***Original Research Article***

# Evaluation of suitable polymers for the development of high-concentrated liquid biofertilizers

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## ABSTRACT

An improvement in the present liquid formulation of biofertilizer is essential to expand its shelf life and enhance the bioefficacy potentials of the inoculated crops. Here, we screened ten different water-soluble polymers for their feasibility as cell protectants in liquid biofertilizers. The physio-chemical properties and *Escherichia coli* survival assay experiments identified five potential polymers: hydroxypropyl methylcellulose, polyvinyl alcohol, and natural polymers extracted from seaweed, red algae, and brown algae. These five polymers' aqueous solutions cope with the standard parameters of liquid biofertilizers. Further, these polymers were compared with standard liquid biofertilizer diluting medium (phosphate buffer with glycerol) for the shelf life of two biofertilizer strains viz., *Azospirillum lipoferum* (Az204) and *Bacillus megaterium* var. *phosphaticum* (Pb1). All the polymers had high cell viability up to 60 days on incubation. In conclusion, the results suggest that these polymers could be effective encapsulating agents to improve the quality of liquid biofertilizers.



*Keywords: Biofertilizer; Liquid formulation; Polymers; Shelf life*


## 1. INTRODUCTION

Biofertilizers (microbial inoculants) are commercial formulations containing specific strain(s) of microorganisms that supply macro- and micronutrients for plant growth upon inoculation through seed, seedling, or soil. These strains colonize the rhizosphere or endosphere of the inoculated plant and enhance nutrient acquisition through various mechanisms [1]. Using such microbial inoculants in sustainable agriculture has been shown to reduce the inorganic fertilizer inputs (nearly 25%) by helping to explore natural resources [2]. Biofertilizers also improve the nutrient use efficiency of various inorganic fertilizers under integrated nutrient management, the quality of the produce, and soil health [3]. The global market for biofertilizers is growing at 12% per annum, had a value of $2.02 billion in 2022, and is expected to reach about $5.02 billion by 2030 [4].

The success of biofertilizer technology depends on two components: the strain used for the commercial preparation and the formulation. The strain should be efficient, competitive colonization, and persistent in the inoculated host plant, and the formulation should hold the strain viable without contaminants until inoculation [5]. The liquid formulation of biofertilizers replaced carrier-based biofertilizers in the past ten years due to the advantages such as higher cell load, longer shelf-life, and zero contaminants [6-9]. The liquid formulation contains amendments that provide low concentrations of nutrients along with cell protectants for maintaining the cell viability of strains until its usage. The minimal medium containing $KH_2PO_4$, $Na_2HPO_4$, $MgSO_4$, NaCl, and $NH_4Cl$ or the phosphate buffer (0.1 M, pH 7.5) is commercially used in liquid biofertilizers to provide nutrients at low concentrations for cell viability. Glycerol, trehalose, and polyvinyl pyrrolidone are the common cell protectants in liquid formulations [10]. Though this formulation is superior to

carrier-based formulations, many disadvantages or issues exist. The major problems are high volume packaging (500 or 1000 ml) of biofertilizers; limitation in increasing the cell load beyond $10^9$ cells per ml; packaging in plastic or polypropylene bottles and their sterilization issues; bloating of the bottles during storage; pH drop of the inoculant; issues with storage, transport, and handling due to large volume. Hence, an improvement in liquid biofertilizers is needed to enhance the biofertilizer-driven benefits in sustainable agriculture.

Before the development of liquid biofertilizers, polymer-entrapped biofertilizer formulation was considered a potential alternative for carrier-based inoculants [11]. Alginate-immobilization was the best encapsulation method that holds the biofertilizer strains with high shelf life, protects the cells from adverse environments, effectively releases the cells in the rhizosphere's vicinity, and is slowly degradable in the soil [12-16]. Due to its high cost, handling issues, and complicated processing during gel entrapment, no polymer-entrapped biofertilizer has been commercialized. Nevertheless, recent developments in the chemistry and processing of polymers allow us to re-think polymer-based commercial formulation of biofertilizers as a better improvement in liquid formulations than the present aqueous products. Several water-soluble synthetic and natural polymers are presently available in the market for various applications [17]. In the present work, an attempt was made to assess the feasibility of these polymers as alternative aqueous solutions to diluent and cell-protectant combinations in liquid formulation. The basic assumption in the present work was that when the polymer is in solution form, it can hold highly concentrated microbial cells viable for a long time, protect them from unfavorable conditions, and increase the volume during field applications. Besides, the polymer-based liquid biofertilizer does not need bottles, as it can easily be packed in spout pouches cheaply. Hence, the current investigation assessed five synthetic and five natural polymers for their physical and chemical properties and suitability for high-concentrated liquid biofertilizers.

## 2. MATERIAL AND METHODS

### 2.1 Polymers

The synthetic polymers such as hydroxypropyl methylcellulose (HPMC), maltodextrin (MD), polyvinyl alcohol (PVA), carboxy methylcellulose (CMC), dextrin white (DW), and natural polymers like guar gum (GGA), bio gel (BG, extracted from seaweed), red algal gel (RAG, extracted from red algae), brown algal gel (BAG, extracted from brown algae), and chitosan (CHI) were used in the present study. All these polymers were solubilized in deionized water as a 1% solution and analyzed. Warm water was used for CMC, MD, and GGA to enhance the solubility. The standard liquid biofertilizer suspension buffer (100 mM phosphate buffer, pH 7.0 with 2% glycerol) was compared with these polymers.

### 2.2 Physio-chemical properties

The pH and electrical conductivity of polymer solutions were measured using pH and EC meter, respectively. The viscosity of polymer solutions was measured by Brookfield spindle viscometer and expressed as centistokes (cSt). A volume of 10 ml of the polymer was transferred to a known-weighed test tube, dried at 80°C overnight, and measured the dry matter content of the polymer.

### 2.3 *Escherichia coli* survival assay

*Escherichia coli* (strain DH5α) harboring pGreenTIR plasmid was used for this study. This strain holds ampicillin resistance (100 µg/ml) and produces colonies with fluorescence under a trans-illuminator. The strain was grown overnight in LB broth with ampicillin (100 µg/ml),

harvested the cells by centrifugation (5000 rpm for five min. at room temperature), and resuspended in phosphate buffer (pH 7.5). A volume of 1 ml of concentrated cells was mixed with each polymer solution and incubated for 24 hours. The population of *E. coli* in each polymer solution was measured using serial dilution and plating in LB + ampicillin plates. The number of fluorescent colonies was counted and expressed as cfu per ml.

### 2.4 Shelf-life of polymer-based biofertilizers

The six selected polymers (HPMC, PVA, BIOP, BAG, RAG) were used to prepare high-concentrated biofertilizers. *Azospirillum lipoferum* (Az204) (N fixer) and *Bacillus megaterium* (Pb1) (Phosphobacteria) being maintained at the Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore were used for this experiment. *Azospirillum* in nitrogen-free malic acid medium and Phosphobacteria in hydroxy apatite medium were grown at 30°C until the cells reached their late log phase. The cells were harvested by centrifugation (5000 rpm for five min. at room temperature), washed twice with phosphate buffer (0.1 M, pH 7.5), and resuspended the same. The population of *Azospirillum* and phosphobacteria was counted by plate count method, and the cell suspension was inoculated with the respective polymer solution to give $10^{15}$ cells per ml. The polymer-based biofertilizer preparations were transferred to sterile containers (HiMedia, India) and stored at room temperature. The population of *Azospirillum* and phosphobacteria were measured at monthly intervals.

### 2.5 Statistical analysis

All the data were statistically scrutinized in R software (Version 4.1.1) (R Core Team, Vienna, Austria). The analysis of variance was performed for assessed traits, and Tukey's honestly significant difference test (Tukey's test) at p = 0.01 was performed to reveal the statistical differences among the treatments. Principal component analysis (PCA) was performed for the polymer traits using the princomp function of the factoextra package of R. Pearson's correlation was conducted among the physical, chemical, and E. coli viability parameters among the polymers and visualized through ggcorrplot package of R software.

## 3. RESULTS

Polymer solutions made from brown algal gel and standard phosphate buffer with glycerol recorded near neutral pH (7.00 – 7.08), followed by CMC (6.42) and HPMC (6.42). BIOG and RAG had slightly acidic pH, 5.60 and 5.59, respectively, while the other polymer solutions had strong acidic pH. Chitosan and dextrin white accounted for a pH of 2.80 (Fig. 1A). Polymer solutions made from red algal gel, chitosan, biogel, CMC, and brown algal gel accounted for high electrical conductivity (> 1 mS/cm), while the rest of the polymer solutions had low EC (0.1 to 0.01). The standard liquid biofertilizer suspension buffer (PBG) and maltodextrin had the lowest EC of 0.035 and 0.020, respectively (Fig. 1B).

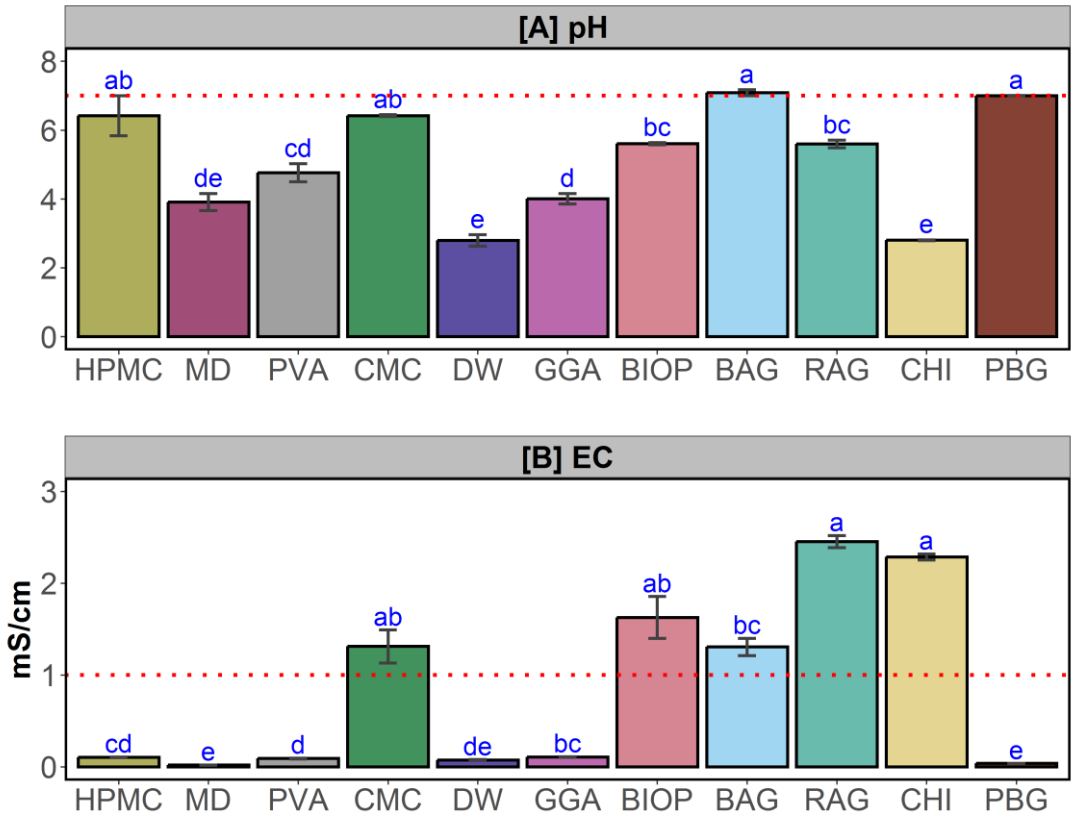


**Fig. 1. pH and electrical conductivity of polymer solutions.** HPMC – hydroxypropyl methylcellulose; MD – maltodextrin; PVA – polyvinyl alcohol; CMC – carboxy methylcellulose; DW – dextrin white; GGA – guar gum; BIOP – biopolymer gel; BAG – brown algal gel; RAG – red algal gel; CHI – chitosan; PBG – phosphate buffer with glycerol. Each panel represents a mean of three replicates, the error bar indicates a standard error, and panels with different letters are significantly different (Tukey test, $p<0.01$). In each panel, the red-dotted line indicates the optimum value of the trait based on the quality standard of liquid biofertilizer.

Among the ten polymers, Biogel, followed by guar gum, made higher viscosity solutions than other polymers evaluated. CMC and chitosan accounted for the viscosity of 140.89 and 128.38 cSt. PVA, HPMC, DW, MD, and BAG accounted for the viscosity with a range of 10-18 cSt, and the RAG and PBG accounted for the lowest viscosity of 9.69 and 9.15 cSt (Fig. 2A). The dry weight of the polymer solution after dried at 80°C represents the total dry matters present in the solution (Fig. 2B). There is no much difference in dry weight of the polymer solutions among the polymer which ranged from 916 mg/ml to 1106 mg/ml.

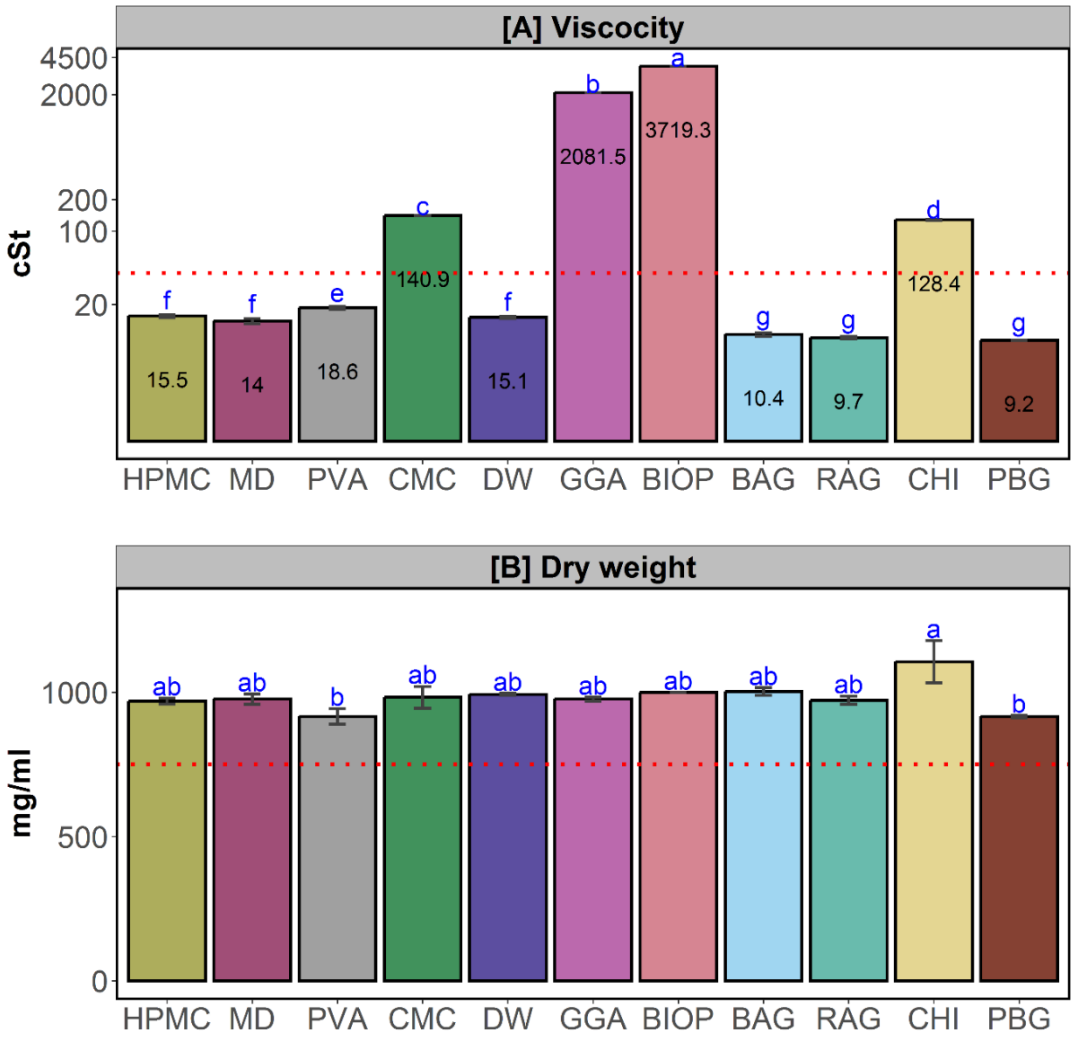


**Fig. 2. Viscosity (A) and dry weight (B) of the polymer solutions.** HPMC – hydroxypropyl methylcellulose; MD – maltodextrin; PVA – polyvinyl alcohol; CMC – carboxy methylcellulose; DW – dextrin white; GGA – guar gum; BIOP – biopolymer gel; BAG – brown algal gel; RAG – red algal gel; CHI – chitosan; PBG – phosphate buffer with glycerol. Each panel represents a mean of three replicates. The error bar indicates a standard error, and panels with different letters significantly differ (Tukey test, $p<0.01$). In each panel, the red-dotted line indicates the optimum value of the trait based on the quality standard of liquid biofertilizer.

All the polymer solutions except chitosan supported the *E. coli* cell viability after 24 hours of incubation. Most of the polymer solutions (BIOG, DW, GGA, HPMC, MD) and standard biofertilizer buffer (PBG) did not reduce the population after 24 hours of incubation. Compared to these polymers, PVA accounted for 1 log reduction ($10^{12}$ cells per ml), and CMC, RAG, and BAG had 2 log reductions ($10^{11}$ cells per ml). *E. coli* did not survive in the chitosan polymer solution after 24 hours of incubation (Fig. 3).

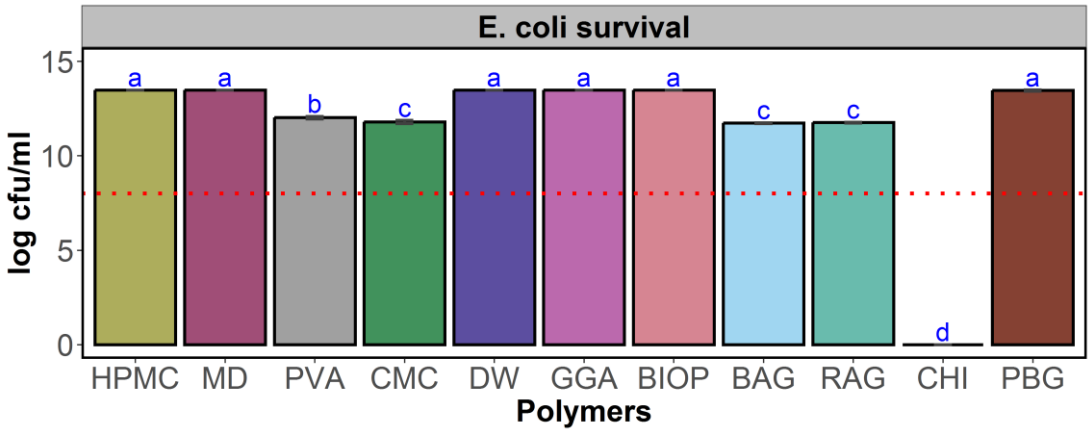


**Fig. 3. Survival of *E. coli* cells in the polymer solutions.** HPMC – hydroxypropyl methylcellulose; MD – maltodextrin; PVA – polyvinyl alcohol; CMC – carboxy methylcellulose; DW – dextrin white; GGA – guar gum; BIOP – biopolymer gel; BAG – brown algal gel; RAG – red algal gel; CHI – chitosan; PBG – phosphate buffer with glycerol. Each panel represents a mean of three replicates. The error bar indicates a standard error, and panels with different letters significantly differ (Tukey test, $p<0.01$). In each panel, the red-dotted line indicates the optimum value of the trait based on the quality standard of liquid biofertilizer.

Among the ten polymer solutions, maltodextrin accounted for 1.13% carbon, followed by HPMC (0.86%) and PVA (0.77%). In contrast, the others, including natural polymers and standard biofertilizer dilutant, accounted for a carbon content ranging from 0.07 to 0.48% (Fig. 4A). BIOG had the highest nitrogen content (81 µg/ml) followed by CMC, GGA, and MD (55.7, 55.3, and 55.4 µg), while DW, PBG, and PVA accounted 42 µg/ml. HPMC and BAG had the least N content of 14 µg/ml (Fig. 4B). The standard biofertilizer buffer (PBG) had 39.7 µg/ml of phosphorus, followed by the polymer solutions, CHI (26.8 µg/ml), BAG (22.51 µg/ml), and RAG (15.7 µg/ml). The least P content of 0.3 µg/ml was recorded in MD (Fig. 4C).

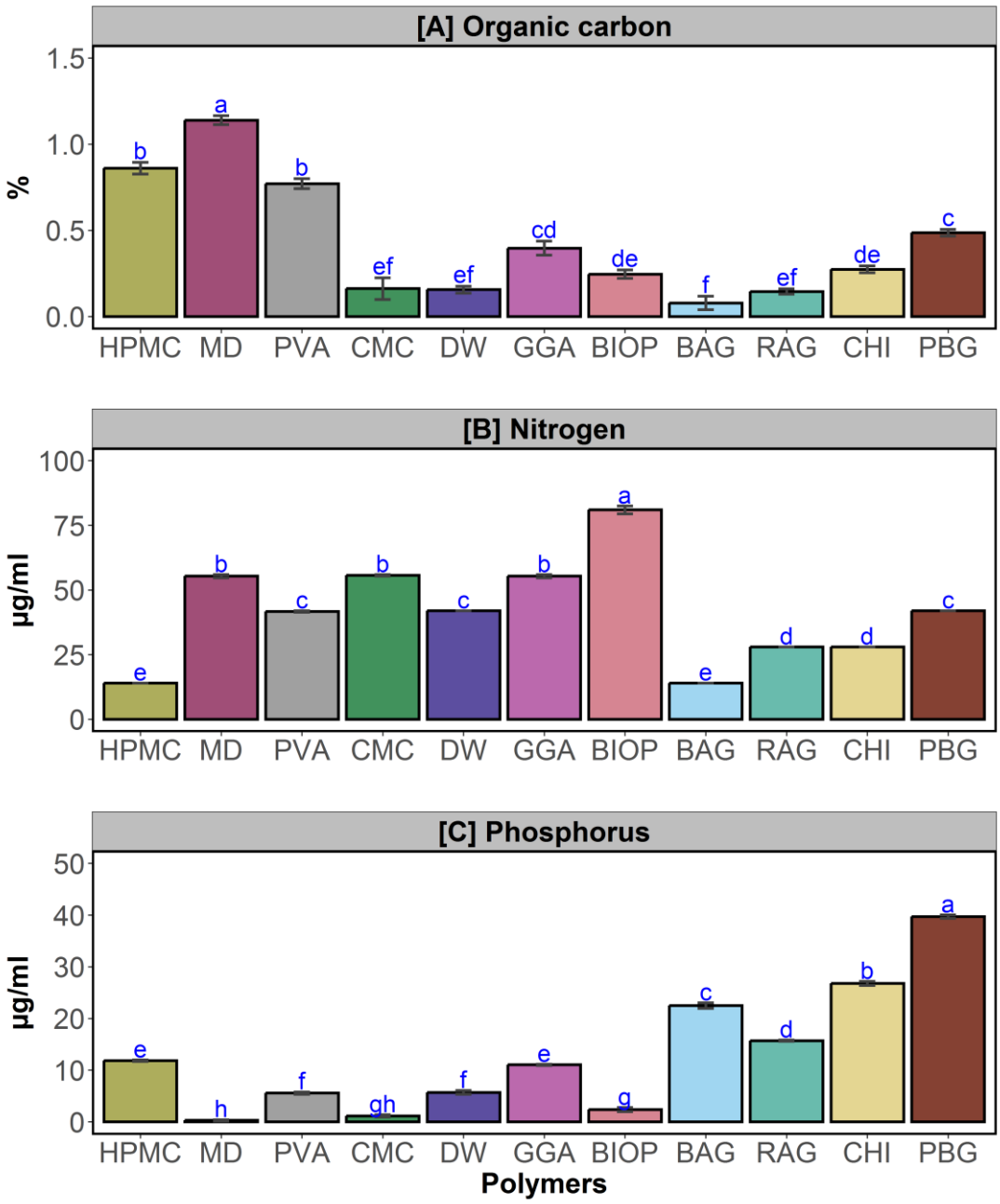


**Fig. 4. Total nutrient contents of the polymer solutions.** HPMC – hydroxypropyl methylcellulose; MD – maltodextrin; PVA – polyvinyl alcohol; CMC – carboxy methylcellulose; DW – dextrin white; GGA – guar gum; BIOG – biogel; BAG – brown algal gel; RAG – red algal gel; CHI – chitosan; PBG – phosphate buffer with glycerol. Each panel represents a mean of three replicates. The error bar indicates a standard error, and panels with different letters significantly differ (Tukey test, $p<0.01$).

The relationships between the polymer solutions and their traits for suitability to use in biofertilizer formulations were visualized through principal component analysis. The first two PCs (Dim1 and Dim2) contributed 33.3% and 25.6% of the total variance of the tested polymers (Fig. 5A and 5B). The traits such as organic carbon, viable cell counts, viscosity, and N contents had positive relations with the polymers and were positioned in either the upper or lower right quadrant (positive for PC1 and PC2; positive for PC1 and negative for PC2) of PCA. The polymers' phosphorus, EC, and dry weight are positioned in the negative quadrant of the PCA. Among the ten polymers, chitosan was orthogonally positioned in the negative quadrant of the PCA. In contrast, the PBG, PVA, HPMC, and MD were positioned in the PC1 and PC2 positive quadrant of PCA, while the BIOG, DW, and GGA were positioned in PC1 +ve and PC2 –ve plot (Fig. 5C). Among the eight traits used, nitrogen, viable cell count, viscosity, electrical conductivity, dry weight had high contribution (>12%) to their respective PCs (Fig. 5D). From the PCA results, the potential synthetic polymers such as HPMC, PVA and natural polymers viz., BIOP, BAG, and RAG were selected for the further studies.

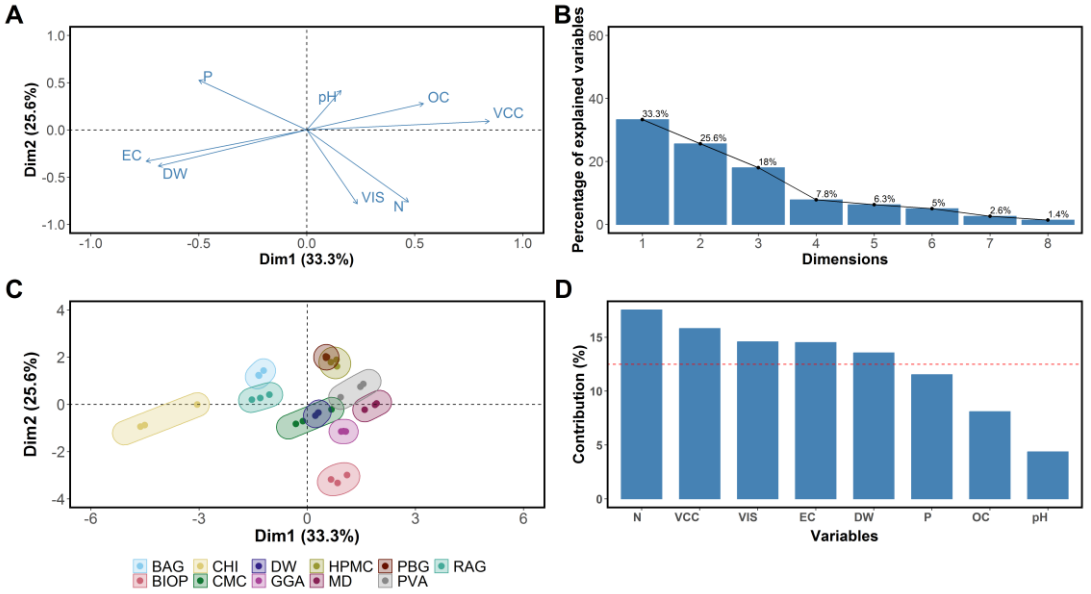


**Fig. 5. PCA relating the traits of ten different polymer solutions.** (A) PCA loading plot showing the orthogonal positions of the observed variables. The percentage variance explained by each principal component (Dim1 and Dim2) is given in parentheses in axes. (B) The percent contribution of each principal component to the cumulative variability in PCA. (C) PCA scoring scatter plots grouped by polymers and the ellipse represent the grouping at a 95% confidence interval. (D) The percent contribution of each variable on the axis is identified by the principal component analysis.

When the five selected polymer solutions (HPMC, PVA, BIOP, BAG, RAG) along with standard biofertilizer dilutant (PBG) tested for biofertilizer preparation and shelf life assessment, all of them supported the survival of *Azospirillum* (Az204) and Phosphobacteria (SP7) for 30 days without any significant reduction in the cell load. All the polymer solutions maintained a cell load of about $10^{15}$ per ml for the first 30 days of incubation and later found slight decline in some of the polymer solutions (Fig. 6). However, HPMC, PVA, and RAG had two-log reductions (100 cells per ml) after 60-days of incubation, while the universal buffer

(PBG) had one-log reduction in the *Azospirillum* and Phosphobacteria population after 60 days.

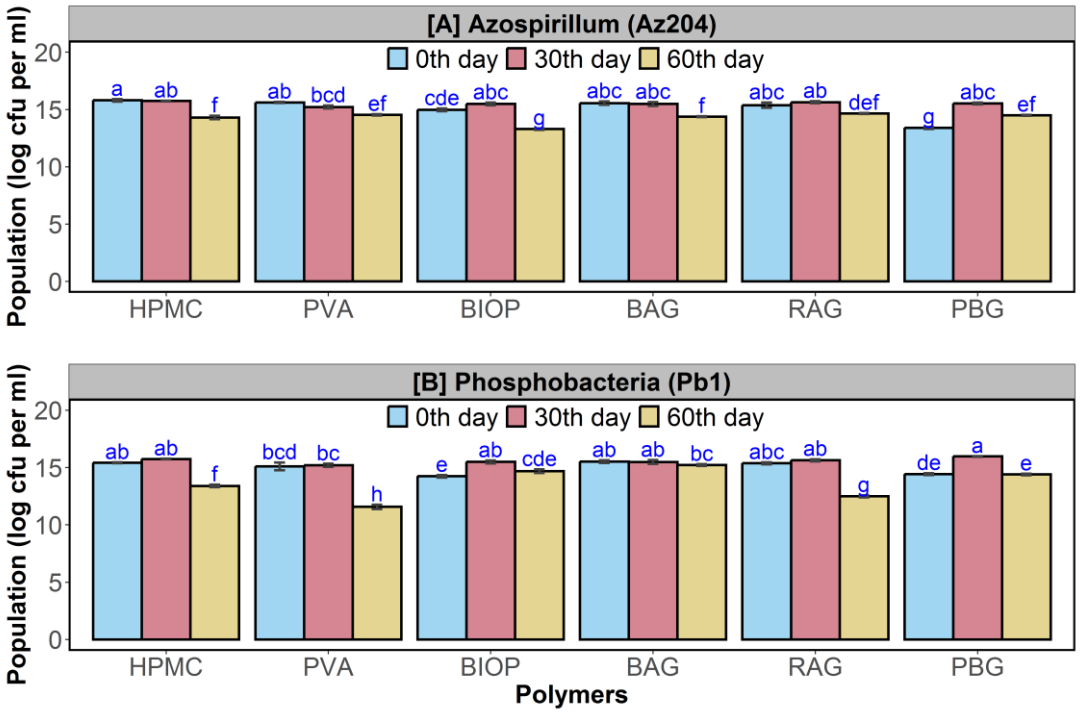


**Fig. 6. Population of biofertilizer strains in polymer-based formulation.** HPMC – hydroxypropyl methylcellulose; PVA – polyvinyl alcohol; BIOP – biopolymer gel; BAG – brown algal gel; RAG – red algal gel; PBG – phosphate buffer with glycerol. Each panel represents a mean of three replicates. The error bar indicates a standard error, and panels with different letters significantly differ (Tukey test, $p<0.01$).

## 4. DISCUSSION

A good biofertilizer formulation should protect the bacterial cells from any stress, create favorable micro-environmental conditions in the container, enhance the cell viability during storage, should effectively deliver the bacterial cells to the soil or crop sphere, and prevent the cells from any stress after introduction to the environment [13]. Though liquid biofertilizers are superior and the best alternative formulation to carrier-based biofertilizers in several aspects, still an upgradation is needed to mitigate the negative aspects of liquid formulations. The liquid formulations were prepared as aqueous cell suspension with limited nutrients and one or two Osmo-protecting chemicals like glycerol, polyvinyl pyrrolidone, and trehalose. Apart from the cell protection from osmotic stress, zero contamination, and enhanced shelf-life, this formulation has not yet fulfilled many other features of a good formulation. For example, the bacterial cells from liquid-formulated biofertilizers deprived of solid-material protection lost their viability quickly after inoculation. Similarly, the long-term storage of liquid formulations often reduced bacterial survival [18]. Hence, the cells in aqueous suspension need some solid matrix or encapsulation to keep the cells more viable and vigor. In the present work, we assessed the feasibility of polymers as an alternative cell protectant or encapsulating matrix for the aqueous formulation.

Formerly, polymer-based biofertilizers were developed using the immobilization principle (physical entrapment in the gel beads). The biofertilizer strains immobilized in alginate beads

are formulated as inoculants for several crops, including rice [19]. However, polymer-immobilized biofertilizers are not commercialized due to labor-intensive and high technological skills. Alternatively, polymer-based encapsulation is now being attempted in several bio inputs formulations [20, 21]. When the polymer and microbial cells are mixed under aqueous conditions, the polymer adsorbed on the cells' surface and protects the cells for prolonged shelf life [7].

In the present work, we used the polymer solutions to encapsulate the bacterial cells, and thereby protection and cell viability could be enhanced. With this background, we assessed five synthetic and natural polymers to protect liquid formulations in the present work. The polymers were first evaluated for their physical and chemical properties to cope with the minimum quality standards of biofertilizers. Near neutral pH, electrical conductivity less than 1.0 mS/cm, low to medium viscosity (10-100 cSt), with adequate carbon, nitrogen, and phosphorus are the desired characteristics to be featured for the biofertilizer formulations. HPMC and PVA were chosen from synthetic polymers at par with recommended traits. Similarly, Biogel, brown algal, and red algal gel polymers were selected from the natural polymers. Maltodextrin and dextrin white showed high acidity (<4.0), while the carboxymethyl cellulose showed high EC (>2.0 mS/cm) and viscosity (>200 cSt). Gaur gum (polymer obtained from pods of *Cyamopsis tetragonoloba*) had low pH and high viscosity issues. The chitosan (extracted from animal chitins) had low pH, high EC, and anti-microbial activity issues. E. coli cells did not survive in chitosan, while all other polymers did not inhibit the E. coli cells. The natural polymers extracted from seaweed (BIOG, BAG, RAG) showed promising results for developing polymer-based biofertilizers. In addition, these five polymers also have adequate carbon and nitrogen contents higher than phosphate buffer with glycerol (standard), with lower phosphorus content.

Eight different polymers (polyethylene glycol 4000 and 6000, polyvinyl alcohol, low and high molecular weight sodium alginate, HPMC, polyvinyl pyrrolidone, and carbopol) were screened for their suitability and compatibility with *Rhizobium* biofertilizer to develop a liquid formulation [22]. HPMC (0.5%) and sodium alginate (1%) showed promising results in improving the shelf life of rhizobia and competitive colonization in host plants. These two polymers were further formulated as polymer-based solid biofertilizers using the fluidized bed technique [23]. The HPMC and Na alginate-based rhizobia showed enhanced nodulation, nitrogen fixation, and plant growth promotion in cowpea upon inoculation. When alginate was amended in the liquid formulation of *Azospirillum*, it enhanced cell viability and stability at high temperatures [24]. The lignite – alginate based beads with hydrogel or starch was developed as *Rhizobium* biofertilizer formulation [25]. In the present work, all the selected polymers (HPMC, PVA, three different polymers from seaweeds) showed high viability of nitrogen-fixing bacterium, *Azospirillum*, and phosphorus solubilizing bacterium, *Bacillus megaterium*, up to 60 days of incubation. These results are similar to the reports by [Rivera [22], 23], in which they identified HPMC as a potential polymer for *Rhizobium* biofertilizer. HPMC is a semisynthetic polymer derived from cellulose containing hydroxypropyl and methyl groups. It is a hydrophilic biodegradable polymer with a wide range of applications. HPMC is an effective bacterial carrier polymer already reported as a stabilizing agent in biofertilizers' liquid formulation [23]. Like HPMC, polyvinyl alcohol is a synthetic, hydrophilic polymer with many applications. Recently, the PVA was used as an adhesive for seed coating the biofertilizer (*Bacillus pumilus* strain TUAT1) on rice [26]. These results support our findings on the feasibility of using HPMC and PVA as polymers for biofertilizer formulations.

The present work also explored the feasibility of the polymers extracted from seaweed extracts. Biogel is a super absorbent polymer from seaweed extract, RAG is the polymer extracted from red algae (*Kappaphycus alvarezii*), and BAG is purified from brown algae

(*Sargassum* spp.). All these three products are commercially available as soil conditioners for moisture conservation and plant growth promotion through bioactive compounds. These three polymers as encapsulating agents in the liquid formulation also performed at par with HPMC and PVA. Several advantages of using these polymers in biofertilizer formulation are natural, biodegradable, eco-friendly, and cheap, and any additional biostimulant chemical molecules can also be delivered to the plant rhizosphere along with biofertilizer strains.

## 5. CONCLUSION

The polymer-based liquid biofertilizer is considered to be the next step progress in liquid biofertilizers for enhanced shelf life and effective delivery of the strains in the vicinity of the crop's sphere. The present results demonstrate that HPMC, PVA, and polymers from seaweed could effectively reduce the biofertilizer volume without affecting the strain's viability. These findings suggest that polymers could be a potential encapsulating agent to improve the liquid biofertilizer's quality and, thereby, its bioefficacy in applied crops.

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