The Bioscan 12(1): 113-116, 2017
AN INTERNATIONAL QUARTERLY JOURNAL OF LIFE SCIENCES
www.thebioscan.com

GROWTH, SURVIVAL AND SHELF LIFE ENHANCEMENT OF PHOSPHATE SOLUBILIZING BACTERIAL LIQUID INOCULANTS FORMULATIONS WITH POLYMERIC ADDITIVES

AARTI YADAV*, SUBHA DHULL, ANJU SEHRAWAT AND SUNITA SUNEJA
Department of Microbiology,
Choudhary Charan Singh Haryana Agricultural University Hisar - 125 004 (Haryana), INDIA
e-mail: yadav.aarti011@gmail.com

KEYWORDS
Cell protectants
Growth promotion
Liquid inoculants
PSB and Shelf life

Received on :
23.11.2016

Accepted on :
18.01.2017

***Corresponding author**

ABSTRACT
Effect of three polymeric additives; polyvinyl pyrrolidone (PVP), gum arabic (GA) and glycerol to promote growth and survival of liquid phosphate solubilizing bacterial (PSB) inoculants (*Pseudomonas* sp. strain P-36) during the storage. Maximum growth and survival of PSB inoculants were observed in Nutrient broth (NB) containing glycerol (1%) (8.225 log number of cells) as compared to standard Pikovaskaya broth (6.000 log number of cells) after 30 days of incubation. On the basis of growth and survival, gum arabic (GA), polyvinyl pyrrolidone (PVP) and glycerol were added @1 and 2% concentration in Nutrient broth (NB) containing 1% glycerol (added after the growth). Higher shelflife of PSB was observed in inoculants amended with GA (2%, 1%) followed by PVP (2%, 1%) and glycerol (2%, 1%). Survival of PSB was higher (8.879 and 8.329 log no. of cells) in inoculant vials amended with 2% GA stored under refrigerated conditions as compared to room temperature conditions (7.784 and 7.304 log no. of cells) at 90 days and 180 days of storage. Hence, amendment of glycerol and GA into the broth maintain the shelf life of bioinoculants up to 6 months and helps to sustain proper level of soil fertility and crop productivity.

INTRODUCTION

Phosphorus (P) is the most critical macronutrient required by both plant and microorganisms, and it is highly deficient in Indian soils. Total P content in agricultural soils lies in the range of 200-500 mg/kg but available 'P' is very low. The average utilization efficiency of added fertilizer P by plants ranges from 15-25 percent (Ali *et al*., 2014). Phosphate solubilizing bacteria (PSB) is one of the most promising bacteria that solubilize the 'P' from insoluble fixed forms by production of organic acids, phenolic compounds, protons and siderophores and make the P available to the plants which enhances its availability during crop growing season and results in increased crop production (Rodriguez *et al*., 2006; Sharma *et al*., 2014). PSB have also known as plant growth promoting rhizobacteria (PGPR) because they colonize the plant roots, promote the growth of the plants by increasing nutrient content in soil and supply phosphate to plants in environment-friendly and sustainable manner (Das and Singh, 2014).

Interest in liquid biofertilizer formulations has grown rapidly all over the world due to their benefits over the traditional carrier-based biofertilizers such as longer shelf life, better survival on seed and better nodulation, cost saving on carrier material i.e. pulverization, neutralization, sterilization, contamination free and convenience of handling, storage and transportation (Hedge, 2008). The success of a biofertilizer is dependent upon the survival of the microbial strain in the soil and it is a big challenge (Xavier *et al*., 2004). The best way to develop a biofertilizer contains resistant microbial strain and able to survive under the wide range of growth and storage conditions. It has been observed that it is possible to make bacteria survive in liquid medium more than six months with the help of additives or cell protectants such as trehalose, arabinose, FeEDTA, glucose and poly vinyl pyrrolidone (PVP), polyethylene glycol (PEG), gum arabic (Panlada *et al*., 2007, Kumaresan and Reetha, 2011, Pindi and Satyanarayna, 2012; Daniel *et al*., 2013). These polymers are normally used as the adhesive when they are applied to the seed. They are also soluble in water and are convenient for seed application, a simple process for farmers.

The selection of ideal polymer is based upon several properties like complex chemical nature, solubility in water and non-toxicity which prevents microorganisms in the soil from rapid degradation. So, a breakthrough is necessary for the current inoculant technology to reinforce shelf life and field efficacy of biofertilizers in India to make them commercially viable and acceptable to the farmers. Therefore, the present study was conducted to increase the survival of the liquid formulations of liquid PSB inoculants (*Pseudomonas* sp. strain P-36) by the addition of different polymers like glycerol, gum arabic and polyvinyl pyrolidone.

MATERIALS AND METHODS

Culture, media and growth conditions

Bacterial strain of PSB (phosphate solubilizing bacterium, *Pseudomonas* sp. strain P-36) was obtained from the

Department of Microbiology, CCS Haryana Agricultural University, Hisar. Nutrient broth and Pikovaskaya's broth (Pikovskaya, 1948) were used to culture phosphate solubilizing bacterium (*Pseudomonas* sp. strain P-36) with selected appropriate concentrations of additives. The freshly prepared inoculum of pure culture of *Pseudomonas* sp. strain P-36 was used for the inoculation and transferred @ 5% to flasks containing nutrient broth with different additives, designated as M1-M9. Different modified media: Standard Pikovskaya Broth [M1], Nutrient Broth without glucose [M2], Nutrient Broth[§] [M3], Nutrient Broth[§] + glycerol (1%) [M4], Nutrient Broth[§] + glycerol (2%) [M5], Nutrient Broth[§] + poly vinyl pyrrolidone (PVP) (1%) [M6], Nutrient Broth[§] + poly vinyl pyrrolidone (PVP) (2%) [M7], Nutrient Broth[§] + gum arabic (GA) (1%) [M8], Nutrient Broth[§] + gum arabic (GA) (2%) [M9]

[§] - Nutrient broth containing 1% glucose

Survival of PSB *Pseudomonas* sp. strain P-36 in amended media

To monitor the growth, a fresh inoculum of PSB was transferred @ 5% to each 250 ml flask containing 100 ml of sterilized medium designated as M1 to M9. All the medium flasks were kept on the shaker at 29 ± 1°C. Three ml sample from each flask was drawn at an interval of 24 hours up to 4 days and optical density was measured at 600 nm. To check the survival of PSB in different amended media all the culture flasks of above growth experiment were shifted to stationary conditions at room temperature after 4 days and 1 ml sample was drawn on 5, 10, 20 and 30 days of storage for taking viable counts. The medium showing maximum viable count of PSB was selected for the next experiment.

Determination of shelf life of PSB *Pseudomonas* sp. strain P-36

To monitor the shelf life of liquid inoculants, the above-selected medium was sterilized and inoculated with fresh PSB inoculums @ 5%. All the medium flasks were incubated at 29 ± 1°C on a rotatory shaker for 2 days. The culture broth was transferred into the sterilized 100 ml capacity plastic vials under aseptic conditions. Before filling the vials, one culture flask was kept as control and the remaining culture flasks were amended with different additives such as glycerol (1% / 2%), PVP (1% / 2 %), GA (1% / 2%). The plastic vials containing liquid inoculants of PSB strain were stored in two sets. One set was stored at room temperature and another set was stored in a refrigerator. One ml sample was drawn aseptically at 0, 15, 30, 45, 60, 90, 120, 150 and 180 day for taking viable counts (Sehrawat *et al*., 2015). Room temperature was monitored during the storage of the inoculant vials which was calculated by taking the average of the maximum and minimum room temperature of one week.

Statistical analysis

Completely randomized design (CRD) was used for experimental data analysis and critical difference (CD) was calculated at 5% level of significance.

RESULTS AND DISCUSSION

To enhance shelf life of *Pseudomonas*. sp. strain P-36 cells in liquid bioinoculant, additives like PVP, glycerol and gum arabic were added as supplements to Nutrient Broth. At 4th day and 30th day maximum growth and survival (8.225 log number of cells) of PSB strain were observed in NB[§] containing 1% glycerol (M4) followed by 2% gum arabic (M9) (Fig. 1 and Table 1). More growth of PSB was observed in NB with or without additives as compared to Pikovskaya broth because NB is rich medium compared to Pikovskaya broth and glucose present in the medium promotes the production of the storage product glycogen (Singleton *et al*., 2002). Our results are similar to Mugilan *et al*. (2011) who reported better survival of PSB (*Pseudomonas striata*) in liquid formulation amended with glycerol.

All the inoculants amended with additives stored at refrigerator showed higher viable count of PSB as compared to inoculant without additive (M1*) as well as inoculants amended with additives stored at room temperature. Maximum viable count was observed in inoculant amended with 2% GA (M7*). Initially 10.303 and 10.90 log no. of viable cells were observed in inoculant amended with 2% GA which decreased to 8.879 and 7.784 log no. of cells at 90 d which further decreased to 8.329 and 7.304 log no. of cells followed by inoculants amended with 1% GA (M6*) with 7.945 and 6.103 log no. of viable cells ml^{-1} respectively at 180 d storage at refrigerator and at room temperature respectively (Table 2 and 3). Our results are similar to Velineni and Brahmaprakash (2011) who studied the survival of PSB (*Bacillus megaterium)* in liquid formulations supplemented with different cell protectants under the influence of high temperature and desiccation stress. Sahai and Chandra (2009) also observed higher shelf life of PSB (*Pseudomonas* sp.) in liquid inoculants stored under

Table 1: Survival of PSB strain P-36 in different media

Medium	Log no. of viable cells ml^{-1} atdifferent days of storage				
	0	5	10	20	30
Pikovskaya Broth (M1)	7.954	8.426	6.727	6.367	6.000
Nutrient Broth without glucose (M2)		9.028	7.668	7.542	7.410
Nutrient Broth* (M3)		10.048	8.263	7.776	7.659
Nutrient Broth* + Glycerol (1%) (M4)		9.514	8.716	8.528	8.225
Nutrient Broth* + Glycerol (2%) (M5)		9.386	8.166	7.278	6.522
Nutrient Broth* + (PVP) (1%) (M6)		9.934	7.921	7.647	7.212
Nutrient Broth* + (PVP) (2%) (M7)		8.724	7.879	7.602	7.569
Nutrient Broth* + (GA) (1%) (M8)		8.602	7.727	7.609	7.587
Nutrient Broth* + (GA) (2%) (M9)		10.024	8.380	8.292	7.775
C.D. at 5%		0.158	0.284	0.111	0.087

*Nutrient broth containing 1% glucose

Table 3: Survival of PSB strain P-36 in different formulated media in sterilized 100 ml plastic vials stored at room temperature

Medium	Log no. of viable cells ml^{-1} at different days of storage								
	0	15	30	45	60	90	120	150	180
Control (M4) {M1*}	9.771	8.762	8.435	7.982	7.563	7.000	6.276	5.683	5.062
M4+ 1% glycerol {M2*}	10.101	8.587	8.345	7.923	7.701	7.070	6.352	6.037	5.64
M4+ 2% glycerol {M3*}	9.951	8.409	8.350	7.876	7.251	7.000	6.640	6.180	5.684
M4+ 1% PVP {M4*}	10.099	8.408	8.122	7.948	7.654	7.154	6.554	6.156	5.767
M4+ 2% PVP {M5*}	9.971	8.816	8.366	8.115	7.869	7.513	6.737	6.084	6.037
M4+ 1% GA {M6*}	9.622	8.217	8.145	8.098	8.030	7.544	6.864	6.406	6.103
M4+ 2% GA {M7*}	10.090	9.340	9.017	8.207	8.135	7.784	7.656	7.433	7.304
C.D. at 5%	0.055	0.125	0.196	N/A	0.211	0.153	0.112	0.012	0.117

M4 - NB + 1% glycerol; M2*-M7*- amendments added after the growth

Table 2: Survival of PSB strain P-36 in different formulated media in sterilized 100 ml plastic vials stored in a refrigerator

Media	Log no. of viable cells ml $^{-1}$at different days of storage								
	0	15	30	45	60	90	120	150	180
Control (M4) {M1*}	10.164	8.735	8.654	8.626	8.239	8.127	8.124	7.518	7.214
M4+ 1% glycerol {M2*}	10.457	8.712	8.378	8.414	8.371	8.182	8.129	7.654	7.217
M4+ 2% glycerol {M3*}	10.184	8.547	8.392	8.265	8.217	8.153	7.982	7.957	7.485
M4+ 1% PVP {M4*}	10.375	8.509	8.328	8.277	8.273	8.262	8.129	7.983	7.878
M4+ 2% PVP {M5*}	10.299	8.747	8.644	8.546	8.414	8.207	7.952	7.862	7.856
M4+ 1% GA {M6*}	9.795	8.664	8.642	8.581	8.628	8.271	8.179	8.106	7.945
M4+ 2% GA {M7*}	10.303	9.552	9.256	9.102	8.935	8.879	8.848	8.408	8.329
C.D. at 5%	0.038	0.106	0.313	0.154	0.117	0.153	0.104	0.150	0.135

M4 - NB + 1% glycerol; M2*-M7*- amendments added after the growth

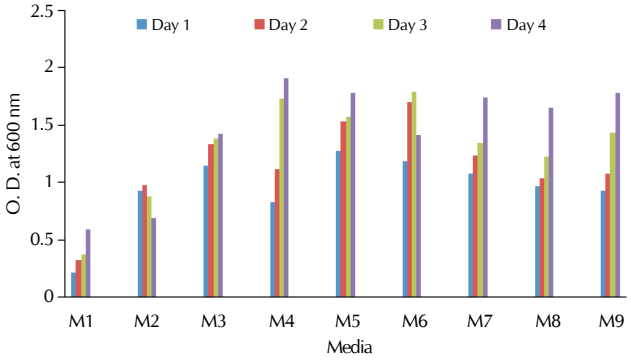

Figure 1: Growth of PSB strain P-36 in different media

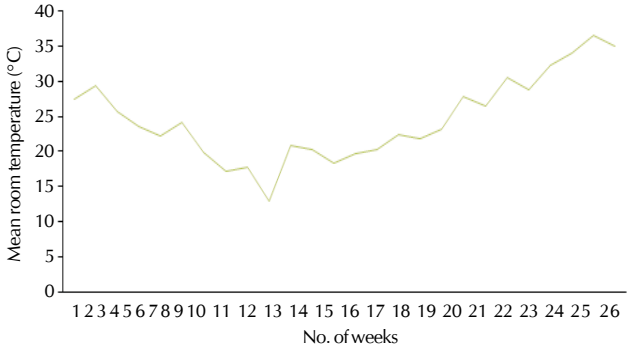

Figure 2: Data of mean room temperature during storage of biofertilizer vials from November 2012 to April 2013

refrigerated conditions as compared to room conditions. Higher survival under refrigerated conditions may be due to the fact that low temperature in refrigerator allows no or little growth with less utilization of nutrients during storage making them available to the organism in optimum concentration for longer period. Low temperature in refrigerator also protects the cell death in inoculums. In contrast, during storage of inoculants at room conditions where temperature may go beyond 30 °C, growth of organism is allowed creating depletion of nutrients and accumulation of toxic metabolites (Tittabutr *et al*., 2007).

Daniel *et al*. (2013) also evaluated the effect of polymeric additives, adjuvants and surfactants for their ability to support growth and shelf-life stability of liquid bioinoculants (*Bacillus megaterium* var. *phosphaticum*, *Azospirillum brasilense* and *Azotobacter chroococcum*). They observed that liquid inoculants formulated with 2% poly vinyl pyrollidone (PVP 30 K), 0.1% carboxy methylcellulose (CMC-high density) and 0.025% Polysorbate 20 promoted long term survival of *Azospirillum, Bacillus megaterium* var. *phosphaticum*, and *Azotobacter* with 1.9x10^8, 5.6 x 10^7, and 3.5x10^7 cfu ml^{-1}, respectively after 480 days of storage at 30 °C. Similarly, Sherawat *et al*. (2015) also made attempts to enhance the survival / shelflife of liquid rhizobial inoculants (*Rhizobium* sp. Strain MB 703) through addition of additives such as glycerol, PVP, GA for their ability to support growth and promote survival in yeast extract mannitol (YEMB) during storage. All liquid rhizobial inoculants prepared in amended media showed higher viable count in comparison to inoculants prepared in YEMB (control) at 180 days of storage. Maximum log no. of cells were obtained in inoculants prepared in YEMB amended with 2% GA followed by YEMB + 1% GA and YEMB + 2% PVP.

Polymers viz., GA or PVP help in maintaining the higher viable count. Bacteria do not use these polymers as an energy source, these polymers have other properties supporting the growth

and survival of cells. PVP is believed to detoxify the fermentation medium by complexing with the phenolic-type, shelf-limiting toxins in the medium. PVP also has protective property known as colloidal stabilization. Gum arabic has sticky consistency due to its adhesive properties which may protect the cells from desiccation and drying (Tittabutr *et al*., 2007). Polymers which are soluble in liquid inoculants formulations make convenient batch processing of inoculants and make seed application a simple process for the farmers.

Room temperature during storge of inoculant vials at room temperature varied from 16 °C to 32 °C (Fig. 2) which could reduce cell survival in all the inoculant vials prepared with or without amendments. From the results discussed, it can be inferred that shelf life of PSB liquid inoculants could be enhanced by growing the bacteria in NB containing 1% glucose amended with 1% glycerol followed by the further addition of PVP or GA up to 6 months in comparison to control (without amendment) as depicted in Tables 1 & 2.

ACKNOWLEDGMENT

The authors are thankful to Professor and Head, Department of Microbiology, College of Basic Sciences and Humanities, CCSHAU, Hisar for providing facilities and support rendered.

REFERENCES

Ali, M. S., Sutradhar, A., Edano, M. L., Edwards, J. T., Girma, K. 2014. Response of winter wheat grain yield and phosphorus uptake to foliar phosphite fertilization. *Int. J. Agro.* DOI: 10.1155/2014/801626.

Daniel, L. A. E., Venkateswarlu, B., Desai, S., Kumar, P. G., Ahmed, A. H. S. K., Sultana, U., Pinisetty, U. and Narasu, L. M. 2013. Effect of polymeric additives, adjuvants, surfactants on survival, stability and plant growth promoting ability of liquid bioinoculants. *J. Plant. Physiol. Pathol.* **1(2):** 1-5.

Das, I. and Singh, A. P. 2014. Effect of PGPR and organic manures on soil properties of organically cultivated Mungbean. *The Bioscan.* **9(1):** 27-29.

Hegde, S. V. 2008. Liquid bio-fertilizers in Indian agriculture. *Biofertil. Newsletter.* pp. 17-22.

Kumaresan, G. and Reetha, D. 2011. Survival of *Azospirillum brasilense* in liquid formulation amended with different chemical additives. *J. Phyt.* **3(10):** 48-51.

Mugilan, I., Gayathri, P., Elumalai, E. K. and Elango, R. 2011. Studies on improve survivability and shelf life of carrier using liquid inoculation of *Pseudomonas striata. International J. Pharmaceutical and Biological. Archives.* **2(4):**1271-1275.

Panlada, T., Payakapong, P. W. and Boonkerd, N. 2007. Growth, survival and field performance of *Bradyrhizobial* liquid inoculant formulations with polymeric additives. *Sci. Asia*. **33:** 69-77.

Pikovskaya, R. I. 1948. Mobilization of phosphates in soil in connection with the vital activities of some microbial species. *Mikrobiologiya.* **17:** 362-370.

Pindi, P. K. and Satyanarayana, S. D. V. 2012. Liquid microbial consortium - A potential tool for sustainable soil health. *J. Biofertil. Biopestici.* **3(4):** 1-9.

Rodriguez, H., Fraga, R., Gonzalez, T. and Bashan, Y. 2006. Genetic of phosphate solubilization and its potential applications for improving plant growth promoting bacteria. *Plant Soil.* **287:** 15-21.

Sahai, P. and Chandra, R. 2009. Shelf life of liquid carrier based *Mesorhizobium* sp. and *Pseudomonas* sp. inoculants under different storage conditions. *J. Food Legumes.* **22(4):** 280-282.

Sehrawat, A., Suneja, S., Yadav, A. and Anand, R. C. 2015. Influence of different additives on shelflife of Rhizobial inoculants for mungbean (*Vigna radiata* L.). *Int. J. Recent Scientific Res.* **6(5):** 4338-4342.

Sharma, R., Rana, S. and Kaur, M. 2014. Isolation and characterization of bacterial isolates for phosphate solubilization and other plant growth promoting activities from apple soil of Himachal Pradesh. *The Bioscan.* **9(1):** 443-448.

Singleton, P., Keyser, H. and Sande, E. 2002. Development and evaluation of liquid inoculants. Australian Centre for International Agriculture Research, Canberra. pp. 52-66.

Tittabutr, P., Payakapong, W., Teaumroong, N., Singleton, P. W. and Boonkerd, N. 2007. Growth, survival and field performance of *Bradyrhizobial* liquid inoculants formulations with polymeric additives. *Sci. Asia.* **33:** 69-77.

Velineni, S. and Brahmaprakash, G. P. 2011. Survival and phosphate solubilizing ability of *Bacillus megaterium* in liquid inoculants under high temperature and desiccation stress. *J. Agri. Sci. Tech.* **13:** 795-802.

Xavier, I. J., Holloway, G. and Leggett, M. 2004. Development of rhizobial inoculants formulations. *Crop Management.* DOI:10.1094/CM-2004-0301-06-RV.