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Effect of Protective Polymers and Storage Temperatures on Shelf Life of Cyanobacterial Liquid Formulation

Aman Jaiswal, Deepak Kumar Koli[*1], Sunil Pabbi[1], Himani Priya[2], Ashish Kumar[3], Rahul Mishra[4], Ganesh Kumar Koli[5] and Bhagwan Singh Dhaked[1]

Department of Microbiology, College of Basic Sciences and Humanities
Dr. Rajendra Prasad Central Agricultural University, Pusa-848 125 India
[1]Division of Microbiology, ICAR-Indian Agricultural Research Institute, New Delhi-110 012, India
[2]ICAR-Indian Agricultural Research Institute, Gauria Karma, Barhi, Hazaribagh- 825 405, India
[3]Division of Genetics, ICAR-Indian Agricultural Research Institute, New Delhi-110 012, India, India
[4]ICAR-Indian Institute of Soil Science Bhopal, Madhya Pradesh- 462 038, India
[5]Department of Genetics and Plant Breeding, Chaudhary Charan Singh Haryana Agricultural University, Hisar-125 004
**E-mail: mr.deepakkoli.iari@gmail.com*

***Abstract*:** The aim of the study was to determine the influence of protective polymers and storage temperatures to prolong the shelf life of cyanobacterial liquid formulation of efficient bio-fertilizer strains of cyanobacteria using different protective polymers and storage conditions. In this experiment, the protective polymers used in formulations were dimethyl sulfoxide (DMSO), glycerol and polyvinyl pyrrolidone (PVP) along with gum Arabic, CMC and Tween-20. The formulation without the addition of protective polymers and no pH was maintained as control. Storage temperature of formulated liquid biofertilizers of cyanobacteria were 4^{0}C, 28^{0}C and 40^{0}C for a period of 18 months (540 days). Most of the formulations showed cell count 2.3x10^{4}to 3.2 x10^{4}cells mL^{-1}, cell dry weight 1.98 to 2.51 mg mL^{-1}, total chlorophyll 3.50 and 6.05 µg mL^{-1} and nitrogenase activity 2.07 to 3.52 µmol C_2H_4 mg^{-1} chl h^{-1} at all the three stored temperatures after 18 months of incubation. The performance of developed liquid formulations was good with protective polymeric additives than without them. The liquid biofertilizer formulation using combination of protective polymers showed maximum cell count and other metabolic activities as compared to alone. At 4^{0}C, liquid inoculant formulated with DMSO and glycerol provided the greatest protection to cells of cyanobacteria and surviving and other growth-promoting ability whereas at 28^{0}C and 40^{0}C, PVP in addition to glycerol retained maximum surviving and other metabolic activities. The combinations of protective polymers *viz*. DMSO (3%) and glycerol (2%) at 4^{0}C and PVP (2%) and glycerol at 28^{0}C and 40^{0}C showed the best conditions to improve shelf life of cyanobacterial liquid biofertilizer formulations. It can be recommended to maintain the shelf life of liquid formulations for storage period of 18 months.

***Keywords*:** Protective polymers, Formulation, DMSO, PVP, Glycerol

The success of any biofertilizer in the field depends on the quality of bioformulations. Microbial based nutrient inputs have emerged as the potential alternative for the productivity, reliability and sustainability of the global food chain. Although, carrier-based biofertilizers are in vogue since long and their use has proved beneficial both for crop yields as well as soil health, but, these are still not popular with the farming community because of varying reasons and mixed results and the main concern has been the viability of the microorganisms. The biofertilizer inoculants should survive up to field application and the shelf-life of inoculants is very important. To overcome such problems with carrier-based biofertilizers; liquid biofertilizers have been developed which seem to be the only alternative for cost-effective sustainable agriculture. Different approaches have been available for the development of liquid formulations of microorganisms, but the basic aim is to have storage stability, easy delivery, increased persistence and protection from harmful and damaging environmental factors. The success of a biofertilizer is dependent upon the survival of the microbial strain in the soil and it is a big challenge (Xavier et al 2004). The selection of ideal polymer is based upon several properties like complex chemical nature, solubility in water and nontoxicity which prevents microorganisms in the soil from rapid degradation. Liquid inoculant formulations may use various broth cultures amended with agents that promote cell survival in the package and after application to soil. It 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 (Hegde et al 2008). Many kinds of polymers such as sodium alginate, methyl cellulose, trehalose, arabinose, gum arabic, starch, glycerol, polyethylene glycol (PEG), polyvinyl pyrrolidone and DMSO (Criste et al 2014) have been used for inoculant production because of their ability to limit heat transfer, their good rheological properties and high

water activities (Pindi and Satyanarayna 2012, Daniel et al 2013). These polymers, are normally used as additives or cell protectants. Hence, we evaluated the optimum concentration of polymers, surfactants and adjuvants for liquid formulation that could sustain the bacterial shelf life. So, a breakthrough is necessary for the current inoculant technology to reinforce the shelf life and field efficacy of biofertilizers in India to make them commercially viable and acceptable to the farmers. Therefore, the present study was conducted to increase the survival of the liquid formulations of cyanobacterial inoculants by the addition of different polymers like gum arabic, CMC, glycerol, polyvinyl pyrrolidone and DMSO.

MATERIAL AND METHODS

Cyanobacterial cultures used in the study: Four cyanobacterial cultures *viz*., *Anabaena variabilis* (CCC441), *Nostoc muscorum* (*CCC442*), *Tolypothrix tenuis* (CCC443) and *Aulosira fertilissima* (CCC444) and one P-solubilizing cyanobacterial culture *i.e., Westiellopsis prolifica* (CCC474) maintained in Culture Collection of Cyanobacteria (CCC) at CCUBGA, Indian Agricultural Research Institute, New Delhi were taken for the study.

Preparation of formulations: Different liquid formulations were prepared using combination of carriers (vegetable oil) at 1%, emulsifiers (Tween-20) at 2% and thickening agents (Gum arabic and CMC) at 5 % with different concentrations (1.5 - 5 %) of protective agents (DMSO, PVP and Glycerol). The experiments were carried out in 100 ml plastic bottle containing amended liquid formulation. A total 10 formulations with triplicates were prepared (Table 1). Initially, pH of all the formulations except F10 were adjusted to 7 by adding drop by drop 1 N NaOH or 1 N HCl. Late log phase (14 days old) cultures of the Cyanobacterial mixture (2.5% v/v) were inoculated into each formulation and stored at three different temperatures 40C, 28^{0}C and 40^{0}C in refrigerator, room condition and incubator respectively. These formulations were characterized for shelf life and metabolic activities up to 18 months at different time intervals *viz*. 30, 60, 90, 150, 180, 270, 360, 450 and 540 days.

Table 1. Different combinations of protective agents used in formulations

Formulations	Protective agents	Concentration (%)
F1	DMSO	3
F2	DMSO	5
F3	Glycerol	3
F4	Glycerol	5
F5	PVP	1.5
F6	PVP	3
F7	Mixture (DMSO+ Glycerol)	3+2
F8	Mixture (PVP+ Glycerol)	2+2
F9	No Protective agents	-
F10	No protective agents, No pH maintained	-

Revival of culture from formulation: Ten ml formulation containing culture was taken in a pre-sterilized centrifuge tube and centrifuged at 5000 X g for 10 minutes. The cell pellet was washed with sterilized distilled water, centrifuged and finally re-suspended in 1ml sterilized BG-11 medium. This re-suspended cell pellet was used as inoculum to inoculate 100 ml nitrogen-free BG-11 medium taken in 250 ml conical Erlenmeyer flasks. The flasks were incubated in a culture room at 28 ± 2°C under a light intensity of 52-55 µmol photon m^{-2} s^{-1} and L: D cycles of 16:8 hours. Representative samples of cyanobacterial cultures were taken after 21days of incubation for analysis of cell population, total chlorophyll, dry weight and nitrogen fixation by ARA.

Cell population: Cell population counting was done using the Neubauer chamber of the Haemocytometer having depth of 0.100 mm and 1 mm^2 area. 10 ml culture was taken in a test tube and homogenized for 60 seconds. 1ml of this was taken through a micropipette (1000µl) and added in 9 ml sterilized BG-11 (N^-medium) and homogenised for 1 minute. This homogenized and diluted culture was taken through micropipette and onto the Neubauer chamber and covered with a coverslip. Cells were observed under compound microscope OLYMPUS-CX40 and counting was performed. Total chlorophyll was estimated by methanol extraction method (McKinney 1941) while Nitrogenase activity was determined by ARA (Acetylene Reduction Assay) given by Hardy et al 1973.

Statistical analysis: Completely randomized design (CRD) was used for experimental data analysis and critical difference (CD) was calculated at 5% level of significance with the help of SPSS-16.0 statistical package.

RESULTS AND DISCUSSION

Shelf life of liquid formulations: Formulations containing protective polymers showed higher viability and metabolic activities of cyanobacteria than formulations without protective polymers. Similarly, Girisha et al (2006) also reported that liquid formulation of cowpea rhizobia prepared with PVP as an osmoprotectant also had higher shelf life than those without PVP amendment. All the formulations amended with additives stored at refrigerator showed higher viable count and other metabolic activities of cyanobacteria as compared to formulations without additive. As compared to initial cell population of 3.5 X 10^4 cells mL^{-1} that was added to each formulation, all formulations maintained a cell population of 2.3x10^4 to 3.2 x10^4 cells mL^{-1} after 18 months of incubation across all the stored temperature. The

combination of protectants gave better result than when they were used alone across the stored temperature. Formulations which contained DMSO and combination of DMSO and glycerol as protective polymers showed higher shelf life and other metabolic activities at 4^0C whereas at 28^0C and 40^0C, formulations contained PVP and combination of PVP and glycerol showed higher shelf life and other metabolic activities. At 4^0C, maximum cell population of 3.2 $x10^4$ cells mL^{-1} was observed in formulation F7 containing mixture of protective polymers *i.e.*, DMSO and glycerol whereas at 28^0C and 40^0C, formulation F8 containing mixture of PVP and glycerol maintained maximum cell population of $3.0x10^4$ and $2.9x10^4$ cells mL^{-1} respectively. However, a cell population of $2.9x10^4$ and $2.8x10^4$ cells mL^{-1} was maintained in formulations F1 and F2 containing protective polymers DMSO only at concentration of 3 and 5% respectively and $2.7x10^4$ and 2.8 $x10^4$ cells mL^{-1} in formulation F3 and F4 containing protective polymers glycerol only at concentration of 3 of 5% respectively after 18 months of incubation at 4^0C (Table 2). Similar results were observed at 28^0C and 40^0C in those formulations containing PVP and glycerol alone. A cell population of $2.8x10^4$ and 2.9 $x10^4$ cells mL^{-1} and $2.7x10^4$ and 2.9 $x10^4$ cells mL^{-1} was observed in formulations F5 and F6 containing PVP at concentration of 1.5% of 3% respectively at 28^0C (Table 3) and formulations F5 and F6 at 40^0C after 18 months of incubation (Table 4). In case of cell dry weight, maximum cell dry weight was observed in culture revived from formulation F7 (2.51 mg mL^{-1}) followed by formulation F2 kept at 4^0C after 18 months of incubation (Table 5). On the other hand, at 28^0C culture from formulation F8 recorded maximum dry weight of 2.20 mg mL^{-1} followed closely by that of formulation F5 (Table 6) whereas at 40^0C, maximum cell

Table 2. Cell count (x 10^4) of BGA culture mixture as revived from selected formulations kept at 4^0C

Treatment	Cell count (x 10^4) at different months of storage									
	1	2	3	4	5	6	9	12	15	18
F1	3.2	3.2	3.2	3.1	3.1	3.1	3.0	2.9	2.9	2.9
F2	3.3	3.2	3.2	3.2	3.2	3.2	3.2	3.1	3.1	2.8
F3	3.2	3.1	3.1	3.1	3.0	3.1	3.0	2.8	2.8	2.7
F4	3.3	3.2	3.2	3.1	3.1	3.1	3.1	3.0	2.8	2.8
F5	3.3	3.3	3.2	3.2	3.1	3.2	3.1	3.0	3.0	2.7
F6	3.4	3.4	3.3	3.3	3.1	3.1	3.0	3.0	3.0	2.7
F7	3.4	3.4	3.3	3.3	3.3	3.3	3.3	3.2	3.2	3.2
F8	3.2	3.1	3.1	3.1	3.0	3.1	3.0	3.0	3.0	2.9
F9	3.3	3.2	3.1	3.1	3.1	3.1	3.0	2.7	2.6	2.6
F10	3.2	3.2	3.1	3.0	3.0	2.8	2.8	2.7	2.6	2.4
CD (P = 0.05)	NS	0.005	0.009	0.127	0.008	0.112	0.107	0.124	0.105	0.12

Cell count of culture revived on 0 day of storage: 3.4 X 10^4

Table 3. Cell count (x 10^4) of BGA mixture as revived from selected formulations kept at 28^0C

Treatment	Cell count (x 10^4) at different months of storage									
	1	2	3	4	5	6	9	12	15	18
F1	3.1	3.1	3.0	3.1	3.1	3.1	3.1	2.9	2.8	2.6
F2	3.1	3.0	3.0	3.0	2.9	2.8	2.8	2.7	2.7	2.5
F3	3.3	3.3	3.2	3.2	3.1	3.1	3.0	2.8	2.8	2.8
F4	3.4	3.3	3.2	3.2	3.1	3.1	3.0	3.0	2.9	2.9
F5	3.4	3.3	3.2	3.2	3.1	3.2	3.1	3.0	2.9	2.8
F6	3.4	3.3	3.3	3.2	3.2	3.1	3.0	3.0	2.9	2.9
F7	3.2	3.1	3.1	3.0	3.0	2.9	2.9	2.8	2.7	2.7
F8	3.4	3.3	3.3	3.3	3.2	3.2	3.1	3.1	3.0	3.0
F9	3.3	3.2	3.1	3.1	3.0	2.9	2.9	2.7	2.7	2.7
F10	3.2	3.2	3.1	3.1	3.0	2.9	2.8	2.7	2.7	2.5
CD (p=0.05)	0.004	0.018	0.102	0.121	0.087	0.112	0.094	0.102	0.023	0.131

Cell count of culture revived on 0 day of storage: 3.4 X 10^4

Table 4. Cell count (x 10^4) of BGA culture mixture as revived from selected formulations kept at 40^0C

Treatment	Cell count at different months of storage									
	1	2	3	4	5	6	9	12	15	18
F1	3.2	3.2	3.0	3.0	2.8	2.7	2.6	2.6	2.6	2.4
F2	3.3	3.2	3.2	3.2	3.2	2.7	2.5	2.5	2.4	2.3
F3	3.2	3.1	3.1	3.1	3.0	3.1	3.0	3.0	2.7	2.7
F4	3.3	3.2	3.2	3.1	3.1	3.1	3.0	2.9	2.9	2.8
F5	3.3	3.3	3.2	3.2	3.1	3.2	3.0	2.8	2.8	2.7
F6	3.4	3.4	3.3	3.3	3.2	3.2	3.0	3.0	2.9	2.9
F7	3.4	3.2	3.1	3.1	3.0	3.0	2.8	2.8	2.7	2.5
F8	3.2	3.1	3.1	3.1	3.0	3.1	3.0	3.0	3.0	2.9
F9	3.3	3.2	3.0	3.0	3.0	2.7	2.7	2.5	2.5	2.5
F10	3.2	3.0	3.0	2.9	2.9	2.7	2.5	2.5	2.5	2.4
CD (p=0.05)	0.009	0.138	0.01	0.127	0.114	0.134	0.107	0.124	0.135	0.128

Cell count of culture revived on 0 day of storage: 3.4 X 10^4

Table 5. Cell dry weight (mg mL^{-1}) of BGA mixture as revived from selected formulations kept at 4^0C

Treatment	Cell dry weight (mg ml^{-1}) at different months of storage									
	1	2	3	4	5	6	9	12	15	18
F1	2.01	1.92	1.89	1.81	1.89	1.97	2.04	2.26	2.31	2.42
F2	2.12	1.97	1.92	1.86	1.98	2.07	2.19	2.38	2.42	2.51
F3	1.94	1.91	1.82	1.94	2.03	2.12	2.2	2.35	2.3	2.24
F4	1.98	1.87	1.84	1.89	1.97	2.1	2.28	2.41	2.36	2.28
F5	1.92	1.89	1.86	1.91	1.97	2.08	2.18	2.31	2.4	2.37
F6	1.97	1.95	1.9	2.01	2.14	2.22	2.32	2.4	2.51	2.46
F7	2.14	2.03	1.97	2.01	2.13	2.2	2.27	2.32	2.39	2.47
F8	2.08	1.98	1.91	1.96	2.01	2.1	2.16	2.2	2.28	2.34
F9	1.91	1.87	1.81	1.88	1.95	2.01	2.12	2.03	1.98	1.93
F10	1.89	1.8	1.76	1.83	1.87	1.92	1.98	2.14	1.97	1.86
CD (p=0.05)	0.101	0.123	0.084	0.072	0.107	0.157	0.132	0.054	0.124	0.093

Cell dry weight on 0 day of storage: 2.18 mg ml^{-1}

Table 6. Cell dry weight (mg mL^{-1}) of BGA mixture as revived from selected formulations kept at 28^0C

Treatment	Cell dry weight (mg ml^{-1}) at different months of storage									
	1	2	3	4	5	6	9	12	15	18
F1	1.98	1.94	1.90	1.85	1.89	1.93	2.09	2.12	2.02	1.97
F2	1.92	1.89	1.84	1.80	1.83	1.97	2.13	2.19	2.22	2.01
F3	2.06	1.94	1.98	2.02	2.05	2.11	2.18	2.22	2.13	2.04
F4	2.11	1.99	2.08	2.13	2.11	2.17	2.26	2.31	2.28	2.2
F5	2.04	1.96	1.99	2.05	1.98	1.92	1.88	1.93	1.99	2.16
F6	2.10	2.02	2.09	2.15	2.12	2.17	2.23	2.3	2.22	2.20
F7	2.09	1.97	1.93	1.96	2.00	2.12	2.19	2.12	2.03	1.97
F8	2.13	2.01	2.11	2.17	2.14	2.11	2.19	2.24	2.28	2.20
F9	1.94	1.89	1.83	1.79	1.75	1.81	1.89	1.92	1.87	1.82
F10	1.91	1.85	1.80	1.76	1.82	1.87	1.95	1.90	1.87	1.82
CD (p=0.05)	0.084	0.141	0.067	0.092	0.160	0.109	0.148	0.083	0.116	0.197

Cell dry weight on 0 day of storage: 2.18 mg ml^{-1}

dry weight was observed in formulation F8 *i.e.* 2.47 mg mL^{-1} followed by formulation F6) after 18 months of incubation (Table 7). Similar results were observed in case of total chlorophyll content. The total chlorophyll varied between 3.50 and 6.05 µg mL^{-1} amongst different formulations and across different temperatures. Maximum total chlorophyll content (5.21 µg mL^{-1}) was observed in culture revived from formulation F7 containing mixture of protective polymers *i.e.,* DMSO and Glycerol kept at 4°C (Table 8) whereas at 28°C (Table 9) and 40°C (Table 10) formulation F8 showed maximum total chlorophyll content 5.06 µg mL^{-1} and formulation 4.84 µg mL^{-1} respectively after 18 months of incubation. Most of the formulations showed nitrogenase activity in terms of ARA in the range of 2.07 to 3.52 µmol C_2H_4 mg^{-1} chl h^{-1} at all the three stored temperatures after 18 months of incubation. At 4°C, culture revived from formulation F7 showed maximum nitrogenase activity *i.e.* 3.52 µmol C_2H_4 mg^{-1} chl h^{-1} (Fig. 1) whereas that of formulation F6 and F8 at 28°C and formulation F8 at 40°C showed maximum nitrogenase activity of 2.81 and 2.62 µmol C_2H_4 mg^{-1} chl h^{-1} respectively after 18 months of incubation (Fig. 2 and Fig. 3). The results are similar to Velineni and Brahmaprakash (2011) and Sahai and Chandra (2009) who observed higher shelf life of PSB (*Pseudomonas* sp.) in liquid inoculants stored under refrigerated conditions as compared to room conditions. A similar finding was reported by Zdenek Hubalek (2003) who observed that DMSO is the most successful cryoprotective agent than glycerol and PVP at frozen storage of microorganisms. Adriana Criste et al. (2014) also studied the efficiency of cryoprotectants *viz*. DMSO (5% and 10%),

Table 7. Cell dry weight (mg mL^{-1}) of BGA mixture as revived from selected formulations kept at 40°C

Treatment	Cell dry weight (mg ml^{-1}) at different months of storage									
	1	2	3	4	5	6	9	12	15	18
F1	1.91	1.89	1.82	1.84	1.88	1.96	2.07	2.21	2.28	2.05
F2	1.86	1.82	1.84	1.88	1.92	2.01	2.18	2.25	2.31	2.26
F3	1.92	1.87	1.79	1.86	1.92	2.01	2.19	2.37	2.48	2.54
F4	1.88	1.85	1.76	1.84	1.92	2.14	2.23	2.29	2.38	2.49
F5	1.92	1.84	1.88	1.90	1.94	2.02	2.21	2.27	2.41	2.37
F6	1.90	1.86	1.90	1.95	1.99	2.14	2.19	2.34	2.48	2.41
F7	1.98	1.92	1.91	1.96	1.99	2.1	2.19	2.2	2.28	2.34
F8	1.90	1.93	1.97	2.02	2.07	2.20	2.29	2.32	2.39	2.47
F9	1.89	1.85	1.76	1.82	1.91	2.09	2.17	2.23	2.07	1.89
F10	1.90	1.83	1.78	1.74	1.79	1.87	1.91	1.89	1.85	1.80
CD (p=0.05)	0.072	0.094	0.126	0.083	0.009	0.091	0.102	0.114	0.083	0.148

Cell dry weight on 0 day of storage: 2.18 mg ml^{-1}

Table 8. Total chlorophyll (µg ml^{-1}) in BGA mixture as revived from selected formulations kept at 4°C

Treatment	Total chlorophyll (µg ml^{-1}) at different months of storage									
	1	2	3	4	5	6	9	12	15	18
F1	5.74	5.46	5.55	5.63	5.75	5.86	5.92	5.96	5.44	5.10
F2	5.66	5.60	5.83	5.88	5.91	5.99	5.89	5.71	5.26	4.48
F3	6.05	5.59	5.50	5.54	5.69	5.75	5.20	5.31	5.20	4.65
F4	5.80	5.44	5.47	5.50	5.64	5.68	5.71	5.82	5.37	5.08
F5	5.64	5.52	5.63	5.73	5.99	5.91	5.69	5.43	4.47	4.16
F6	5.55	5.43	5.45	5.66	5.75	5.69	5.43	5.34	4.38	4.05
F7	6.01	5.92	5.98	6.05	6.19	6.23	6.27	6.31	5.49	5.21
F8	5.99	5.78	5.90	5.98	6.09	6.15	5.98	5.44	4.57	4.19
F9	5.70	5.41	5.80	5.93	5.76	5.27	4.46	4.22	4.13	4.01
F10	5.50	5.27	5.43	5.46	5.24	4.63	4.39	4.19	4.00	3.87
CD (p=0.05)	0.10	0.08	0.08	0.05	0.08	0.08	0.11	0.16	0.19	0.12

Total chlorophyll on 0 day of storage: 6.97 µg mL^{-1}

ethylene glycol (5 and 10%) and glycerol (5 and 10%) on long term microorganisms conservation and they found that among the four cryoprotectants, the best results were given by DMSO or combination of DMSO and glycerol utilization. Santhosh (2015) reported that liquid biofertilizers formulated with PVP in addition to glycerol at the rate of 0.5% retained maximum number of colonies *of Rhizobium, Azotobacter, Azospirillum* and PSB at 28±2 °C for a period of 180 days. Daniel et al (2013) and Gopal and Baby (2016) have reported the liquid inoculants formulations using 2% PVP and 0.1% CMC promoted long term survival of *B. megaterium, Azospirillum* and *Azotobacter* after 480 days at 30^{0}C. Our results are also similar to Mugilan et al (2011) reported better survival of PSB (*Pseudomonas striata*) in liquid formulation amended with PVP and glycerol stored at room temperature. Aarti Yadav et al (2017) observed higher shelf life of PSB in inoculants amended with PVP (2%, 1%) followed by glycerol (2% and 1%) during the storage at room temperature. Kumaresan and Reetha (2011) reported that liquid *Azospirillum* bioinoculant formulated with glycerol (10 mM) followed by gum arabica (0.3%) and PVP (2%) promoted long term survival of *Azospirillum* and supported 10^8 cells/mL up to 11 months of storage under ambient temperature (28°C to 32°C). Babu et al (2002) also found higher population of *Azospirillum* due to the addition of PVP at both 1 and 2% levels. Similarly, Sherawat et al. (2015) reported that liquid inoculants prepared in amended additives such as glycerol, PVP, GA media showed higher viable count in comparison to inoculants prepared in YEMB (control) at 180 days of storage. Sridhar et al (2004) developed liquid inoculant of

Table 9. Total chlorophyll (µg mL^{-1}) in BGA mixture as revived from selected formulations kept at 28^{0}C

Treatment	Total chlorophyll (µg ml^{-1}) at different months of storage									
	1	2	3	4	5	6	9	12	15	18
F1	5.31	5.19	5.04	4.9	4.84	4.87	4.76	4.6	4.12	4.09
F2	5.15	4.99	4.93	4.72	4.65	4.56	4.44	4.3	3.97	3.92
F3	6.01	5.74	5.76	5.8	5.81	5.86	5.99	5.94	5.66	5.01
F4	5.83	5.63	5.5	5.56	5.6	5.71	5.81	5.94	5.26	4.5
F5	5.77	5.72	5.75	5.79	5.8	5.82	5.85	5.87	5.17	4.73
F6	5.62	5.57	5.63	5.65	5.69	5.61	5.34	5.43	4.89	4.25
F7	5.42	5.31	5.39	5.41	5.21	5.06	4.96	4.89	4.78	4.37
F8	5.84	5.75	5.79	5.82	5.87	5.98	6.01	5.8	5.33	5.06
F9	5.98	5.48	5.44	5.5	5.58	5.5	5.29	4.98	4.46	4.18
F10	5.53	5.33	5.29	5.44	5.02	4.98	4.52	4.15	3.92	3.5
CD (p=0.05)	0.17	0.15	0.16	0.18	0.20	0.15	0.18	0.15	0.16	0.12

Total chlorophyll on 0 day of storage: 6.97 µg mL^{-1}

Table 10. Total chlorophyll (µg ml^{-1}) in BGA mixture as revived from selected formulations kept at 40^{0}C

Treatment	Total chlorophyll (µg ml^{-1}) at different months of storage									
	1	2	3	4	5	6	9	12	15	18
F1	5.54	5.46	5.31	5.1	4.85	4.53	4.44	4.12	3.9	3.83
F2	5.51	5.44	5.17	4.99	4.44	4.23	4.14	3.98	3.52	3.46
F3	5.7	5.67	5.6	5.39	5.5	5.58	5.39	5.09	4.43	4.27
F4	5.66	5.52	5.43	5.28	5.3	5.33	5.15	4.94	4.32	4.24
F5	5.74	5.68	5.63	5.4	5.41	5.47	5.22	5.04	4.45	4.33
F6	5.76	5.71	5.66	5.48	5.59	5.6	5.45	5.13	4.82	4.7
F7	5.57	5.54	5.41	5.14	4.91	4.85	4.69	4.48	3.96	3.92
F8	6.01	5.86	5.8	5.69	5.75	5.81	5.75	5.32	5.00	4.84
F9	5.62	5.54	5.44	5.25	5.04	4.91	4.59	4.3	3.92	3.88
F10	5.51	5.44	5.23	5.07	4.57	4.44	4.22	4.11	3.8	3.5
CD (p=0.05)	0.12	0.11	0.17	0.13	0.18	0.12	0.09	0.06	0.08	0.05

Total chlorophyll on 0 day of storage: 6.97 µg mL^{-1}

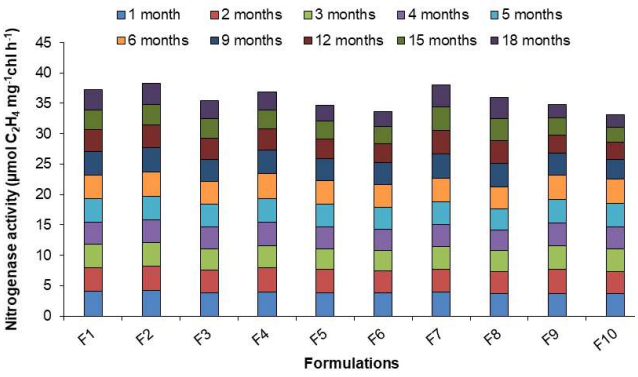

Fig. 1. Nitrogenase activity in terms of ARA (µmol C_2H_4 mg^{-1} chl h^{-1}) in BGA culture mixture as revived from selected formulations kept at 4^{0}C

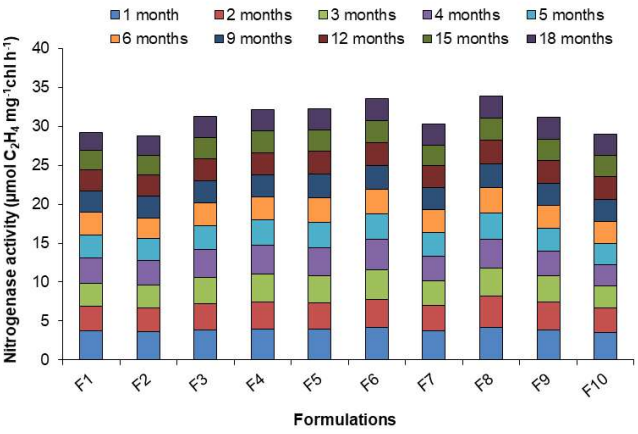

Fig. 2. Nitrogenase activity in terms of ARA (µmol C_2H_4 mg^{-1} chl h^{-1}) in BGA culture mixture as revived from selected formulations kept at 28^{0}C

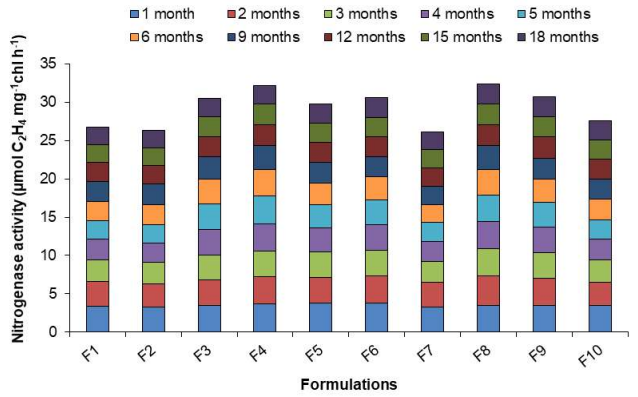

Fig. 3. Nitrogenase activity in terms of ARA (µmol C_2H_4 mg^{-1} chl h^{-1}) in BGA culture mixture as revived from selected formulations kept at 40^{0}C

Bacillus megaterium using osmoprotectants PVP, glycerol and glucose and found supported higher viable population up to 6 months storage period than formulations where they used alone. *Mahdi* et al (2010) also reported that the shelf-life of liquid formulations could be as high as two years.

Temperature is important for the shelf life of microbial products and can affect their normal activity. Cultures used in these formulations normally grow at 28^{0}C±2°C but since the temperature in many parts of our country goes around 10^{0}C in winter and above 40°C in summer so it was necessary to test the formulations at these lower and higher temperature. Liquid biofertilizer preparations contain special cell protectants or additives which promote longer shelf life and tolerance to adverse conditions (Hegde 2008). A number of types of polymers has been used for inoculant preparations because of their ability to limit heat transfer, good rheological properties and high water activities. Polymers like sodium alginate, gum arabic, dimethyl sulfoxide (DMSO), polyvinyl pyrrolidone (PVP), polyvinyl alcohol (PVA) and polyethylene glycol (PEG) have normally been used as thickener/adhesive or protectants (Leo Daniel et al., 2013). Higher survival under refrigerated conditions may be due to the fact that low temperature in refrigerator allows no or little growth with less utilization of nutrients during storage making them available to the organism in optimum concentration for longer period. Low temperature in refrigerator also protects the cell death in inoculums. Glycerol and DMSO decrease the freezing-point of water and biological fluids by colligative action (up to 46^{0}C and 73^{0}C respectively). Therefore lessen the concentration of salts dissolved in solutions, in turn inhibiting osmotic shock and bind intracellular water colligatively which prevents excessive dehydration, reduces salt toxicity and prevents the formation of large ice crystals within the cell (Mugilan et al 2011, Singleton et al 2002) Glycerol has high water binding capacity and may protect cells from the effect of desiccation by reducing the rate of drying (Lorda and Balatti 1996). In contrast, during storage of inoculants at room conditions where temperature may go beyond 30^{0}C, growth of organism is allowed creating depletion of nutrients and accumulation of toxic metabolites (Tittabutr et al 2007). In the present study, formulations were also kept at higher temperature to study the shelf life and other metabolic activities of inoculated culture. Hoe and Rahim (2010) reported that liquid formulations can tolerate the temperature as high as 55°C. Similar reports were observed by many researchers (Panlada et al 2007, Vendan and Thangaraju 2006). In the present study, at higher storage temperature PVP and combination of PVP and glycerol polymeric additives maintain higher shelf life and other metabolic activities. At higher temperature, PVP act as stabilizing polymers in

reducing the extent of protein precipitation or coagulation of cells (Deaker et al 2004, Gaind et al 1990). PVP help in maintaining the higher viable count as bacteria do not use these polymers as an energy source, these polymers have other properties supporting the growth and survival of cells. PVP is believed to detoxify the fermentation medium by complexing with the phenolic-type, shelf-limiting toxins in the medium. (Temprano et al 2002, Errington et al 2002) PVP also has colloidal 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 and maintain water around the cells for their metabolism (Singleton et al 2002, Deaker et al 2004, Tittabutr et al 2007). This polymer is absorbed in a thin molecular layer on the surface of the individual colloidal particles resulting in a stabilized suspension that prevents coalescence of cells, which might block the O_2 and nutrient diffusion from media to cells. PVP has also ability to limit heat transfer. These might be the mechanisms that improve the survival of cyanobacterial cells in formulations stored at higher temperature.

CONCLUSIONS

Present study revealed that the performance of developed liquid formulations was good with protective polymeric additives than without them. The liquid biofertilizer formulation using combination of protective polymers showed maximum cell count and other metabolic activities as compared to alone. At 4^0C, liquid inoculant formulated with DMSO and glycerol provided the greatest protection to cells of cyanobacteria and surviving and other growth promoting ability whereas at 28^0C and 40^0C, PVP in addition to glycerol retained maximum surviving and other metabolic activities. Hence, the combinations of protective polymers viz. DMSO (3%) and glycerol (2%) at 4^0C and PVP (2%) and glycerol at 28^0C and 40^0C showed best conditions to improve shelf life of cyanobacterial liquid biofertilizer formulations. It can be recommended to maintain the shelf life of liquid formulations for storage period of 18 months. Further, in-depth analysis of the formulations is needed to understand the role of protectants for maintenance under various storage conditions and needs to be standardised.

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