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ENHANCED SHELF-LIFE OF *AZOSPIRILLUM* AND PSB THROUGH ADDITION OF CHEMICAL ADDITIVES IN LIQUID FORMULATIONS

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Abstract: A study was undertaken to standardize liquid formulation for *Azospirillum* (KAU isolate) and phosphate solubilizing bacteria (KAU isolate) with chemical amendments so as to increase the shelf-life of the inoculants for the benefit of farmers in Kerala. The main objective was to standardize liquid formulation and enhance the shelf-life of liquid formulation containing *Azospirillum* and PSB. The highest population of *Azospirillum* (1.77 $\times 10^8$ cfu/ml) was recorded in the case of trehalose (15 mM) whereas, PSB population (3.77×10^8 cfu /ml) was highest in the case of PVP (2.5%). Hence, trehalose (15 mM) and PVP (2.5%) were the most suitable chemical additive for enhancing the shelf life of *Azospirillum* sp. and PSB respectively upto 9 months with a population of 10^8 cfu/ml. These results indicated that the shelf-life of *Azospirillum* sp. and PSB could be enhanced upto 9 months at room temperature. However, further studies are needed to evaluate its efficiency under field conditions.
Keywords: Liquid formulations, *Azospirillum* sp. phosphate solubilizing bacteria, chemical amendments.

Introduction

The success of any biofertilizer in field depends on the quality of bioformulations. Most of the biofertilizer available in India are of inferior quality. The biofertilizer inoculants should survive up to field application and the shelf-life of inoculants is very important. However, majority of the bioinoculants produced are the carrier-based formulations (solid substrates). The biofertilizer which are solid carrier based inoculants suffer from short shelf-life, poor quality, high contamination and low field performances (Hegde, 2002). It has some limitations like short-shelf life, unavailability of good quality carrier material in the local area, labour intensive, sensitivity to temperature, contamination etc. To overcome the problems of carrier based formulations, liquid formulations are preferred these days due to its advantages like no contamination, better survival on seeds, longer shelf-life, less dosage and easy to use**.** Liquid biofertilizers (LB) contain a desired organism and their nutrients with special cell protectants or substances that encourage longer shelf life and tolerance to adverse conditions

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(Krishan Chandra *et.al*., 2005). The liquid formulations have a shelf-life of 18-24 months (Sharma *et al*., 2010). Several kinds of polymers have been used for inoculant production because of their ability to restrict heat transfer and high water activities (Mugnier and Jung, 1985).

Azospirillum is a free-living, plant-growth-promoting bacteria which is capable of improving the growth and yield of several plant species due to its ability to produce various phytohormones (Dobbelaere *et al*. 2001). The *Pseudomonas, Bacillus* (Illmer and Schinner 1992) *Aspergillus* and *Penicillium* fungi (Wakelin *et al*. 2004) are well known P-solubilizers. *Azospirillum* and phosphate solubilizing bacteria (PSB) are the two most popular biofertilizers in Kerala but no systematic work has been done on developing liquid formulations of *Azospirillum* and PSB. Literature on liquid formulations of native biofertilizers in Kerala is also scanty. Therefore, a study was undertaken to standardize liquid formulation for native *Azospirillum* and PSB isolates with chemical amendments so as to increase the shelf-life of the inoculants for the benefit of farmers in Kerala. The main objectives were to standardize liquid formulation and enhance the shelf-life of liquid formulation containing *Azospirillum* and PSB.

Materials and Methods

The identified isolates of *Azospirillum* and PSB were obtained from the repository of Dept. of Agricultural Microbiology, CoA, Vellayani and CoH, Vellanikkara respectively.

The Okon's broth (*Azospirillum*) and Pikovskaya's broth (Phosphate solubilizing bacteria) were prepared using standard protocol. Okon's broth (*Azospirillum)* and Pikovskaya's broth (phosphate solubilizing bacteria) were supplemented with Trehalose (Control, 5 mM, 10 mM, 15 mM and 20 mM), Glycerol (Control, 5 mM, 10 mM,15 mM and 20 mM) and PVP (Control,1,1.5,2,2.5 %) at different concentrations. Trehalose was prepared as stock solution and added separately into Okon's broth (100 ml) and Pikovskaya's broth (100 ml) by filter sterilization. Polyvinylpyrrolidone (PVP) and glycerol were added into Okon's broth (100 ml) and Pikovskaya's broth (100 ml) at different concentrations separately before sterilization.

The enumeration of *Azospirillum* and PSB were done at monthly interval using serial dilution and plate count method (Johnson and Curl, 1952) under aseptic conditions.

Results and Discussion

Effect of chemical additives on the *Azospirillum* sp. population in Okon's broth

In general, the population of *Azospirillum* sp. declined from x 10^{10} cfu /ml to x 10^5 cfu/ml at 14 MAI (Table 1). The population of *Azospirillum* sp. was highest in trehalose and glycerol when compared with PVP as supplement. The highest population of *Azospirillum* (1.77 $\times 10^8$ cfu/ml) was maintained in the case of trehalose (15 mM) upto 9 MAI and after that the population declined to 10^6 cfu/ml by 14 MAI. Hence, trehalose (15 mM) was the most suitable supplement for enhancing the shelf life of *Azospirillum* sp. with the population at 10^8 cfu/ml, which is also the quality standard for liquid formulation of *Azospirillum* sp. These results are in concurrence with earlier studies where enhanced survival of *Azospirillum* cells in the liquid formulation has been reported due to the action of chemical amendments added in the medium. In the present studies, the trehalose recorded highest population upto 9 months which might be due to its capacity to enhance cell tolerance to desiccation, osmotic pressure and temperature stress (Streeter 1985). Similarly, Vendan and Thangaraju (2006) developed liquid formulation of *Azospirillum brasilense* amended with trehalose, glycerol and PVP in NFb malate broth and reported 10^{8} cells/ml upto 10 months storage under room temperature, which is in agreement with the present studies.

Effect of chemical additives on the PSB population in Pikovskaya's broth

In general, the population of PSB declined from 10^{10} cfu /ml at 1 MAI to 10^6 cfu/ml at 14 MAI (Table 2). The population of PSB was highest in PVP when compared with other supplements added. The highest population of PSB (3.77$\times 10^8$ cfu /ml) was maintained in the case of PVP (2.5 %) upto 9 MAI and after that the population declined to 10^6 cfu/ml by 14 MAI. Hence, PVP (2.5 %) was the most suitable supplement for enhancing the shelf life of PSB with 10^8 cfu/ml which is also the quality standard for liquid formulation of PSB. Polyvinylpyrrolidine (PVP) has a high water binding capacity, which could maintain water around the cells for their metabolism (Singleton *et al.,* 2002, Deaker *et al.,* 2004). PVP and gum arabic have been reported to protect cells against toxic factors (Mugnier and Jung, 1985). Moreover, PVP have property known as colloidal stabilization which protects the bacteria in colloids (Deaker *et al.* 2004). Similarly, Sridhar *et.al.*(2004) developed a liquid inoculant using osmoprotectants for *Bacillus megaterium* in which PVP, glycerol and glucose supported higher viable population ($\log_{10}$ 10.50 CFU/ml) and endospores ($\log_{10}$ 9.21 CFU/ml) up to 6 months storage period. Liquid inoculant formulation of cowpea rhizobia prepared with PVP as an osmoprotectant also had higher shelf life than those without PVP amendment (Girisha *et. al.,* 2006). These studies are in agreement with present studies where PVP supplement recorded highest population (10^8 cfu/ml) upto 9 MAI.

Conclusion

The highest population of *Azospirillum* (1.77×10^8 cfu/ml) was recorded in trehalose (15 mM) whereas, PSB population (3.77×10^8 cfu /ml) was highest in the case of PVP (2.5 %) upto 9 MAI. Hence, trehalose (15 mM) and PVP (2.5%) were the most suitable chemical additives for enhancing the shelf life of *Azospirillum* sp. and PSB respectively upto 9 months with a population of 10^8 cfu/ml. These results indicated that the shelf-life of *Azospirillum* sp. and PSB could be enhanced upto 9 months at room temperature when compared to shelf-life of carrier based inoculant. However, further studies are needed to evaluate its efficiency under field conditions.

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Table 1: Effect of different chemical additives on the population of *Azospirillum* in Okon's broth at monthly interval

Chemical Additives	**Population of *Azospirillum* (cfu/ml)**															
	I MAI	**2 MAI**	**3 MAI**	**4 MAI**	**5 MAI**	**6 MAI**	**7 MAI**	**8 MAI**	**9 MAI**	**10 MAI**	**11 MAP**	**12 MAP**	**13 MAP**	**14 MAP**	**15 MAP**	**16 MAP**
Trehalose (5 mM)	0.06×10^{7}	0.332×10^{7}	1×10^{7}	0.66×10^{8}	0.99×10^{7}	300×10^{8}	6×10^{8}	1.44×10^{6}	0.33×10^{5}	0.11×10^{5}	0	0	0	0	0	0
Trehalose (10 mM)	0.462×10^{7}	19.6×10^{8}	1.6×10^{8}	2.33×10^{7}	0.33×10^{7}	0.44×10^{8}	0.11×10^{8}	0.11×10^{8}	0	0	0	0	0	0	0	0
Trehalose (15 mM)	0.66×10^{9}	0.33×10^{7}	10.6×10^{8}	1.33×10^{8}	0.66×10^{8}	0.66×10^{8}	1.43×10^{8}	26.5×10^{8}	1.77×10^{8}	0.33×10^{6}	0.55×10^{5}	0	0	0	0	0
Trehalose (20 mM)	0.66×10^{9}	0.332×10^{7}	71×10^{8}	2×10^{7}	0.66×10^{8}	0.33×10^{8}	7.98×10^{8}	0.44×10^{8}	0.22×10^{7}	0.11×10^{7}	1.55×10^{5}	1.33×10^{5}	0	0	0	0
PVP (1%)	0.266×10^{9}	0.52×10^{8}	23.3×10^{8}	3.66×10^{6}	0.33×10^{6}	300×10^{8}	1.11×10^{8}	0.11×10^{8}	0.55×10^{5}	0.33×10^{4}	0.66×10^{3}	0.77×10^{4}	0.22×10^{3}	0	0	0
PVP (1.5%)	0.66×10^{9}	0.80×10^{10}	0.66×10^{8}	1.66×10^{8}	4×10^{6}	0.33×10^{8}	0.11×10^{8}	0.11×10^{8}	0.11×10^{4}	0.22×10^{4}	0.66×10^{3}	1.55×10^{4}	0.11×10^{4}	1.99×10^{3}	0	0
PVP (2%)	0.264×10^{7}	0.32×10^{7}	2.33×10^{7}	1.33×10^{7}	1.66×10^{6}	0.66×10^{8}	1.33×10^{8}	0.33×10^{5}	0.22×10^{4}	0.22×10^{4}	0.66×10^{4}	0	0	0	0	0
PVP (2.5%)	0.38×10^{7}	0.32×10^{7}	0.66×10^{8}	1×10^{7}	1.66×10^{6}	1.88×10^{8}	0.55×10^{8}	0.11×10^{4}	0.33×10^{4}	0.55×10^{3}	0	0	0	0	0	0
Glycerol (5 mM)	0.64×10^{10}	0.6×10^{10}	5.33×10^{7}	0.66×10^{7}	0.33×10^{6}	2×10^{7}	0.55×10^{8}	0.22×10^{7}	0.66×10^{6}	0.11×10^{5}	0.18×10^{5}	0.11×10^{5}	0.11×10^{5}	2.11×10^{4}	0	0
Glycerol (10mM)	0.26×10^{10}	0.38×10^{10}	6.33×10^{7}	0.33×10^{8}	0.33×10^{7}	0.11×10^{8}	0.11×10^{6}	0.11×10^{5}	0.22×10^{5}	4.22×10^{5}	$0..22\times10^{5}$	0.33×10^{5}	0	0	0	0
Glycerol (15 mM)	0.12×10^{10}	0.3×10^{7}	2.66×10^{7}	1×10^{8}	1.66×10^{7}	8.44×10^{8}	0.33×10^{4}	0.77×10^{5}	0.44×10^{4}	1.44×10^{4}	0.22×10^{5}	0	0	0	0	0
Glycerol (20 mM)	0.18×10^{10}	0.06×10^{8}	0.66×10^{8}	7.66×10^{7}	1.33×10^{7}	3.33×10^{6}	0.22×10^{7}	0.22×10^{7}	0.11×10^{7}	0.22×10^{4}	3.1×10^{5}	2.66×10^{5}	0.11×10^{5}	0.55×10^{5}	0	0
Control without additives	0.67 X10^{7}	0.47×10^{7}	8.00 X10^{4}	0	0	0	0	0	0	0	0	0	0	0	0	0

Each value represents a mean of three replication

Table 2: Effect of different chemical additives on the population of phosphate solubilizing bacteria in Pikovskaya's broth at monthly interval

Chemical Additives	**Population of PSB (cfu/ml)**															
	I MAI	**2 MAI**	**3 MAI**	**4 MAI**	**5 MAI**	**6 MAI**	**7 MAI**	**8 MAI**	**9 MAI**	**10 MAI**	**11 MAP**	**12 MAP**	**13 MAP**	**14 MAP**	**15 MAP**	**16 MAP**
Trehalose (5mM)	0.11×10^{9}	6.66×10^{7}	0.44×10^{8}	0.77×10^{8}	1.88×10^{5}	0.44×10^{5}	0.11×10^{5}	0	0	0	0	0	0	0	0	0
Trehalose (10mM)	0.33×10^{7}	0.33×10^{7}	0.99×10^{8}	0	0	0	0	0	0	0	0	0	0	0	0	0
Trehalose (15mM)	0.33×10^{8}	0.33×10^{6}	1.66×10^{7}	0.77×10^{8}	0.66×10^{7}	0.11×10^{5}	0.22×10^{5}	0	0	0	0	0	0	0	0	0
Trehalose (20mM)	0.11×10^{8}	0.66×10^{8}	0.11×10^{8}	0.11×10^{5}	0	0	0	0	0	0	0	0	0	0	0	0
PVP (1%)	0.33×10^{6}	0.33×10^{8}	1.33×10^{7}	0.11×10^{8}	0.11×10^{8}	0.22×10^{6}	0.55×10^{4}	0.33×10^{5}	0	0	0	0	0	0	0	0
PVP (1.5%)	0.33×10^{8}	1×10^{8}	0.33×10^{8}	0.33×10^{8}	0	0	0	0	0	0	0	0	0	0	0	0
PVP (2%)	0.33×10^{10}	2.33×10^{7}	1×10^{8}	0.66×10^{8}	0.77×10^{8}	0.55×10^{8}	0.22×10^{8}	0.55×10^{6}	6.44×10^{6}	1.10×10^{6}	6.55×10^{6}	5.22×10^{6}	5.2×10^{6}	0.66×10^{6}	0	0
PVP (2.5%)	1.33×10^{10}	0.33×10^{9}	0.11×10^{9}	6.66×10^{7}	0.33×10^{8}	0.33×10^{7}	0.33×10^{7}	0.33×10^{8}	3.77×10^{8}	0.44×10^{5}	1.33×10^{6}	2.88×10^{6}	0.44×10^{4}	0	0	0
Glycerol (5 mM)	0.55×10^{4}	4×10^{8}	0.33×10^{7}	0.11×10^{8}	0.11×10^{8}	0.11×10^{7}	0.33×10^{6}	0.11×10^{6}	0.33×10^{6}	0.11×10^{6}	0	0	0	0	0	0
Glycerol (10mM)	4×10^{8}	2.33×10^{7}	0.66×10^{8}	0.33×10^{8}	0.11×10^{8}	0.88×10^{8}	1.22×10^{7}	0.22×10^{7}	16.22×10^{6}	0.22×10^{6}	0	0	0	0	0	0
Glycerol (15 mM)	0.22×10^{5}	0.33×10^{8}	1.33×10^{7}	1.33×10^{6}	0.44×10^{8}	0.99×10^{8}	1.55×10^{7}	1.88×10^{6}	2.33×10^{6}	0.11×10^{6}	1.66×10^{6}	0	0	0	0	0
Glycerol (20 mM)	0.11×10^{9}	2×10^{6}	1.66×10^{8}	1.33×10^{8}	0.33×10^{8}	1.21×10^{8}	0.11×10^{8}	18.33×10^{6}	0	0	0	0	0	0	0	0
Control without additives	$0.67 \text{ X}10^{6}$	0.33×10^{5}	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Each value represents a mean of three replication MAI: Months after inoculation