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RESEARCH ARTICLE

STUDIES TO IMPROVE THE SHELF LIFE OF *AZOSPIRILLUM LIPOFERUM* IMMOBILIZED IN ALGINATE BEADS

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ABSTRACT

One of the successful, safe and effective methods to introduce bioinoculants in soil is encapsulation of cells in biodegradable gel matrices which not only releases the microorganisms to the soil gradually but also helps to increase the survival rate by protecting them against many environmental stresses. The present study to develop the gel based formulation of *Azospirillum* bioinoculant to reduce chemical fertilizers that cause environmental problems. This new gel based formulation consists of dry beads containing nitrogen fixing *Azospirillum* cells. They were produced by quick encapsulation and drying processes, are made of alginate (2.5%) and enriched with different additives like skimmed milk poweder (8.0%), starch (25.0%) and humic acid (0.8%) and can contain up to 10^8 CFU g^{-1} of dry beads at the end of 360 days. This gel based formulation have been formulated to reduce the production cost, favour its storage and easy its application in the field and the results showed that gel based formulation is far better than the carrier formulation (lignite)

INTRODUCTION

In the context of increasing international concern for food and environmental quality, the use of plant growth-promoting rhizobacteria (PGPR) for reducing chemical inputs in agriculture is a potentially important issue. The PGPR were applied to various crops to enhance growth, seed emergence and crop yield, and a few such applications have been commercialized (Dey *et al*, *2004*; Herman *et al*., *2008*; Minorsky, 2008). Diazotrophic bacteria are also PGPR, because of their competitive advantage in C- rich and N-poor environments (Kennedy *et al*., 2004). Diazotrophic bacteria were reported to secrete growth promoting hormones like auxin, gibberellins and cytokinin into their culture media (Fuentes-Ramirez *et al.*, 1993).

Azospirillum is one of the potential plant growth promoting rhizobacterial (PGPR) bio-fertilizer. Its positive impacts on plant growth through several mechanisms include enhancement of root development, production of growth regulators and nitrogen fixation. Microbial survival following introduction to natural soils depends on both abiotic and biotic factors (Van Veen *et al*., 1997). The population of the inoculated bacteria declines progressively preventing the buildup of a sufficiently large PGPB population in rhizosphere (Bashan, 1998). In the soil, the survival of the inoculated bacteria largely depends on the availability of the empty niche; withstand competition with the often better-adapted native microflora and predation by protozoans or by other micro-invertebrates. A major role of inoculant formulation is to provide more suitable microenvironment for the prolonged survival in the soil. Inoculum strategies should include application of carrier materials aimed at providing protective niche together with the provision of nutrient sources. It is opined that the encapsulation method helps to increase the survival rate and easy delivery of bacterial cultures. It also helps in segregating the bacterial cells from adverse environment thereby reducing cell loss (Rekha *et al*., 2007). Advantages of microencapsulation of PGPB are described extensively by Cassidy *et al*., (1996). One of the successful, safe and effective methods to introduce bioinoculants in soil is encapsulation of cells in biodegradable gel matrices (Vassilev *et al*., 2001).In the present study, experiments were conducted to develop the gel based formulation of *Azospirillum lipoferum* Maz-3 bioinoculant by enriched with different additives *viz.*, humic acid, skimmed milk powder and starch.

MATERIALS AND METHODS

Microorganisms

A. lipoferum MAZ-3 isolate has been obtained from the maize rhizosphere soil in Cuddalore district of Tamilnadu, India.

Medium

N_2- free malate (NFb) medium containg (g l^{-1}) 5.0 malic acid, 0.5 K_2HPO_4, 0.2 $MgSO_4$ $7H_2O$, 0.1 NaCl, 2.0 $CaCl_2$, 4.0 ml Fe-EDTA (1.64% w/v aquous), 2.0 ml trace element solution, 2.0 ml bromothymol blue (0.5% alcoholic solution), 1.0 ml vitamin solution, 4.0 KOH, 15.0 Agar and pH 6.8 was used.

Preparation of inoculums

*A. lipoferum (*MAZ-3) isolate was inoculated in 100 ml of

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Nfb broth and incubated in shaker incubator (200 rpm) at 30 ±2°C for 48 h to obtain inoculum concentration of 10^9CFU ml^{-1}. This culture was centrifuged for 10 min at 8720 x g at 4°C. The cell pellet was then washed thoroughly with 10 ml (0.8%) of NaCl solution and re-suspended in 1ml of 1% peptone solution.

Production of gel based formulation of A. lipoferum (MAZ-3) bioinoculant

Encapsulation of *Azospirillum* cells within beads was carried out under sterile conditions in a laminar airflow hood (LAF). Sodium alginate solution (2.5 % w/v), skimmed milk powder (8.0%), starch (25.0%) and humic acid (0.8%) and calcium chloride (0.1 M) were autoclaved separately. Two ml of each conc. of each additive were mixed with 1.0 ml of *Azospirillum* cell pellet and 2.0 ml of 2.5% sodium alginate solution and stirred gently for 1 h in shaking incubator. The mixture was vigorously stirred to allow a homogenous dissolution of alginate. Then the mixture was extruded through sterile syringe (syringe size; 10 ml, needle; 0.65 mm × 22 mm) into gently stirred, sterilized 0.1 M $CaCl_2$ at room temperature. The beads were kept in $CaCl_2$ solution at room temperature for 1-3 h to obtain regular solid beads. The $CaCl_2$ solution was drained and the beads were washed twice with the sterile tap water. After washing step, beads were incubated in fresh growth medium (nutrient broth) for an additional 24 h in shaker incubator at 30 ± 2°C to allow the bacteria to multiply inside the beads. Then the beads were washed twice with distilled water, collected and allowed to dry overnight in a LAF. These beads were further used for *in vitro* experiments.

Drying

Drying the capsules is one of the ways of improving the survival of bacteria during storage. The requirement for the stability of bacteria during storage is water content less than 10% in wet beads. The wet beads were spread on a 10 mesh sieve and dried using two different techniques. The first was kept beads bed in an oven at 40°C and 35% RH for 48h. The second was by crossing the beads bed with a dry air stream (5% RH) at room temperature (about 25°C) and at air velocities (2 m/s) for 3h.

Enumerating the viable cell population in gel based formulation of Azospirillum inoculants

Azospirillum entrapped in the beads were counted after ten bead samples were dissolved in 10 ml potassium phosphate buffer (0.25 M, pH 6.8 ± 0.1) in a test-tube for 16-24 h at 30 ± 2 °C. No bacterial multiplication occurred under these incubations (Bashan, 1986). To facilitate solubility, the beads were shaken for 5 min at top speed on a Vortex mixer. The released *Azospirillum* cells were counted by the plate count method on nutrient agar. The cell count was recorded at 30 days interval up to 360 days

Bead size and weight measurements

The average diameter and weight of beads were measured in wet and dry conditions and expressed in mm and mg respectively

Slow release of entrapped Azospirillum cells

A portion of 20 washed beads containing immobilized bacteria was transferred into 75 ml of sterile saline solution (0.85% [w/v] NaCl) and gently shaken at 30 ± 2°C for 24 h. Then triplicate samples of 0.5 ml of saline solution were collected, and the number of released bacteria was determined by the plate count method on nutrient agar plates. Then the beads were rinsed twice with sterile tap water and transferred into a fresh saline solution, and the procedure was repeated after an additional 24 h up to 6 days.

RESULTS

Development of gel based formulation of A. lipoferum MAZ-3 by sodium alginate with selected concentration of various additives

The results of gel formulation of *A. lipoferum* MAZ-3 with sodium alginate (2.5%) plus selected concentrations of various additives *viz.*, SMP (8.0%), starch (25.0%) and humic acid (0.8%) for longer survival of *Azospirillum* cells are presented in Table-1.

Among the treatments, the lower reduction of *Azospirillum* cells was observed in sodium alginate along with humic acid (0.8%) followed by starch (25.0%) and SMP (8.0%) and SA (2.5%) alone when compare to control. The highest population of 1.33 $x10^9$ CFU g^{-1} beads was recorded in SA (2.5%) + humic acid (0.8%) followed by SA (2.5%) + starch (25.0%) (0.87$x10^9$ CFU g^{-1} beads) at 360^{th} day. Whereas lowest population of 0.0009$x10^9$ CFU g^{-1} was recorded in carrier formulation (lignite).

Survival of A. lipoferum MAZ- 3 and characteristics of wet and dry beads enriched with various additives

The results of the present study revealed that all the treatments recorded higher *A. lipoferum* MAZ- 3 population in dry beads inoculants when compared to wet beads inoculants (Table-2). Among the treatments, the maximum population of 0.43 $x10^9$ and 19.33 $x10^9$ CFU g^{-1} beads in wet and dry beads inoculant was recorded in SA (2.5%) + humic acid (0.8%) during the 6^{th} month, respectively. Whereas the minimum population of 0.10 $x10^9$ and 7.67 $x10^9$ CFU g^{-1} beads in wet and dry beads observed in SA (2.5%) respectively.

In contrast to *A. lipoferum* MAZ- 3 population, the average bead diameter and average bead weight were higher in wet bead when compared to dry bead. The maximum of 3.2mm and 10.3mg (average bead diameter and weight respectively) recorded in SA (2.5%) + Starch (25.0%) in wet bead and lowest values (1.3mm and 0.5mg) were recorded in dry bead of sodium alginate (2.5%) alone.

Release of A. lipoferum MAZ-3 cells from gel formulation enriched with various additives

The release of *Azospirillum* cells from the gel the formulation was assessed up to six days. It was found that, showed a decrease of *Azospirillum* cells released from gel beads initial to 6^{th} day (Table-3).

Among the various treatments, higher amount of *Azospirillum* cells (8.5 $x10^8$ and 1.67$x10^5$ CFU g^{-1} beads) was released in SA (2.5%) +humic acid (0.8%) (T_4) at initial and 6^{th} day of sampling respectively, followed by SA (2.5%) +

25% of starch (T_3) (7.00 x108 and 9.33 x104 $CFUg^{-1}$ beads). The lower amount of *Azospirillum* cells (4.67×10^8 and $4.64 \times 10^4 CFUg^{-1}$ beads) in SA 2.5% (T_1) alone gel formulation at initial and 6^{th} day of sampling respectively.

In practical terms, formulation determines potential success of inoculants (Fages, 1994). Entrapment of microbial cells has been reported to improve their metabolic activities and enhance the production of several hydrolic enzymes (El-Katatny *et al*., 2003; El-Katatny *et al*., 2004).

Table 1 Survival of *A. lipoferum* (MAZ-3) in gel based formulation amended with selected concentrations different chemical additives

Days	*Azospirillum* population (× 10^9 CFU g^{-1}) Period of storage (Days)				
	Carrier formulation (lignite)	Sodium alginate (2.5%)	Sodium alginate (2.5%) + SMP (8.0%)	Sodium alginate (2.5%) + Starch (25.0%)	Sodium alginate (2.5%) + Humic acid (0.8%)
Initial	3.53 (9.55)	17.00 (10.23)	24.67 (10.39)	27.33 (10.44)	28.67 (10.46)
30	4.67 (9.67)	15.67 (10.20)	23.00 (10.36)	26.33 (10.42)	27.67 (10.44)
60	3.80 (9.58)	14.67 (10.17)	22.67 (10.35)	24.67 (10.39)	26.33 (10.42)
90	3.03 (9.48)	12.67 (10.10)	20.33 (10.31)	22.00 (10.34)	24.00 (10.38)
120	2.16 (9.33)	11.00 (10.04)	18.67 (10.27)	21.33 (10.33)	23.67 (10.37)
150	1.33 (9.12)	9.33 (9.97)	17.00 (10.23)	19.00 (10.28)	21.00 (10.32)
180	0.86 (8.94)	7.67 (9.88)	15.67 (10.19)	17.33 (10.24)	19.33 (10.29)
210	0.43 (8.63)	5.33 (9.73)	12.33 (10.09)	14.67 (10.17)	17.00 (10.23)
240	0.17 (8.23)	4.00 (9.60)	9.00 (9.95)	11.00 (10.04)	14.33 (10.16)
270	0.073 (7.86)	2.33 (9.37)	6.67 (9.82)	9.33 (9.97)	11.00 (10.04)
300	0.026 (7.14)	0.83 (8.92)	4.33 (9.64)	6.67 (9.82)	8.67 (9.94)
330	0.006 (6.78)	0.57 (8.76)	1.00 (9.00)	3.33 (9.52)	5.00 (9.70)
360	0.0009 (5.95)	0.23 (8.36)	0.63 (8.80)	0.87 (8.94)	1.33 (9.12)
SEd	0.041	0.010	0.002	0.003	0.001
CD(p=0.05)	0.085	0.021	0.005	0.006	0.003

Values in parenthesis are $\log_{10}$ transformed values

Table 2 Bead characteristics and survival of *A. lipoferum* (MAZ-3) isolate in wet and dry alginate beads enriched with different additives

Beads *		*Azospirillum* population (× 109 CFU g -1)		Average bead diameter (mm)	Average weight bead -1 (mg)
		Initial	6th month		
Wet bead	SA (2.5%)	0.57 (8.75)	0.10 (8.00)	2.9	9.8
	SA (2.5%) + SMP (8.0 %)	0.83 (8.92)	0.33 (8.51)	3.0	10.1
	SA (2.5%) +Starch (25.0 %)	0.87 (8.94)	0.37 (8.57)	3.2	10.3
	SA (2.5%) + Humic acid (0.8%)	0. 94 (8.97)	0.43 (8.60)	2.9	9.8
Dry bead	SA (2.5%)	17.00 (10.23)	7.67 (9.88)	1.3	0.5
	SA (2.5%) + SMP (8.0 %)	23.67 (10.37)	14.33 (10.16)	1.3	0.6
	SA (2.5%) + Starch (25.0 %)	27.33 (10.44)	17.00 (10.23)	1.4	0.7
	SA (2.5%) + Humic acid (0.01%)	28.67 (10.46)	19.33 (10.29)	1.3	0.5

*- alginate beads enriched with different additives
Values in parenthesis are $\log_{10}$ transformed values

DISCUSSION

The success of inoculation technology depends on two factors such as the microbial strain and inoculant formulation.

Alginate immobilization has also been used as inoculant for plant growth promoting bacteria for over more than two decades (Bashan, 1986).

Table 3 Release of *A. lipoferum* (MAZ-3) cells from alginate beads enriched with different additives

Alginate beads with additives	*Azospirillum* population (CFU g -1 of beads)					
	Period of incubation in days					
	Initial	2nd	3rd	4th	5th	6th
T1 - Sodium alginate (2.5%)	4.67 × 107 (7.67)	3.33 × 106 (6.52)	6.67 × 105 (5.82)	2.00 × 105 (5.30)	8.67 × 104 (4.94)	4.33 × 104 (4.64)
T2 - Sodium alginate (2.5%) + SMP (8.0 %)	6.33 × 108 (8.80)	6.00 × 107 (7.78)	4.33 × 106 (6.64)	9.67 × 105 (5.99)	3.33 × 105 (5.52)	8.00 × 104 (4.90)
T3 - Sodium alginate (2.5%) + starch (25.0 %)	7.00 × 108 (8.85)	7.67 × 107 (7.88)	7.00 × 106 (6.85)	2.33 × 106 (6.37)	5.00 × 105 (5.69)	9.33 × 104 (4.97)
T4 - Sodium alginate (2.5%) + humic acid (0.8%)	8.5 × 108 (8.93)	9.33 × 107 (7.97)	9.33 × 106 (6.97)	5.67 × 106 (6.75)	7.5 × 105 (5.88)	1.67 × 105 (5.52)
SEd	0.020	0.018	0.018	0.016	0.015	0.014
CD(p=0.05)	0.046	0.042	0.041	0.038	0.035	0.032

Values in parenthesis are $\log_{10}$ transformed values

The gel-like matrix allows the cells to remain viable and with its catalytic ability for longer duration. Several studies thus far have used alginate as the encapsulating material as it forms microbeads instantaneously in presence of polyvalent cations by binding the cation to guluronic acid units (Witter, 1996) in one step with sufficient mechanical strength. Moreover, alginate beads are capable of entrapping sufficient number of bacteria (Fenice *et al*., 2000; Zohar-Perez *et al.*, 2002).

In the present study, the survival of *A. lipoferum* (MAZ-3) in the gel based formulation amended with sodium alginate(SA) (2.5%) and different concentrations of additives *viz.*, skimmed milk powder (SMP), starch and humic acid was studied up to 360 days of storage period. The maximum number of *Azospirillum cells* was recorded in SA (2.5%) with humic acid (0.8%) followed by SA (2.5%) with starch (25.0%) and SA (2.5%) with SMP (8.0%). The gel based formulations developed with additives were supported higher survival of *Azospirillum* population when compared to SA (2.5%) alone and carrier based formulation (lignite).

The sodium alginate with humic acid supported higher *Azospirillum* population; it might due to the porous gel matrix providing the space and the humic acid providing nutrients facilitating the bacterial multiplication within the bead environment (Young *et al.,* 2006). Additional supplementation of the gel beads with nutrient compounds is presumed to enhance the stability, provides protection and nutrition to the encapsulated cells (Bashan, 1986). But, not all the amendments provide advantageous effects on the microbial cells, for example, Vivekananda and Jauhri (2000) found that charcoal-soil mixed with alginate, adversely affected the initial cell loading and also survival of P-solubilizing bacteria. In this respect, the use of humic acid as a supplementary nutrient for encapsulated *Azospirillum* in this study has offered several advantages over the existing enriching compounds. Humic acid in general is the most versatile organic compound.

This is mainly due to its natural origin from soil processes, contains chemical structures which can oxidize or reduce elements, photosensitize chemical reactions and enhance or retard the uptake of toxic compounds or micronutrients to plants and microorganisms thereby greatly benefiting plant growth (Bacilio *et al.,* 2003; Nardi *et al.,* 2002). Humic acid is colloidal in nature with particles of different size and 60% particles were of the size 5 mm and remaining 40% of the size range between 0.04 and 0.5 mm indicating that bacteria may utilize these sub-micron particles for its immediate survival in the encapsulated state (Young *et al.,* 2006).

Use of skim milk and clay are among the most applied amendments for gel entrapped soil microbial inoculants that showed better performance in soil (Vassilev *et al.,* 2001). Entrapment of microbial cells has been reported to improve their metabolic activities and enhance the production of several hydrolytic enzymes (El-Katatny *et al.,* 2003). Furthermore, Vassilev *et al.* (1997) found that *Enterobacter* sp. encapsulated in alginate gel enriched with 3% skim milk powder demonstrated better establishment and improved phosphate solublilization activity and Minaxi and Saxena (2011) reported that *Pseudomonas fluorescens* BAM-4 and *Burkholderia cepacia* BAM-12 were immobilized using sodium alginate and alginate *Azospirillum* skim milk as carrier to check the phosphate solubilization *in vitro* and were found to have significantly higher activity than control.

Gel formulation consists of macrocapsules (large size beads, about 3 to 4 mm diameter) containing nitrogen fixing bacteria: *Azospirillum* and produced by quick encapsulation and drying processes, are made of sodium alginate (3%), standard starch (44.6%) and modified starch (2.4%) and can contain up to 10^6 CFU capsule (Ivanova *et al.,* 2005).

CONCLUSIONS

Gel based formulation of *Azospirillum* was developed by sodium alginate(2.5%) with selected concentration of additives *viz.,* humic acid(0.8%), starch (25%) and skimmed milk powder (8.0%) that supported higher *Azospirillum* cells when compared to carrier (lignite) formulation up to 360 days. Among the additives, humic acid (0.8%) with sodium alginate (2.5%) was found to be sustained highest survival in gel based formulation.

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