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Cell Protectants, Adjuvants, Surfactant and Preservative and their Role in Increasing the Shelf Life of Liquid Inoculant Formulations of *Pseudomonas fluorescens*

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ABSTRACT

The cell protectants viz., polyvinlypyrrolidone (PVP, 2%), polyethylene glycol (PEG, 1%), gum arabic (0.8%) and sodium alginate (0.1%), adjuvants like xanthan gum (0.3%) and carboxymethyl cellulose (CMC, 0.1%), Tween 20 (0.5%) as a surfactant and potassium sorbate (0.2%) as a preservative were used in preparation of liquid inoculant formulation of Pseudomonas fluorescens. These liquid inoculants were stored in BOD incubator at 28±2 °C for a period of 180 days. The inoculant containing cell protectant, polyvinlypyrrolidone (2%), adjuvant xanthan gum (0.3%), Tween 20 (0.5%) as surfactant and potassium sorbate (0.2%) as preservative retained 1.76 X 10^{10} CFU/ml at the end of 180 days of storage. These formulated liquid inoculants had both extended shelf life and viability.

Key words: *Formulation, Liquid inoculant, Pseudomonas fluorescens, Shelf-life.*

INTRODUCTION

Biofertilizers usually need a carrier as medium for the microbial inoculants. A suitable carrier material needs to be inexpensive, easily available, and high in organic matter content, and should have a high water-holding capacity and a favourable H^+ concentration[5]. A good quality carrier should be free from microbial contamination, and should optimise the growth of biofertilizer microorganisms[10]. However, it is not easy to get a carrier that meets all these desired qualities. Further, the problem with the carrier based biofertilizers is lower shelf-life, inconsistent field performance and poor survival under adverse environmental conditions. There have been many attempts to produce a suitable inoculant that would overcome the problems of carrier based biofertilizers. In this context, liquid biofertilizer serves as the best solution to the aforesaid problems.

Liquid inoculant formulation contains not only the desired microorganisms and their nutrients but also special cell protectants or additives that promote for longer shelf-life and tolerance to adverse conditions[7].

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Liquid inoculants are formulated using polymeric additives that are known to enhance their shelf-life and increase their performance under field condition, besides the polymers added will help in adherence of the inoculants to the seeds[11]. The added polymers will help in survivability of inoculants by the virtue of their high water holding capacity, thicky consistency, and viscous nature.

These polymeric additives are soluble in water and non-toxic in nature. These polymeric additives have been proven best in maintaining the viable population individually, but work on using them in different combinations was lacking. Combination of polymeric additives at optimum concentration can serve the purpose of increasing the shelf-life of liquid inoculant. The present investigation was aimed to formulate and study the shelf-life of a liquid inoculant of *Pseudomonas fluorescens*.

MATERIAL AND METHODS

Maintenance of culture and formulation of *Pseudomonas fluorescens*

King's B broth (peptone: 20g/L; glycerol: 15ml/L; K_2HPO_4: 1.5 g/L; $MgSO_4.7H_2O$: 1.5 g/L) was used to culture *Pseudomonas fluorescens*. Sterilized King's B broth was inoculated with the *Pseudomonas fluorescens* and incubated at 28±2°C on a reciprocatory shaker for 24 hrs. Different liquid inoculant formulations were prepared by amending the King's B broth using polymeric additives *viz*., cell protectants, adjuvants, surfactant and preservative. Cell protectants *viz.*, polyvinlypyrrolidone (PVP; 2%), polyethylene glycol (PEG; 1%), gum arabic (0.8%) and sodium alginate (0.1%) were used. Adjuvants used were xanthan gum (0.3%) and carboxymethyl cellulose (CMC; 0.1%). Tween 20 (0.5%) was used as a surfactant and potassium sorbate (0.2%) as a preservative. One ml of a day old culture of *Pseudomonas fluorescens* was used to inoculate the media prepared using polymeric additives and incubated in BOD incubator at 28±2 °C.

A total of 11 formulations were prepared for this study. Eight liquid inoculant formulations (T_4-T_{11}) were prepared using cell protectants, adjuvants, a surfactant and a preservative in different combinations. One formulation (T_3) was prepared by amending King's B broth with Tween 20 and potassium sorbate. King's B broth was maintained without addition of polymeric additives in treatment T_1. A talc based formulation (T_2) was prepared by employing finely powdered talc whose pH was adjusted to 6.5-7.0 by using calcium carbonate ($CaCO_3$).

Shelf life studies of liquid inoculants

Liquid inoculant formulations prepared were packed in UV sterilized high density polyethylene (HDPE) bottles of 100 ml capacity. The formulated inoculants were stored in BOD incubator at 28±2 °C and assessed for their shelf-life at monthly intervals upto 180 days after storage (DAS) using standard plate count. Values obtained were means of three replications ± standard deviation and were statistically analysed using Duncan's multiple range test ($p<0.05$).

RESULTS AND DISCUSSION

Survival of *Pseudomonas fluorescens* without polymeric additives

The data regarding the survival of *Pseudomonas fluorescens* upto 180 days in King's B broth, talc based formulation and King's B broth amended with surfactant and preservative is presented in Table 1. In treatment T_1 (King's B broth only) the highest number of population was observed on zero day (2.43 X 10^{10} CFU/ml) and the number of colonies decreased thereafter with the lowest number of colonies observed on 120th (DAS; 0.05 X 10^{10} CFU/ml). The next highest number of colonies observed after zero day was on 30 DAS with 1.45 X 10^{10} CFU/ml followed by 60 DAS with 1.18 X 10^{10} CFU/ml and 90 DAS with 1.08 X 10^{10} CFU/ml. Thereafter no colonies were observed on 150 and 180 DAS.

In treatment T_2 (talc based formulation) the highest number of colonies were observed at zero day (0.19 X 10^{10} CFU/g) and the lowest number of colonies were found at 180 DAS (0.11 X 10^{10} CFU/g).

There was a gradual decrease in number of colonies over the period of storage and the decrease was found to be significantly different. The number of colonies observed at zero day was followed by 30 (0.18 X 10^{10} CFU/g), 60 (0.17 X 10^{10} CFU/g), 90 (0.16 X 10^{10} CFU/g), 120 (0.15 X 10^{10} CFU/g) and 150 DAS (0.13 X 10^{10} CFU/g).

King's B broth (T_1) and talc based formulation (T_2) were poor in supporting the survival of *Pseudomonas fluorescens*. This could be due to depletion of nutrients from the media, loss of moisture content and desiccation stress during the storage period. The other possible reason for poor survival of *Pseudomonas fluorescens* in these formulations could be due to absence of cell protectants, resulting in the failure of bacteria to protect them against desiccation. The death of bacteria due to desiccation could be attributed to the changes in membrane permeabilities and quantities of water retained at a known relative pressure[3]. The outcome of the work of Vidyashekaran and Muthamilan[13] suggested that the talc based formulation could not sustain higher population of *Pseudomonas fluorescens* more than 4 months of storage.

In treatment T_3 (King's B broth + 0.5% Tween 20 + 0.2% potassium sorbate) the highest number of colonies was observed on zero day (2.14 X 10^{10} CFU/ml) and the lowest number was observed on 180 DAS (1.17 X 10^{10} CFU/ml). There was a gradual decrease and were significantly different in the order of number of colonies found at 30 (1.87 X 10^{10} CFU/ml), 60 (1.77 X 10^{10} CFU/ml), 90 (1.67 X 10^{10} CFU/ml), 120 (1.57 X 10^{10} CFU/ml) to 150 DAS (1.37 X 10^{10} CFU/ml). This was because this treatment was prepared by amending King's B broth with Tween 20 as surfactant and potassium sorbate as preservative. The added chemicals might have enhanced the survival when compared to using only King's B broth (T_1).

Survival of *Pseudomonas fluorescens* in polyvinlypyrrolidone

The data on the effect of polyvinlypyrrolidone (PVP) as cell protectant in combination with adjuvants, surfactant and preservative on survival of *Pseudomonas fluorescens* in liquid inoculants is shown in Table 2.

Treatment T_4 (King's B broth + 2% PVP + 0.1% CMC + 0.5% Tween 20 + 0.2% potassium sorbate) showed highest number of colonies at zero day (2.36 X 10^{10} CFU/ml) followed by 30 (2.28 X 10^{10} CFU/ml), 60 (2.17 X 10^{10} CFU/ml), 90 (2.07 X 10^{10} CFU/ml), 120 (1.97 X 10^{10} CFU/ml) and 150 DAS (1.77 X 10^{10} CFU/ml). The lowest number of colonies was found at 180 DAS (1.57 X 10^{10} CFU/ml). The colonies gradually decreased as the number of days increased and were significantly different.

Similarly in T_5 (King's B broth + 2% PVP + 0.3% xanthan gum + 0.5% Tween 20 + 0.2% potassium sorbate) the same trend was followed, wherein zero day (2.45 X 10^{10} CFU/ml) showed maximum number of colonies followed by 30 (2.40 X 10^{10} CFU/ml), 60 (2.30 X 10^{10} CFU/ml), 90 (2.20 X 10^{10} CFU/ml), 120 (2.09 X 10^{10} CFU/ml) and 150 DAS (1.89 X 10^{10} CFU/ml). Lowest number of colonies was found at 180 DAS (1.76 X 10^{10} CFU/ml).

These two treatments maintained best colony counts upto 180 DAS because they were formulated using PVP as cell protectant. It is a synthetic polymer of vinyl groups with pyrrole ring. It is a high molecular weight compound (40000), water soluble with stabilization and adhesive properties, with high water holding capacity that appears to slow down the drying rate of media, thus maintaining the moisture level in the media[4]. Polyvinylpyrrolidone is also known to bind toxins that are constantly released into the media during stationary growth phase of bacteria.

Survival of *Pseudomonas fluorescens* in polyethylene glycol

The data pertaining to the effect of polyethylene glycol (PEG) as cell protectant in combination with adjuvants, surfactant and preservative on survival of *Pseudomonas fluorescens* in liquid inoculants is discussed in Table 3. At zero day (2.37 X 10^{10} CFU/ml) treatment T_6 (King's B broth + 1% PEG + 0.1% CMC + 0.5% Tween 20 + 0.2%

potassium sorbate) showed maximum number of colonies followed by 30 (2.12 X 10^{10} CFU/ml), 60 (2.02 X 10^{10} CFU/ml), 90 (1.92 X 10^{10} CFU/ml), 120 (1.82 X 10^{10} CFU/ml) and 150 DAS (1.62 X 10^{10} CFU/ml). The lowest number of colonies was observed at 180 DAS (1.42 X 10^{10} CFU/ml). The number of colonies at different DAS was found to be significantly different from each other.

In treatment T_7 (King's B broth + 1% PEG + 0.3% xanthan gum + 0.5% Tween 20 + 0.2% potassium sorbate) similar trend was followed as in case of T_6. Maximum number of colonies were found at zero day (2.42 X 10^{10} CFU/ml) and minimum colony count was observed at 180 DAS (1.51 X 10^{10} CFU/ml). From 30 days (2.21 X 10^{10} CFU/ml) to 150 days (1.71 X 10^{10} CFU/ml) there was a gradual decrease in the colony count and were found to be significantly different.

Polyethylene glycol added into these two treatments helped better survival of *Pseudomonas fluorescens*. Polyethylene glycol is small molecular weight (3000), water soluble compound with adhesive property[8, 12]. Polyethylene glycol has adhesive property and has a sticky consistency which will enhance cell adherence to seed and its viscous nature will slow the drying process of the inoculant.

Survival of *Pseudomonas fluorescens* in gum arabic

Table 4 represents the effect of gum arabic as cell protectant, in combination with surfactant and preservative on the survival of in liquid inoculants. Treatment T_8 (King's B broth + 0.8% Gum arabic + 0.1% CMC + 0.5% Tween 20 + 0.2% Potassium sorbate) at zero day (2.23 X 10^{10} CFU/ml) maintained highest colony counts followed by 30 (2.04 X 10^{10} CFU/ml), 60 (1.95 X 10^{10} CFU/ml), 90 (1.85 X 10^{10} CFU/ml) upto 150 DAS (1.55 X 10^{10} CFU/ml). The lowest number of colonies was observed at 180 DAS (1.29 X 10^{10} CFU/ml). The colony counts when statistically analysed at different DAS were found to be significantly different.

In treatment T_9 (King's B broth + 0.8% Gum arabic + 0.3% Xanthan gum + 0.5% Tween 20 + 0.2% Potassium sorbate) also the similar results were obtained wherein, maximum number of colonies was obtained at zero day (2.30 X 10^{10} CFU/ml) and lowest at 180 days (1.35 X 10^{10} CFU/ml). Colony counts decreased from 30 (2.05 X 10^{10} CFU/ml) to 150 DAS (1.56 X 10^{10} CFU/ml) and were significantly different from each other.

Both these treatments were prepared using gum arabic as cell protectant. Gum arabic, is a biopolymer, with large molecular weight with adhesive, emulsification and stabilization properties which limits heat transfer and has high water activity[1, 6, 9].

Survival of *Pseudomonas fluorescens* in sodium alginate

The effect of Sodium alginate as cell protectant in combination with adjuvants, surfactant and preservative on survival of *Pseudomonas fluorescens* in liquid inoculants in explained in Table 5. When T_{10} (King's B broth + 0.1% Sodium alginate + 0.1% CMC + 0.5% Tween 20 + 0.2% Potassium sorbate) was stored upto 180 days, the maximum number of colonies were observed at initial day (0 DAS; 2.16 X 10^{10} CFU/ml). Gradually the colony counts decreased from 30 (1.92 X 10^{10} CFU/ml) to 150 DAS (1.42 X 10^{10} CFU/ml) and were significantly different. Lowest number was observed on 180 DAS (1.21 X 10^{10} CFU/ml).

Similarly, in treatment T_{11} (King's B broth + 0.1% Sodium alginate + 0.3% Xanthan gum + 0.5% Tween 20 + 0.2% Potassium sorbate), maximum colonies were found at zero day (2.16 X 10^{10} CFU/ml) followed by 30 (1.99 X 10^{10} CFU/ml) upto 150 DAS (1.49 X 10^{10} CFU/ml) and on 180 DAS lowest colony count of 1.22 X 10^{10} CFU/ml was obtained.

Sodium alginate was used as cell protectant in the above treatments, it is a large molecular weight non-toxic compound with adhesive property, limits heat transfer, has high water activity; and these properties are useful in supporting long term survival of inoculant[1, 2, 9].

From the experiment on formulation and shelf life studies of liquid inoculants formulations of *Pseudomonas fluorescens*, it

could be said that the chemicals added into the media while preparation helped in increasing the shelf life of biofertilizer inoculant. There was no contamination observed during the period of storage and shelf life studies.

Table 1: Survival of *Pseudomonas fluorescens* in King's B broth, talc based formulation and, King's B broth amended with surfactant and preservative

Storage (days)	Population density (X 10^{10} CFU/ml or g)		
	Inoculant formulations		
	T_1	T_2	T_3
0	2.43^{a} (±0.246)	0.19^{a} (±0.001)	2.14^{a} (±0.005)
30	1.45^{b} (±0.05)	0.18^{b} (±0.002)	1.87^{b} (±0.020)
60	1.18^{c} (±0.028)	0.17^{c} (±0.002)	1.77^{c} (±0.020)
90	1.08^{c} (±0.028)	0.16^{d} (±0.002)	1.67^{d} (±0.020)
120	0.05^{d} (±0.004)	0.15^{e} (±0.002)	1.57^{e} (±0.020)
150	0^{e} (±0.00)	0.13^{f} (±0.001)	1.37^{f} (±0.020)
180	0^{e} (±0.00)	0.11^{g} (±0.002)	1.17^{g} (±0.020)

Note: T_1 = King's B broth; T_2 = talc based formulation; T_3 = King's B broth + 0.5% Tween 20 + 0.2% Potassium sorbate.

Table 2: Effect of PVP as cell protectant in combination with adjuvants, surfactant and preservative on survival of *Pseudomonas fluorescens* in liquid inoculants

Storage (days)	Population density (X 10^{10} CFU/ml)	
	Liquid inoculant formulations	
	T_4	T_5
0	2.36^{a} (±0.076)	2.45^{a} (±0.05)
30	2.28^{b} (±0.03)	2.40^{b} (±0.01)
60	2.17^{c} (±0.025)	2.30^{c} (±0.01)
90	2.07^{d} (±0.025)	2.20^{d} (±0.01)
120	1.97^{e} (±0.025)	2.09^{e} (±0.005)
150	1.77^{f} (±0.025)	1.89^{f} (±0.005)
180	1.57^{g} (±0.025)	1.76^{g} (±0.055)

Note: T_4 = King's B broth + 2% PVP + 0.1% CMC + 0.5% Tween 20 + 0.2% Potassium sorbate; T_5 = King's B broth +2% PVP + 0.3% Xanthan gum + 0.5% Tween 20 + 0.2% Potassium sorbate

Table 3: Effect of PEG as cell protectant in combination with adjuvants, surfactant and preservative on survival of *Pseudomonas fluorescens* in liquid inoculants

Storage (days)	Population density (X 10^{10} CFU/ml)	
	Liquid Inoculant formulations	
	T_6	T_7
0	2.37^{a} (±0.02)	2.42^{a} (±0.025)
30	2.12^{b} (±0.025)	2.21^{b} (±0.028)
60	2.02^{c} (±0.025)	2.11^{c} (±0.028)
90	1.92^{d} (±0.025)	2.01^{d} (±0.028)
120	1.82^{e} (±0.025)	1.91^{e} (±0.028)
150	1.62^{f} (±0.025)	1.71^{f} (±0.028)
180	1.42^{g} (±0.025)	1.51^{g} (±0.028)

Note: T_6 = King's B broth + 1% PEG + 0.1% CMC + 0.5% Tween 20 + 0.2% Potassium sorbate; T_7 = King's B broth + 1% PEG + 0.3% Xanthan gum + 0.5% Tween 20 + 0.2% Potassium sorbate

Table 4: Effect of Gum arabic as cell protectant in combination with adjuvants, surfactant and preservative on survival of *Pseudomonas fluorescens* in liquid inoculants

Storage (days)	Population density (X 10^{10} CFU/ml)	
	Liquid Inoculant formulations	
	T_8	T_9
0	2.23^{a} (±0.0.032)	2.30^{a} (±0.020)
30	2.04^{b} (±0.037)	2.05^{b} (±0.030)
60	1.95^{c} (±0.030)	1.97^{c} (±0.026)
90	1.85^{d} (±0.030)	1.86^{d} (±0.036)
120	1.75^{e} (±0.030)	1.76^{e} (±0.036)
150	1.55^{f} (±0.030)	1.56^{f} (±0.036)
180	1.29^{g} (±0.011)	1.35^{g} (±0.030)

Note: T_8 = King's B broth + 0.8% Gum arabic + 0.1% CMC + 0.5% Tween 20 + 0.2% Potassium sorbate; T_9 = King's B broth + 0.8% Gum arabic + 0.3% Xanthan gum + 0.5% Tween 20 + 0.2% Potassium sorbate

Table 5: Effect of Sodium alginate as cell protectant in combination with adjuvants, surfactant and preservative on survival of *Pseudomonas fluorescens* in liquid inoculants

Storage (days)	Population density (X 10^{10} CFU/ml)	
	Liquid Inoculant formulations	
	T_{10}	T_{11}
0	2.16^{a} (±0.020)	2.16^{a} (±0.015)
30	1.92^{b} (±0.025)	1.99^{b} (±0.011)
60	1.82^{c} (±0.025)	1.89^{c} (±0.011)
90	1.72^{d} (±0.025)	1.79^{d} (±0.011)
120	1.62^{e} (±0.025)	1.69^{e} (±0.011)
150	1.42^{f} (±0.025)	1.49^{f} (±0.011)
180	1.21^{g} (±0.028)	1.22^{g} (±0.025)

Note: T_{10} = King's B broth + 0.1% Sodium alginate + 0.1% CMC + 0.5% Tween 20 + 0.2% Potassium sorbate; T_{11} = King's B broth + 0.1% Sodium alginate + 0.3% Xanthan gum + 0.5% Tween 20 + 0.2% Potassium sorbate.

CONCLUSION

The results showed that, when PVP was used as cell protectant along with adjuvants, surfactant and preservative, the liquid formulation sustained good population of *Pseudomonas fluorescens* even upto 180 days. Next best cell protectant was PEG followed by gum arabic and sodium alginate. Liquid inoculant formulation using only King's B broth (T_1) maintained good population only upto 90 days, and after that there was a decrease in CFU at 120 DAS while, at 150 and 180 days, no colonies were observed. Hence, PVP could serve as best protectant when compared with other cell protectants such as PEG, gum arabic and sodium alginate. Similarly, xanthan gum would serve as best adjuvant when compared to CMC. The use of Tween 20 as surfactant and, potassium sorbate as preservative (T_3) had an added advantage in enhancing the shelf-life of liquid formulation of *Pseudomonas fluorescens*.

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