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Enhancing the shelf life of *Azotobacter and Azospirillum* bioinoculants by development of liquid formulations

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ABSTRACT
Liquid bioinoculant formulation has become the preferred technology to solve the problems associated with shorter shelf life, high contamination, poor quality, low field performance and processing solid carrier in carrier based bioinoculant formulation. In the present study was conducted to formulate and determine the shelf-life of liquid biofertilizers of efficient strains of Azotobacter chroococcum and Azospirillum brasilense using different cell protectants and nutrients in liquid broth. The cell protectants used were glycerol (10 mM), polyvinyl pyrrolidone (PVP, 2.0%), trehalose (10 mM),polyethylene glycol (PEG (1%),PVA (0.5%) and gum arabic (GA, 0.3%). The treatments without addition of cell protectants (only broth) and carrier (lignite) based formulation were maintained as check.The formulated liquid biofertilizers of Azotobacter and Azospirillum were stored in BOD incubator at 28±2 °C for a period of 12 months days and colony forming units were determined at monthly intervals. Liquid Azotobacter and Azospirillum bioinoculants formulated with trehalose (10mM) promoted long term survival of Azotobacter and Azospirillum followed by glycerol (10 mM) gum arabic (0.3%) and PVP (2%) and they supported 108 cells/ml up to 11 months of storage under ambient temperature (28°C to 32°C), whereas PEG (1%), PVA (0.5%) and control (lignite carrier) recorded the same population upto 8 months, 6 months and 5 months respectively. The results of the present study clearly indicated that the liquid formulation of Azotobacter and Azospirillum could be used more effectively than the carrier based formulation.
Keywords: bioinoculants, Azotobacter and Azospirillum, liquid biofertilizers

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INTRODUCTION

Microbial inoculants represent an emerging technology designed to improve the productivity of Agricultural systems in the long run. They can be seen as a technology aligned with principles of sustainable agriculture, as opposed to the increased use of pesticides and fertilizers in recent times. *Azotobacter* and *Azospirillum*are potential plant growth promoting rhizobacteria (PGPR). Its positive impacts on plant growth through several mechanisms which include enhancement of root development, production of growth regulators and nitrogen fixation. The content of nitrogen, phosphorus, potassium and various micronutrients is higher in plants inoculated with *Azospirillum* [4]. [3] reported that most of the international producers of biofertilizers are engaged in the production of carrier-based inoculants. Peat is the most frequently used carrier for rhizobial inoculant industry because it has characteristics such as high water holding capacity and high surface area that support rhizobial growth and survival in large numbers. However, peat is not available in many countries, especially in tropics, and will be depleted in many areas in future [14].

The carrier based microbial inoculants produced in India are generally lignite, coal (or) Charcoal based. The major disadvantages associated with these carriers are shorter shelf life, poor quality, high contamination and unpredictable field performance. The cost of solid carrier based inoculant production is high as it is labour and energy intensive process, involving milling, sieving and correcting PH [15]. Liquid inoculant formulation is one solution to the problems associated with processing of solid carriers. The use of various broth cultures amended substance that promotes cells survival in the package and after application for seed (or) soil. Additives to liquid inoculant formulations should have a role in protecting microbial cells on seed at high temperature and during desiccation. Many kinds of polymers have been used for inoculant production because of their ability to limit heat transfer, their good rheological properties and high water activities [11]. In the present study, experiments were conducted to increase the survival of the liquid formulations of Azospirillum and Azotobacter bioinoculants by the addition of different polymers like gum arabic, polyvinyl pyrollidone(PVP), glycerol, trahalose, polyethylene glycol (PEG) and poly vinylalcohol (PVA).

MATERIALS AND METHODS:

Microorganisms and Medium used

The strains used for liquid biofertilizer formulation were *Azotobacter chroococcum* AC-5,*Azospirillum brasilense* AZ-15. Waksman medium No.77 broth and Dobereiner's malic acid broth with NH4Cl (1g per liter) medium were used to culture *Azotobacter* and *Azospirillum* respectively. The sterilized broths were inoculated with the respective strains and incubated at 28±2°C on a reciprocatory shaker for 24 hrs. The cell protectants *viz*., glycerol (10 mM), polyvinyl pyrrolidone (PVP, 2.0%), trehalose (10 Mm), polyethylene glycol (PEG (1%),PVA (0.5%) and gum arabic (GA, 0.3%) were added to the broths during the preparation of media. The prepared media was inoculated with 1.0 ml log phase culture and incubated in BOD incubator at 28±2 °C.

Enumerating the viable cell population:

The Waksman medium No.77 and Dobereiner's malic acid with NH_4Cl (1g per liter) medium were prepared, sterilized and plated in sterile petriplates. The plates were kept at room temperature for 48h.Eight equal sectors on the outside bottom of the petridishes were radially marked. Four sectors were used for replication of one dilution and four for another, allowing two dilutions per plate. Serial dilutions were prepared by transfer of 1 ml each of inoculum into 9 ml sterile water blanks to get 10^{-1} dilutions. Similarly, the dilutions were made serially upto 10^{-10}. From the dilutions, 5µl was pipetted out and placed on the respective quadrant in the Petri plate. The plates were incubated at 28 ± 2°C without any disturbance and individual colonies were counted through this drop plate method [15].

Liquid inoculant production and survival of *Azospirillum* duringprolonged storage

For developing liquid formulation of *Azotobacter* and *Azospirillum*, Waksman medium No.77 broth and Dobereiner's malic acid broth with NH4Cl (1g per liter) medium were prepared and standardized dosage of chemical amendments *viz*,PVP (2%), glycerol (10mM), gum arabic (0.3%), trehalose (10mM),PEG (1.0%) and PVA (0.5%) were added to one litre of broth separately. One ml of log phase culture of *Azotobacter chroococcum* and *Azospirillum brasilense* were inoculated individually in each broth and flasks were incubated at room temperature. The broth cultures were analyzed for viable cell population and pH at monthly intervals upto 12 months.

RESULTS AND DISCUSSION

To enhance the shelf life of *Azospirillum* cells in liquid bioinoculant, certain chemicals *viz*., PVP, glycerol, gum arabic, trehalose, PEG and PVA were added as supplements to Waksman medium No.77 broth and Dobereiner's malic acid broth with NH_4Cl (1g per liter) medium.

Survival of liquid bioinoculant of *Azospirillum*

Liquid formulation of *Azospirillum,* was developed inDobereiner's malic acid broth with NH_4Cl (1g per liter) medium amended with PVP (2.0%), glycerol (10 mM), gumarabic (0.3%), trehalsoe (10 mM), PEG (1.0%) and PVA (0.5%)separately. The addition of the chemical amendments like trehalose, glycerol, gum arabic and PVP allowed to maintenance of 10^8 cells upto 11 months of storage, followed by, PEG upto 8 months and PVA upto 7 months

whereas the control (lignite carrier based formulations) recorded the population level of 10^8 only up to 5months. Among the amendments, trehalose supported highest number of *Azospirillum* cells throughout the observation period followed by glycerol, gum arabic, PVP, PEG and PVA (Table 1).

Survival of liquid bioinoculant of *Azotobacter*

Liquid formulation of *Azotobacter,* was developed in Waksman medium No.77 broth amended with PVP (2.0%), glycerol (10mM), gumarabic (0.3%), trehalsoe (10 mM), PEG (1.0%) and PVA (0.5%)separately. The addition of the chemical amendments like PVP,trehalose, glycerol and gum arabic and allowed to maintenance of 10^8 cells upto 11 months of storage, followed by, PEG upto 8 months and PVA upto 7 months whereas the control (lignite carrier based formulations) recorded the population level of 10^8 only up to 5months. Among the amendments, PVP supported highest number of *Azotobacter* cells throughout the observation period followed by trehalose, glycerol, gum arabic, PEG and PVA (Table 2).

Trehalose is an enigmatic compound, which act as a reserve carbohydrate that may be mobilized during stress [7]. It is widely reported to enhance cell tolerance to desiccation, osmatic and temperature stress. It acts by stabilizing both enzymes and cell membranes. The possible effect of trehalose's protective action is that it may be incorporated into the cell (or) may induce the synthesis of metabolites that protect against stress [6], which might be the reason for the higher population of *Azospirillum* cells in the trehalose treatments. Next totrehalose, 10 mM glycerol supported greater number of *Azospirillum*in liquid formulation. This may be due to high water binding capacity and may protect cells from the effect of desiccation by reducing therate of drying [10, 9]. [16] also found higher population of *Azospirillum* due to the addition of PVP at both 1 and2% levels. It might be due to its high water binding capacity. Various polymers, such as PVP, PEG and gum arabic have adhesive properties. They have sticky consistency, which may enhance cell adherence to seed, and their viscous nature may slow the drying process of the bioinoculants. PVP also has a high water binding capacity, which could maintain water around the cells for their metabolism [13]. PVP and gum arabic have been reported to protect cells against toxic seed coat factors. Biopolymers such as Cassava starch, alginate and gum arabic have the ability to limit heat transfer and also have high water activities [11].

Liquid inoculant formulation of cowpea rhizobia prepared with PVP as an osmoprotectant been observed to have higher shelf life than those without PVP amendment [5]. Some of the polymers and chemicals which can be used as additives and protectants in liquid inoculants include PVP, methyl cellulose, gumarabica, trehalose, glycerol, sodium alginate, poly ethylene glycol, polyvinyl alcohol and tapioca flour [12].[8] developed liquid formulation of *Pseudomonas fluorescens* amended with trehalose, glycerol and PVP in King'B broth and reported 10^8 cells/ml upto 10 months storage under room temperature. [13] developed liquid formulations of *Rhizobium* by adding various additives in the yeast extract mannitol media and claimed cell numbers of 1 x 10^{10} cells/ml in the liquid inoculant. Enhanced survival of *Azospirillum* cells in the liquid formulation may be due to the action of chemical amendments added in the medium. Trehalose is capable of enhancing cell tolerance to desiccation, osmotic pressure and temperature stress and stabilizing both enzymes and cell membranes. Moreover, some polymeric additives such as PVP,PVA and starch have polymeric properties. This protective property known as colloidal stabilization. The improvement of survival is analogous to the protective colloid effect where bacteria represent one colloid and the suspension the other [1].

Liquid bioinoculant formulation could be produced by simple fermentation process with minimum labour, space and energy, as the culture from the fermentor is directly packed under aseptic conditions and stored. The cost of production of liquid formulation could be lesser than that of carrier formulation. From this study, ithas been concluded that liquid formulation of *Azotobacter* and *Azospirillum* bioinoculants has a shelf life of one year compared to the carrier based inoculant. Among the different chemical additives trehalose(10mM) performed well in liquid formulation of *Azospirillum* whereas PVP (2%)) performed well in liquid formulation of *Azotobacter* and hence this two can be used in the formulation of liquid bioinoculant.

Table 1: Efficacy of liquid formulation of inoculants amended with selected concentrations of different additives on the survival of *Azospirillum brasilense*

Days	*Azospirillum* population (× 10^9 CFU ml $^{-1}$)						
	Control (lignte carrier)	**Glycerol (10 mM)**	**Trehalose (10 mM)**	**Gum arabic (0.3%)**	**PVP (2.0 %)**	**PEG (1.5 %)**	**PVA (1.0%)**
0	0.867 (8.94	43.00 (10.63)	45.00 (10.65)	41.67 (10.62)	50.00 (10.69	38.33 (10.58)	34.33 (10.3)
30	2.00 (9.3)	42.67 (10.63)	44.67 (10.65)	40.00 (10.60)	49.67 (10.69)	37.00 (10.57)	33.33 (10.52)
60	0.76 (8.88)	41.00 (10.61)	43.00 (10.63)	37.67 (10.57)	46.33 (10.66)	35.33 (10.55)	31.67 (10.50)
90	0.56 (8.75)	37.67 (10.57)	38.00 (10.58)	34.33 (10.53)	44.00 (10.64)	32.67 (10.51)	29.00 (10.46)
120	0.36 (8.56)	33.67 (10.53)	34.67 (10.54)	29.67 (10.47)	38.33 (10.58)	27.00 (10.43)	25.00 (10.39)
150	0.13 (8.11)	30.00 (10.48)	33.00 (10.52)	24.00 (10.38)	33.67 (10.53)	22.67 (10.35)	19.67 (10.29)
180	0.08 (7.9)	26.33 (10.42)	30.57 (10.49)	21.67 (10.33)	31.00 (10.49)	19.00 (10.27)	13.33 (10.12)
210	0.004 (6.6)	22.67 (10.36)	25.67 (10.41)	19.00 (10.28)	27.67 (10.44)	15.33 (10.19)	11.67 (10.07)
240	0.0002 (5.3)	18.00 (10.26)	23.33 (10.37)	16.33 (10.21)	23.00 (10.36)	9.67 (9.99)	8.00 (9.91)
270	0.00003 (4.48)	15.00 (10.18)	19.33 (10.29)	12.67 (10.10)	20.67 (10.32)	6.87 (9.84)	4.87 (9.69)
300	0.000002 (3.3)	10.33 (10.01)	15.67 (10.20)	9.33 (9.97)	16.33 (10.21)	2.63 (9.42)	1.23 (9.08)
330	-	7.67 (9.88)	10.00 (10.00)	6.67 (9.82)	13.67 (10.14)	0.67 (8.82)	0.067 (7.83)
360	-	4.67 (9.67)	7.73 (9.82)	2.13 (9.32)	9.00 (9.95)	0.27 (8.43)	0.013 (7.11)
SEd	0.010	0.008	0.008	0.009	0.002	0.015	0.004
CD(p=0.05)	0.032	0.017	0.018	0.020	0.006	0.032	0.010

Values in parenthesis are $\log_{10}$ transformed value

Table 2: Efficacy of liquid formulation of inoculants amended with selected concentrations of different additives on the survival of *Azotobacter chroococcum*

Days	*Azotobacter* population (× 10^9 CFU ml $^{-1}$)						
	Control (lignte carrier)	**Glycerol (10 mM)**	**Trehalose (10 mM)**	**Gum arabic (0.3%)**	**PVP (2.0 %)**	**PEG (1.5 %)**	**PVA (1.0 %)**
0	0.80 (8.9)	40.33 (10.61)	42.67 (10.63)	36.67 (10.56)	45.67 (10.66)	33.67 (10.53)	29.67 (10.47)
30	1.67 (9.22)	39.67 (10.59)	42.00 (10.62)	35.33 (10.55)	44.00 (10.64)	32.67 (10.51)	25.33 (10.4)
60	0.63 (8.79)	36.00 (10.56)	40.33 (10.61)	33.67 (10.53)	42.67 (10.63)	30.00 (10.48)	20.67 (10.32)
90	0.467 (8.65)	33.00 (10.52)	36.33 (10.56)	31.00 (10.49)	37.00 (10.57)	29.00 (10.46)	15.00 (10.18)
120	0.30 (8.48)	27.33 (10.44)	33.00 (10.52)	26.00 (10.41)	34.00 (10.53)	25.67 (10.41)	11.00 (10.04)
150	0.09 (7.95)	23.00 (10.36)	29.00 (10.46)	20.67 (10.32)	32.67 (10.51)	19.00 (10.28)	6.67 (9.82)
180	0.04 (7.6)	20.33 (10.31)	25.33 (10.40)	17.67 (10.25)	30.33 (10.48)	12.67 (10.11)	3.00 (9.48)
210	0.0013 (6.11)	17.67 (10.25)	21.33 (10.33)	13.67 (10.14)	25.00 (10.39)	11.00 (10.04)	0.70 (8.85)
240	0.0008 (5.9)	14.67 (10.17)	16.67 (10.22)	8.33 (9.92)	22.67 (10.36)	7.67 (9.88)	0.23 (8.36)
270	0.00009 (4.95)	10.67 (10.03)	14.00 (10.15)	3.67 (9.56)	18.00 (10.26)	3.67 (9.56)	0.09 (7.95)
300	-	8.33 (9.92)	9.67 (9.99)	1.00 (9.00)	14.33 (10.16)	1.23 (9.09)	0.008 (6.9)
330	-	5.00 (9.69)	6.33 (9..80)	0.67 (8.83)	9.33 (9.97)	0.10 (8.0)	0.001 (6.0)
360	-	1.67 (9.22)	3.33 (9.52)	0.16 (8.20)	6.67 (9.82)	0.023 (7.63)	0.0009 (5.95)
SEd	0.014	0.009	0.006	0.009	0.007	0.015	0.003
CD(p=0.05)	0.030	0.021	0.017	0.020	0.016	0.032	0.010

Values in parenthesis are $\log_{10}$ transformed value

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