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FORMULATION AND SHELF LIFE OF LIQUID BIOFERTILIZER INOCULANTS USING CELL PROTECTANTS

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Abstract
The study was conducted to formulate and determine the shelf-life of liquid biofertilizers of efficient biofertilizer strains of HK region using different cell protectants and nutrients in liquid broth. The cell protectants used were glycerol (0.5%), polyvinyl pyrrolidone (PVP, 0.5%), polyethylene glycol (PEG, 0.5%), gum arabic (GA, 0.5%) and sodium alginate (SA, 0.1%). The treatments without addition of cell protectants (only broth) and carrier (lignite) based formulation were maintained as check. The formulated liquid biofertilizers of *Rhizobium, Azotobacter, Azospirillum* and PSB (*Bacillus megaterium*) were stored in BOD incubator at 28±2 °C for a period of 180 days and colony forming units were determined at monthly intervals. The liquid biofertilizers formulated using PVP in addition to glycerol at the rate of 0.5% each retained maximum number of colonies in all strains followed by PEG, GA and SA.

Keywords: Cell protectants, Formulation, Liquid biofertilizer, Shelf-life.

Introduction:

In the carrier-based (solid) bio-fertilizers, the microorganisms have a shelf life of only six months. They are not tolerant to UV rays and temperatures of more than 30 degrees. The population density of these microbes is only 10^8 (10 crores) c.f.u/ml at the time of production. This count reduces day by day. In the fourth month it reduces to 10^6 (10 lakhs) c.f.u/ml and at the end of 6 months the count is almost nil. That's why the carrier-based bio-fertilizers are not effective and had not become popular among the farmers.

These defects can be rectified and fulfilled in the case of liquid bio-fertilizers. The shelf life of the microbes in these liquid bio-fertilizers is higher than carrier based biofertilizers without considerable loss in viable counts. They are tolerant to high temperatures (55 degrees) and ultra violet radiations. This is especially feasible in Hyderabad-Karnataka where there is prevalence of high average temperatures. The viable cell count is as high as 10^9 c.f.u/ml, which is maintained constant during the period. So, the application of 1 ml of liquid bio-fertilizers is equivalent to the application of 1 kg of 5 months old carrier based bio-fertilizers (1000 times). Since these are liquid formulations the application in the field is also very simple and easy. They are applied using hand sprayers, power sprayers, fertigation tanks and as basal manure mixed along with FYM etc. The present study was undertaken to study the effect of different cell protectants *viz.*, glycerol (0.5%), polyvinyl pyrrolidone (PVP, 0.5%), polyethylene glycol (PEG, 0.5%), gum arabic (GA, 0.5%) and sodium alginate (SA, 0.1%) on shelf life of different liquid biofertilizer inoculants *viz.*, *Rhizobium*, *Azotobacter*, *Azospirillum* and PSB (*Bacillus megaterium*).

Material and Methods

Formulation of liquid biofertilizer inoculants

The strains used for liquid biofertilizer formulation were *Rhizobium, Azotobacter, Azospirillum* and PSB (*Bacillus megaterium*). Yeast extract mannitol broth, Waksman medium No.77 broth, Dobereiner's malic acid broth with NH_4Cl (1g per liter), and Pikovskaya medium were used to culture *Rhizobium*, *Azotobacter, Azospirillum* and PSB (*Bacillus megaterium*) 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 (0.5%), polyvinyl pyrrolidone (PVP, 0.5%), polyethylene glycol (PEG, 0.5%), gum arabic (GA, 0.5%) and sodium alginate (SA, 0.1%) were added to the broth during the preparation of media. The prepared media was inoculated with 1.0 ml overnight grown mother culture and incubated in BOD incubator at 28±2 °C.

There were a total of seven liquid biofertilizers formulations for every biofertilizer strain used. Out of which, four (T_2-T_6) were prepared using cell protectants in optimum concentrations. Only broth was maintained without addition cell protectants in treatment T_1. The lignite based formulation (T_7) was prepared by mixing the media with sterile lignite powder at 1: 2.5 ratio.

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. Yeast extract mannitol broth, Waksman medium No.77 broth, Dobereiner's malic acid broth with NH_4Cl, and Pikovskaya medium were used to enumerate *Rhizobium*, *Azotobacter, Azospirillum* and PSB (*Bacillus megaterium*) respectively. 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 liquid bioinoculant of *Rhizobium*

Table 1 represents the results on the survivability of *Rhizobium* at different intervals. At zero days, the highest number of colonies was observed in T_2 (broth + 0.5 % glycerol; 2.14 x 10^{10} cfu/ml) followed by T_5 (broth + 0.5 % glycerol + 0.5 % GA; 2.05 x 10^{10} cfu/ml) and the lowest number of colonies was observed in T_7 (lignite based formulation; 0.20 x 10^{10} cfu/g). The treatments T_3 (broth + 0.5 % glycerol + 0.5 % PVP), T_4 (broth + 0.5 % glycerol + 0.5 % PEG) and T_6 (broth + 0.5 % glycerol + 0.1 % SA) were on par with each other. T_1 (only broth) recorded 1.71 x 10^{10} cfu/ml. At 30 days after storage (DAS), all the treatments were found to be significant. The highest number of colonies was observed in T_3 (1.97 x 10^{10} cfu/ml) followed by T_4, T_5, T_6, T_2, T_1 and the lowest colonies were retained in T_7 (0.16 x 10^{10} cfu/g). The similar trend was observed at 60 and 120 DAS. At 90 days after storage, T_3 (1.76 x 10^{10} cfu/ml) maintained highest number of colonies while the lowest number of colonies was observed in T_7. Treatments T_1 and T_2 were found to be on par with each other. At 150 and 180 DAS, all the treatments were found to be significant. The highest number of colonies was observed in T_3(1.02 x 10^{10} and 0.76 x 10^{10} cfu/ml respectively), followed by T_4, T_5, T_6, T_2 and T_7. Treatment T_1 could not retain any colonies after 150 DAS.

Survival of liquid bioinoculant of *Azotobacter*

The survivability of *Azotobacter* at different days of storage is explained in Table 2. At zero DAS treatment T_3 (2.17 x 10^{10} cfu/ml) showed highest number of colonies and lowest number of colonies were observed in T_7 (0.21 x 10^{10} cfu/g). Treatments T_2, T_4 and T_5 were on par with each other. At 30 DAS highest number of colonies were found in T_3 (1.91 x 10^{10} cfu/ml) and T_4 (1.86 x 10^{10} cfu/ml) which were found to be non significant while, lowest colony forming units observed in T_7. The next best treatments were T_5, T_6, T_2 and T_1. At 60 DAS all the treatments were found to be significant wherein the highest number of colonies were observed in T_3 followed by T_4, T_5, T_6, T_2 and T_1. Treatment T_7 maintained lowest colony forming units. At 90 and 120 DAS the same trend was followed as in case of 60 DAS. The results of colony forming units observed during 150 and 180 DAS shared the same trend as T_3 (1. 14 x 10^{10} and 0.83 x 10^{10} cfu/ml) maintained highest number of colonies while T_1 showed no colony forming units. Treatment T_3 was followed by treatments T_4, T_5, T_6, T_2 and T_7.

Survival of liquid bioinoculant of *Azospirillum*

Table 3 explains the survivability of *Azospirillum* at different days after storage. At zero DAS T_3 (2.16 x 10^{10} cfu/ml) showed highest number of colonies and in contrast lowest number of colonies were observed in T_7 (0.19 x 10^{10} cfu/g). Treatments T_2, T_4 and T_5 were found to be on par with each other. At 30 DAS, treatment T_3 (1.94 x 10^{10} cfu/ml) showed maximum retention of colony forming units and T_7 showed lowest number of colonies. The next best treatments after T_3 were T_4, T_5, T_6. At 60 DAS, T_3 (1.80 x 10^{10} cfu/ml) showed highest number of colonies and lowest number of colonies were observed in T_7 and treatments T_5 and T_6 were found to be non significant with each other. At 90 DAS treatment T_3 (1.68 x 10^{10} cfu/ml) retained highest number of colony forming units while T_7 showed lowest number of colonies. Treatments T_4 and T_5 were found to be on par with each other. At 120 DAS the maximum number of colonies were observed in T_3 (1.36 x 10^{10} cfu/ml) and minimum number of colonies were observed in T_7 while the treatments T_2 and T_7 showed on par results in maintaining colony forming units. At 150 DAS all the treatments were significant to each other in retaining colony forming units wherein the maximum of colonies were observed in T_3 (0.92 x 10^{10} cfu/ml) and there were no colony forming units recorded as in case of T_1. At 180 DAS T_3 (0.78 x 10^{10} cfu/ml) recorded highest number of colonies while, treatments T_6 and T_7 were found to be on par with each other.

Survival of liquid bioinoculant of PSB (*Bacillus megaterium*)

Table 4 explains the survivability of PSB (*Bacillus megaterium*) at different days after

storage. At zero DAS treatment T_3 (2.01 x 10^{10} cfu/ml) showed maximum number of colonies and minimum number of colonies were observed in T_7 (0.18 x 10^{10} cfu/g). Treatments T_2 and T_6 were non significant with each other. At 30 DAS all the treatments were found to be significant with each other. The highest number of colonies was observed in case of T_3 (1.78 x 10^{10} cfu/ml) in contrast the T_7 showed the lowest colony count. The next best treatments after T_3 were T_4, T_5, T_6, T_2 and T_1. The results for the colony forming units observed at 60, 90 and 120 DAS followed the same trend as it is in case of 30 DAS. The observations recorded for colony counts at 150 and 180 DAS shared the same wherein the highest number of colonies were observed in T_3 (1.04 x 10^{10} and 0.96 x 10^{10} cfu/ml respectively) while no colony was recorded in T_1. The treatment T_3 was followed by T_4, T_5, T_6, T_2 and T_7.

Polyvinylpyrrolidone is a synthetic polymer of vinyl groups with pyrrole ring. It is a high molecular weight compound (40000), it is a water soluble compound 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 (Singleton *et al.,* 2002; Deaker *et al.,* 2004). Polyvinylpyrrolidone also has a capacity to bind bacterial toxins that were constantly released into the media, when bacterial cells were in stationary phase (Errington *et al.*, 2002). In addition, as concentrations of salts increase in the cell environment with the drying liquid inoculant, stabilizing polymers like PVP may be useful in reducing the extent of protein precipitation or coagulation of cells. Maintenance of macromolecular structure may improve biological integrity, thus leading to improved survival.

Polyethylene glycol is a small molecular weight (3000), water soluble compound with adhesive and sticky consistency. The adhesive property of PEG enhances cell adherence to seed, and its viscous nature will slow the drying process of the inoculant (Temperano *et al.,* 2002; McAneney *et al.,* 1982).

Gum arabic is a biopolymer with large molecular weight, adhesive, emulsifier and stabilization property which limits heat transfer and has high water activity (Mugnier and Jung, 1985; Vincent *et al.,* 1962; Hale and Mathers, 1977).

Sodium alginate 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 (Mugnier and Jung, 1985; Bashan and Gonzalez, 1999; Bashan, 1986).

Table 1. Effect of cell protectants on survival of liquid bioinoculant of *Rhizobium*

Inoculant formulations	**Population density (X 10^{10} CFU/ml or g)**						
	Days after storage (DAS)						
	0	**30**	**60**	**90**	**120**	**150**	**180**
T_1	1.71^{d} (±0.025)	1.34^{f} (±0.045)	1.20^{f} (±0.020)	1.02^{e} (±0.015)	0.60^{f} (±0.010)	0.00^{g} (±0.000)	0.00^{g} (±0.000)
T_2	2.14^{a} (±0.040)	1.59^{e} (±0.026)	1.39^{e} (±0.005)	1.03^{e} (±0.020)	0.89^{e} (±0.010)	0.73^{e} (±0.020)	0.42^{e} (±0.015)
T_3	1.90^{c} (±0.026)	1.97^{a} (±0.015)	1.76^{a} (±0.015)	1.63^{a} (±0.030)	1.37^{a} (±0.010)	1.02^{a} (±0.020)	0.76^{a} (±0.010)
T_4	1.91^{c} (±0.025)	1.88^{b} (±0.015)	1.67^{b} (±0.026)	1.50^{b} (±0.025)	1.27^{b} (±0.026)	0.94^{b} (±0.02)	0.67^{b} (±0.026)
T_5	2.05^{b} (±0.037)	1.75^{c} (±0.015)	1.54^{c} (±0.025)	1.41^{c} (±0.035)	1.11^{c} (±0.030)	0.90^{c} (±0.010)	0.60^{c} (±0.025)
T_6	1.86^{c} (±0.035)	1.67^{d} (±0.026)	1.44^{d} (±0.020)	1.27^{d} (±0.020)	1.02^{d} (±0.026)	0.85^{d} (±0.011)	0.54^{d} (±0.030)
T_7	0.20^{e} (±0.015)	0.16^{g} (±0.010)	0.15 ^{g} (±0.010)	0.12^{f} (±0.017)	0.08^{g} (±0.010)	0.06^{f} (±0.005)	0.04^{f} (±0.005)

Note: T_1: Yeast extract mannitol broth; T_2: Yeast extract mannitol broth + 0.5 % glycerol; T_3: Yeast extract mannitol broth + 0.5 % glycerol + 0.5 % PVP; T_4: Yeast extract mannitol broth + 0.5 % glycerol + 0.5 % PEG; T_5: Yeast extract mannitol broth + 0.5 % glycerol + 0.5 % GA; T_6: Yeast extract mannitol broth + 0.5 % glycerol + 0.1 % SA; T_7: lignite based formulation;
PVP = polyvinylpyrrolidone; PEG = polyethylene glycol; GA = gum arabic; SA = sodium alginate;
Values are the mean of three replications ±SD;
Means values followed by the same letter are not significantly different based on Duncan's multiple range test ($p<0.05$), a> b > c.

Table 2. Effect of cell protectants on survival of liquid bioinoculant of *Azotobacter*

Inoculant formulations	Population density (X 10^{10} CFU/ml or g)						
	Days after storage (DAS)						
	0	30	60	90	120	150	180
T_1	1.78^{c} (±0.010)	1.21^{e} (±0.031)	1.04^{f} (±0.020)	0.69^{f} (±0.015)	0.49^{f} (±0.010)	0.00^{g} (±0.000)	0.00^{g} (±0.000)
T_2	2.04^{b} (±0.036)	1.53^{d} (±0.045)	1.27^{e} (±0.020)	1.05^{e} (±0.015)	0.84^{e} (±0.030)	0.66^{e} (±0.025)	0.38^{e} (±0.010)
T_3	2.17^{a} (±0.020)	1.91^{a} (±0.040)	1.71^{a} (±0.025)	1.52^{a} (±0.030)	1.25^{a} (±0.010)	1.14^{a} (±0.021)	0.83^{a} (±0.021)
T_4	2.01^{b} (±0.035)	1.86^{a} (±0.021)	1.52^{b} (±0.021)	1.44^{b} (±0.030)	1.13^{b} (±0.040)	0.95^{b} (±0.021)	0.65^{b} (±0.026)
T_5	2.02^{b} (±0.050)	1.73^{b} (±0.026)	1.42^{c} (±0.030)	1.34^{c} (±0.017)	1.03^{c} (±0.021)	0.81^{c} (±0.150)	0.51^{c} (±0.020)
T_6	1.77^{c} (±0.020)	1.65^{c} (±0.015)	1.36^{d} (±0.025)	1.25^{d} (±0.020)	0.96^{d} (±0.015)	0.72^{d} (±0.015)	0.42^{d} (±0.030)
T_7	0.21^{d} (±0.035)	0.18^{f} (±0.006)	0.17^{g} (±0.010)	0.13^{g} (±0.010)	0.08^{g} (±0.010)	0.06^{f} (±0.012)	0.04^{f} (±0.006)

Note: T_1: Waksman No.77 broth; T_2: Waksman No.77 broth + 0.5 % glycerol; T_3: Waksman No.77 broth + 0.5 % glycerol + 0.5 % PVP; T_4: Waksman No.77 broth + 0.5 % glycerol + 0.5 % PEG; T_5: Waksman No.77 broth + 0.5 % glycerol + 0.5 % GA; T_6: Waksman No.77 broth + 0.5 % glycerol + 0.1 % SA; T_7: lignite based formulation;
PVP = polyvinylpyrrolidone; PEG = polyethylene glycol; GA = gum arabic; SA = sodium alginate;
Values are the mean of three replications ±SD;
Means values followed by the same letter are not significantly different based on Duncan's multiple range test ($p<0.05$), a> b > c.

Table 3. Effect of cell protectants on survival of liquid bioinoculant of *Azospirillum*.

Inoculant formulations	Population density (X 10^{10} CFU/ml or g)						
	Days after storage (DAS)						
	0	30	60	90	120	150	180
T_1	1.72^{d} (±0.015)	1.23^{f} (±0.031)	0.94^{e} (±0.040)	0.88^{e} (±0.031)	0.57^{e} (±0.025)	0.00^{g} (±0.000)	0.00^{e} (±0.000)
T_2	1.96^{b} (±0.025)	1.64 ^{e} (±0.031)	1.45^{d} (±0.031)	1.28^{d} (±0.025)	0.98^{d} (±0.015)	0.49^{e} (±0.015)	0.48^{d} (±0.035)
T_3	2.16^{a} (±0.010)	1.94^{a} (±0.035)	1.80^{a} (±0.015)	1.68^{a} (±0036)	1.36^{a} (±0.036)	0.92^{a} (±0.025)	0.78^{a} (±0.010)
T_4	1.93^{b} (±0.035)	1.83^{b} (±0.015)	1.72^{b} (±0.031)	1.58^{b} (±0.060)	1.25^{b} (±0.025)	0.85^{b} (±0.040)	0.65^{b} (±0.031)
T_5	1.95^{b} (±0.025)	1.79^{c} (±0.010)	1.63^{c} (±0.036)	1.52^{b} (±0.044)	1.14^{c} (±0.021)	0.79^{c} (±0.025)	0.60^{c} (±0.032)
T_6	1.79^{c} (±0.015)	1.72^{d} (±0.031)	1.58^{c} (±0.035)	1.40^{c} (±0.015)	1.02^{d} (±0.021)	0.64^{d} (±0.030)	0.57^{c} (±0.020)
T_7	0.19^{e} (±0.006)	0.17^{g} (±0.015)	0.13^{f} (±0.020)	0.11^{f} (±0.021)	0.07^{f} (±0.015)	0.06^{f} (±0.010)	0.04^{e} (±0.010)

Note: T_1: Dobereiner's malic acid broth; T_2: Dobereiner's malic acid broth + 0.5 % glycerol; T_3: Dobereiner's malic acid broth + 0.5 % glycerol + 0.5 % PVP; T_4: Dobereiner's malic acid broth + 0.5 % glycerol + 0.5 % PEG; T_5: Dobereiner's malic acid broth + 0.5 % glycerol + 0.5 % GA; T_6: Dobereiner's malic acid broth + 0.5 % glycerol + 0.1 % SA; T_7: lignite based formulation;
PVP = polyvinylpyrrolidone; PEG = polyethylene glycol; GA = gum arabic; SA = sodium alginate;
Values are the mean of three replications ±SD;
Means values followed by the same letter are not significantly different based on Duncan's multiple range test ($p<0.05$), a> b > c.

Table 4. Effect of cell protectants on survival of liquid bioinoculant of PSB (*Bacillus megaterium*).

Inoculant formulations	Population density (X 10^{10} CFU/ml or g)						
	Days after storage (DAS)						
	0	30	60	90	120	150	180
T_1	1.73^{e} (±0.015)	1.42^{f} (±0.030)	1.02^{f} (±0.015)	0.90^{f} (±0036)	0.61^{f} (±0.030)	0.00^{g} (±0.000)	0.00^{g} (±0.000)
T_2	1.79^{d} (±0.020)	1.54^{e} (±.020)	1.28^{e} (±0.026)	1.10^{e} (±0.015)	0.80^{e} (±0.025)	0.65^{e} (±0.020)	0.49^{e} (±0.020)
T_3	2.01^{a} (±0.025)	1.78^{a} (±0.015)	1.67^{a} (±0.026)	1.50^{a} (±0.020)	1.30^{a} (±0.030)	1.04^{a} (±0.030)	0.96^{a} (±0.015)
T_4	1.90^{c} (±0.025)	1.72^{b} (±0.041)	1.57^{b} (±0.026)	1.36^{b} (±0.015)	1.17^{b} (±0.010)	0.94^{b} (±0.025)	0.81^{b} (±0.030)
T_5	1.95^{b} (±0.020)	1.65^{c} (±0.015)	1.40^{c} (±0.025)	1.26^{c} (±0.025)	1.01^{c} (±0.036)	0.85^{c} (±0.026)	0.66^{c} (±0.026)
T_6	1.81^{d} (±0.020)	1.60^{d} (±0.032)	1.34^{d} (±0.020)	1.17^{d} (±0.025)	0.95^{d} (±0.015)	0.79^{d} (±0.010)	0.53^{d} (±0.020)
T_7	0.18^{f} (±0.005)	0.17^{g} (±0.011)	0.14^{g} (±0.005)	0.12^{g} (±0.015)	0.10^{g} (±0.010)	0.07^{f} (±0.015)	0.05^{f} (±0.015)

Note: T_1: Pikovskaya broth; T_2: Pikovskaya broth + 0.5 % glycerol; T_3: Pikovskaya broth + 0.5 % glycerol + 0.5 % PVP; T_4: Pikovskaya broth + 0.5 % glycerol + 0.5 % PEG; T_5: Pikovskaya broth + 0.5 % glycerol + 0.5 % GA; T_6: Pikovskaya broth + 0.5 % glycerol + 0.1 % SA; T_7: lignite based formulation
PVP = polyvinylpyrrolidone; PEG = polyethylene glycol; GA = gum arabic; SA = sodium alginate;
Values are the mean of three replications ±SD;
Means values followed by the same letter are not significantly different based on Duncan's multiple range test ($p<0.05$), a> b > c.

Conclusions

The experiment showed that, liquid biofertilizer inoculants developed using 0.5% PVP in addition to 0.5% glycerol (T_3) increased the shelf-life of all the biofertilizer inoculants tested when the liquid formulations were stored for 180 days. The next best treatment was found to be T_4 prepared using 0.5% PEG in addition to 0.5% glycerol as cell protectant. Treatment T_4 was followed by the T_5 prepared using 0.5% gum arabic in addition to 0.5% glycerol as cell protectant. These treatments were followed by treatments T_6 (broth + 0.5% glycerol + 0.5% sodium alginate) and T_2 (broth + 0.5% glycerol). The least population density was observed in treatment T_1 (only broth) followed by T_7 (lignite based formulation). The superiority of cell protectants were in the order 0.5%PVP > 0.5%PEG > 0.5%gum arabic > 0.1%SA in increasing the shelf life of liquid inoculants when compared to using glycerol alone. Further, the population data revealed that the liquid formulations prepared using above cell protectants can be maintained beyond 180 DAS. Carried based formulation harbored lowest cell counts when compared to liquid formulations containing cell protectants. Liquid broth without cell protectants could not support life after 150 DAS for all the biofertilizer inoculants tested.

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