DEVELOPMENT OF LIQUID FORMULATION AMENDED WITH DIFFERENT CHEMICAL ADDITIVES ON THE SURVIVAL OF *PSEUDOMONAS FLUORESCENS*

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Abstract

Liquid biofertilizer are special liquid formulation containing not only the desired micro organisms and their nutrients, but also special cell protectants or substances that encourage formation of resting spore or cyst for longer shelf life and tolerance to advanced condition. In this experiment, the authors evaluated different concentrations of four different chemical amendments *viz,* polyvinyl pyrollidone (PVP), glycerol, gum arabica, and trehalose for their ability to support growth and promote survival of *Pseudomonas fluorescens* in King's B broth during the storage. Some concentrations of various additives to King's B broth promoted higher *Pseudomonas* population compared to *Pseudomonas* cells in King's B broth alone. Liquid *Pseudomonas* bioinoculant formulated with trehalose (10mM) promoted long-term survival of *Pseudomonas* followed by glycerol (10mM) gum arabica (0.3%) and PVP (2%) and they supported 10^8 cells/ml up to 11 months of storage under ambient temperature (28°C to 32°C), whereas control (talc powder carrier) recorded the same population upto 5 months only. The results indicated that the liquid formulation of *Pseudomonas* could be used more effectively than the carrier based formulation.

Key words: Bioinoculants, *Pseudomonas fluorescens,* King's B broth, trehalose, liquid formulation, etc.

1. Introduction

Liquid biofertilizer are special liquid formulation containing not only the desired micro organisms and their nutrients, but also special cell protectants or substances that encourage formation of resting spore or cyst for longer shelf life and tolerance to advanced condition. 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. *Pseudomonas fluorescens* is a plant growth promoting rhizobacteria (PGPR). The group of bacteria that colonize roots or rhizosphere soil and beneficial to crop are referred as plant growth promoting rhizobacteria. The PGPR are referred to as biostimulant and the phytohormone they

produce indole acetic acid, cytokinins, gibberellins and inhibitors of ethylene productions. The fluorescent *Pseudomonas* have found to be associated with higher plants such as pomegranate, wheat and rice, which also play an important role in controlling *Fusarium oxysporum, Rhizoctonia solani*. *Pythium* spp., *Phytophthora megasperma*, *Alternaria alternate* and also increase the growth of the plants. The fluorescent *Pseudomonas* produces the antifungal compounds like siderophores (pyoveidine), cyanie, antibiotic-3, phenazines.

The PGPR inoculant currently commercialized that seem to promote growth through at least one mechanism, suppression of plant disease (termed as bioprotect ants), improved nutrient acquisition (termed as biofertilizer) or phytohormone production (termed as biostimulant) species of *Pseudmonas* can produce as yet not well characterized. Phytohomones or growth regulators that cause crops to have greater amount of fine roots which have the effect of increasing the absorptive surface of plant roots for uptake of water and nutrient. *Pseudomonas* sp. produce antibiotics, that controls major soil borne plant pathogens. Apart from their role in transport of iron (III), siderophores may act as growth factors and some are potent antibiotics. The ability of synthetic chelators such as EDTA to mimic the biological action of suppressive *Pseudomonas*. They suggested that siderophores induced iron limiting conditions which help to account in part for pathogen suppression. 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 inoculat production is high as it is labour and energy intensive process, involving milling, sieving and correcting pH. Liquid inoculant formulation is one solution to the problems associated with processing of solid carriers. The use of various broth cultures amended with 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 *P. fluorescens* 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. In the present study, experiments were conducted to increase the survival of the liquid formulations of *P. fluorescens* bioinoculant by the addition of different polymers like gum arabic, polyvinyl pyrollidone (PVP), glycerol and trehalose.

2. Materials and methods

Micro organisms used: *Pseudomonas fluorescence* TPS-3 isolate has been obtained from the screening studies and used in liquid formulation.

Culture Medium: King'B medium containing Protease peptone: 20 g/ l, dipotassium hydrogen ortho phosphate (K2HPO4): 1.5 g/ l, glycerol: 10 ml/ l, magnesium sulphate (MgSO4 7H2O): 1.5 g/, agar: 20 g/ l, and distilled water: 1000 ml and pH: 7.0

Chemical amendments: King's B broth was tried in combination with different chemicals to increase the survival of *P. fluorescens* cells in a liquid formulation. To standardize the optimum quantity of the amendments, the chemicals like PVP 1.0, 1.5, 2.0, 2.5 and 3.0%, glycerol 5.0, 7.5, 10, 12.5 and 15.0 mM, gum arabica 0.1, 0.3, 0.5, 0.8 and 1.0%, and trehalose 1, 5, 10, 15 were added to one liter of Kings'B broth broth separately. One ml of log phase culture of *P. fluorescens* was inoculated individually in each broth. Control (without any chemical addition) was also maintained and the flasks were incubated at room temperature. The broth cultures were analysed for viable cell population at 30 days interval up to six months.

Enumerating the viable cell population: The King's B medium was prepared, sterilized and plated in sterile petri plates. The plates were kept at room temperature for 48 h. 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 up to 10-10. From the dilutions, 5 µl 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.

Liquid inoculant production and survival of *P. fluorescens* during prolonged storage: For developing liquid formulation of *P. fluorescens*, King's B broth was prepared and standardized dosage of chemical amendments *viz*, PVP (2%), glycerol (10mM), gum arabica (0.3%) and trehalose (10mM), were added to one litre of broth separately. One ml of log phase culture of *P. fluorescens* was 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.

3. Results

To enhance the shelf life of *P. fluorescens* cells in liquid bioinoculant, certain chemicals *viz*., PVP, glycerol, gum arabica and trehalose were added as supplements to King's B broth. Experiments were carried out to standardize the optimum concentrations of different chemical additives in liquid formulation of *P. fluorescens* to support more viable population for longer period. The effects of different concentrations of chemical additives on the survival of *P. fluorescens* are presented in Figures 1 to 4. The results showed that maximum population was recorded in trehalose (10 mM) 4.00 x 10^9 followed by glycerol (10mM) 3.33 x 10^9, gum arabica (0.3%) 2.67x$10^9$and PVP (2%) 2.33x10^9 cells/ml of *P. fluorescens* liquid inoculants during 6th month of storage at room temperature (28 ± 2°C) whereas minimum population 6.33 x 10^2 was recorded in control (without chemical additives) King's B broth alone during 5th month. Hence, the concentration level of different chemical additives *viz*. PVP (2 %), glycerol (10 mM), gum arabic (0.3%) and trehalose (10 mM) were taken for further study. Liquid formulation of *P. fluorescens*, was developed the King's B broth was amended with PVP (2.0%), glycerol (10 mM), gum arabic (0.3%), trehalsoe (10 mM), PEG (1.0%) and PVA (0.5%) separately. The addition of the chemical amendments like trehalose, glycerol, and gum arabic allowed the maintence of 10^8 cells upto 12 months of storage, followed by, gum arabic upto 11 months and gum arabic upto 10 months where as the control (talc carrier based formulations) recorded the population level of 10^8 only upto 5 months. Among the amendments, trehalose supported highest number of *P. fluorescens* cells throughout the observation period followed by glycerol, gum Arabic and PVP (Table 1).

4. Discussion

Trehalose is an enigmatic compound which acts as a reserve carbohydrate that may be mobilized during stress. 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 stres, which might be the reason for the higher population of *P. fluorescens* cells in the trehalose treatments. Next to trehalose, 10 mM glycerol supported greater number of *P. fluorescens* in liquid formulation. This may be due to high water binding capacity and may protect cells from the effect of desiccation by reducing the rate of drying. The 2% PVP treatment gave a marginal increase in population compared to control. Suresh Babu, *et al*. found higher population of *P. fluorescens* due to the addition of PVP at both 1 and 2% 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. 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. Liquid inoculant formulation of cowpea rhizobia prepared with PVP as an osmo protectant been observed to have higher shelf life than those without PVP amendment. Some of the polymers and chemicals which can be used as additives and protectants in liquid inoculants include PVP, methyl cellulose, gum arabica, trehalose, glycerol, sodium alginate, poly ethylene glycol, polyvinyl alcohol and tapioca flour. 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. 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 *P. fluorescens* cells in the liquid formulation may be due to the action of chemical amendments added in the medium.

5. Conclusion

P. fluorescens 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. It is concluded that liquid formulation of *P. fluorescens* bioinoculant has a shelf life of one year compared to the carrier based inoculant. Among the different chemical additives, trehalose (10mM) performed well and hence this can be used in the formulation of liquid bioinoculant.

7. Reference

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TABLE 1

Efficacy of liquid formulation of inoculants amended with selected concentrations of different additives on the survival of *P.fluorescens* isolate (TPS-3)

Days	*Pseudomonas* population (× 10^9 CFU ml^{-1})			
	PVP (2.0 %)	Gum arabica (0.4%)	Glycerol (10 mM)	Trehalose (10 mM)
30	45.67 (10.63)	43.67 (10.65)	41.00 (10.60)	48.67 (10.69)
60	41.00 (10.61)	43.00 (10.63)	37.67 (10.57)	47.33 (10.66)
90	37.67 (10.57)	38.00 (10.58)	34.33 (10.53)	45.00 (10.64)
120	34.67 (10.53)	34.67 (10.54)	29.67 (10.47)	39.33 (10.58)
150	31.00 (10.48)	33.00 (10.52)	24.00 (10.38)	34.67 (10.53)
180	26.33 (10.42)	30.57 (10.49)	21.67 (10.33)	31.00 (10.49)
210	21.67 (10.36)	25.67 (10.41)	19.00 (10.28)	27.67 (10.44)
240	18.00 (10.26)	23.33 (10.37)	16.33 (10.21)	23.00 (10.36)
270	17.00 (10.18)	19.33 (10.29)	12.67 (10.10)	20.67 (10.32)
300	10.33 (10.01)	15.67 (10.20)	9.33 (9.97)	17.33 (10.21)
330	7.67 (9.88)	10.00 (10.00)	6.67 (9.82)	14.67 (10.14)
360	7.73 (9.82)	6.67 (9.67)	2.13 (9.32)	8.00 (9.95)
SEd	0.008	0.008	0.011	0.004
CD(p=0.05)	0.016	0.017	0.023	0.008

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

Fig. 1: Effect of different concentrations of trehalose on the survival of *P. fluorescens* isolate (TPS-3)

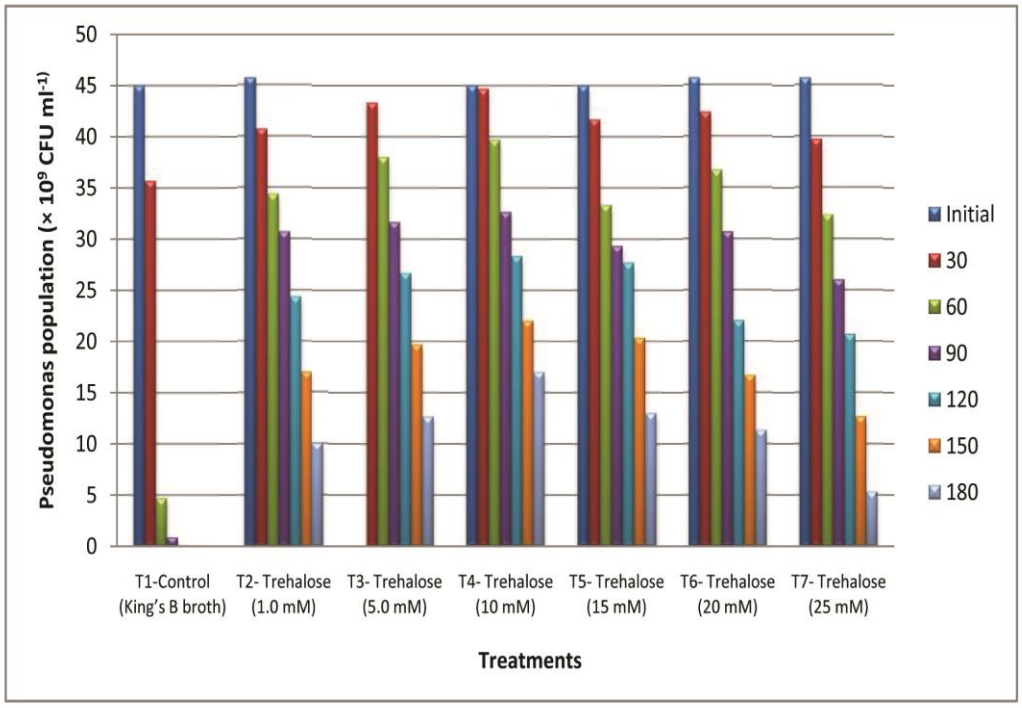

Fig. 2: Effect of different concentrations of polyvinyl pyrollidone (PVP) on the survival of *P. fluorescens* isolate (TPS-3)

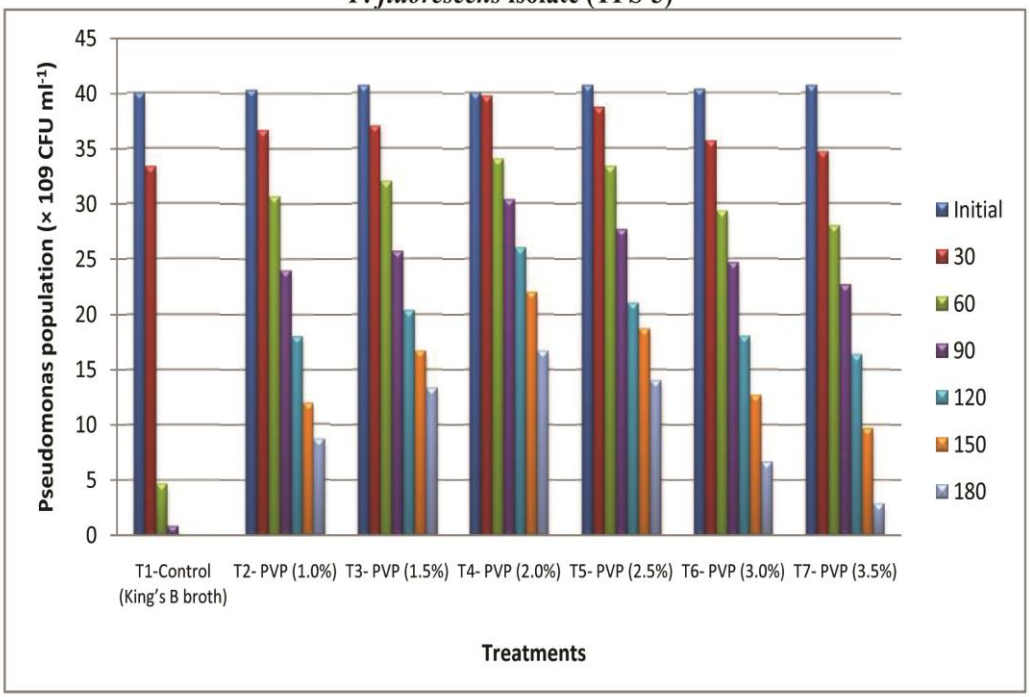

Fig. 3: Effect of different concentrations of gum arabica on the survival of *P. fluorescens* isolate (TPS-3)

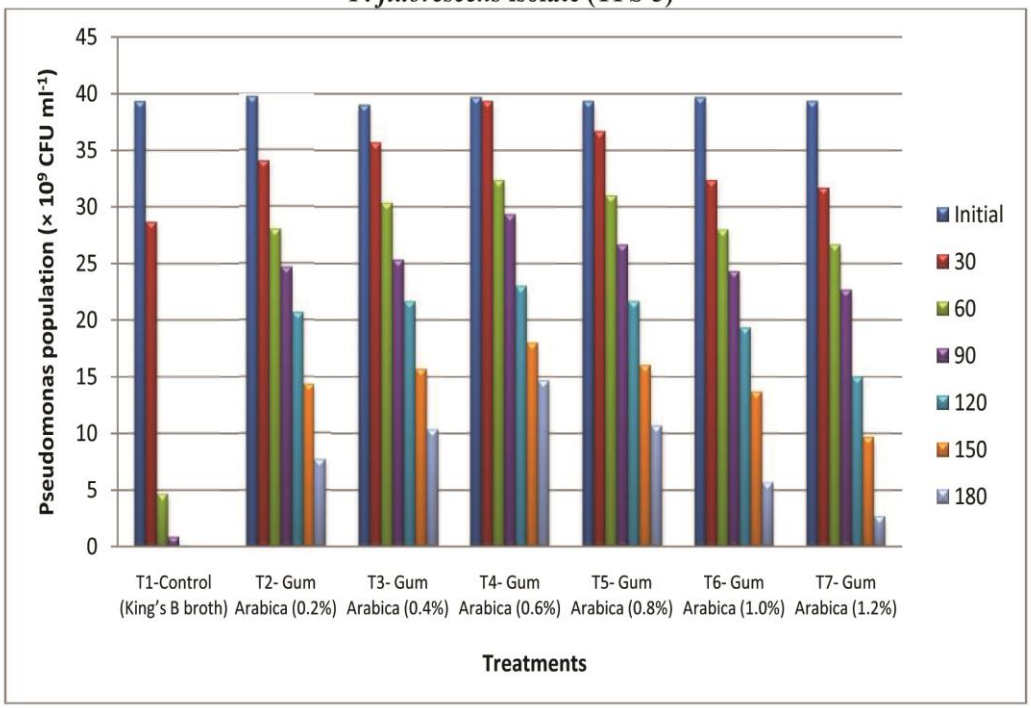

Fig. 4: Effect of different concentrations of Glycerol on the survival of *P. fluorescens* isolate (TPS-3)