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Research Article

EVALUATION OF LOW COST LIQUID FORMULATION OF PGPR INOCULANTS WITH PROTECTIVE SUBSTANCES

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ABSTRACT

Plant growth promoting rhizobacteria (PGPR) are a specific group of multifunctional bacteria interacting beneficially with plants. They help to improve soil quality, promote crop growth, and sustain soil health. Among the known PGPRs, the *Azospirillum, Azotobacter, Pseudomonas* have been highlighted in both growth promotion and control of diseases. Ensuring the stability of the microorganism during production, formulation, distribution and storage has been a challenge for these species. In this context, the objective of this work was to develop liquid formulations, through a simplified process, that allows increase in the shelf life of these rhizobacteria for commercial application. The liquid formulation of efficient PGPR was prepared in sterilized liquid manure *matkha khaad* with the addition of four protective substances glycerol (2%), polyethylene glycol [PEG – 400 1%], polyvinylpyrrolidone-30 [PVP 1 %], and trehalose (1%), and their capacities for maintaining cell viability during storage in low, medium, and high temperature and pH ranges were evaluated. Trehalose (1 %) was chosen as a potential additive because it could maintain a relatively high population and conferred greater microbial vitality under various storage conditions *matkha khaad* is considered a safe, low-cost, and easy-to-process material, and this formulation would facilitate the practical use of PGPR in agriculture.

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INTRODUCTION

Liquid biofertilizers are one solution to the problems associated with solid carrier based biofertilizers and it enhance yield of treated plants markedly. Liquid inoculant formulations use various broth cultures amended with agents that promote cell survival in the package and after application to seed or soil (Girisha *et al*., 2006; Tittabutr *et al*., 2007; Kumaresan and Reetha, 2011).

Carrier is an important component of biofertilizer technology and is defined as the vehicle carrying efficient microbial strains from the laboratory to the field with minimum damage to the viable cell population. To facilitate introduction of high cell numbers and increased survival of microorganisms in soil, preparation of carrier based microbial inoculants is pre-requisite (Bashan 1998). Solid carrier based preparations generally suffer from short shelf-life, poor quality, high contamination and low and unpredictable field performances (Vendan and Thangaraju 2006). To overcome these problems, the liquid carrier based formulations have been introduced (Gupta 2005; Albareda *et al*., 2008). Liquid bioinoculants are special liquid formulations containing not only the desired microorganisms and their nutrients, but also, special cell protectants or substances that encourage the longer shelf life and tolerance to adverse conditions (Vora *et al*., 2008). Also, a liquid inoculant formulation made from local low cost material may be useful to the small producers especially in overcoming some of problems associated with processing of the carrier (Singleton *et al*., 2002). Before recommending a bioinoculant for crop production, its shelf life in different carrier materials needs to be addressed.

Low cost formulation technology has now become a main concern of research in developing countries where, a major role of inoculants formulation is to provide a more suitable microenvironment to prevent the rapid decline of introduced bacteria in the soil. In recent years many of the formulatios of the liquid based inoculants are introduced which are less expensive, enhance shelf life of PGPR and tolerate adverse environmental conditions in a better way in comparision to solid carrier based preperations. (Vora; 2008 and Sandra and Rebeca,2015)

The negative impact of chemical fertilizers on the global environment and the cost of production have lead to research with objective of replacing chemical fertilizers with organic

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amendment completely or partially. The application of microbial products to replenish the reserve nutrients is now increasing throughout the world (Kumar *et al.,* 2012). Plant growth promoting rhizobacteria have the potential to contribute to sustainable plant growth promotion. The PGPR retain more soil organic N and other nutrients in the plant – soil system, thus reducing the need for fertilizer N

In liquid microbial cultures or suspensions amended with substances that may improve stickiness, stabilization, and surfactant and dispersal abilities (Singleton *et al.,* 2002). The main advantage of these inoculants over solid inoculants is that they are easy to handle. Unlike solid carrier-based inoculants, liquid formulations allow the manufacturer to include sufficient amounts of nutrients, cell protectants, and inducers responsible for cell/spore/cyst formation to improve performance. While the shelf life of common solid carrier-based inoculants is ~ 6 months (or in6 months (or in the best of cases, 12–18 months in Australia), another advantage of a liquid formulation is that its shelf life could be as high as 2 years (Stephens and Rask, 2000).

Thus in the present investigation, the establishment of bacteria in liquid manure *matkha khaad* carriers for their survival was studied. The liquid manures amended with protective substances were used in this study to maintain the viability of microbial cells.

MATERIALS AND METHODS

Mass production of efficient strains

For mass production of inoculant, 500 ml of respective growth medium (Nfb broth for *Azospirillum sp*, Ashby's mannitol broth for *Azotobacter sp* and Nutrient broth for *Pseudomonas sp*) was inoculated with 5ml of efficient microbial cultures and incubated for 48-72 h at their optimum growth temperatures under shaking (120 rev/ min.) conditions. Following incubation, the cells were harvested by centrifugation (10000 rpm) for 10 min at 4°C, washed twice with 100 mM potassium phosphate buffer solution and resuspended in 100 mM potassium phosphate buffer, so as to achieve the final concentration of 10^9 cfu/ml. This served as mother culture for inoculating with liquid carriers.

Preparation of liquid inoculants of PGPR in matka khaad with different protective substances

Preparation of natural medium

matkha khaad, a liquid manure was prepared by mixing cow dung, cow urine, water and jaggery (1:1:1: 0.25) in an earthen pot. The mixture was incubated for 10 days at room temperature and used for further studies (Chadha et al., 2012).

Nutrient profile analysis of matka khaad

matkha khaad was sterilized twice by autoclaving at 121°C with 15psi for 20 min so as to eradicate its natve microflora. Nitrogen and phosphorous content was measured by Olsens method and potassium content was determined by Flame photometry method. Micronutrients such as magnesium,Iron calcium, manganese and Zinc content were analysed in Atomic absorption spectrophotometer by DTPA (Diethylene Triamine Penta –acetic acid) method (Lindsay,1978).

Mixing of mother culture with the carrier material

Three active PGPR namely *Azospirillum, Azotobacter*, and *Pseudomonas sp* were selected for liquid inoculant formulation. The above prepared bacterial suspension was mixed thoroughly with 100 ml of liquid carrier at the rate of 1 per cent (%). The inoculated flasks were kept at room temperature for different intervals of time (Kanwar *et al.,* 2017). Six treatments (Control, four liquid inoculants formulations and one lignite formulation) were selected for survival studies. The liquid carrier *matkha khaad* were tried in combination with different chemicals to study the survivability of each efficient PGPR. To develop liquid formulations t,he amendments viz., trehalose (1% W/v), polyvinylpyrollidone (PVP) (1%), glycerol (2%) and polyethyleneglycol (PEG - 400) (1 %) were added separately to different liquid carriers. These liquid carriers were then sterilized at 121°C for 15 minutes in an autoclave.

Enumeration of viable cell population

The population of inoculated bacteria in liquid carriers was enumerated at monthly intervals up to decline phase by serial dilution technique by taking 1 ml of the carrier material and aseptically transferring it to 9 ml distilled water and shaking it vigorously, so as to form homogeneous suspension. The enumeration was carried out using spread plate technique using appropriate growth medium. These plates were incubated at appropriate growth temperature for 24-48 h. Plates containing 30-300 colonies were recorded and the counts were expressed as log cfu per ml of carrier material.

Effect of stress conditions on liquid formulation

The best formulation was subjected to various stress conditions viz., temperature (15, 25, 40 and 50 °C), pH (4.5, 5.5, 6.5 and 7.5). The best formulation was amended with different chemicals and inoculated as described in above. The viable populations of bacterial cultures were enumerated at regular intervals of time.

Effect of temperature stress on Survivability

The inoculated formulation was incubated at different stress temperatures and was enumerated for viable cell population at regular intervals of time.

Effect of P^H stress on Survivability

The best formulation prepared was subjected to varying PH, and incubated at their optimum growth temperature. The population of viable cells was enumerated at regular intervals of time.

Table 1 Nutrient profile of *matkha khaad* used in the study

Parameters	Mean value
1.Carbon(%)	1.9
2.Nitrogen (%)	2.3
3.Micronutrients (ppm)	
Fe^{2+}	0.25
Ca^{2+}	0.46
Mn^{2+}	0.02
Zn^{2+}	0.04
Mg^{2+}	1.05

Table 2 Survival of *Azospirillum* sp. in liquid formulation (*matkha khaad*) with protective substances

Inoculant Formulations	Population density (CFU/ ml or g) at different sampling intervals Duration of storage (Days)									
	5	30	60	90	120	150	180	210	260	290
Control	$7x10^{9}$	$4x10^{9}$	$2x10^{8}$	$28x10^{6}$	$30x10^{6}$	$25x10^{5}$	$24x10^{4}$	$17x10^{4}$	$15x10^{4}$	$12x10^{3}$
Glycerol (2%)	$21x10^{9}$	$15x10^{9}$	$14x10^{9}$	$60x10^{8}$	$54x10^{7}$	$45x10^{6}$	$38x10^{6}$	$42x10^{5}$	$39x10^{5}$	$28x10^{4}$
PEG 400 (1%)	$17x10^{9}$	$13x10^{9}$	$10x10^{9}$	$22x10^{8}$	$16x10^{8}$	$40x10^{7}$	$35x10^{7}$	$38x10^{6}$	$40x10^{5}$	$35x10^{4}$
PVP K 30 (1%)	$15x10^{9}$	$8x10^{9}$	$12x10^{9}$	$20x10^{8}$	$14x10^{8}$	$54\ x10^{7}$	$50\ x10^{7}$	$47x10^{7}$	$59x10^{6}$	$48x10^{5}$
Trehalose (1%)	$23x10^{9}$	$19x10^{9}$	$17x10^{9}$	$15x10^{9}$	$13x10^{9}$	$40x10^{8}$	$35x10^{8}$	$29x10^{8}$	$35x10^{7}$	$28x10^{7}$
Carrier based inoculants	$14x10^{9}$	$8x10^{9}$	$7x10^{9}$	$10x10^{8}$	$5x10^{7}$	$3x10^{6}$	$4x10^{5}$	$3x10^{4}$	$2\ x10^{4}$	$2x10^{2}$

Table 3 Survival of *Azotobacter* sp. in liquid formulation (*matkha khaad*)with protective substances

Inoculant Formulations	Population density (CFU/ ml or g) at different sampling intervals Duration of storage (Days)									
	5	30	60	90	120	150	180	210	260	290
Control	$10x10^{7}$	$7x10^{6}$	$13x10^{5}$	$9x10^{5}$	$8x10^{4}$	$6x10^{4}$	$13x10^{3}$	$7x10^{3}$	$15x10^{2}$	$12x10^{2}$
Glycerol (2%)	$14x10^{7}$	$13x10^{7}$	$12x10^{7}$	$7x10^{7}$	$6x10^{7}$	$4x10^{6}$	$3x10^{5}$	$14x10^{4}$	$8x10^{4}$	$10x10^{3}$
PEG 400 (1%)	$8\ x10^{7}$	$8x10^{7}$	$10x10^{6}$	$9x10^{6}$	$16x10^{5}$	$12\ x10^{7}$	$9x10^{6}$	$13x10^{5}$	$8x10^{4}$	$5x10^{3}$
PVP K 30 (1%)	$12x10^{7}$	$10x10^{7}$	$8x10^{7}$	$6x10^{6}$	$6x10^{6}$	$15x10^{5}$	$8x10^{5}$	$22x10^{4}$	$18x10^{4}$	$28x10^{3}$
Trehalose (1%)	$16x10^{9}$	$12x10^{9}$	$10x10^{9}$	$15x10^{8}$	$32x10^{8}$	$25x10^{8}$	$22x10^{8}$	$20x10^{7}$	$17x10^{7}$	$15x10^{7}$
Carrier based inoculants	$15x10^{7}$	$8x10^{6}$	$7x10^{6}$	$6x10^{5}$	$5x10^{5}$	$3x10^{4}$	$8x10^{3}$	$7x10^{2}$	$5x10^{2}$	$2x10^{2}$

Table 4 Survival of *Pseudomonas* sp. in liquid formulation (*matkha khaad*) with protective substances

Inoculant Formulations	Population density (CFU/ ml or g) at different sampling intervals Duration of storage (Days)									
	5	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**260**	**290**
Control	$21x10^{9}$	$19x10^{9}$	$17x10^{9}$	$12x10^{8}$	$45x10^{6}$	$42x10^{6}$	$13x10^{4}$	$12x10^{4}$	$15x10^{2}$	$12x10^{2}$
Glycerol (2%)	$34x10^{9}$	$33x10^{9}$	$29x10^{9}$	$23x10^{8}$	$9x10^{8}$	$18x10^{7}$	$10x10^{6}$	$8x10^{6}$	$9x10^{5}$	$10x10^{4}$
PEG 400 (1%)	$31\ x10^{9}$	$23x10^{9}$	$21x10^{9}$	$17x10^{8}$	$8x10^{8}$	$20x10^{7}$	$12x10^{6}$	$9x10^{6}$	$3x10^{5}$	$5x10^{4}$
PVP K 30 (1%)	$45x10^{7}$	$70x10^{7}$	$58x10^{7}$	$40x10^{6}$	$27x10^{6}$	$20x10^{6}$	$23x10^{5}$	$20x10^{5}$	$18x10^{4}$	$15x10^{4}$
Trehalose (1%)	$65x10^{9}$	$64x10^{9}$	$52x10^{9}$	$17x10^{9}$	$13x10^{9}$	$48x10^{8}$	$19x10^{8}$	$8x10^{8}$	$9x10^{7}$	$5x10^{7}$
Carrier based inoculants	$42x10^{9}$	$40x10^{9}$	$37x10^{9}$	$32x10^{8}$	$37x10^{7}$	$32x10^{7}$	$30x10^{6}$	$12x10^{6}$	$8x10^{6}$	$8x10^{5}$

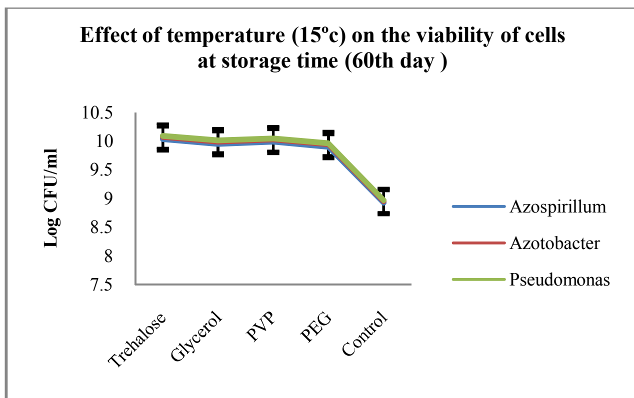
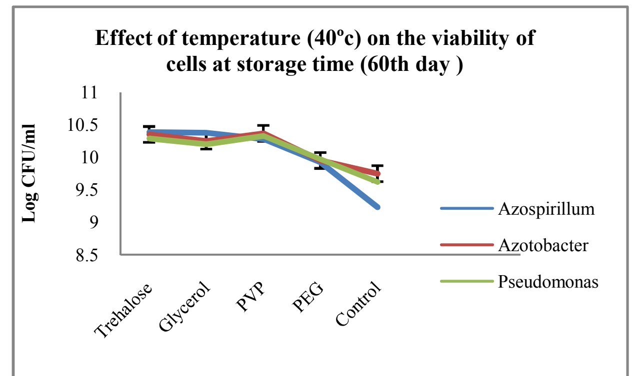


Fig 1



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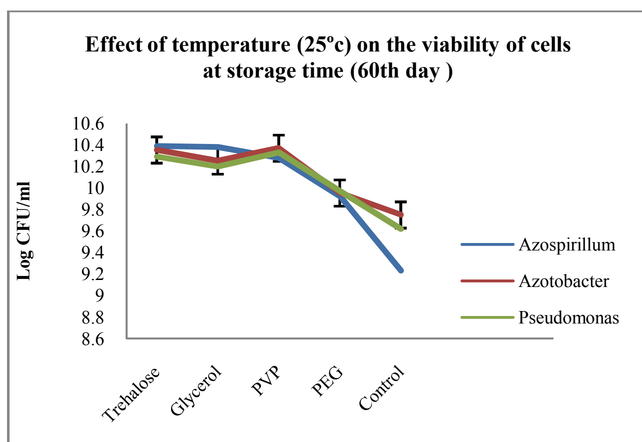

Fig 2

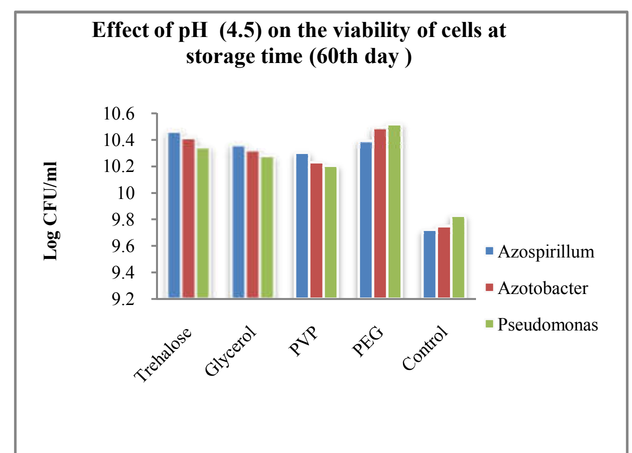

Fig 4

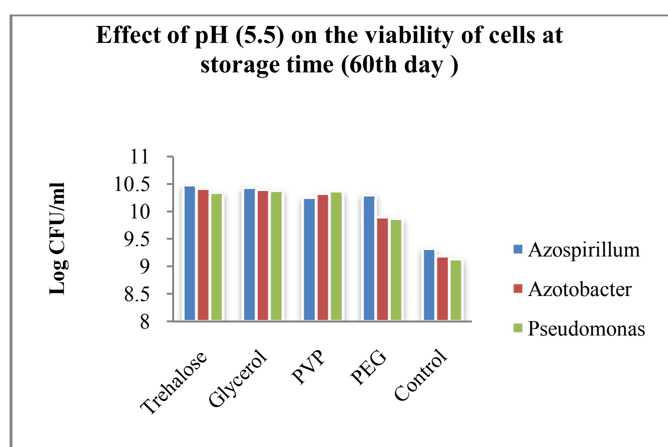

Fig 5

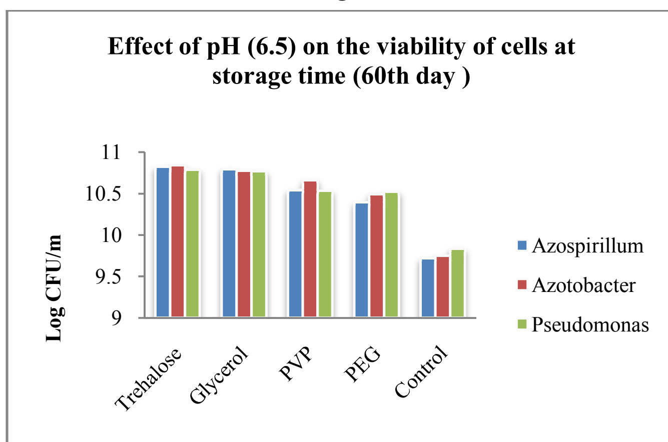

Fig 6

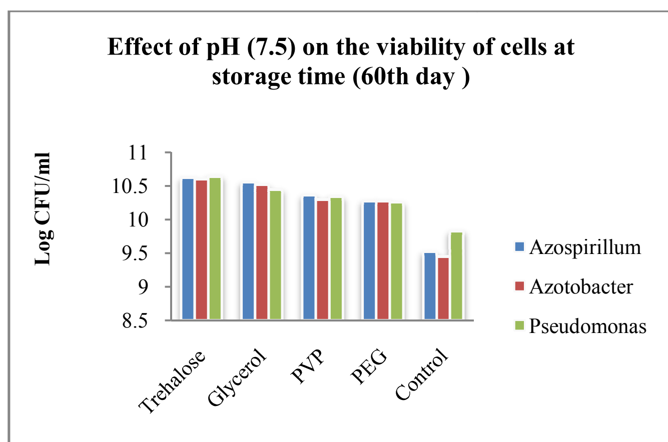

Fig 7

RESULT AND DISCUSSION

Preparation of matka khaad and its nutrient analysis

Our studies demonstrated that after 10 days of incubation at room temperature, *matka khaad* was collected as brown colored liquid organic manure. After nutrient analysis it was noticed that *matka khaad* was rich in carbon (1.9), nitrogen (2.3) iron (0.25ppm), calcium (0.46 ppm ppm) manganese (0.02 ppm) zinc (0.02 ppm) and magnesium (1.05 ppm) (Table 1).

Shelf – life study of efficient PGPR in matka khaad

The results clearly showed that *matka khaad* was the effective liquid carrriers in maintaining higher microbial load. The results were presented in Table 2, 3 and 4. *matka khaad* on 30 th day of incubation (Table 2-4), the treatments viz., trehalose and glycerol were statistically at par with each other, whereas at 60-290 days of incubations, all the treatments were significantly different. *matka khaad* with trehalose maintained microbial population of *Azosprillum sp* $15X10^9$cfu/ml, *Azotobacter* sp 22 $X10^8$ cfu/ml and *Pseudomonas sp* $17x10^9$on 90th day of incubation. After 290 days of incubation *Azospirillum* showed 28 $X10^7$ cfu/ml, *Azotobacter* $15X10^7$cfu/ml, *Pseudomonas* 5 $X10^7$ cell populations as compared to other treatments. Whereas, glycerol as an additive maintained microbial population of $14X10^9$cfu/ml and $28X10^4$cfu/ml on 30th and 210th day of incubation, respectively. Polyvinylpyrrolidone (PVP) was also effective additive but less efficient than glycerol. This treatment maintained $48X10^4$ cfu/ml of inoculated strains on 210 th day of incubation. After PVP, Polyethylene glycol (PEG) was found effective in maintaining higher microbial load of $35X10^4$ cfu/ml on 210 th day of incubation. The control treatment was found to be least effective in maintaining higher microbial load of inoculated efficient strains. It maintained microbial population up to $13X10^4$ log cfu/ml on 180th day of incubation and thereafter the population decreased very rapidly. In the present study *Azotobacter* and *Azospirillum* was found to be most efficient and trehalose treatment was found to be effective in maintain statistically higher microbial load as compared to other treatments, except on 30th day of incubation at which trehalose treatment was at par with glycerol treatment in *matka khaad*.

The population density of PGPR in carrier based inoculant formulation and liquid formulation without any osmolytes significantly decreased during the first 30 days of storage, whereas, such a decrease was not observed in the remaining liquid inoculant formulations (Table 2 , 3 and 4).

Among various additives tested, trehalose (Table 2-4) was found to be most effective in maintaining higher microbial load for longer period as compared to other additives used. Similar findings made by Vendan and Thangaraju (2006), He developed liquid formulation of *Azospirillum* by using various cell protectants and found trehalose to be most effective in maintaining higher population. Other workers also used additives to improve the shelf life of formulation (Larena *et al.,* 2005; Streeter 2006; Tittabutr *et al,.* 2007) .Trehalose is an enigmatic compound which act as a reserve carbohydrate that may be mobilized during stress (Hounsa et al., 1988). It is widely reported to enhance cell tolerance to desiccation, osmatic and temperature stress. It acts by stabilizing both enzymes and cell membranes (Fillinger *et al.,* 2001). 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 (Gomez *et al.,* 2003) which might be the reason for the higher population of *Azospirillum* cells in the trehalose treatments. Next to trehalose, 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 the rate of drying (Lorda and Balatti 1996).

Effect of Temperature

The effect of temperature viz., 15, 25, 40 and 50 ºC on the survivability of the efficient strains in the *matka khaad* is

shown in Figure 1- 4. It was observed that at 15 ºC temperature the efficient PGPR showed highest survivability upto 45 days of incubation [*Azotobacter sp* (10.113 log cfu/ml), *Azospirillum sp* (10.133 log cfu/ml) and *Pseudomonas* (10.091 log cfu/ml)]. Whereas at 25 ºC (Figure 3), the highest survivability was observed upto 60th day of incubation [(*Azotobacter sp* (10.636 log cfu/ml), *Azospirillum sp* (10.671 log cfu/ml) and *Pseudomonas* (10.607 log cfu/ml)] and the efficient isolates showed highest survivability at 15th day of incubation [(*Azotobacter sp* (9.924 log cfu/ml), *Azospirillum sp* (9.949 log cfu/ml) and *Pseudomonas sp* (9.892 log cfu/ml)] at 40 ºC (Table 4.82 to Table 4.85). At 50 ºC, none of the efficient isolates was able to grow. The treatment with trehalose was found to be the most effective in maintaining high microbial load as compared to the other treatments at different incubation intervals. Only at 15 ºC, the treatment with trehalose and glycerol was found to be statistically at par upto 30th day of incubation. The interaction between treatments and microorganisms was found to be non-significant at 15 ºC (Fig - 1), 25 ºC (Fig - 2) and 40 ºC (Fig -3) on the incubation day at which isolates showed highest survivability. It was observed that (Fig -1- 4) population of efficient strains was low at 15 ºC and 40 ºC as compared at 25 ºC. This might be due to the fact that organisms grow well and multiply at 25 ºC which was nearer to optimum growth temperature.

Effect of pH

pH is an important aspect of bacterial cell physiology over which the cell exerts relatively tight regulation (Booth 1985). To examine the effect of different pH on the survivability of the efficient PGPR in the *matka khaad*, the selected bacterial isolates were grown in varying pH viz., 4.5, 5.5, 6.5 and 7.5. Figure 4 to 7 depict the effect of different pH on the shelf life of the efficient isolates. It was observed that on 30th day of incubation all the isolates showed highest survivability at all the tested pH values. The least survival was observed at pH 4.5 (Fig - 4). The highest survivability at various tested pH was observed at pH 6.5 which was 10.697 log cfu/ml, 10.727 log cfu/ml and 10.743 log cfu/ml (Fig - 6) for *Azotobacter sp*, *Pseudomonas sp* and *Azospirillum sp*, respectively on 30th day of incubation. Trehalose was found to be best additive that maintained statistically higher microbial load as compared to the other additives used, except at pH 4.5 on 30th days of incubation (Fig - 5) where trehalose and glycerol treatments were statistically at par. On 30th day of incubation, the interaction between treatments and microorganisms was found to be significant at pH 4.5 whereas, it was non-significant at other pH values (4-7). Figure showed that pH 4.5, 5.5 and 7.5 maintained lower microbial load as compared to pH 6.5. This could be due to the fact that pH 6.5 is closer to neutral and is optimum for microbial growth as compared to other tested pH values.

Effect of stress conditions on liquid formulation Stress is an inevitable part of the life for all organisms. The bulk soil is generally a very poor, nutrient-diluted and hostile environment for many microorganisms. In soil, microorganisms are exposed to a range of variable biotic and abiotic stresses, such as competition, predation, changes in temperature, osmolarity, availability of water etc. (Miller and Wood 1996; van Veen et al. 1997). The performance of inoculants is severely affected by these stress factors (Zahran 1999; Vriezen et al. 2006). The important properties of a good inoculant are having a strain with high plant growth promoting potential, capability of surviving in stressful conditions such as acidity, salinity, different temperatures, moisture deficiency, etc. and able to adapt to the formulation and storage conditions with minimal population reduction (Khavazi *et al*., 2007).

CONCLUSION

The present study revealed that the *matka khaad* liquid manure with trehalose maintained the viability of efficient PGPR namely, *Azotobacter, Azosprillum* and *Pseudomonas* at different storage conditions. The results of these studies suggest that the liquid formulations of beneficial organisms generally have higher population density and permit higher survival rate than that of lignite based formulations and further research is needed to optimize their effectiveness under field conditions.

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