

Influence of form and Concentration of the Osmolytes in Liquid Inoculants Formulations of Plant Growth Promoting Bacteria

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Abstract- Laboratory investigations were conducted to identify suitable form and to determine the suitable dose of application of osmolytes for liquid inoculant formulations of plant growth promoting rhizobacteria (PGPR) viz., *Azotobacter* sp., *Azospirillum* sp., *Acinetobacter* sp., *Bacillus* sp. and *Pseudomonas* sp. Osmolytes such as four grades of polyvinyl pyrrolidone (PVP), four grades of polyethylene glycol (PEG), and glycerol were added to broth at three different concentrations (0.5%, 1.0% and 2.0%). Supplementing the specific media with 2 % Glycerol for *Azotobacter* sp., 2 % PVP K-15 for *Bacillus* sp., 1 % PEG 400 for *Azospirillum* sp., 2 % PVP K-15 for *Pseudomonas* sp., and 2 % PEG 4000 for *Acinetobacter* sp. resulted in the highest population densities.

Index Terms- concentration, liquid inoculants, osmolytes, PGPR

I. INTRODUCTION

Liquid inoculant formulations generally contain certain compounds which serve as cell protectants in addition to all other constituents of specific nutrient media used for the growth of PGPR in laboratory and have been observed to serve as effective inoculants in case of rhizobia (Deaker *et al.*, 2004). The results of several investigations have shown that the performance of liquid rhizobial formulations is comparable to that of peat-based products under field conditions in terms of growth and yield (Hynes *et al.*, 1995, 2001). On the contrary, liquid formulations support high population density of bacteria under varying environmental conditions in pure culture. Hence, the possibility of developing liquid formulations of microbial inoculants is being explored. Liquid formulations typically are aqueous, oil, or polymer-based products. Polysaccharides such as gums, carboxymethylcellulose and polyalcohol derivatives are commonly used to alter the fluid properties of liquid formulations (Paau, 1988). Several liquid formulations available today support high viable rhizobial numbers for extended periods of time. Tittabutr *et al.*, (2007) determined the effectiveness of bradyrhizobial liquid inoculant formulations with gum arabic, sodium alginate, polyvinyl pyrrolidone (PVP), polyethylene glycol (PEG), polyvinyl alcohol (PVA) and cassava starch under field conditions and found that the effectiveness of liquid inoculant was as good as peat based inoculant. Liquid cultures containing cell protectants not only maintain high microbial numbers but also promote the formation of resting cells such as, cysts and spores which offer higher resistance to abiotic stresses,

thus increasing the survivability of bacteria. Liquid formulations containing different concentrations of arabinose, trehalose, glycerol and polyvinyl pyrrolidone (PVP) were devised for *Rhizobium* sp., *Azospirillum* sp. and phosphate solubilising *Bacillus megaterium*.

II. MATERIAL AND METHODS

Plant growth promoting bacteria and Media used

Five plant growth promoting bacteria were used in this study representing nitrogen fixing (*Azotobacter* sp. and *Azospirillum* sp.), phosphate solubilizing (*Bacillus* sp. and *Acinetobacter* sp.) and growth promoting (*Pseudomonas* sp.).

Okon's N- free semisolid malate media was used to grow nitrogen fixing bacterium, *Azospirillum* sp. and its chemical composition was malic acid, 5.0 g, KOH/NaOH, 3.0 g, K₂HPO₄, 0.5 g, FeSO₄·7H₂O, 0.05 g, MnSO₄, 0.01 g, MgSO₄·7 H₂O, 0.1 g, NaCl, 0.02 g, Na₂MoO₄·2H₂O, 0.002 g, agar, 3 g, distilled water, 1000 ml, pH, 6.6-7.0, BTB (0.5% alcohol), 2 ml.

Azotobacter sp. was grown on media recommended by Senior *et al.*, (1972), Solution A containing glucose, 20.0, MgSO₄·7H₂O, 0.4, CaCl₂, 0.11, FeSO₄·7H₂O, 0.012 and Na₂MoO₄·2H₂O, 0.01(g/l) and Solution B containing K₂HPO₄, 2.0 and NaCl, 0.4 (g/l) were prepared. These two solutions were autoclaved separately and mixed in equal proportions after cooling. For solid medium 2% agar (w/v) was added.

Phosphate solubilizing bacteria, *Bacillus* sp. and *Acinetobacter* sp. were grown on modified Sperber's medium. This medium contained glucose, 10.0 g, yeast extract, 0.5 g, MgSO₄·7H₂O, 0.25 g, North Carolina Rock Phosphate (NCRP), 1.57% (w/v), distilled water, 1000 ml and agar, 20.0 g. *Pseudomonas* sp. was grown on media which contained peptone 20.0 g, K₂HPO₄ 1.5 g, MgSO₄·7H₂O 1.5 g, glycerol 10.00 ml, distilled water, 1000 ml and agar, 20.0 g.

Four grades of polyvinyl pyrrolidone (PVP) such as, PVP K-15, PVP K- 25, PVP K- 30, and PVP K- 90, four grades of polyethylene glycol (PEG), such as PEG 400, PEG 600, PEG 4000 and PEG 6000 and glycerol were used at three different concentrations (0.5 %, 1.0 % and 2.0 %). Media unamended with osmolyte served as standard control. Media were inoculated with a day old culture of bacteria. The incubation time varied with different species of bacteria, 15 hours for *Azotobacter* sp., 48 hours for *Azospirillum* sp., 24 hours for *Bacillus* sp., 12 hours for *Acinetobacter* sp. and 10 hours for *Pseudomonas* sp. The population density of bacteria was determined by SPC. Petri

plates were incubated at 30° C for 48 hours and data on number of colonies were recorded.

III. RESULT AND DISCUSSION

Attempts have been made to develop liquid inoculant formulations of *Rhizobium* sp. and *Bradyrhizobium* sp. by amending media with some polymeric compounds such as polyvinyl pyrrolidone (PVP), polyethylene glycol (PEG), polyvinyl alcohol (PVA), gum arabic, sodium alginate and tapioca flour or with diluents (Tittabutr *et al.*, 2007; Rice *et al.*, 2000 and Somasegaran, 1985). In most of these studies, concentrations selected were based on the population density of bacteria rather than the effectiveness of bacteria. Research on effect of such polymeric additives on population density of PGPB, such as those used in this study is rare. Further, the effect

of polymeric additives, such as PVP and PEG formulations differing in molecular weight has not been assessed. In order to define the optimum concentration and appropriate form of polymeric additives this study was conducted by employing four formulations of PVP and four formulations of PEG depending on molecular weight at three different concentrations on growth of PGPB such as *Azospirillum* sp., *Azotobacter* sp., *Pseudomonas* sp., *Bacillus* sp. and *Acinetobacter* sp.

In general, the population density of *Azotobacter* sp. was 3-4 times higher than control in all PVP formulations (Fig 1). Application of PEG 400 did not influence population density of *Azotobacter* sp. irrespective of its concentration. Further, amending medium with 0.5 % of PEG 600, PEG 6000 and glycerol did not influence population density. Application of glycerol at 2% resulted in highest population density.

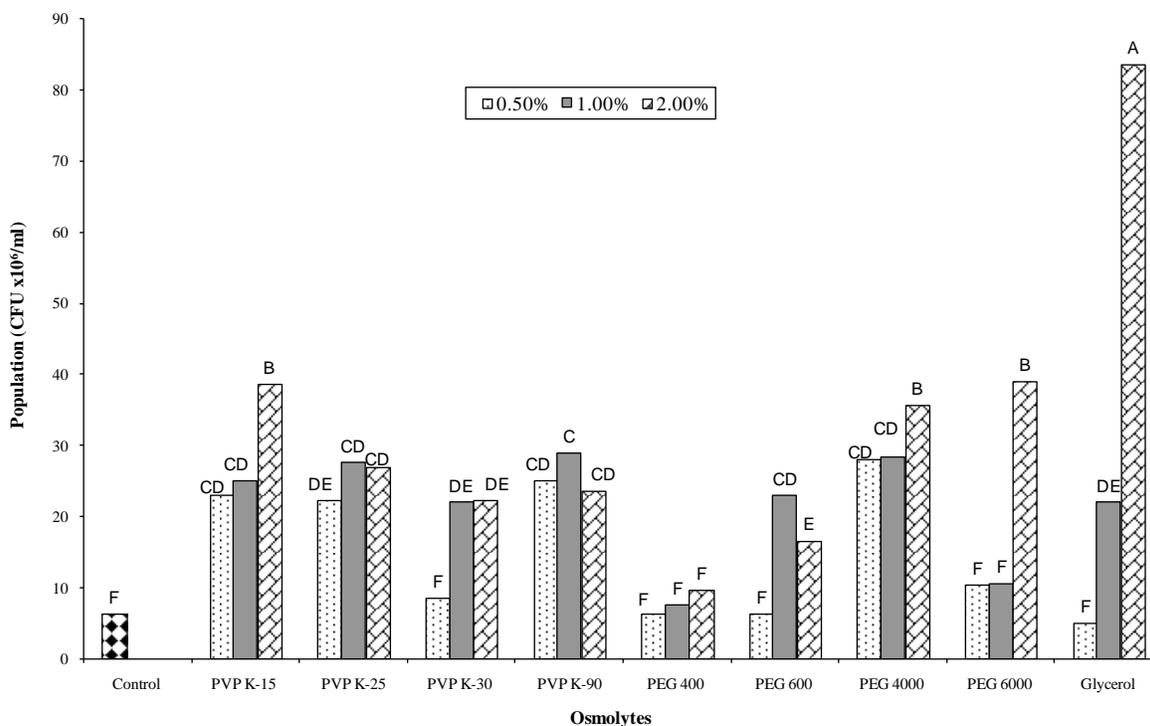


Fig. 1. Population density of *Azotobacter* in N- free minimal medium as influenced by the addition of osmolytes

Means with same letter do not differ significantly at the 5% level of significance

Control-without any osmolytes; PVP- Polyvinyl pyrrolidone; PEG- Polyethylene glycol

In contrast, the population density of *Bacillus* sp. was not influenced by the incorporation of glycerol into the medium (Fig 2). The addition of PVP K-15 caused significant increase in

population density at all concentrations compared to control. Media amended with 2 % PVP K-15 contain the highest number of CFU of *Bacillus* sp. In general, the effect of PVP formulations was more pronounced than that of PEG formulations.

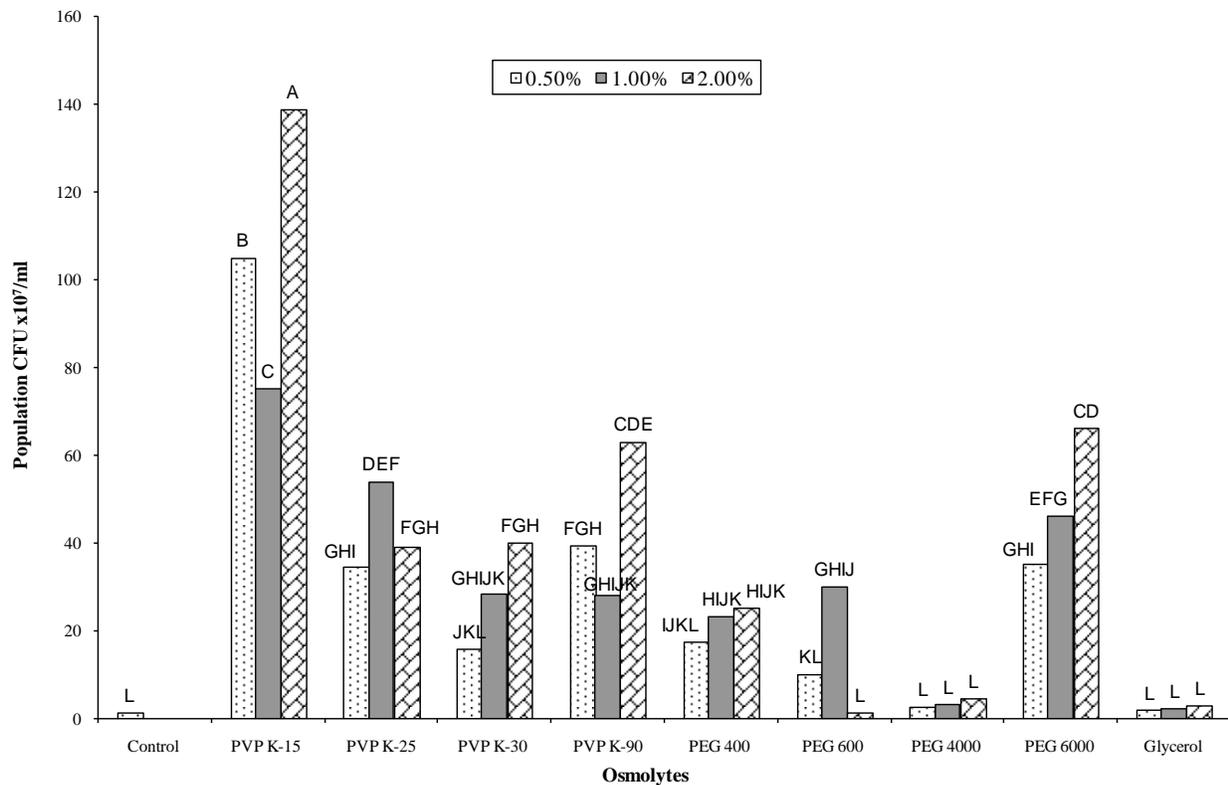


Fig.2 Population density of *Bacillus* in modified Sperber's Medium as influenced by the addition of osmolytes

Means with same letter do not differ significantly at the 5% level of significance

Control-without any osmolytes; PVP- Polyvinyl pyrrolidone; PEG- Polyethylene glycol

the addition of PVP K-25, PVP K-30, PEG 6000 and glycerol at the 2% level and by the application of PVP K-30, PEG 400 and PEG 600 at the 1 % level.

Data on changes in population density of *Azospirillum* sp. as influenced by the incorporation of osmolytes in N free malate medium are presented in **Fig 3**. The addition of osmolytes, PVP K-25, PVP K-30, PVP K-90 and PEG 4000 caused a significant reduction in population density when added at the 0.5 % level. The population density increased nearly 4 times than control by

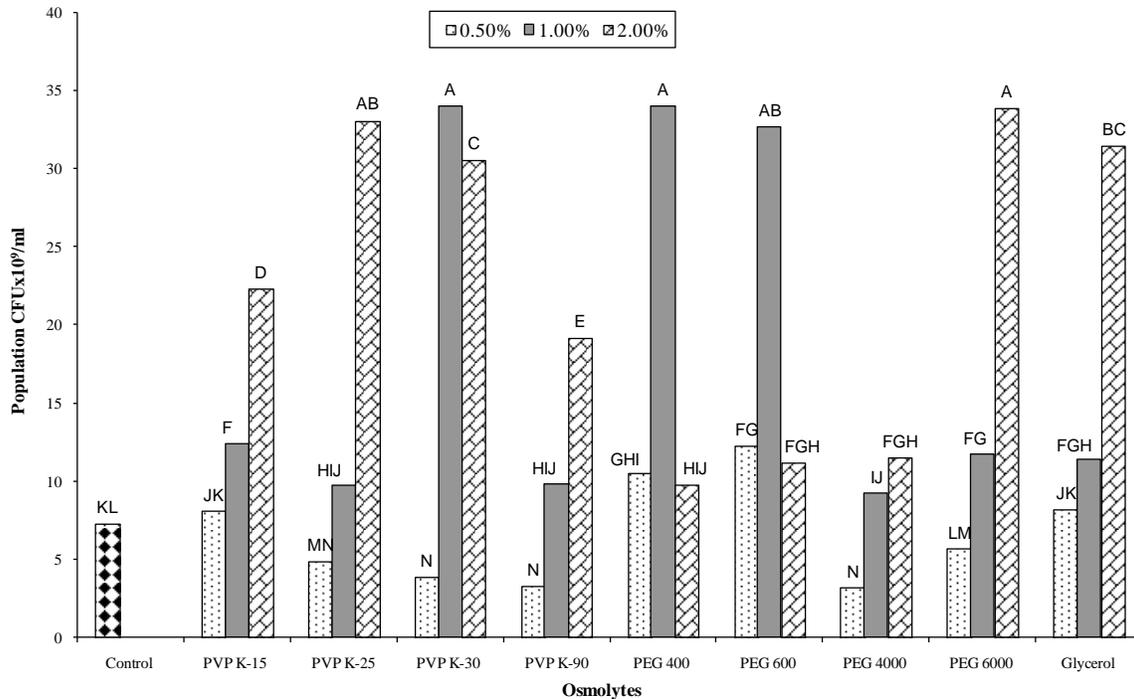


Fig. 3. Population density of *Azospirillum* in N-free malate medium as influenced by the addition of osmolytes

Means with same letter do not differ significantly at the 5% level of significance

Control-without any osmolytes; PVP- Polyvinyl pyrrolidone; PEG- Polyethylene glycol

The population density of *Pseudomonas* sp. significantly increased by the addition of PVP K-15 at all concentrations and the extent of increase was almost seven times by the addition of PVP K-15 at the 2 % level (Fig 4). The addition of PVP K-25, PVP K-30, PEG 400 and glycerol at the 2% level also caused an increase in population density but the magnitude of increase was lower than that was noticed with PVP K-15. The amendment of media with all other formulations, in general, caused marked reduction in population density compared to unamended control.

Among PGPB used in this study, the response of *Acinetobacter* sp. to the addition of osmolytes was unique (Fig 5). In general, PVP formulations did not influence the population density of *Acinetobacter* sp. at all concentrations. The addition of PEG 4000 at the 2 % level increased the population density of *Acinetobacter* sp. significantly to the extent of 9 times compared to control. Further, amending media with PEG 400 at all concentrations, PEG 600 and PEG 6000 at the 2 % level and glycerol at the 0.5 % level increased population density substantially.

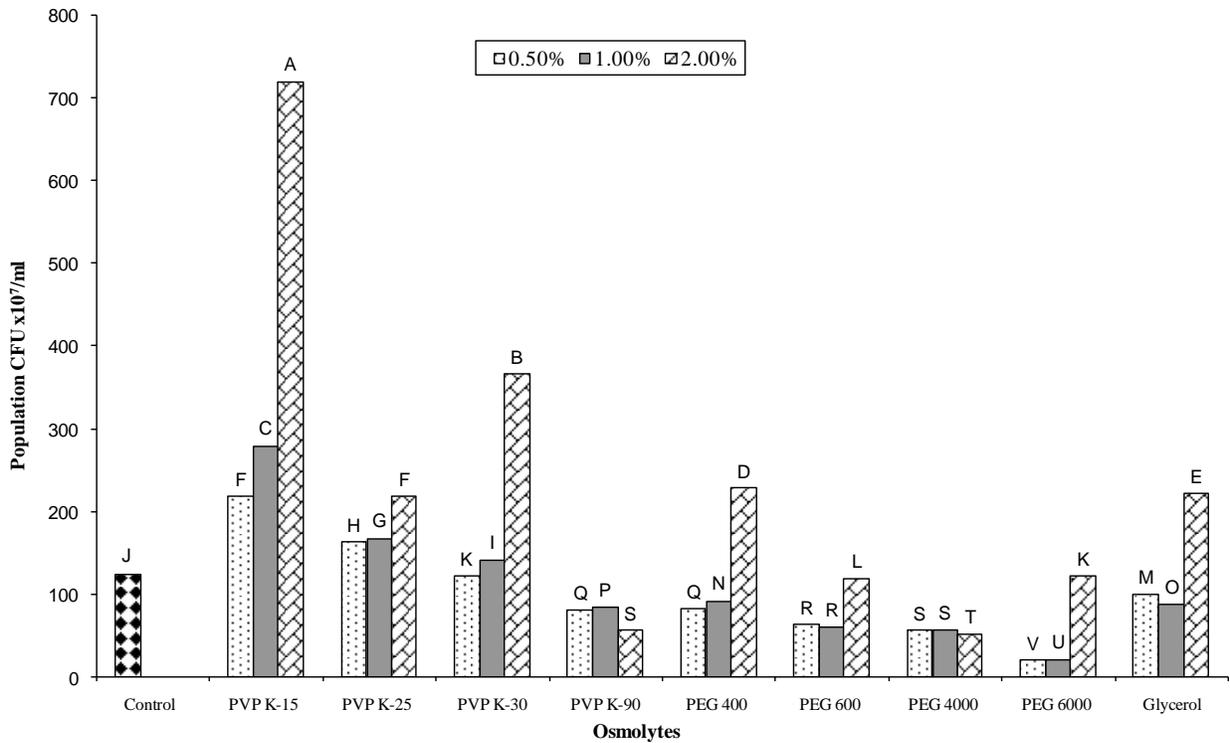


Fig. 4. Population density of *Pseudomonas* in King's B medium as influenced by the addition of osmolytes

Means with same letter do not differ significantly at the 5% level of significance
 Control-without any osmolytes; PVP- Polyvinyl pyrrolidone; PEG- Polyethylene glycol

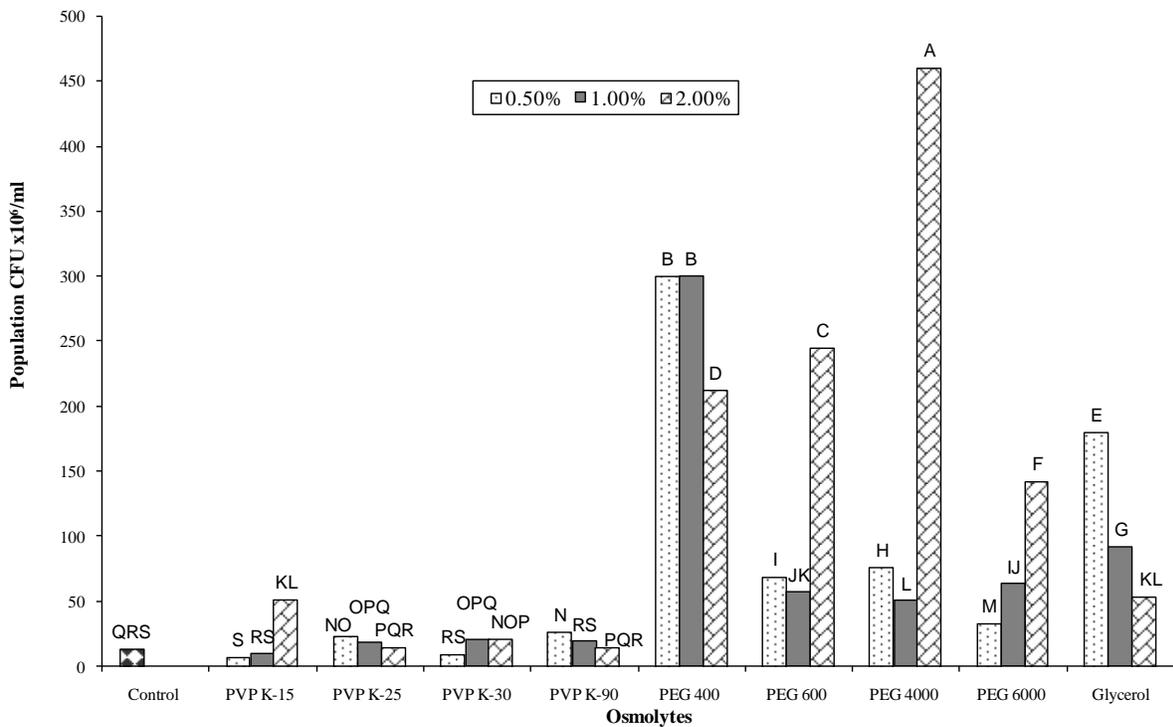


Fig 5. Population density of *Acinetobacter* in modified Sperber's medium as influenced by the addition of osmolytes

Means with same letter do not differ significantly at the 5% level of significance
 Control-without any osmolytes; PVP- Polyvinyl pyrrolidone; PEG- Polyethylene glycol

Although the mechanism of action is not clear, PVP amendment maintained higher population density in the medium and to permit the survival of higher number of bacteria per seed (Tittabutr *et al.*, 2007 and Singleton *et al.*, 2002). These formulations, PVP and PEG cannot be used as either carbon source or energy source by the PGPB, hence it can be concluded that other properties of these additives are responsible for maintaining higher population density in the respective media, particularly with *Azotobacter* sp., *Bacillus* sp. and *Azospirillum* sp. Both these polymeric additives are soluble in water and in other polar solvents. Further, they have the capacity to bind polar and hydrophobic molecules, function as complexing agents reducing the toxicity of compounds and could be used to create high osmotic potential in liquids (Errington, *et al.*, 2002; McAneney *et al.*, 1982 and Coiffer *et al.*, 2001). It is possible that these compounds influence population density level of these three PGPB, either by one of these or by a combination of these mechanisms.

The positive response pattern of two gram-negative proteobacterial members, *Pseudomonas* sp. to application of PVP and that of *Acinetobacter* sp. to PEG, particularly to low molecular weight of these formulations is interesting as well as intriguing. However, the findings of Mugnier and Jung (1985) on the effect of solutes at different water activity levels on population density of gram negative bacteria suggests that the number of viable cells was positively correlated with molecular weight of polyalcohols. Their research results also suggest that viability could also be related to stearic and structural properties of these solutes. The only important variation between this study and that of Mugnier and Jung (1985) is the solutes used by them could be used by organism as a source of carbon and energy but PVP and PEG used in this study cannot be used by these bacteria as source of carbon and energy. Hence, it is possible that the effect of PVP / PEG is modulated by altering water activity of media, more favourably when the formulation of low molecular weight were present compared to formulation of higher molecular weight. These compounds may also be influencing the movement of solutes in medium as well as across the cell membrane.

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