter-mud carrier media. One gram of each sample was taken for estimating viable cells on the first day and 15, 30, 45, 60, 75 and 90 days after storage using the dilution plating method on PVK medium and incubated at 28°C for 5-7 days. The number of apparent PSB colonies were counted and the viability of the cells was estimated.

Survival in distillery spent-wash

The distillery spent-wash samples were collected from the distillery of Sevanagala sugar factory and carried to the laboratory of the Sugarcane Research Institute, Uda Walawe. The spent wash samples of five concentrations (5%, 10%, 15%, 20% and 100%) were prepared and sterilised by autoclaving at 121°C for 20 minutes. The selected bacteria with high phosphate-solubilising ability were inoculated into each sample.

One ml of each sample was taken for estimating viable cells on the first day and, 15, 30, 45, 60, 75 and 90 days after storage using the dilution plating method on PVK agar medium and incubated at 28°C for 5-7 days. The number of apparent PSB colonies were counted and the viability of the cells was estimated.

Statistical analysis

All experiments were carried out in triplicate to analyse the significance of the differences in phosphate solubilisation ability of the isolated bacteria by one-way analysis of variance (ANOVA). The mean differences of the phosphate solubilisation index were tested using Tukey's test at the 5% level of probability. MINITAB 16 was used for the statistical analysis.

RESULTS AND DISCUSSION

Isolation of PSB strains

Thirty two bacterial strains were isolated from the soil samples collected from various locations. Only 27, numbered from B1to B27 were able to solubilise actively phosphate *in-vitro* (Figure 1) and showed persistence of this capability after five or more subcultures (Table 1).These 27 PSB strains were selected for further characterisation.

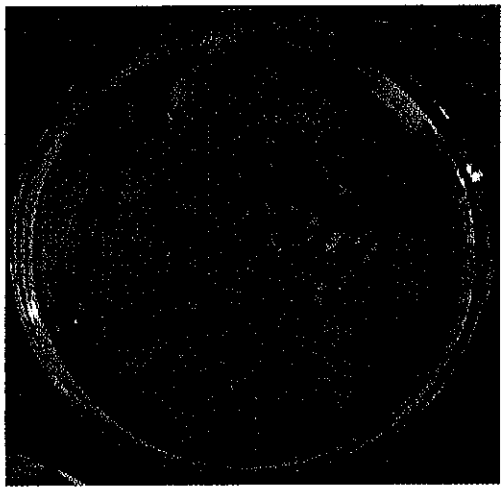


Figure 1 Halo Zone around the colony on PVK media confirms phosphate solubilising bacteria

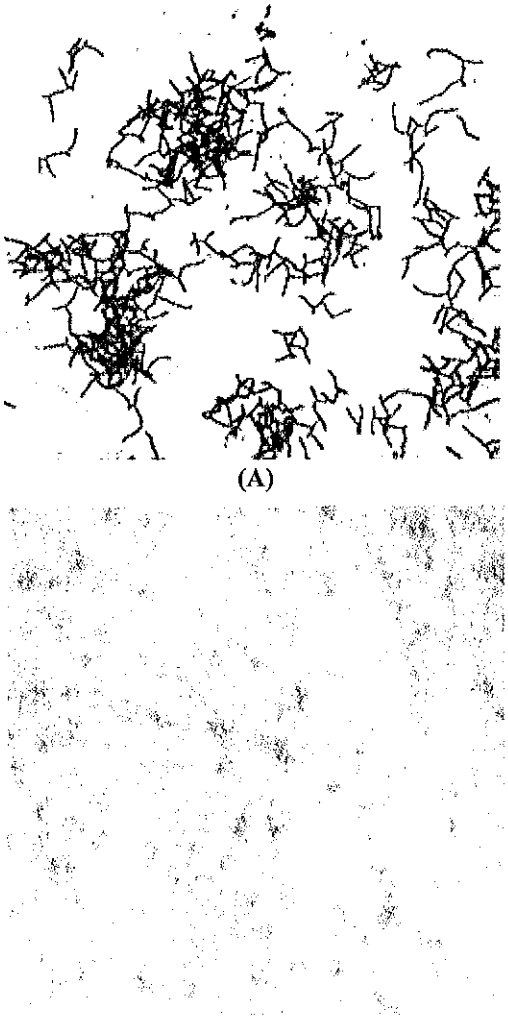
Morphological characterisation

Most of the isolated colonies exhibited round shape with smooth margins and flat elevation. The colour of the colonies showed a wide variation ranging from white to yellow and brown. The results are summarised in Table 1.

Identification of bacterial isolates

The results of the biochemical tests carried out to identify bacteria are shown in Table 2.

Fifteen bacteria isolates were Gram-positive and twenty isolates were Gram-negative. The Gram-positive bacteria appear purple as stained by crystal violet, which is trapped within their thick cell walls. The Gram-negative bacteria appear pink as stained by the safranin counter stain, as thin cell walls allow the crystal violet to wash out during decolourisation (Figure 2).



(A)

(B)

Figure 2 Appearance Bacterial isolates as Gram-Positive (A), Gram-Negative (B) under light microscope

Table 1 Colony characteristics of the individual isolates

Isolate Number (Bacteria)	Isolated Source	Configuration	Margin	Elevation	Colour
B1	Soil from fertiliser applied (before 3 months) sugarcane field-UdaWalawe	Round	Smooth	Flat	Yellow
B2	Soil from sugarcane cultivated field-UdaWalawe	Round	Smooth	Flat	Cream
B3	Soil from sugarcane harvested field-UdaWalawe	Round	Smooth	Convex	Yellow
B4	Soil from land prepared sugarcane field-UdaWalawe	Irregular	Lobate	Umbonate	Cream
B5	Soil from sugarcane cultivated field-UdaWalawe	Round	Smooth	Convex	Yellow
B6	Sugarcane roots from sugarcane cultivated field-UdaWalawe	Round with radiant margin	Branching	Convex	White
B7	Sugarcane roots from sugarcane harvested field-UdaWalawe	Round	Smooth	Convex	Cream
B8	Soil from sugarcane cultivated field-UdaWalawe	Round	Smooth	Convex	Cream
B9	Rhizosphere of the finger millet plant	Round	Smooth	Convex	White
B10	Rhizosphere of the finger millet plant	Round	Smooth	Convex	Yellowish orange
B11	Soil from bagasses decomposing area- Sugarcane research institute	Round	Smooth	Umbonate	Yellow
B12	Soil from Ginigal-pelessa	Irregular	Wavy	Flat	Yellow
B13	Soil from sugarcane harvested field - Pelwatte	Round	Smooth	Raised	Yellow
B14	Soil from sugarcane cultivated field –Kilinochchi	Round	Smooth	Flat	Yellow
B15	Soil from maize field	Round	Smooth	Flat	White
B16	Soil from sugarcane cultivated field –Hingurana	Round	Smooth	Convex	White
B17	Soil from Badulla	Round	Wavy	Flat	Yellowish brown
B18	Soil from Badulla	Round	Smooth	Flat	Yellow
B19	Soil from Badulla	Filamentous	Branching	Flat	White
B20	Soil from Badulla	Round	Smooth	Convex	Yellow
B21	Soil from Badulla	Round	Wavy	Flat	Yellow
B22	Rhizosphere soil from sugarcane cultivated field – Pelwatte	Round	Smooth	Flat	Whitish yellow
B23	Sugarcane roots from Sugarcane cultivated field – Pelwatte	Round	Smooth	Convex	White
B24	Soil from sugarcane nursery field - Pelwatte	Round	Smooth	Flat	Cream
B25	Sugarcane roots from sugarcane nursery field –Pelwatte	Round	Smooth	Convex	Yellow
B26	Sugarcane roots from sugarcane harvested field – Pelwatte	Round	Smooth	Flat	Yellowish brown
B27	Soil from Badulla	L- form	Smooth	Umbonate	Cream

Fourteen bacteria isolates were able to produce catalase (catalase-positive) and other thirteen bacteria could not produce catalase (catalase-negative).The catalase enzyme serves to neutralise the bactericidal effects of hydrogen peroxide(H_2O_2). Catalase expedites the breakdown of hydrogen peroxide into water and oxygen ($2H_2O_2$ + Catalase $\rightarrow$ $2H_2O + O_2$). Positive reactions were evident by the rapid formation of bubbles (Figure 3).

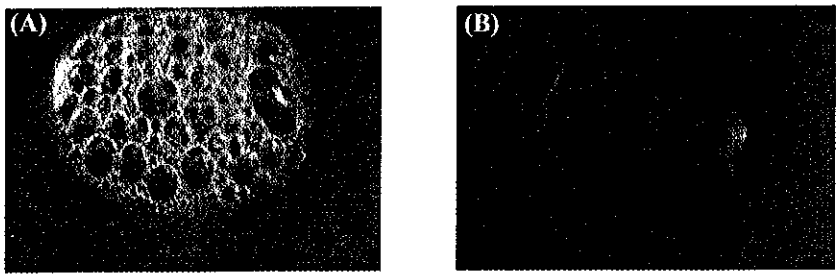


Figure 3 Slide catalase test results. Positive reaction (A), Negative reaction (B)

Table 2 Biochemical characterisation of the isolates

Isolate Number (Bacteria)	Gram Staining	Catalase Test	Starch Hydrolysis Test
B1	-	+	-
B2	+	-	+
B3	+	+	-
B4	+	+	+
B5	-	-	-
B6	-	+	-
B7	+	-	+
B8	-	-	-
B9	+	+	-
B10	+	+	+
B11	+	+	-
B12	-	-	-
B13	+	+	+
B14	-	+	-
B15	-	-	-
B16	+	+	+
B17	-	-	+
B18	+	-	+
B19	+	-	-
B20	-	+	+
B21	+	-	-
B22	-	+	-
B23	-	-	+
B24	+	+	-
B25	-	+	+
B26	+	-	-
B27	+	-	+

Twelve bacteria isolates hydrolysed starch and fifteen bacteria isolates could not hydrolyse starch. The positive test was showed by clear zone around the bacteria colony. This zone indicates the starch broken down to dextrins, maltose, and glucose/alpha-amylase.

Phosphate solubilisation ability of the bacteria isolates

The results of phosphate-solubilising ability were expressed as solubilisation index. All PSB isolates were able to solubilise phosphate in the culture media (Figure 4) at different levels. Phosphate solubilisation index values of the isolated bacteria are shown in Table 3.

Table 3 Phosphate solubilisation index of the bacteria isolates

Isolate Number	Phosphate Solubilisation Index						
	Days after Inoculation						
	2	4	6	8	10	12	14
B1	1.026	1.035	1.038	1.041	1.046	1.067	1.060
B2	1.333	1.214	1.226	1.264	1.154	1.123	1.086
B3	1.022	1.020	1.014	1.013	1.011	1.010	1.010
B4	1.042	1.063	1.066	1.076	1.082	1.100	1.100
B5	1.500	1.078	1.154	1.211	1.160	1.111	1.100
B6	1.875	2.017	1.886	1.703	1.473	1.386	2.300
B7	1.550	1.667	1.756	1.833	1.874	1.872	1.804
B8	1.575	1.552	1.848	2.056	2.771	2.784	2.792
B9	2.000	2.350	2.430	2.653	2.254	2.000	2.000
B10	1.232	1.121	1.321	1.554	1.567	1.567	1.233
B11	1.544	1.324	1.356	1.634	1.766	1.823	1.867
B12	2.422	2.666	2.588	2.200	2.230	2.000	2.000
B13	2.171	2.714	2.600	2.817	2.731	2.720	2.720
B14	2.014	1.888	2.000	2.122	1.312	1.310	1.300
B15	1.875	2.200	2.690	2.693	2.701	2.700	2.688
B16	1.750	1.688	1.866	2.000	2.022	2.000	2.000
B17	1.900	1.294	1.444	1.476	1.513	1.522	1.500
B18	2.700	2.714	2.055	2.173	2.220	2.320	2.320
B19	2.375	2.200	2.650	2.660	2.652	2.650	2.600
B20	2.600	2.302	1.836	2.000	2.200	2.230	2.220
B21	2.600	2.571	2.000	2.131	2.220	2.212	2.022
B22	2.630	2.750	2.771	2.874	2.845	3.000	3.000
B23	2.383	2.714	2.200	2.321	2.320	2.430	2.000
B24	2.167	1.710	1.597	2.000	2.466	2.580	2.694
B25	1.674	1.722	2.000	2.200	2.201	2.200	2.200
B26	1.662	1.705	1.537	1.624	1.522	1.600	1.621
B27	2.533	2.243	1.667	1.660	1.444	1.220	1.200

Note: Data values are means of three replicates.

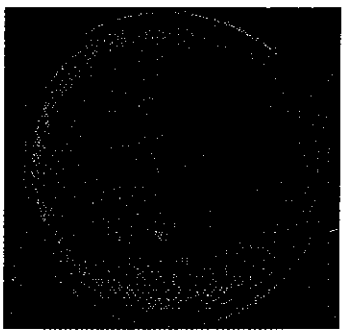


Figure 4 Phosphate solubilisation on PVK media plates

According to the results, the bacteria isolate B22 was recorded the highest phosphate-solubilising index of 3.0 followed by B13 (2.817), B8 (2.792), B18 (2.714), B23 (2.714) and B15 (2.700) (Figure 5). The isolate B3 was recorded the lowest phosphate-solubilising index from the evaluated isolates. After 10 days, phosphate-solubilising index of B3 was 1.0. Solubilisation index of the isolates B1, B4, B8,

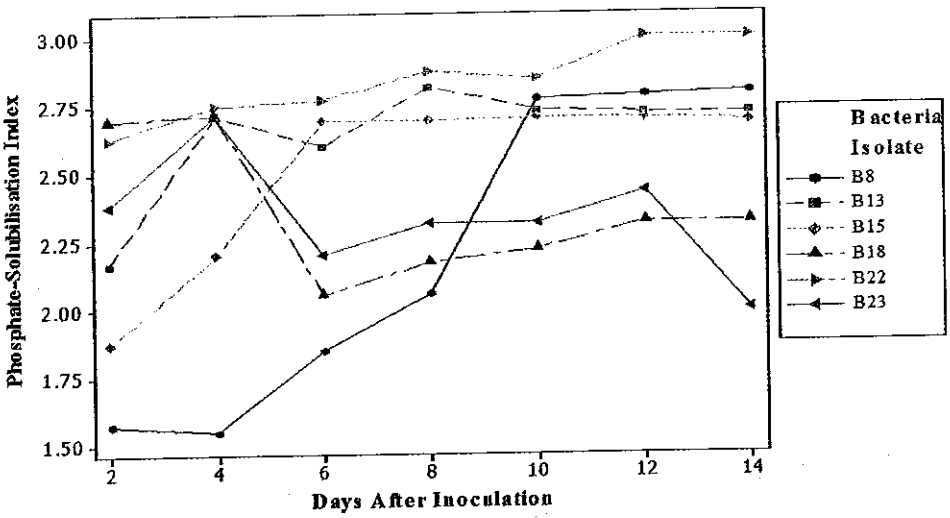


Figure 5 Bacteria isolates with high phosphate solubilisation index

B11, B15, B16, B22 and B25 gradually increased with time. The solubilisation index of the isolates B2, B5,B9, B13 and B14 increased up to 8th day of incubation, and then it remained unchanged although the colony was still growing. Therefore, the solubilisation index started to decrease after 10th days of incubation.

Most of the recent literature concerning microorganisms capable to form a clear zone due to organic acids production in the media plates and are selected as potential phosphate solubilisers (Singal *et al.*, 1991) and the clear zone formation could be due to the activity of phosphatase enzyme in bacterial isolates (Goldstein, 1995).The use of phosphate-solubilising bacteria as bio-fertilisers in crop production has been encouraging (Khan *et al.*, 2007).

Survival ability of PSB in the prepared carrier media

The results showed that the population of bacteria in the filter-mud media increased gradually up to 90 days (Table 05).

Initially, the bacterial population was high in all five concentrations of spent-wash media. After 60 days of incubation, the bacterial population in the spent- wash media declined rapidly (Table 6).

According to the results, the spent wash solution with 20% concentration supported significantly higher number of viable bacterial cells than the other samples on the first day. After 30 days, there was no significant difference in the survival ability in different concentration of spent wash. PSB can survive in both filter-mud and spent-wash carrier media more than two months. The filter-mud medium showed a higher survival ability of PSB than the spent-wash medium.

Table 5 Survival of PSB on filter-mud media during 6 months

Days after Inoculation	0	15	30	45	60	75	90
Viable Cell (log_{10}CFU/g)	12.234	12.436	13.237	13.877	14.216	14.975	15.132

Table 6 Survival of PSB on different concentration of spent-wash solution during 6 months period

Concentration of the Spent-wash solution (%)	Viable Cell (log_{10} CFU/g)						
	Days after inoculation						
	0	15	30	45	60	75	90
5	12.251	13.703	14.142	13.743	12.815	11.532	10.282
10	12.173	14.813	14.323	13.662	12.606	11.325	09.616
15	12.212	14.501	12.671	13.763	12.442	11.424	09.708
20	12.323	14.871	14.961	13.871	12.831	11.653	10.715
100	12.241	14.672	13.843	13.765	12.107	11.574	09.525

Conclusion

The bacteria isolate B22 was the most efficient in solubilising P on PVK agar medium with a solubilisation index 3.000 at 12 and 14 days after inoculation. The isolates B13, B8, B18, B23 and B15 showed relatively high solubilisation index. According to the results, PSB can survive in both filter-mud and spent-wash media. Therefore, sugar factory filter-mud and distillery effluent can be used as carrier media for the bio-fertiliser production This *in-vitro* potential of the bacteria needs to be further tested under natural field conditions to confirm their actual phosphate-solubilising ability for the production of bio-fertiliser.

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