

Effect of Different Concentrations of Glycerol on survival of *Azotobacter chroococcum* Local strains isolated from Wild Grasses

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ABSTRACT

The plant growth promoting rhizobacterias inoculants used in various formulations for different purpose. In addition to rhizobacterias, the formulation may also contain various additives. Furthermore, it is important to understand the interaction between bacteria and formulation materials. The formulation materials serve as cell protectants which enhance the shelf-life of bacteria. It is proved that the Rhizobium is the effective inoculants along with specific nutrient media for survival. Many researchers have shown that liquid rhizobial formulations are more beneficial than solid bio-fertilizer formulations. In the present work the survival of *Azotobacter* in liquid formulations were evaluated by using Glycerol at different concentration in Jenson's broth. It was noted that survival of *Azotobacter* was concentration correlated. Lowest number of colonies in 5 mM in the medium containing glycerol (23 colonies at zero days and 5 colonies on 360th day) and highest number of colonies in 25 mM in the medium containing glycerol (38.66 colonies on zero day and 22 colonies on 360th day) (109 CFU/ml).

Keywords: Glycerol, Survival, *Azotobacter chroococcum*.

Introduction

Azotobacter bio-fertilizer can be used in the form of liquid or solid formulations for crop improvement and growth. The Indian government and private sectors have been trying to increase the production and application of bio fertilizers along with modern agrochemicals (Mohd Mazid and Taqi Ahmed Khan 2014). Liquid bio-fertilizers are special liquid formulations containing not only the desired microorganisms and their nutrients but also special cell protectants or chemicals that promote formation of resting spores or cysts for longer shelf life and tolerance to adverse conditions (Ghosh, 2004). A particular efficient strain of the N-fixing/P-solublizing microorganism selection for bio-fertilizer is a big task (Gandhi and Saravanakumar 2009). It was revealed that *Azotobacter* could be one of the bio-fertilizer options for sustainable and environmental ecofriendly for maize production where chemical fertilizer is limited (Baral and Adhikari, 2013). In India, the bio-fertilizers are mostly lignite, coal, peat based. The microbial inoculants are prepared with the above carried based. Generally it called solid formulations. But this has many disadvantages such as shorter shelf life, poor quality, high contamination and poor performance. In addition to this the carrier based inoculants production is tedious, energy consuming activity. It involves milling, sieving and correcting pH (Somasengaran and hoben 1994). The liquid inoculants formulation is one solution to the problem associated within processing of solid carriers. The use of various broths cultures amended with substances that promote the cell survival in the package and after applications for seed or soil. Additives to liquid inoculants formulations should have a role in protecting *Azotobacter* cells on seeds at high temperature and during desiccation. Many kinds of polymer have been used for inoculants production because of their ability to limit heat transfer, their good rheological properties and high water activities (Mungier and Jung 1985).

In the present work the survival of *Azotobacter* in liquid formulations were evaluated by using Glycerol at different concentration. *Azotobacter* cells in Jenson's broth alone were used as control. The glycerol at different

concentration was tried in formulation to understand the survival life of *Azotobacter* and interaction between *Azotobacter* and formulation materials.

Material and Methods

Jensen's Basal Medium: The N₂ free Jensen's medium (Jensen 1954) containing 20 gm sucrose, 1.0 gm K₂HPO₄, 0.5 gm MgSO₄.7H₂O, 0.5 gm NaCl, 2 gm CaCO₃, 0.005 gm Na₂MoO₄, and 0.1 gm FeSO₄ dissolved in 1 lit Distilled water and pH was adjusted to 7.1

Liquid State Formulation with polymer cell protectants: For the standardization of proper quantity of amendments and to find out the survival time of *Azotobacter chroococcum* the polymer cell protectants were added with Jensen's basal medium. These various formulation were as follows

(1) Basal medium (Control).

(2) Basal medium + Glycerol with 5, 10, 15, 20, 25 mM.

The slant culture of *Azotobacter* along with above formulation was incubated for 4 days on shaker at 121 rpm. The optical densities 0.25 were adjusted at 0.25 with the help of spectrophotometer. This 10 ml culture of *Azotobacter chroococcum* was inoculated in each flask of above 5 types of liquid formulation under aseptic condition. The broths were kept for continuous shaking on rotary shaker for 72 hours at 121 rpm, and then the cultures were transferred in sterilized glass bottle, plugged with cotton and stored at room temperature. These broth cultures were tested for total viable count at 30 days interval up to 12 months.

(3) Basal Medium + Glycerol 25 mM only- This formulation was prepared to compare the growth and survival of *Azotobacter chroococcum* A1 *Azotobacter chroococcum* A2, *Azotobacter chroococcum* NCIM 5576 isolates. These isolates were inoculated (10 ml of culture) separately in each flask under aseptic condition. The broths were incubated on rotary shaker for 72 hours at 121 rpm, and then cultures were transferred in sterilized glass bottle, plugged and stored at 30⁰C temperature. These broth cultures were tested for total viable count at 30 days interval up to 12 months.

Table1. Effect of Glycerol (CFU/ml at 10⁹) in Jensen's broth on survival of *Azotobacter chroococcum* A1 colonies

| Days | Broth Alone (control) | Glycerol 5 mM | Glycerol 10 mM | Glycerol 15 mM | Glycerol 20 mM | Glycerol 25 mM | CD | at 0.05% |
|------|-----------------------|------------------------|--------------------------|-------------------------|-------------------------|--------------------------|------|----------|
| 0 | 22.66a±0.51 | 23.00a±0.58 (1.50) | 30.00b±0.58 (32.39) | 31.00c ±0.58 (36.80) | 34.66d±0.51 (50.04) | 38.66e±0.51 (70.61) | 0.9 | 4.20E-10 |
| 30 | 20.33a±0.58 | 23.00a±0.58 (13.13) | 28.00c ±0.58 (37.73) | 29.00d±0.58 (42.65) | 34.33e±0.69 (68.86) | 38.33f±0.69 (88.54) | 0.47 | 8.66E-10 |
| 60 | 16.66a±0.38 | 21.00b±0.58 (26.05) | 26.00c±0.58 (56.06) | 28.00d±0.58 (68.07) | 32.00c±0.58 (92.08) | 37.00f± 0.58 (122.09) | 0.42 | 7.84E-11 |
| 90 | 14.00a±0.58 | 20.00b±0.58 (42.86) | 24.00c±0.58 (71.43) | 25.00d±0.58 (78.57) | 30.00e±0.58 (114.29) | 35.00f±0.32 (150.00) | 0.44 | 1.35E-10 |
| 120 | 10.00a±0.58 | 17.00b±0.58 (70.00) | 22.00c ±0.58 (120.00) | 22.00c±0.58 (120.00) | 28.00d±0.58 (180.00) | 33.66e±0.32 (236.60) | 0.42 | 1.71E-11 |

| | | | | | | | | |
|-----|------------|--------------------------|--------------------------|-------------------------|-------------------------|-------------------------|------|----------|
| 150 | 8.00a±0.58 | 16.00b±0.58 (100.00) | 20.00c ±0.58 (150.00) | 21.00d±0.58 (162.50) | 25.00e±0.58 (212.50) | 33.33f±0.51 (316.63) | 0.43 | 2.00E-11 |
| 180 | 5.33a±0.31 | 14.00b± 0.58 (162.66) | 20.00c ±0.58 (275.23) | 21.66d±0.50 (306.38) | 25.00e±0.58 (369.04) | 32.00f±0.58 (500.38) | 0.4 | 3.52E-12 |

Data presented are means of ten readings; values within the same row with different letters are significantly different at 0.05% P-level by Single factor ANOVA test followed by CD & Tukey's test. [Figures in parentheses indicate % increase (+) and % decrease (-) over control; ± standard error of mean; CD:critical difference; P-value/alpha value at 0.05%; CFU/ml At 10⁹: Colony forming unit per gram per ml of Glycerol plus Jensen's broth).

Table 2. Effect of Glycerol (CFU/ml at 10⁹) in Jensen's broth on survival of *Azotobacter chroococcum* A1 colonies

| Days | Broth Alone (control) | Glycerol 5 mM | Glycerol 10 mM | Glycerol 15 mM | Glycerol 20 mM | Glycerol 25 mM | CD | at 0.05% |
|------|-----------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|------|----------|
| 210 | 4.00a±0.58 | 12.00b±0.58 (200.00) | 17.00c±0.58 (325.00) | 21.33d±0.62 (433.25) | 23.66e ±0.61 (491.50) | 30.00f±0.58 (650.00) | 0.45 | 1.30E-11 |
| 240 | 3.00a±0.58 | 10.66b± 0.20 (255.33) | 16.00c±0.58 (433.33) | 20.00d±0.58 (566.67) | 23.33e±0.56 (677.67) | 28.00f±0.58 (833.33) | 0.84 | 4.45E-12 |
| 270 | 1.33a±0.07 | 10.33b± 0.46 (676.69) | 15.00c±0.58 (1027.82) | 18.00d±0.58 (1253.38) | 22.00e±0.58 (1554.14) | 27.00f±0.58 (1930.08) | 0.39 | 2.61E-12 |
| 300 | 0.00a±0.00 | 9.00b±0.58 | 13.00c±0.58 | 17.00d±0.58 | 20.00e±0.58 | 25.00f±0.58 | 0.4 | 5.54E-12 |
| 330 | 0.00a±0.00 | 7.00b±0.58 | 10.00c±0.58 | 15.00d±0.58 | 18.00e±0.58 | 23.00f±0.58 | 0.4 | 1.24E-11 |
| 360 | 0.00a±0.00 | 5.00b±0.58 | 9.00c±0.58 | 14.00d±0.58 | 17.00e±0.58 | 22.00f±0.58 | 0.4 | 1.53E-11 |

Data presented are means of ten readings; values within the same row with different letters are significantly different at 0.05% P-level by Single factor ANOVA test followed by CD & Tukey's test. [Figures in parentheses indicate % increase (+) and % decrease (-) over control; ± standard error of mean; CD: critical difference; P-value/alpha value at 0.05%; where CFU/ml AT 10⁹: Colony forming unit per gram per ml of Glycerol plus Jensen's broth).

Table 3. Effect of Glycerol in Jensen's broth on survival of A1,A2 and NCIM 5576 strains of *Azotobacter chroococcum* during Storage, at 10⁹ CFU/ml

| Name of the bacterium | Survival of colonies (average number) | Days |
|------------------------------------|--|----------|
| <i>Azotobacter chroococcum</i> A1 | 40.69a±2.90 | 0 to 360 |
| <i>Azotobacter chroococcum</i> A2 | 46.85b±2.85 | 1 to 360 |
| <i>Azotobacterchroococcum</i> NCIM | 33.00c±1.18 | 2 to 360 |
| CD at 0.05% | 3.1 | |
| P-value at 0.05% | 0.001 | |

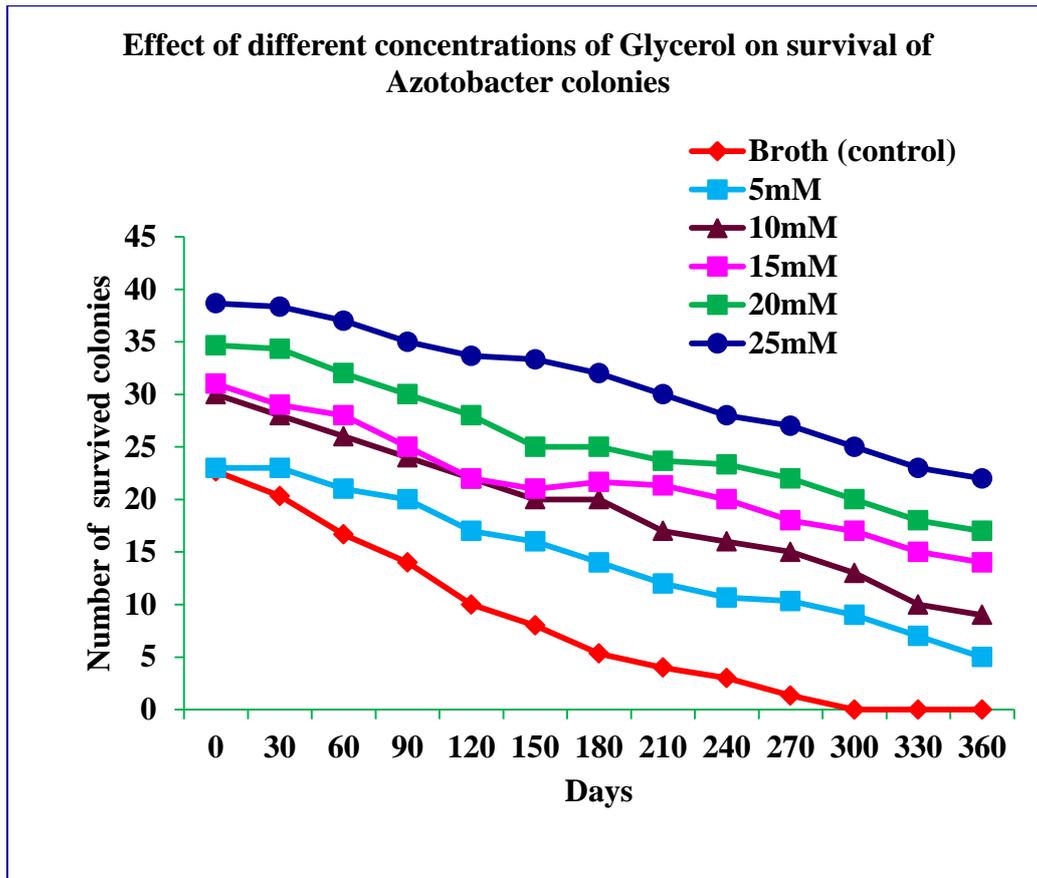


Fig.1. Effect of different concentrations (5 mM to 25 mM) of Glycerol in Jensen's broth on survival of *Azotobacter chroococcum* colonies

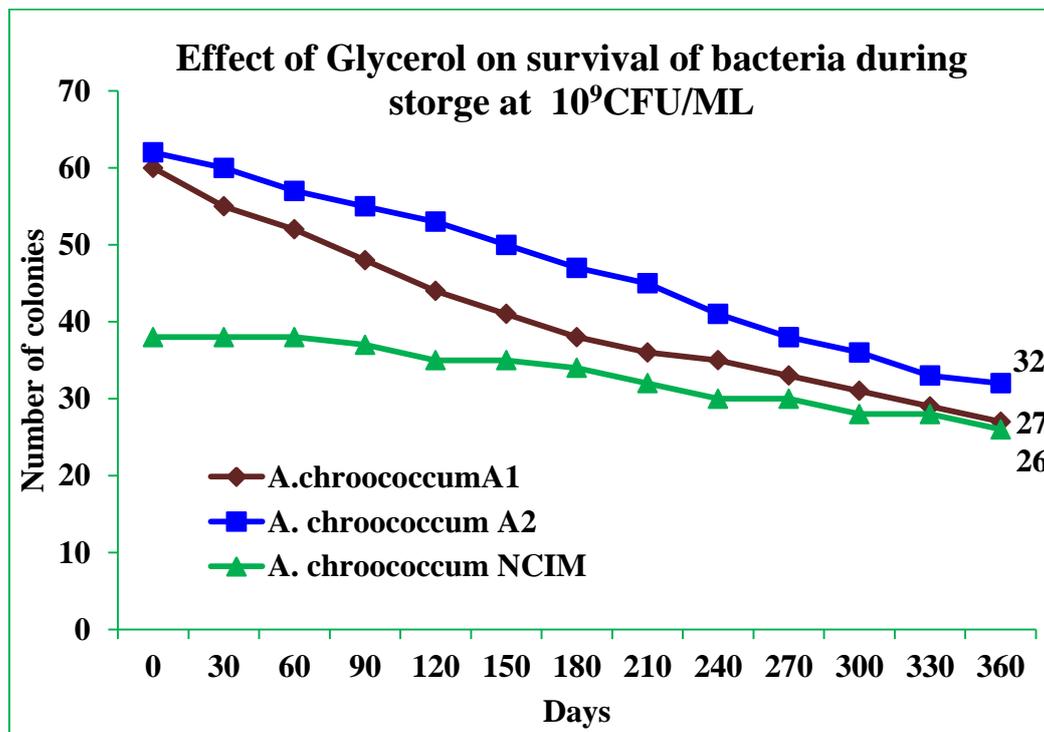


Fig.2. Effect of Glycerol in Jensen's broth on survival of A1, A2 and NCIM 5576 strains of *Azotobacter chroococcum* during Storage, at 10⁹ CFU/ml

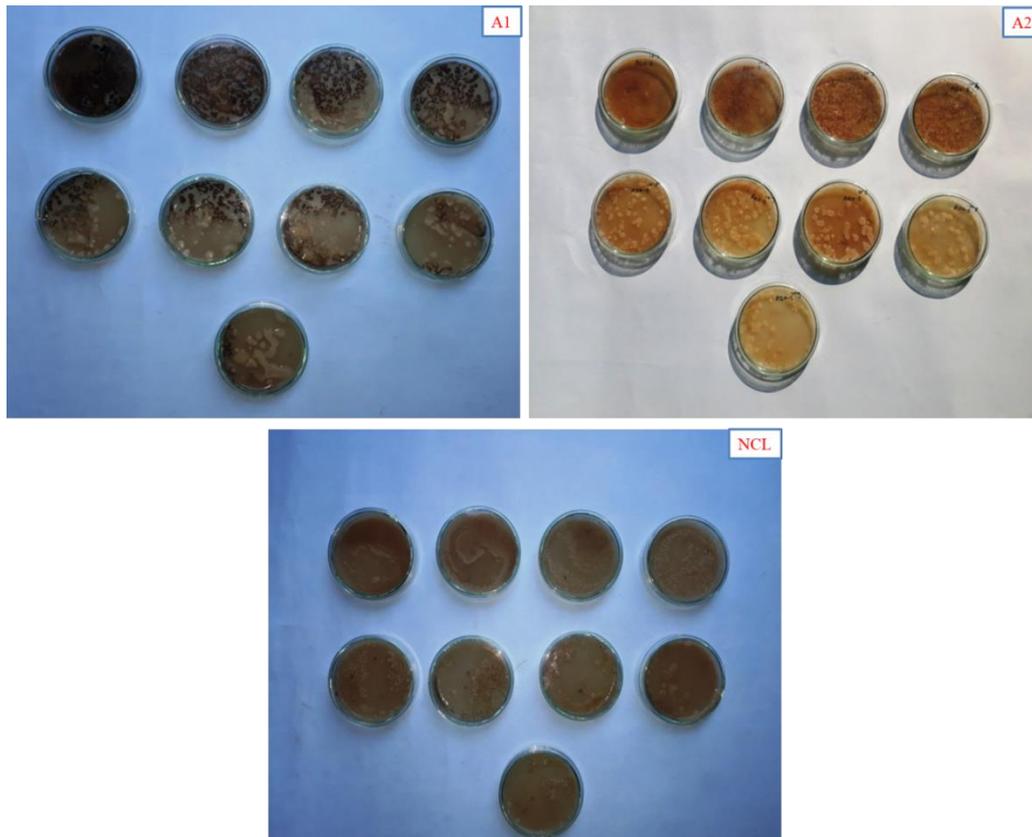


Photo plate 1. Effect of Glycerol in Jensen's broth on survival of A1, A2 and NCIM 5576 strains of *Azotobacter chroococcum* during Storage, at 10^9 CFU/ml

Result and Discussion

The experimental work was carried out for the survival of *Azotobacter* in liquid and solid formulations. Many researchers have shown that liquid rhizobial formulations are more beneficial. Hynes et al (1995, 2001) revealed that the results of liquid rhizobial formulations for the growth and yield of crops are more suitable than that of peat-based products. Maniknandan et al (2010) worked on standardization of liquid formulation of *Pseudomonas fluorescens* by using , trehalose, polyvinylpyrrolidone and glycerol for development of liquid formulation. Among these, glycerol amendment maintained the greater population level of *P. fluorescens* Pf1 up to 6 months of storage. Cortes-Patino Sandra and Bonilla Ruth Rebeca (2015) worked on Polymers selection for a liquid inoculant of *Azospirillum brasilense* based on the Arrhenius thermodynamic model they used a method of accelerated degradation to select a polymer and a concentration to maintain cell stability of a liquid inoculant based on the strain C16 *Azospirillum brasilense*. A screening at 45°C was made to compare the protectant effect of five polymers on the viability of the strain (p/v): carrageenan (1.5%), sodium alginate (1%), trehalose (10 mM), polyvinylpyrrolidone (2%), glycerol (10 mM) and phosphate saline buffer as control. conclude that these polymers can be used for a formulation of a liquid inoculant based on the strain C16 *Azospirillum brasilense*.

It was evaluated by using polymers like polyvinyl pyrrolidone (PVP), Glycerol, trehalose, separately and PVP, Glycerol, trehalose together. Simultaneously the survival was also evaluated by using different carbon sources along with Jensen's basal medium. The results are depicted in following tables. Data was tabulated and analyzed

statistically, presented in graphical form also. *Azotobacter* cells in Jensen's broth alone were used as control for liquid formulation and lignite was used for solid formulation. The different additives were tried in formulation to understand the survival life of *Azotobacter* and interaction between *Azotobacter* and formulation materials.

(1) Effect of glycerol on survival of *Azotobacterchroococcum* A1 colonies grown in Jensen's medium (Table No 15, 16, Figure No 19): Glycerol is a sweet-tasting, non-toxic substance that contain a propane molecule attached to three -Oh groups. Different concentrations viz. 10 mM to 25 mM were prepared and added to the culture of *Azotobacter chroococcum* grown in Jensen's medium. Jensen's broth/medium without addition of glycerol was treated as control. Effect of all concentrations on survival of *Azotobacter* colonies was significant at 0.05% probability level. On zero days highest number of colonies was recorded in medium containing 25 mM Trehalose followed by 20 mM, 15 mM, 10 mM and 5 mM in the medium containing glycerol. Survival of the *Azotobacter* was maintained up to 360 days. It was noted that survival of *Azotobacter* was concentration correlated. Lowest number of colonies in 5mM in the medium containing glycerol (23 colonies at zero days and 5 colonies on 360th day) and highest number of colonies in 25mM in the medium containing glycerol (38.66 colonies on zero day and 22 colonies on 360th day) (10^9 CFU/ml). Santhosh (2015) recorded similar results. Colonies (22.66 on zero day, 1.33 colonies on 270th day) in pure broth could be maintained. Effect of glycerol on survival of *Azotobacterchroococcum* colonies grown in Jensen's medium has shown better result of colony count (22 colonies on 360th day) over control at room temperature.

Stock culture of *Azotobacter* (SR-4) was maintained by Misbahud Dineet al (2019) on nutrient agar slants and glycerol cultures in nutrient broth and was stored at -80°C . Rojas-Tapias et al (2013) stated that use of preservation techniques is usually accompanied with use of protective agents, which can increase effectiveness of the technique preventing cell damage. For this reason, selection of the protective agent like glycerol depends on the preservation method and type of bacteria.

As per the previous records, our efforts for survival of *Azotobacter* are successful and economical by using Glycerol as preservative. Lorda and Balatti (1996) recorded that glycerol (10 mM) showed better population survival of *Azotobacter* next to trehalose may which may be due to high water holding capacity and may protect cell from the effect if desiccation by reducing rate of drying.

(2) The result indicate that, maximum population of *Azotobacter* was recorded in, glycerol 25 mM 22×10^9 cfu/ml, followed by pvp 3% 16×10^9 cfu/ml, trehalose (25 Mm) 14×10^9 cfu/ml, combine use of trehalose, and glycerol 6×10^7 cfu/ml where as minimum population was recorded in control(Jensen broth only) 4×10^4 cfu/ml during storage of 12 months.

Santhosh (2015) formulated *Rhizobium*, *Azotobacter*, *Azospirillum*, and *Bacillus megaterium* and stored these formulations in BOD incubator for 180 days and total viable count was analyzed at 30 days interval he recorded 0.5% polyvinylpyrrolidone in addition with 0.5% glycerol recorded maximum cell count in all cultures followed by polyethelene glycerol, gum Arabic and sodium alginate. And also reported that, the survival of bacteria in liquid formulation was increased due to the improvement in biological integrity, this was because polyvinyl pyrrolidone which helps to reduce extra protein coagulation of bacterial cell and macromolecular structure maintenance.

Polyvinyl pyrrolidone helps to maintain moisture level in medium (Singleton *et al* 2002; Deaker *et al* 2004). Also Polyvinyl pyrrolidone binds with bacterial toxins released in medium, during stationary phase of bacteria (Errington *et al* (2002), Deaker *et al* (2004), C.F. Dayamani and Brahmaaprakash (2014).

Conclusion

It was concluded that survival of *Azotobacter* along with different concentrations of glycerol was concentration correlated. Lowest number of colonies in 5 mM in the medium containing glycerol (23 colonies at zero days and 5 colonies on 360th day) and highest number of colonies in 25 mM in the medium containing glycerol (38.66 colonies on zero day and 22 colonies on 360th day) at (10^9 CFU/ml) was recorded.

Declarations

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This research did not receive any grant from funding agencies in the public, commercial, or not-for-profit sectors.

Competing Interests Statement

The authors declare no competing financial, professional and personal interests.

Consent for publication

Authors declare that they consented for the publication of this research work.

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