DEVELOPMENT OF ENCAPSULATED FORMULATION OF PGPR MIX- I AND ITS EVALUATION

by

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THESIS

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DECLARATION

I, hereby declare that this thesis entitled **"DEVELOPMENT OF ENCAPSULATED FORMULATION OF PGPR MIX- I AND ITS EVALUATION"** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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LIST OF ABBREVIATIONS AND SYMBOLS USED

ABBREVIATIONS/ SYMBOLS	TERMS
ABA	Abscisic acid
et al.	And other co workers
B	Boron
$CaCl_2$	Calcium chloride
cm	Centimetre
cfu	Colony forming units
cfu g^{-1}	Colony forming units per gram
cfu ml^{-1}	Colony forming units per milliliter
CRD	Completely Randomised Design
Cu	Copper
CD	Critical Difference
$^{\circ}C$	Degree Celsius
df	Degrees of freedom
FYM	Farm Yard Manure
Fig.	Figure
GA3	Gibberellic acid
g	Gram
ha	Hectare
hrs	Hours

IAA	Indole-3- Acetic Acid
Fe	Iron
kg	Kilogram
kg N ha^{-1} yr^{-1}	Kilogram Nitrogen per hectare per year
kg ha^{-1}	Kilogram per hectare
µg	Microgram
µg ml^{-1}	Microgram per milliliter
mg	Milligram
mL	Millilitre
mm	Millimetre
mM	Millimolar
min	Minutes
M	Molarity
viz.	Namely
nm	Nanometre
N	Nitrogen
NFB	Nitrogen Free Bromothymol Blue
No.	Number
ppm	Parts per million
%	Per cent

P	Phosphorous
PSI	Phosphorous Solubilization Index
PGPB	Plant Growth Promoting Bacteria
PGPR	Plant Growth Promoting Rhizobacteria
K	Potassium
lbs	Pound-Mass or Pound
NaCl	Sodium chloride
sp.	Species
SE	Standard Error
i.e.	That is
vol	Volume
wt	Weight
Zn	Zinc

INTRODUCTION

1. INTRODUCTION

Crop fertilization is a major concern in the aspect of rising population, food scarcity, high price of mineral fertilizers and less target capacity of conventional mineral fertilization applications. Use of microbial inoculants to the soil can magnify the assimilation of nutrients by crop plants and rise the efficiency of mineral fertilizers. Biofertilizer technology is architechtured to enhance the productivity of agricultural structures globally in a sustainable manner.

Biofertilizers consists of live microorganisms and meant for seed, soil or phyllosphere application and thereby colonizes the rhizospheric or phyllospheric region of the crop plants and enhances growth by contributing enhanced supply of essential nutrients (Vessey, 2003). Biofertilizers upholds a innate technique to provide nutrients to crops by an integration of phosphorus solubilization, nitrogen fixation and production of plant growth promoting substances. The microorganisms included in biofertilizers enhances the soil natural nutrient cycle and aids in establishing organic matter content of soil and nurture soil fertility. Plant growth promoting rhizobacteria is the one of the beneficial bacteria that has acquired global wide acceptance.

PGPR are one among the most studied aggressively root colonizing soil microorganisms which can promote plant performance as plant growth promoters and biocontrol agents. Inoculation of crop plants with certain strains of PGPR at an early stage of development improves biomass production through direct effects on root and shoot growth. Many strains in the genera such as *Azoarcus, Azospirillum, Azotobacter, Arthrobacter, Bacillus, Clostridium, Enterobacter, Gluconoacetobacter, Pseudomonas* and *Serratia* have been reported to have PGPR mechanisms (Hurek and Hurek, 2003).

There are several mechanisms by which PGPR encourage plant growth such as ability to synthesize various growth promoting substances (phytohormones, organic acids, siderophores) and enhancing the processes of biological nitrogen fixation, phosphate solubilization and production of antibiotics that suppress deleterious rhizobacteria (Arshad and Frankenberger, 1998).

PGPR mix-I is a consortium of compatible soil beneficial microorganisms such as nitrogen fixers, P and K solubilizers developed by the Department of Agricultural Microbiology,

College of Agiculture, Vellayani (KAU, 2017). It contains strains of *Azospirillum lipoferum* and *Azotobacter chroococcum* (Nitrogen fixers), *Bacillus megaterium* (P solubilizer) and *Bacillus sporothermodurans* (K solubilizer). These bacteria aggressively colonize the roots of plants and can act as biofertilizers and help plant growth.

Azospirillum is one of the promising plant growth promoting rhizobacteria. Its positive impact on plant growth through several mechanisms such as enhancement of root development, production of growth regulators and nitrogen fixation has been well established (Garcia *et al.,* 1996). *Azotobacter* is a free living nitrogen fixing bacteria present in soil capable of synthesizing plant growth promoting substances like IAA, gibberellins, antifungal antibiotics etc. Phosphate (P) and potassium (K) solubilizing bacteria enhance mineral uptake by plants through solubilizing insoluble P and releasing K from silicate in soil. The *Pseudomonas*, *Bacillus* (Illmer and Schinner, 1992) *Aspergillus* and *Penicillium* (Wakelin *et al.*, 2004) are well known P-solubilizers. Addition of K solubilizers had significantly increased the K content of shoot clearly revealing the favourable effect of K solubilizers on K availability and nutrition of plants (Sakthidharan, 2011).

PGPR mix-I developed by KAU supplements all the three basic NPK elements to crops along with its phytostimulation and other growth promoting activities. This carrier (talc) based formulation of PGPR mix-I has been greatly accepted by the farmers of Kerala. Application of PGPR mix-I has considerably reduced the use of chemically bagged synthetic fertilizers since it act as a sensible substitute for chemical fertilizers which is also suggested as a profitable and effective technology.

Biofertilizers are generally applied as solid carrier based or liquid inoculants to the soil or to the seed directly. The solid carrier based inoculants suffer from inferior quality, high rate of contamination and low field performances (Cassidy *et al.,* 1996).

Liquid inoculants show quick and high decrease of viability during storage and transportation besides the high risk of contamination and low survival of bacteria in the soil. Bacterial survival is inferior in the liquid type of inoculants as bacteria lack carrier protection, has short shelf life and difficult to transport (Albareda *et al*., 2008). Here comes the necessity of encapsulated microbial inoculants that rectifies the demerits associated with traditional peat or talc inoculants and liquid formulations which cause greater variability in quality.

According to Ding and Shah (2009), cell immobilization by encapsulation or entrapment involves coating or entrapping microbial cells within a polymeric material to produce beads which are permeable to nutrients, gases and metabolites for maintaining cell viability within the beads. Bioencapsulation is the approach of coating a protective shell surrounding the active element or microbial cells (John *et al*., 2011). According to Schoebitz *et al*. (2012), one of the widely used substance for the encapsulation of microorganisms is sodium alginate. Bioencapsulation of microbial inoculants is done with the addition of an active element into a matrix followed by a mechanical operation and finally stabilization by a chemical or physical-chemical process. In the agricultural industry, microbial cells can be encapsulated mainly by processes such as spray drying, interfacial polymerization or cross-linking.

Benefits of formulating encapsulated formulation of biofertilizers consists of controlled and gradual release of bacteria into the soil, adequate protection of encapsulated microorganisms in the soil against biotic and abiotic stresses and reduction of contamination during storage and transport. It can protect the bacterial cells from bacteriophages, hydrogen peroxide, short chain fatty acids, carbonyl-aromatic compounds and drying (Mortazavian *et al*., 2007). This forbearance was largely due to the increased modification of the cell membrane (Groboillot *et al*., 1994).

Since encapsulated formulations have several merits over carrier based formulations, the present study was undertaken to standardize encapsulated formulation of PGPR mix–I with major thrust on the following aspects:

1. Standardization of encapsulated formulation of PGPR mix–I with different filler materials.

2. Evaluation of standardized encapsulated formulation of PGPR mix–I for its shelf life, moisture content, rate of release and biodegradation.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Microbial inoculants technology designed to boost the productivity of agricultural systems in the long haul. Microbial inoculant technology is aligned with principles of sustainable agriculture, as opposed to the increased use of pesticides and fertilizers in recent times. The application of selected plant beneficial microorganisms individually or as microbial consortia with multifunctional properties is an important tool to promote crop health and productivity (Ahmad *et al*., 2018; Maron *et al*., 2018). Plant growth promoting rhizobacteria (PGPR) is the most widely studied aggressively root colonizing soil microflora which can enhance crop overall performance (Klopper *et al.,* 1980). PGPR have attained great attentiveness in research since it has considerable effects on stimulation of crop growth, crop yields and being harmless to the soil and plant habitat.

PGPR has been given reasonable consideration in recent times as it seems to substitute agrochemicals and supports crop growth and health by a combination of various mechanisms. These mechanisms include development of sustainable soil structure, organic matter decomposition, recycling of essential elements, solubilization of mineral nutrients, synthesis of several plant growth regulating substances, degradation of pollutants having organic origin, enhanced root growth essential for soil fertility, biocontrol of plant pathogens.

For sustainable crop production, proper understanding of plant growth promoting rhizobacteria and their interactions with biotic and abiotic factors is necessary. Also there is a necessity to develop efficient microencapsulated formulations of agriculturally important microorganisms for better agricultural application and production in order to achieve the targeted role of microbial inoculants in sustainable agriculture. Utilization of various types of matrices and filler materials of biological origin and evaluation of survival during storage and application are also necessary.

2.1 Plant Growth Promoting Rhizobacteria (PGPR)

Rhizosphere is the narrow region of soil unique to the root system (Dobbelaere *et al*., 2003). The accumulation of various plant secretions such as amino acids and sugars have transformed this area into a rich carbon source zone that promotes the growth of various microbes involved in nutrient transformation (Gray and Smith, 2005).

Studies by Weller and Thomashow in 1994 found that microbial population is usually 10 to 100 times higher around the rhizospheric area than that of bulk soil. According to Schroth and

Hancock in 1982, a variety of beneficial microorganisms and bacterial population called rhizobacteria inhabit rhizosphere. The rhizoshperic region consists of large number of microorganisms mainly bacteria. Based on the plant growth effects, bacterial population may be classified as beneficial, deleterious and neutral groups (Dobbelaere *et al*., 2003).

Klopper *et al*. (1980) reported that plant growth rhizobacteria (PGPR) are one of the most studied soil microorganisms that can promote plant performance by vigorously colonizing plant roots. Plant growth promoting rhizobacteria (PGPR) are beneficial bacteria that can colonize plant roots, either through direct action or by biological regulation of plant diseases. PGPR enhances crop growth by a variety of mechanisms and are mainly classified as direct and indirect mechanisms. PGPR are either plant growth promoters or biocontrol agents (Kloepper and Schroth, 1978). Based on the studies conducted Kloepper *et al*. in 1989 reported that plant growth promoting rhizobacteria (PGPR) are free-living bacteria in soil which are beneficial for crop growth, yield and quality.

Strains in the genera such as *Serratia, Pseudomonas, Burkholderia, Agrobacterium, Erwinia, Xanthomonas, Azospirillum, Bacillus, Enterobacter, Rhizobium, Alcaligenes, Arthrobacter, Acetobacter, Acinetobacter, Achromobacter, Aerobacter, Azotobacter, Clostridium, Klebsiellla, Micrococcus, Rhodobacter* and *Flavobacterium* are reported to have PGPR activity (Rodriguez and Fraga, 1999; Bloemberg and Lugtenberg, 2001; Esitken *et al*., 2003).

Mechanisms exhibited by PGPR to promote plant growth includes its ability to generate several growth promoting substances (phytohormones, organic acids, siderophores), biological fixation of nitrogen, solubilization of Phosphorous and biocontrol activity by production of antibiotics that inhibits noxious rhizobacteria. Ability to produce biologically active substances like plant growth regulators (PGRs) is the one of the vital mechanisms through which PGPR induce plant growth and development (Arshad and Frankenberger, 1998).

Application of PGPR biofertilizer has reported noticeable changes in terms of crop growth, health and yield. The final result of PGPR inoculation is highly favoured by crop age and chemical, physical and biological properties of the soil (Kundan *et al*., 2015).

2.2 Effect of PGPR Formulations on Soil Microbial Population

For better understanding of how PGPR application affects the soil properties, the changes in rhizosphere population of microorganisms should also be taken into account. In this regard

several researchers studied the effect of application of PGPR formulations on rhizosphere microflora.

Inoculation of *Azospirillum, Azotobacter* and *Glomus fasciculatum* were found to increase the soil beneficial microbial population which in turn results in increased activities of dehydrogenase and phosphatase enzymes (Aseri and Rao, 2000).

Increase in rhizosphere population of bacteria, fungi and actinomycetes was observed when soil was inoculated with phosphate solubilizing bacteria enriched vermicompost and PGPR mix-I enriched vermicompost with different doses of NPK fertilizers (Sathyan, 2013).

Vijendrakumar *et al*. (2014) investigated that maximum population of beneficial micro flora in terms of cfu g^{-1} was reported as a result of dual and triple inoculation of bio-fertilizers in the soil. Mary *et al*., (2015) reported luxuriant growth of bacteria in all the biofertizer treated rhizosphere in the order of FYM < Phosphobacteria < Vermicompost. Khipla *et al*., (2017) reported that application of 100 per cent chemical N and P with consortium of *Azotobacter* and phosphate solubilizing bacteria was recorded highest soil microflora population and enzyme activities.

2.3 PGPR mix-I - A Consortium of Efficient Plant Growth Promoting Bacteria

PGPR mix-I is a consortium of compatible soil beneficial microorganisms such as nitrogen fixers, P and K solubilizers developed by the Department of Agricultural Microbiology, College of Agiculture, Vellayani (KAU, 2017). It contains strains of *Azospirillum lipoferum* and *Azotobacter chroococcum* (Nitrogen fixers), *Bacillus megaterium* (P solubilizer) and *Bacillus sporothermodurans* (K solubilizer). These bacteria aggresively colonize the roots of plants and can act as biofertilizers and help plant growth.

Talc is a widely used carrier material for the development of formulations as it is inert and easy procurable as raw material from the soap stone industries. The potentiality of talc to be used as a carrier for formulating rhizobacteria was revealed by Kloepper and Schroth in 1981. Many scientific workers have reported the efficiency of talc based PGPR mix-I developed by KAU (Sathyan, 2013; Raj *et al*., 2013; Mohanan, 2016). Several studies in KAU have established the positive effect of carrier based formulation of PGPR mix-I in various crops (Akshay, 2011; Raj *et al*., 2013; Sathyan, 2013; Yadav, 2013; Mohanan, 2016).

Application of PGPR mix-I was found to increase the leaf width (9.66 cm), number of suckers per plant (4.25), number of ray florets (69.6) and length of ray florets (5.51 cm) in the flowering plant *Gerbera jamesonii* (Mohanan, 2016).

In order to increase the yield and save chemical fertilizers in paddy, basal application of PGPR mix-I (2 kg ha^{-1}) with prescribed half dose of chemical fertilizer (45:22.5:7.5 kg ha^{-1} NPK) and lime top dressing (250 kg ha^{-1}) was found to be the best combination (Raj *et al*., 2013).

PGPR mix-I enriched vermicompost increased the availability of micronutrient status (Fe, Cu, B), biological properties of soil viz., dehydrogenase activity, cellulase and protease activities and biometric characters of bhindi such as plant height and number of fruits (Sathyan, 2013).

2.4 Plant Growth Promoting Rhizobacteria as Biofertilizers

Plant growth promoting rhizobacteria is well known for crop growth promotion due to various rhizobacterial characteristics. PGPR employs various mechanisms under different environmental conditions in order to stimulate crop growth and development.

Modification of overall microbial population in the rhizosphere occurs through the development of specific substances in the case of PGPR induced plant growth promotion.

Azospirillum is one of the most studied plant growth promoting rhizospheric bacterium which is able to improve crop growth and yield of several species due to its capacity to synthesize several phytohormones (Dobbelaere *et al*., 2001). Pandiarajan *et al*. (2012) reported that strains of *Azospirillum* aid the plants in utilization of several soil resources for better growth and used as efficient biofertilizers in crop plants all over the world.

Azospirillum sp. enhance plant growth by multiple effect on plants including synthesis of phytohormones, nitrogen fixation, nitrate reductase activity and enhanced mineral uptake as reported by El- Komy (2004). This nitrogen fixing bacterium is known to produce the plant growth promoting phytohormone IAA. Studies by Kavitha (2001) reported that the IAA production by *Azospirillum* sp. isolated from chilli roots under *in vitro* conditions ranged between 21 and 55 μg ml^{-1}.

Faruq *et al*. (2015) investigated the potential of *Azospirillum* sp. for improving shoot and root growth of Malaysian sweet corn variety (j 58) under *in vitro* conditions and it was found that

Azospirillum brasilense strains inoculated corn seedlings produced longer and highest number of roots, lateral and tertiary root formation and biomass.

The beneficial effect of *Azospirillum* can be accrued from its nitrogen fixation and stimulating effect on root development (Noshin *et al*., 2008). It has also been reported that *Azospirillum* - plant association induces certain biochemical changes in roots which results in plant growth promotion and tolerance to low soil moisture. The bacteria induce crop growth even under stressed conditions such as drought.

Many reports suggested that *Azotobacter chroococcum*, a plant growth promoting rhizobacterium can act as potential plant growth promoter. Inoculation of *Azotobacter* sp. induces many plant growth promotion activities in plants. Eklund (1970) reported that germination and seedling growth of tomato and cucumber were enhanced due to the presence of *Azotobacter chroococcum* in the rhizosphere. *Azotobacter* sp. are non-symbiotic heterotrophic bacteria capable of fixing an average of 20 kg $Nha^{-1}year^{-1}$. Bacterization by *Azotobacter* sp. helps to improve plant growth and soil nitrogen through nitrogen fixation by utilizing carbon for its metabolism (Moniba *et al*., 1979).

Investigations of Brakel and Hilger (1965) reported that *Azotobacter* produced indole-3-acetic acid (IAA) in a medium containing tryptophan and also reported that maize seeds coated with *Azotobacter* caused stimulated germination to a significant level.

Rajaee *et al*. (2007) observed that inoculation of wheat seeds with *Azotobacter* helped in uptake of N, P and micronutrients like Fe and Zn. Besides nitrogen fixation, *Azotobacter* produces plant growth promoting substances like thiamine, riboflavin, nicotine, indole-3-acetic acid and gibberellin.

There are several reports which firmly support crop growth promoting activities of *Azospirillum* and *Azotobacter* (Okon et al., 1976; Sasikumar, 1996; Kavitha, 2001; Kizilkaya, 2009). Meenakumari *et al*. (2018) isolated 25 isolates of *Azospirillum* and 12 isolates of *Azotobacter* from the soil samples collected from undisturbed forest areas of Attappady hill tracts. All the isolates have shown to produce IAA and it ranged from 14.83 to 49.74 $\mu g\ ml^{-1}$ and 28.95 to 49.81 $\mu g\ ml^{-1}$ of culture filtrate for *Azospirillum* sp. and *Azotobacter* sp. respectively.

PGPR stimulate the nutrient availability to host plants by solubilization of phosphorus in the rhizosphere. According to the studies by Nautiyal *et al*. (2000) phosphate-solubilizing

bacteria are common in rhizospheres and improves nutrition to plants by phosphorous solubilization of rhizosphere soil.

Banerjee *et al*. (2005) reported that among the soil bacterial communities most powerful phosphate solubilizers belong to the genera *Bacillus*, *Rhizobium* and *Pseudomonas*. The most important P solubilizers include *Bacillus megaterium, Bacillus circulans, Bacillus subtilis, Bacillus polymyxa, Bacillus sircalmous, Pseudomonas striata* and *Enterobacter* (Kucey *et al*., 1989).

Phosphate mobilizing bacteria inoculation in plants showed increased plant growth and yield not only in field conditions but also under glasshouse conditions as well (Zaidi *et al*., 2009; Khan *et al*., 2010). Investigations of Meenakumari *et al*. (2008) reported that P solubilizers isolated from Kerala soils were highly efficient in releasing the soil phosphorus.

Investigations of Singh and Reddy (2011) reported that in wheat and maize under field condition phosphorous solubilizing microorganisms reduced the need of chemical or organic fertilizers. Karpagam and Nagalakshmi (2014) observed that the highest Phosphate Solubilization Index (PSI) for 8 microbial isolates of *Pseudomonas, Bacillus* and *Rhizobium* out of 37 isolates and the range of PSI was from 1.13 - 3.0.

Plant growth promoting rhizobacteria can produce organic acids which in turn solubilize potassium rock. Potassium solubilizing PGPR strains such as *Acidothiobacillus ferrooxidans*, *Bacillus edaphicus*, *Bacillus mucilaginosus*, *Pseudomonas* sp. are capable of releasing potassium in available form. Therefore addition of potassium solubilizing biofertilizers for crop production can greatly deduct the use of chemicals (Sakthidharan, 2011; Shanware *et al*., 2014).

Investigations of Sakthidharan (2011) reported that application of K solubilizers developed by KAU increased beta carotene, vitamin C and crude protein content in amaranthus. Chandra *et al*. (2005) observed that application of potash solubilizer in combination with other biofertilizers like *Rhizobium*, *Azospirillum*, *Azotobacter*, *Acetobacter* and phosphorous solubilizing microorganisms (PSM) in yam and tapioca increased the yield by 15-20 per cent.

Considerable release of K from muscovite by *Bacillus muciloginosus* was documented by Sugumaran and Janarthanam (2007). Use of these potential agents can enhance crop growth and productivity without losing soil quality. Inoculation of potash solubilizing bacteria in brinjal significantly enhanced the yield, plant height and K uptake compared to control as per the studies of Ramarethinam and Chandra (2006).

Most predominantly studied mechanism to describe the positive effects of PGPR on crop growth is their capacity to synthesize auxin. As per the studies of Patten and Glick (1996), plant growth hormone auxin was synthesized and secreted by 80% of rhizospheric microflora as a secondary metabolite. In addition to the aforementioned plant growth promoting mechanisms, many investigations reported the ability of PGPR to produce several other plant growth promoting substances including GA3, zeatin, ABA (Perrig *et al*., 2007) and siderophores (Beneduzi, 2012). Furthermore, they promote plant growth through production of antibiotics (Hill *et al*., 1994; Souza *et al*., 2003), hydrolytic enzyme production (Neeraja *et al*., 2010), induced systemic resistance (Van Loon *et al*., 1998) and exo polysaccharides production (Lloret *et al*., 1996; Rehm and Valla, 1996).

2.5 Bioencapsulated formulation

Considering the demerits associated with carrier based and liquid formulations, researches have focussed on development of encapsulated formulation of efficient plant growth promoting rhizobacteria without losing their ability to increase plant growth easier to handle for agricultural applications.

According to studies of Ding and Shah (2009) cell immobilization by enacapsulation or entrapment involves entrapping microbial cells or tissues within a polymeric substance to synthesize bead formulation which are permeable to nutrients, gases and metabolites for sustaining cell viability within it. According to the studies by John *et al*. (2011), bioencapsulation is the method of coating a protective shell surrounding the active substance or microbial cells.

The entrapment of microbial cells into a biopolymer such as alginate or xanthan gum helps to protect cells from environmental stresses and ensures slow release of immobilized microbes into the soil (Dommergues *et al*., 1979). This technique has been applied with success to *Azospirillum* (Bashan 1986; Fages 1992).

According to the studies of Bashan (1986) and Kim *et al*. (2012), the basic principle of bioencapsulation of beneficial rhizobacteria is to protect the immobilized bacteria applied to the soil and to promise a gradual and prolonged release.

Investigations by Trivedi and Pandey (2008) reported the long term survival of sodium alginate beads. Rhizobacteria such as *Bacillus subtilis* and *Pseudomonas corrugata* encapsulated

in sodium alginate biopolymer beads was found viable with 10^8 cfu g^{-1} without losing the capability to stimulate plant growth even after 3 years of storage at 4ºC.

Sodium alginate based encapsulated formulations of microbial inoculants have reported higher cell concentration than conventional formulations even after longer period of storage. The survival rate of *Azospirillum lipoferum* immobilized in dry alginate encapsulated beads was recorded 10^{10} cfu g^{-1} after 1 year of storage at room temperature condition and this concentration was higher when compared with the conventional solid carrier based and liquid microbial inoculants (Fages, 1992; Trejo *et al.*, 2012).

Several alginate based encapsulated formulations of *Bacillus subtilis* and *Pseudomonas corrugata* were found superior over liquid inoculants or charcoal-based inoculants for inoculating maize plants under low temperatures in the Indian Himalayas (Trivedi *et al*. 2008).

Inoculation with alginate macro bead formulations of *Trichoderma harzianum* strain 24 and *Azospirillum brasilense* reported remarkable enhancement in the growth variables of *Solanum lycopersicum* seedlings. The growth parameters of *Azospirillum brasilense* or *Trichoderma harzianum* strain 24 treated seeds using dry immobilized macro beads were significantly higher than those of the untreated control (Katatny, 2010).

2.5.1 Advantages of bioencapsulation over conventional formulations

The bioinoculants are generally applied as solid carrier based or liquid inoculants to the soil or to the seed directly. The solid carrier based inoculants suffer from inferior quality, high rate of contamination and low field performances (Cassidy *et al*., 1996).

Investigations of Fages (1992) reported that important objectives of a suitable biofertilizer formulation is the optimization of the cell viability during a long period of storage to ensure good protection of bacteria in soil and to provide a product easy to apply.

Various organic carriers experimented for formulation evolution include peat, turf, talc, lignite, kaolinite, pyrophyllite, zeolite, montmorillonite, alginate, pressmud, sawdust, vermiculite etc. An investigation by Vendan and Thangaraju (2006) reported that solid carrier based preparations generally suffer from short shelf-life, inferior quality, high rate of contamination and uncertain field performances.

Studies conducted by John *et al*. (2011) reported that talc based powder formulations have shorter shelf-life and reduced efficiency during longer storage periods which demands for the development of feasible alternate formulations with greater shelf life. The application of talc

based bioformulations through micro irrigation techniques encountered problems such as blockage of nozzles and rutted distribution of bio-inoculants. The major disadvantages linked with talc based inoculants are shorter shelf life, inferior quality, high rate of contamination, uncertain field performance and high cost as it is labour and energy intensive process, involving milling, sieving and correcting pH (Somasegaran and Hoben, 1994).

A solid based formulation usually contains peat as a carrier material. Peat is generally used because of its high content of organic matter and water holding capacity. But the studies of Cassidy *et al*. (1996) showed that talc and peat based inoculants expressed a higher variation in initial and final cell count and truncated survival on storage. Peat and talc formulations are not capable of ensuring high cell density and reported negative environmental impacts on peat available ecosystems (Hynes *et al*., 1995). Peat based microbial inoculant formulations couldnot promise a high cell concentration. Studies of Fallik and Okon (1996) showed deduction of bacterial population (10^{10} to 10^5 cfu g^{-1}) in peat inoculant of *Azospirillum brasilense* after 6 months of storage.

Considering the demerits of carrier based bioformulations many researches have already conducted experiments on development of alternative formulations such as liquid formulation of biofertilizers using different additives (Lorda and Balatti, 1996; Sridhar *et al*., 2004; Santhosh, 2015; Velineni and Brahmaprakash, 2011; Gopal and Baby, 2016). Liquid formulations that can rectify the disadvantages of powder formulation were introduced by many studies. Liquid formulations provides higher cell load in rhizosphere. Shelf life of liquid carriers can be increased up to 2 years if stored under refrigerated conditions and addition of antimicrobial agents reduces the chances of contamination. However liquid inoculants show quick and high decrease of viability during storage and transportation besides low survival of bacteria in the soil as it lacks carrier protection (Deaker *et al*., 2004).

Bacteria lack carrier protection in liquid formulations of inoculants and therefore survival of bacteria in such formulations is lesser with shorter shelf life. Increased cost of liquid inoculants and difficult to transport limit their use in developed countries as reported by Stephens and Rask (2000). PGPR liquid formulation could display a deduction in the number of viable cells from the initial concentration (Haggag and Singer, 2012).

Here comes the necessity of encapsulated microbial inoculants that rectifies the demerits associated with traditional peat or talc inoculants and liquid formulations which cause greater variability in quality (Albareda *et al.,* 2008).

Studies of Kim *et al* (1996) reported that bioencapsulation stabilizes microbial cells by greatly potentially increasing the viability and maintaining stability during production, storage and handling process. It also ensures a further protection in the course of rehydration. Moreover microbial cells trapped in the interstitial space in alginate gels can remain physiologically active and eventually grow within the beads.

The viability of microorganisms in encapsulated formulations has noticeably improved due to its shielding effect against deleterious environmental factors such as changes in pH and the noxious agents bring about during the process of bioencapsulation. It can also safeguard the bacterial cells from bacteriophages and other chemicals including hydrogen peroxide, short chain fatty acids, carbonyl-aromatic compounds, alcohols and drying (Mortazavian *et al*., 2007).

2.5.2 Bioencapsulation materials

Bioencapsulation of rhizobacteria have been done with the use of both natural and synthetic polymers. Hydrogels such as alginate, carrageenan, agar-agar and agarose which are extracted from seaweed are regarded as natural biopolymers and are synthesized by the processes of polymerization. Synthetic polymers used for bioencapsulation of microbial cells consists of polyacrylamides and polyurethane (Trevors *et al*., 1993; Cassidy *et al*., 1996).

According to the studies of *Chan et al*. (2009) immobilized beads provides adequate protection, controlled release and stabilization of bacteria within the beads. Calcium alginate based beads are architectured as network of loose structure holding large amount of water. Thus preparation of encapsulated beads with alginate alone without the use of any filler material may finally gives distorted beads and fails to provide adequate protection. Investigations of Bashan *et al*. (2002) reported that addition of base materials including starch or clay to the formulation may improve the dry matter content in the beads thereby increasing mechanical resistance and allows a progressive release of cells into the soil.

The entrapment of microbial cells into a biopolymer such as alginate or xanthan gum helps to protect cells from environmental stresses and ensures slow release of immobilized microbes into the soil (Dommergues *et al*., 1979; Jung *et al*., 1982).

Studies of Reineccius (1991) pointed out that carbohydrates such as starches, maltodextrins, corn syrup solid and acacia gums are used widely in spray-dried encapsulations. Different filler materials used for bioencapsulation includes talc (Kloepper and Schroth, 1981), alginate (Bashan, 1986), carrageenan (Cassidy *et al*., 1996), vermiculite and perlite (Temprano *et al*., 2002), alginate-starch (Schoebitz *et al*., 2012). Ivanoa *et al.* (2006) has developed alginate plus carrier based formulation for nitrogen fixing bacteria *Azospirillum* and found that the starch performed better than other filler materials mainly since it has increased the dry matter which is needed for stable microbial population during storage. Similar results were also obtained by Schoebitz *et al*. (2012) who stated that immobilized alginate beads amended with starch as filler material makes the stable manufacturing of alginate beads with a high cfu value.

2.6 Bioencapsulation techniques

Different techniques are used to encapsulate microbial cells and bioactive molecules which can be divided into three kinds of process such as physical, chemical and physiochemical methods. Physical methods of encapsulation include spray drying (Picot and Lacroix, 2003) and fluidized bed spray coating (Jacquot and Pernetti, 2003). Molecular inclusion (Godshall, 1997) and interfacial polymerization are included under chemical methods of encapsulation. Physiochemical methods of encapsulation include coacervation (Park and Chang, 2000) and ionic gelation (Lim and Sun, 1980).

Spray drying and cross linking are the major techniques of bioencapsulation applicable for agricultural industry inorder to bioencapsulate biofertilizer bacteria and biocontrol agents (Watanabe *et al*., 2002).

2.6.1 Ionic gelation or cross linking technique of bioencapsulation

Ionotropic gelation is the capability of polyelectrolytes to cross link in the presence of counter ions to develop hydrogel beads by dripping a cell-loaded solution of polymer into the aqueous solution of polyvalent cations. The cations diffuses into the cell-loaded polymeric drops, forming a three dimensional lattice of ionically crosslinked beads**.** Sodium alginate acts as the polyelectrolyte and calcium chloride as the counter ion (Lim and Sun, 1980). As per this technique of encapsulation, culture containing the microbial cells and sodium alginate (2%) is dripped into 0.1M calcium chloride solution to form the beads (Lim and Sun, 1980). Membrane of calcium alginate develops spontaneously around the drop, sustaining the bead shape in the calcium chloride aqueous system. Calcium gellify the whole bead drop through diffusion and

then kept as such in CaCl$_2$ solution for at least 30 minutes to 2 hours to cure the beads. Then the cured beads are stained out from CaCl$_2$ solution and are washed with sterile water and dried at room temperature. The major benefit of this technique is the biocompatibility, eventhough scaling up is hectic and the beads are frequently pervious to cells (Lacroix *et al*., 1990).

Covarrubias *et al*. (2012) have investigated that under unfavourable conditions alginate beads maintain cells within the beads and thus restrict harmful microbes in the soil from entering inside the beads. As per the studies of Schoebitz *et al*. (2012) addition of materials such as starch improves the shelf life of the alginate beads.

2.7 Evaluation of shelf life of encapsulated beads

Bashan (1986) has outlined the procedure for evaluation of shelf life of encapsulated beads. According to his studies beads were immersed in 0.1M concentration of potassium phosphate buffer (pH 6.8) which is known for its capability to solubilize alginate beads and incubated for 30 to 60 minutes at 30⁰C. Inorder to make ease the solubility, the encapsulated beads were strenuously shaken on a vortex mixer and the released bacteria were counted by the plate count method on appropriate agar plate.

2.8 Evaluation of slow release of entrapped bacteria from encapsulated beads

Release of bacteria from encapsulated beads of PGPR mix-I was determined at weekly intervals as per the procedure described by Bashan (1986). Beads containing immobilized bacteria were taken into 75 ml of sterile saline solution (0.85% [wt/vol] NaCl) and moderately shaken at $30^{°}$C for 24 hours. Triplicate samples of 0.5 ml of saline solution were collected and the number of released bacteria was determined by the plate count method in respective selective medium. The beads were rinsed twice with sterile water and transferred into a fresh sterile saline solution. The sequence of same procedure was repeated for another 24 hours and the beads were placed on a thin layer of water at $4^{°}$C for 30 days. After this 30 days of incubation, the same procedure was repeated and the number of released bacteria was determined by the plate count method.

2.9 Bioencapsulation of microorganisms for agricultural applications

The soil application of encapsulated bacterial beads composed of biopolymers such as polyacrylamide (Dommergues *et al*., 1979) or polysaccharides such as alginate (Diem *et al*., 1988; van Elsas *et al*., 1992) at the time of sowing is oftenly practiced for encapsulated biocontrol and biofertilizer. Studies by Bashan (1986) reported that the native microflora in the

soil acts upon the encapsulated beads and gradually degrades the polymer, resulting in the release of immobilized bacteria into the soil where crops that requires infection by PGPB are present. According to Liakos *et al*. (2014) biodegradation occurred possibly through the breaking of covalently linked (1-4) glycoside bonds of sodium alginate composed of unbranched chains of β-d-mannuronate (M) and α-l-guluronate (G) residues by the enhanced loss of the matrix with extended incubation time in natural soil conditions possibly by the combined action of soil microflora, soil moisture content, residual enzymatic activity and physical properties of soil.

2.9.1 Biocontrol activity of bioencapsulated formulations

Several studies have conducted by many researchers on disease and pest control potential of alginate based encapsulated formulation of microbial inoculants. Aino *et al*. (1997) reported that *Pseudomonas fluorescens* implanted in beads of sodium alginate origin had the capacity to generate biocontrol effect against bacterial wilt disease of *Solanum lycopersicum* by its establishment on the root. Sodium alginate pellets containing spores of the biocontrol strain *Trichoderma viride* ATCC 5244 effectively controlled soil borne plant pathogens like *Fusarium oxysporum*, *Phytophthora capsici* and *Verticillium dahliae* (Cho and Lee, 1999). Studies by Cote *et al*. (2001) pointed out an prolonged duration of mortality of *Choristoneura rosaceana* with an entrapped formulation of *Bacillus thuringiensis* var. *kurstaki* in comparison to an accepted *Bacillus thuringiensis* var. *kurstaki* based ordinary formulation.

2.9.2 Plant growth promotion of bioencapsulated formulations

Many researches have reported the plant growth promotion activity of alginate based encapsulated formulation of microbial inoculants over conventional formulations even after extended period of storage. Encapsulated strains of *Azospirillum brasilense* Cd and *Pseudomonas fluorescens* 313 have recorded population in the range of 10^5-10^6 cfu g^{-1} even after 14 years of storage and did not disssipate the capacity to promote growth of *Triticum aestivum* (Bashan and Gonzalez, 1999).

Katatny (2010) conducted studies on encapsulation of agriculturally important microorganisms and reported that inoculation with alginate macro bead formulations of *Trichoderma harzianum* and *Azospirillum brasilense* showed remarkable increment in the growth variables of *Solanum lycopersicum* seedlings.

2.9.3 Survival rate of bioencapsulated formulations

The survival rate of encapsulated formulation of biofertilizers and biocontrol agents are found to be high as compared to solid carrier based and liquid formulations. Studies conducted by many researchers pointed out that the viability of microorganisms in encapsulated formulation did not lose even after several years of storage under room and controlled conditions. The survival and metabolic activities of cells can be retained for prolonged time period with regulated release of bacteria into the natural habitat.

The investigations by Fages (1992) reported that after 1 year of storage at room temperature, the survival rate of *Azospirillum lipoferum* immobilized in alginate beads dry was 10^{10} cfu g^{-1}. The concentration in this situation was higher than the traditional microbial inoculants. After 3 years on storage conditions at 4⁰C, the survival rate of *Bacillus subtilis* and *Pseudomonas corrugata* immobilized in sodium alginate beads was 10^8 cfu g^{-1} without losing their capability to perform as plant biocontrol agents (Trivedi and Pandey, 2008).

Studies of Anith and Roystephen (2009) found that application of alginate encapsulated formulation of *Pseudomonas fluorescens* resulted in increased colonization of roots and crop growth in black pepper. They also investigated an enhanced number of primary roots and beneficial bacterial population when inoculated the black pepper roots with alginate beads of *Pseudomonas fluorescens* than conventional microbial inoculants of the same rhizobacteria.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The experiment on "Development of encapsulated formulation of PGPR mix-I and its evaluation", was carried out during the period from 2018-2020 in the Department of Agricultural Microbiology, College of Agriculture, Vellayani, Thiruvananthapuram. The details of the materials used and methods followed in the present study are presented in this chapter.

3.1 Standardization of protocol for preparation of calcium alginate based encapsulated bead formulation of PGPR mix-I.

3.1.1 Procurement of cultures of PGPR mix- I and maintenance in specific medium

The component cultures of PGPR mix-I viz., *Azospirillum lipoferum*, *Azotobacter chroococcum*, *Bacillus megaterium* and *Bacillus sporothermodurans* were procured from the Department of Agricultural Microbiology, College of Agriculture, Vellayani and the cultures were maintained in Nitrogen Free Bromothymol Blue (NFB) medium (Plate 1) (Dobereiner and Day, 1976), Jensenʹs medium (Plate 2) (Jensen, 1942), Pikovaskayaʹs medium (Plate 3) (Rao *et al.*, 1984), Nutrient Agar medium (Plate 4) (Salfinger and Tortorello, 2015) respectively as mother cultures. These cultures were maintained as the mother cultures of each of the component organisms of PGPR mix-1. All the cultures were preserved on slants of the respective selective medium at 4°C in a refrigerator for further use.

3.1.2 Preparation of encapsulated bead formulation of PGPR Mix-1

Encapsulated bead formulation of PGPR mix-I was prepared following standard procedures (Ivanova *et al*., 2006). A loopful of constituent cultures of PGPR mix-I on slants which were procured from the Department of Agricultural Microbiology were streaked on Petri dishes of the respective selective medium of each of the component cultures.

After 48 hours of incubation at 27°C, a well isolated colony of each of the component cultures of PGPR mix-1 from respective plates were inoculated to respective sterile broths of each of the selective medium in order to prepare the mother cultures. After 72 hours of incubation at 27°C, each bacteria inoculated broth showed turbidity which is an indication of bacterial growth in the broth. Along with turbidity, the colour of *Azospirillum lipoferum* inoculated Nitrogen Free Bromothymol Blue (NFB) medium has changed from green colour to blue colour which was a clear indication of growth of *Azospirillum lipoferum* in Nitrogen Free Bromothymol Blue (NFB) medium.

Two ml each of the mother cultures of component cultures of PGPR mix-I containing 7.5×10^9 cfu ml^{-1} of *Azospirillum lipoferum* , 8.2×10^9 cfu ml^{-1} of *Azotobacter chroococcum,* 1.50×10^{10} cfu ml^{-1} of *Bacillus megaterium* and 1.62×10^{10} cfu ml^{-1} of *Bacillus sporothermodurans* were inoculated to 100ml of the sterile PGPR mix-I medium and incubated for 72 hours at $27^{\circ}C$ (Sivaprasad, 2011). Sodium alginate and filler materials in dry powder form was autoclaved at 15 lbs pressure and $121^{\circ}C$ for 15 min (Thilagavathi *et al*., 2015., Bashan 1986). Two grams of sodium alginate (dry powder) was sterilized separately in 25mL glass vials. Filler materials i.e.; standard starch, wheat flour and talc, each at two different concentrations (10% and 15%) were also sterilized separately in glass vials. After 72 hours of incubation at $27^{\circ}C$, both sterile sodium alginate (2g) and filler material as per treatment dosage was added to 100 ml grown culture of PGPR mix-I medium and mixed thoroughly to form encapsulation matrix (Plate 5). The control containing sodium alginate alone without any filler material was also prepared.

For the preparation of beads, this mixture was dropped slowly into sterile solution of 0.1M calcium chloride using a sterile micropipette or automatically by an equipment layout by Anith (1993)**.** Membrane of calcium alginate developed spontaneously around the drop, sustained the bead shape in the calcium chloride aqueous system (Plate 6). Calcium gellified the whole bead drop through diffusion and then kept as such in $CaCl_2$ solution for 30 minutes to cure the beads (Plate 7). Then the cured beads were stained out from $CaCl_2$ solution and were washed with sterile water. The water was drained off completely and the beads were spread on a clean tray and kept inside the laminar air flow chamber for 15 hours for drying. After complete drying, the beads were stored hermetically in sterilized screw cap bottles (Ivanova *et al*., 2006).

3.1.3 Details of the Experiment

An experiment was carried out in completely randomized design with the following treatments with three replications each. The encapsulation matrix was standardized with the following filler materials at two different concentrations along with sodium alginate. Appropriate control treatment without filler material was also retained.

Design   : Complete Randomized Design

Treatments   : 7 [(3x2) +1]

Replications   : 3

Treatments

T1 - 10% Standard starch (Plate 8)

Plate 1. Mother culture of *Azospirillum lipoferum* in Nitrogen Free Bromothymol Blue (NFB) broth

Plate 2. Mother culture of *Azotobacter chroococcum* in Jenson's broth
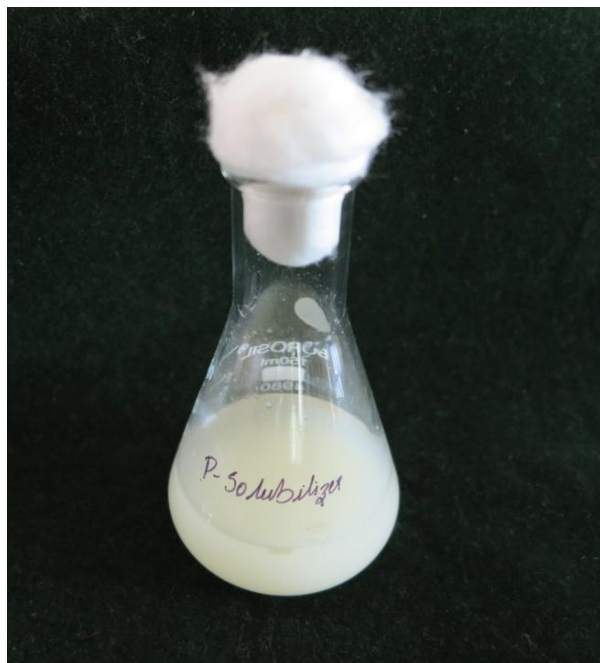
Plate 3. Mother culture of *Bacillus megaterium (P solubilizer)* in Pikovaskaya's broth
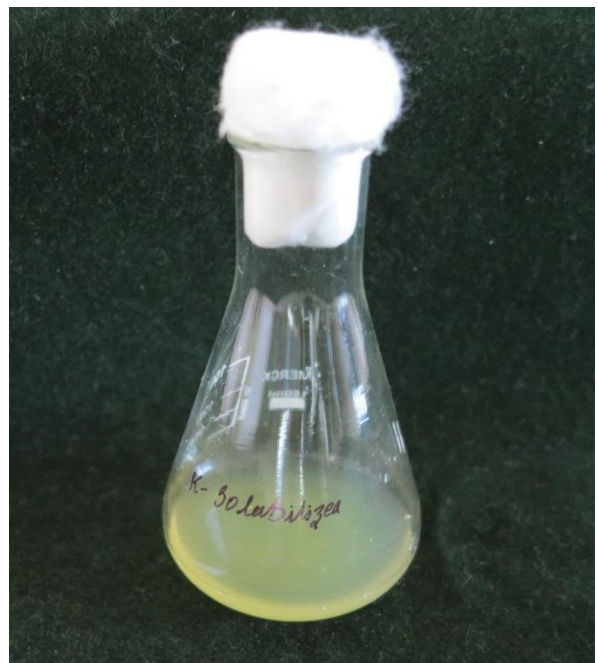
Plate 4. Mother culture of *Bacillus sporothermodurans* in Nutrient Agar broth
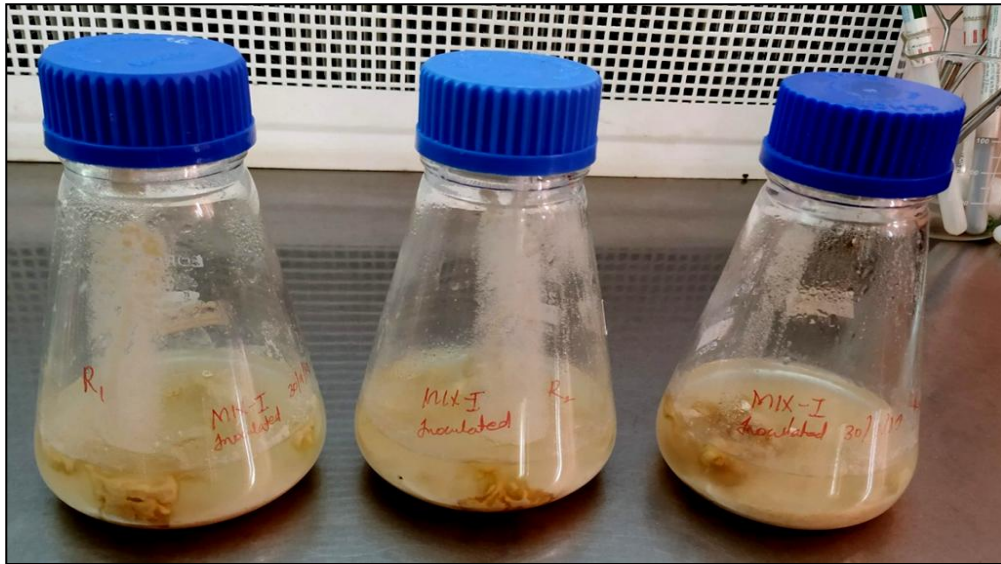
Plate 5. Culture of PGPR mix-I amended with 2% Sodium alginate and 10% Standard starch
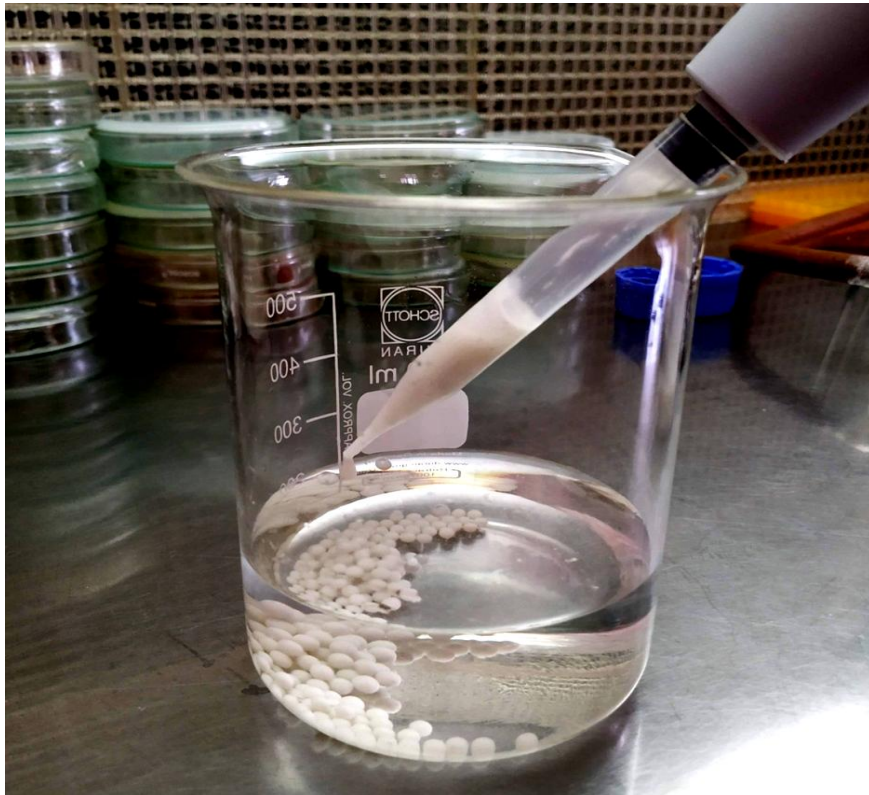
Plate 6. Bead formation in CaCl$_2$ solution

Plate 7. Encapsulated beads of PGPR mix-I in CaCl₂ solution

T2 - 15% Standard starch (Plate 12)

T3 - 10% Wheat flour (Plate 9)

T4 - 15% Wheat flour (Plate 13)

T5 - 10% Talc (Plate 10)

T6 - 15% Talc (Plate 14)

T7 - 2% Sodium Alginate alone (control) (Plate 11)

From mother cultures of *Azospirillum lipoferum*, *Azotobacter chroococcum*, *Bacillus megaterium* and *Bacillus sporothermodurans* in Nitrogen Free Bromothymol Blue (NFB) broth, Jenson's broth, Pikovaskaya's broth, Nutrient broth respectively, 2 ml of each of them were inoculated to 100 ml of sterile PGPR mix-1 medium (Sivaprasad, 2011). After 72 hours of incubation at $27^{\circ}C$, 2% sodium alginate and respective filler material were added to PGPR mix-1 culture which has attained the log phase and the contents were mixed thoroughly.

The population of each of the component cultures in each treatment was enumerated at monthly intervals for a period of 3 months in appropriate medium by serial dilution technique and plate count method (Timonin, 1940). The beads were dissolved in 0.1M of sterile potassium phosphate buffer having pH 6.8 (Bashan, 1986) and incubated at 30⁰C for 60 min. In order to facilitate the solubility, the beads were vigorously shaken in a vortex mixer for 2 minutes and the number of released bacteria were determined by serial dilution and plate count method in the respective selective medium. The experiment design was completely randomized design with three treatments replicated thrice. Based on the population of component cultures of PGPR mix-I, the best filler material was selected.

3.1.4 Estimation of moisture content of beads

The moisture content in prepared beads was determined by Gravimetric method. A clean, dry watch glass was weighed and 5g of beads were taken in the watch glass. Weight of watch glass along with beads was taken before oven drying and then heated in an oven for 5 hours at $65^{\circ}C$ to constant weight and cooled in a desiccators. The weight of watch glass along with beads was taken after drying. Moisture content of beads was estimated as percentage loss in its weight. The moisture content of beads was estimated using the formula

Moisture percentage by weight = 100(B-C)/B-A

A = weight of the watch glass

B = weight of the watch glass + weight of the beads before drying

C = weight of the watch glass + weight of the beads after drying

3.2 Evaluation of shelf life of encapsulated beads of PGPR Mix-I

Treatment one which was identified as the best treatment from the experiment 3.1 was further taken for evaluation of shelf life. The survival of constituent cultures of PGPR mix-I in the encapsulated beads which were stored in screw capped bottles was monitored at monthly intervals at room temperature as well as refrigerated conditions for a period of 6 months by serial dilution and plate count method (Timonin, 1940).

The beads were dissolved in 0.1M of sterile potassium phosphate buffer having pH 6.8 (Bashan, 1986) and incubated at 30⁰C for 60 min. Inorder to make ease the solubility, the encapsulated beads were strenuously shaken on a vortex mixer and the released bacteria were counted by the plate count method on appropriate agar plate. The experiment design was completely randomized design with three treatments replicated thrice.

3.3 Evaluation of rate of release of immobilized bacteria from beads.

Release of bacteria from encapsulated beads was determined as per the procedure described by Bashan (1986). Twenty beads containing immobilized bacteria was taken into 75 ml of sterile saline solution (0.85% [wt/vol] NaCl) in 150ml Erlenmeyer flask (Plate 15) and moderately shaken at 30⁰C for 24 hours. Then triplicate samples of 1ml solution containing encapsulated beads were collected and serially diluted to $10^{-7.}$ The number of released bacteria was determined by the plate count method in respective selective medium.

The beads were then rinsed with sterile water and again transferred into a fresh 75ml of sterile saline solution (0.85% [wt/vol] NaCl) in 150ml Erlenmeyer flask and moderately shaken at 32⁰C for another 24 hours. Triplicate samples of 1ml of saline solution were collected and serially diluted to $10^{-7.}$ The number of released bacteria was determined by the plate count method in respective selective medium as done before.

The jelly mucoid beads were then kept at 4⁰C on a Petri dish containing 1cm layer of water. After 30 days of incubation, the jelly mucoid beads were rinsed with sterile water and transferred into a fresh 75ml of sterile saline solution (0.85% [wt/vol] NaCl) in 150ml Erlenmeyer flask and gently shaken at 32⁰C for 24 hours. Triplicate samples of 1ml of saline solution were collected and serially diluted to $10^{-7.}$ The number of released bacteria was

Plate 8. Encapsulated beads of PGPR mix-I amended with 10% Standard starch
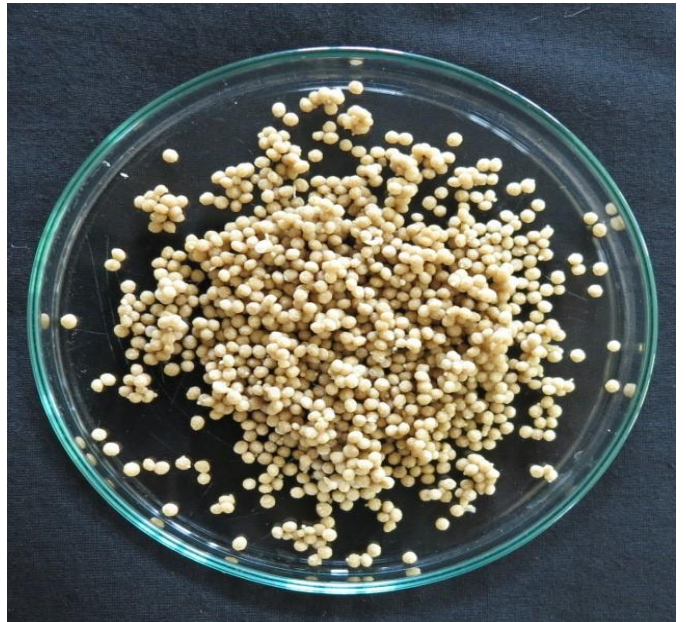
Plate 9. Encapsulated beads of PGPR mix-I amended with 10% Wheat flour

Plate 10. Encapsulated beads of PGPR mix-I amended with 10% Talc

Plate 11. Encapsulated beads of PGPR mix-I amended with 2% Sodium alginate

Plate 12. Encapsulated beads of PGPR mix-I amended with 15% Standard starch

Plate 13. Encapsulated beads of PGPR mix-I amended with 15% Wheat flour

Plate 14. Encapsulated beads of PGPR mix-I amended with 10% Talc

Plate 16. Encapsulated beads of PGPR mix-I amended with 10% Standard starch in sterile saline solution

determined by the plate count method as done before in respective selective medium. The experiment design was completely randomized design with three treatments replicated thrice.

3.4 Evaluation of biodegradation of encapsulated beads of PGPR mix-I.

The biodegradation of encapsulated PGPR mix-I was studied at weekly intervals for four weeks in sterile and non-sterile soil (Bashan, 1986). Hundred beads containing component cultures of PGPR mix-1 were placed in nylon nets having mesh size of 1.2mm, tied with thread and then buried in sterile and non-sterile soil with three replications each (Plate 16).

Beads were also prepared using sterile PGPR mix-1 media added with 2% sterile sodium alginate and 10% sterile standard starch without component cultures of PGPR mix-1. As done with beads inoculated with component cultures of PGPR mix-I, beads without component cultures of PGPR mix-I were also placed in nylon nets having mesh size of 1.2mm, tied with thread and then buried in sterile and non-sterile soil with three replications each.

Both the sets were observed weekly for their rate of biodegradation. From each replication, out of 100 beads, 25 beads were randomly selected and observed for their biodegradation rate at weekly intervals. As per biodegradation scale, values such as 0, >0-0.5, >0.5-1, >1-2, >2-2.5 or 3 were assigned according to the degree of visible degradation as indicated below (Bashan, 1986).

0- no visible degradation

>0-0.5- onset of degradation, undistinguishable

>0.5-1- slight visible degradation on bead edges

>1-2 - one-half to three-fourth of the beads degraded

>2-2.5 - 90% of beads become mushy

3 - Full degradation, beads are disintegrated into small pieces or not found in the nylon bag

Each of the randomly selected 25 beads of each replication was observed individually and a value was assigned as per biodegradation scale depending on their visible degradation. Then the average of sum of the values of biodegradation assigned to individual bead was taken. The value thus calculated represents the value of biodegradation of respective replication. Kruskal-Wallis rank sum was done test and there was a significant difference between treatments and so multiple comparison was done using Dunn test for week wise and treatment wise evaluation of biodegradation of beads.

3.5 STATISTICAL ANALYSIS

The data obtained from the present investigation were statistically analyzed using Analysis of Variance Technique (ANOVA) as applied to Completely Randomized Design described by Panse and Sukhatma (1985).

Non parametric data were analyzed using Kruskal-Wallis rank sum test (Kruskal and Wallis, 1952) and post hoc with Dunn's multiple comparison test for significance.

Plate 15. General view of Biodegradation study

RESULTS

4. RESULTS

The present study on "Development of encapsulated formulation of PGPR mix-I and its evaluation." was conducted during the period from 2018-20 in the Department of Agricultural Microbiology, College of Agriculture, Vellayani, Thiruvananthapuram, Kerala. The results based on statistically analyzed data pertaining to the experiment conducted during the course of investigation are presented below:

4.1 Standardization of protocol for preparation of calcium alginate based encapsulated bead formulation of PGPR mix-I.

4.1.1 Population of *Azospirillum lipoferum*, *Azotobacter chroococcum*, *Bacillus megaterium* and *Bacillus sporothermodurens* in encapsulated formulation of PGPR mix-I (cfu g^{-1}).

4.1.1.1 Population after 24 hours

After 24 hours of drying of encapsulated formulation of PGPR mix-I, the treatment T1 recorded the maximum significant population of *A. lipoferum*, *A. chroococcum*, *B. megaterium* and *B. sporothermodurens* of 1.46×10^{10} cfu g^{-1}, 1.75×10^{10} cfu g^{-1}, 1.87×10^{10} cfu g^{-1} and 1.75×10^{10} cfu g^{-1} respectively, which was significantly superior to all other treatments except in the case of population of *B. megaterium* which was statistically on par with that in the treatment T3 (1.74×10^{10} cfu g^{-1}). The control treatment recorded 7.5×10^{9} cfu g^{-1}, 1.08×10^{10} cfu g^{-1}, 1.52×10^{10} cfu g^{-1} and 1.13×10^{10} cfu g^{-1} of *A. lipoferum, A. chroococcum, B. megaterium* and *B. sporothermodurens* respectively (Table 1).

4.1.1.2 Population after first month

One month after inoculation, the treatment T1 recorded the maximum total viable count of *A. lipoferum* (1.37×10^{10} cfu g^{-1}) which was significantly superior to the control treatment which recorded 3.5×10^{9} cfu g^{-1}. The maximum colony count of *A. chroococcum* was observed in treatment T1 (1.69×10^{10} cfu g^{-1}). This was significantly superior to the control treatment which recorded 3.0×10^{9} cfu g^{-1}.

The treatment T1 (1.79×10^{10} cfu g^{-1}) recorded the highest population of *B. megaterium* which was significantly superior to the control treatment which recorded 5.8×10^{9} cfu g^{-1}. The maximum colony count of *B. sporothermodurens* was observed in treatment T1 (1.65×10^{10} cfu g^{-1}). This treatment was significantly superior to the control treatment which recorded 3.6×10^{9} cfu g^{-1} (Table 2).

4.1.1.3 Population after second month

From the observation on second month, it was noticed that the treatment T1 recorded the highest population of *A. lipoferum* (1.32×10^{10} cfu g^{-1}) which was significantly superior to the control treatment which recorded 2.8×10^{9} cfu g^{-1}. The maximum viable count of *A. chroococcum* was observed in treatment T1 (1.62×10^{10} cfu g^{-1}). This was significantly superior to the control treatment (2.8×10^{9} cfu g^{-1}). The treatment T1 (1.65×10^{10} cfu g^{-1}) recorded the highest population of *B. megaterium* which was significantly superior to the control treatment (5.3×10^{9} cfu g^{-1}).

Population of *B. sporothermodurens* was highest in treatment T1 (1.50×10^{10} cfu g^{-1}). This was followed by T5 (9.1×10^{9} cfu g^{-1}), T2 (8.8×10^{9} cfu g^{-1}) and T6 (8.8×10^{9} cfu g^{-1}). All these treatments were statistically on par with each other, these were also significantly superior to control treatment (3.2×10^{9} cfu g^{-1}) (Table 3).

4.1.1.4 Population after third month

Observations on third month indicated that the maximum population of *A. lipoferum* was observed in treatment T1 (1.26×10^{10} cfu g^{-1}) followed by T3 (3.2×10^{9} cfu g^{-1}). Both these treatments were significantly superior to control treatment (2.4×10^{9} cfu g^{-1}).The treatments T2 (2.8×10^{9} cfu g^{-1}), T5 (2.8×10^{9} cfu g^{-1}), T6 (2.8×10^{9} cfu g^{-1}) and T4 (2.7×10^{9} cfu g^{-1}) were statistically on par with both the treatments T3 (3.2×10^{9} cfu g^{-1}) and control treatment (2.4×10^{9} cfu g^{-1}).

Similarly, maximum population of *A. chroococcum* was observed in treatment T1 (1.55×10^{10} cfu g^{-1}) followed by T3 (5.9×10^{9} cfu g$^{-1)}$) and T2 (4.5×10^{9} cfu g^{-1}). All these treatments were significantly superior to control treatment (2.7×10^{9} cfu g^{-1}). Treatments T4 (3.0×10^{9} cfu g^{-1}), T5 (2.9×10^{9} cfu g^{-1}), T6 (3.2×10^{9} cfu g^{-1}) were statistically on par with the control treatment (2.7×10^{9} cfu g^{-1}).

The treatment T1 (1.62×10^{10} cfu g^{-1}) recorded the highest population of *B. megaterium* which was significantly superior to the control treatment (4.7×10^{9} cfu g^{-1}). Treatment T3 (7.2×10^{9} cfu g^{-1}) recorded the second highest population. Control treatment (4.7×10^{9} cfu g^{-1})

recorded the third highest population which was statistically on par with the treatment T2 $(4.6 \times 10^9$ cfu g$^{-1})$. Treatment T5 $(4.0 \times 10^9$ cfu g$^{-1})$ was statistically on par with the treatment T4 $(3.8 \times 10^9$ cfu g$^{-1})$ and the treatment T4 $(3.8 \times 10^9$ cfu g$^{-1})$ was statistically on par with both the treatments T5 $(4.0 \times 10^9$ cfu g$^{-1})$ and T6 $(3.5 \times 10^9$ cfu g$^{-1})$.

The maximum colony count of *B. sporothermodurens* was observed in treatment T1 $(1.52 \times 10^{10}$ cfu g$^{-1})$ which was significantly superior to the control treatment which recorded 2.5×10^9 cfu g^{-1}. The second highest population was recorded in treatment T6 $(7.5 \times 10^9$ cfu g$^{-1})$ which was statistically on par with the treatment T2 $(7.1 \times 10^9$ cfu g$^{-1})$ (Table 4).

Table 1. Population of component organisms of PGPR mix-1 in encapsulated formulation with different filler materials after 24 hours

TREATMENTS	VIABLE COUNT AFTER 24 HOURS (log cfu g^{-1})			
	Azospirillum lipoferum	*Azotobacter chroococcum*	P solubilizer (*Bacillus megaterium*)	K solubilizer (*Bacillus sporothermodurens*)
T1-10% STANDARD STARCH	10.163 ^{a}	10.243^a	10.273^a	10.240^a
T2-15% STANDARD STARCH	9.753^e	9.927^c	9.927^e	10.097^b
T3-10% WHEAT FLOUR	9.923^c	10.033^b	10.240^a	10.083^b
T4-15% WHEAT FLOUR	9.857^d	9.813^d	10.130^c	10.073^b
T5-10% TALC	10.077^b	10.050^b	10.087^d	10.060^b
T6-15% TALC	9.580^f	9.777^d	9.863^f	10.047^b
T7-2% SODIUM ALGINATE(CONTROL)	9.873^{cd}	10.033^b	10.180^b	10.053^b
SEm ($\pm$)	0.021	0.022	0.011	0.017
CD (0.05)	0.064	0.066	0.034	0.052

Each value represents a mean of 3 replications

Figures in a column followed by same letters do not differ significantly at p≥0.05

Table 2. Population of component organisms of PGPR mix-1 in encapsulated formulation with different filler materials after first month of inoculation

TREATMENTS	VIABLE COUNT AFTER FIRST MONTH (log cfu g^{-1})			
	Azospirillum lipoferum	*Azotobacter chroococcum*	P solubilizer (*Bacillus megaterium*)	K solubilizer (*Bacillus sporothermodurens*)
T1-10% STANDARD STARCH	10.137^a	10.227^a	10.253^a	10.217^a
T2-15% STANDARD STARCH	9.650^c	9.863^b	9.813^d	10.033^b
T3-10% WHEAT FLOUR	9.580^{cde}	9.847^b	9.943^c	9.823^c
T4-15% WHEAT FLOUR	9.617^{cd}	9.667^c	9.823^d	9.877^c
T5-10% TALC	10.033^b	9.920^b	10.060^b	10.027^b
T6-15% TALC	9.550^{de}	9.727^c	9.820^d	10.013^b
T7-2% SODIUM ALGINATE(CONTROL)	9.540^e	9.473^d	9.763^d	9.557^d
SEm (±)	0.023	0.025	0.022	0.023
CD (0.05)	0.070	0.076	0.067	0.069

Each value represents a mean of 3 replications

Figures in a column followed by same letters do not differ significantly at p≥0.05

Table 3. Population of component organisms of PGPR mix-1 in encapsulated formulation with different filler materials after second month of inoculation

TREATMENTS	VIABLE COUNT AFTER SECOND MONTH (log cfu g^{-1})			
	Azospirillum lipoferum	*Azotobacter chroococcum*	P solubilizer (*Bacillus megaterium*)	K solubilizer (*Bacillus sporothermodurens*)
T1-10% STANDARD STARCH	10.120^a	10.207^a	10.217^a	10.177^a
T2-15% STANDARD STARCH	9.573^C	9.777^b	9.787^c	9.943^b
T3-10% WHEAT FLOUR	9.560^c	9.800^b	9.910^b	9.800^c
T4-15% WHEAT FLOUR	9.547^C	9.560^d	9.727^d	9.783^c
T5-10% TALC	9.767^b	9.823^b	9.943^b	9.960^b
T6-15% TALC	9.453^d	9.667^c	9.797^c	9.943^b
T7-2% SODIUM ALGINATE(CONTROL)	9.453^d	9.453^e	9.727^d	9.500^d
SEm (±)	0.024	0.020	0.016	0.017
CD (0.05)	0.072	0.061	0.047	0.051

Each value represents a mean of 3 replications

Figures in a column followed by same letters do not differ significantly at p≥0.05

Table 4. Population of component organisms of PGPR mix-1 in encapsulated formulation with different filler materials after third month of inoculation

TREATMENTS	VIABLE COUNT AFTER THIRD MONTH (log cfu g^{-1})			
	Azospirillum lipoferum	*Azotobacter chroococcum*	P solubilizer (*Bacillus megaterium*)	K solubilizer (*Bacillus sporothermodurens*)
T1-10% STANDARD STARCH	10.097^a	10.190^a	10.207^a	10.183^a
T2-15% STANDARD STARCH	9.443^{bc}	9.650^c	9.660^c	9.853^b
T3-10% WHEAT FLOUR	9.500^b	9.767^b	9.863^b	9.740^c
T4-15% WHEAT FLOUR	9.427^{bc}	9.473^d	9.580^{de}	9.587^d
T5-10% TALC	9.453^{bc}	9.467^d	9.597^d	9.823^{bc}
T6-15% TALC	9.443^{bc}	9.507^d	9.537^e	9.877^b
T7-2% SODIUM ALGINATE(CONTROL)	9.377^c	9.430^d	9.667^c	9.393^e
SEm ($\pm$)	0.025	0.025	0.018	0.028
CD (0.05)	0.077	0.077	0.054	0.086

Each value represents a mean of 3 replications

Figures in a column followed by same letters do not differ significantly at p≥0.05

4.1.2 Estimation of moisture content of encapsulated beads (%)

The moisture content of encapsulated beads of each treatment of experiment 4.1.1 was monitored for a period of three months at monthly intervals at room temperature and it showed a significant variation among treatments in each month. A reduction in moisture content of beads was observed from the first month to the end of third month and the results are presented (Table 5).

4.1.2.1 Moisture content of beads after 24 hours

After 24 hours of drying, highest moisture content of 28.53% was recorded in control treatment T7. Second highest moisture content of 13.73% was recorded in treatment T2. Treatment T1 (13.37%) was statistically on par with the treatment T4 (13.34%) and recorded the third highest percentage of moisture content (Table 5).

4.1.2.2 Moisture content of beads after first month of storage

Moisture content recorded after first month of storage showed significantly a higher moisture content of 17.66% in control treatment T7. Second highest moisture content of 12.07% was recorded in treatment T1. Treatment T5 (6.59%) and treatment T6 (6.42%) were statistically on par with each other. Treatment T2 recorded the third highest percentage of moisture content (11.44%) (Table 5).

4.1.2.3 Moisture content of beads after second month of storage

After second month of storage, highest moisture content of 12.53% was recorded in control treatment T7. Second highest moisture content of 11.72% was recorded in treatment T1. Treatment T5 (6.03%) recorded the least moisture content (Table 5).

4.1.2.4 Moisture content of beads after third month of storage

Moisture content recorded after third month of storage showed significantly superior moisture content of 11.45% in treatment T1. Second highest moisture content of 10.40% was recorded in treatment T2. Control treatment T7 (9.05%) recorded the third highest percentage of moisture content. Treatment T5 recorded least percentage of moisture content (5.73%) (Table 5).

Table 5. Moisture content of encapsulated formulation of PGPR mix-I during three months of standardization

TREATMENTS (%)	MOISTURE CONTENT (%)			
	AFTER 24 HOURS	FIRST MONTH	SECOND MONTH	THIRD MONTH
T1-10% STANDARD STARCH	13.37 ^{c}	12.07 ^{b}	11.72 ^{b}	11.45 ^{a}
T2-15% STANDARD STARCH	13.73 ^{b}	11.44 ^{c}	11.08 ^{c}	10.40 ^{b}
T3-10% WHEAT FLOUR	12.70 ^{d}	9.53 ^{d}	8.12 ^{d}	7.60 ^{d}
T4-15% WHEAT FLOUR	13.34 ^{c}	8.43 ^{e}	7.35 ^{e}	7.05 ^{e}
T5-10% TALC	6.53 ^{f}	6.59 ^{f}	6.03 ^{g}	5.73 ^{g}
T6-15% TALC	7.35 ^{e}	6.42 ^{f}	6.37 ^{f}	6.25 ^{f}
T7-SODIUM ALGINATE(CONTROL)	28.53 ^{a}	17.66 ^{a}	12.53 ^{a}	9.05 ^{c}
SEm ($\pm$)	0.106	0.108	0.077	0.088
CD (0.05)	0.322	0.328	0.233	0.268

Each value represents a mean of 3 replications

Figures in a column followed by same letters do not differ significantly at p$\geq$0.05

4.2 Evaluation of shelf life of encapsulated beads of PGPR mix-I

The best treatment selected from experiment 1 viz., 10% Standard starch amended alginate beads, was monitored for survival of component cultures of PGPR mix-I at monthly intervals at room temperature condition (Treatment 1) and refrigerated condition at 4^0C (Treatment 2) for a period of 6 months by serial dilution and plate count method. The moisture content of encapsulated beads (%) at monthly intervals at room temperature condition (Treatment 1) and refrigerated condition (Treatment 2) for a period of 6 months was also monitored.

Initial population of component organisms of PGPR mix-I in encapsulated formulation with 10% Standard starch after 24 hours of drying was recorded before evaluating the shelf life at room temperature and refrigerated condition for a period of 6 months. After 24 hours of drying of encapsulated formulation of PGPR mix-I, 10% standard starch amended alginate beads recorded a population of *A. lipoferum*, *A. chroococcum*, *B. megaterium* and *B. sporothermodurens* of 1.46×10^{10} cfu g^{-1}, 1.75×10^{10} cfu g^{-1}, 1.87×10^{10} cfu g^{-1} and 1.75×10^{10} cfu g^{-1} respectively.

4.2.1 Population of *Azospirillum lipoferum*, *Azotobacter chroococcum*, *Bacillus megaterium* and *Bacillus sporothermodurens* in encapsulated formulation of PGPR mix-I (cfu g^{-1})

4.2.1.1 Viable count after first month of storage

After one month of storage, the treatment T1 recorded the maximum significant population of *A. lipoferum*, *A. chroococcum*, *B. megaterium* and *B. sporothermodurens* of 1.37×10^{10} cfu g^{-1}, 1.69×10^{10} cfu g^{-1}, 1.79×10^{10} cfu g^{-1} and 1.65×10^{10} cfu g^{-1} respectively, which was significantly superior to treatment T2 which recorded 5.50×10^9 cfu g^{-1}, 7.40×10^9 cfu g^{-1}, 7.80×10^9 cfu g^{-1} and 1.50×10^{10} cfu g^{-1} of *A. lipoferum, A. chroococcum, B. megaterium* and *B. sporothermodurens* respectively (Table 6).

4.2.1.2 Viable count after second month of storage

Treatment T1 recorded the maximum significant population of *A. lipoferum*, *A. chroococcum*, *B. megaterium* and *B. sporothermodurens* of 1.32×10^{10} cfu g^{-1}, 1.61×10^{10} cfu g^{-1}, 1.65×10^{10} cfu g^{-1} and 1.50×10^{10} cfu g^{-1} respectively after second month of storage, which was significantly superior to treatment T2 which was recorded 4.7×10^9 cfu g^{-1}, 6.5×10^9 cfu g^{-1}, 7.5×10^9 cfu g^{-1} and 1.31×10^{10} cfu g^{-1} of *A. lipoferum, A. chroococcum, B. megaterium* and *B. sporothermodurens* respectively (Table 7).

4.2.1.3 Viable count after third month of storage

Treatment T1 recorded the maximum significant population of *A. lipoferum*, *A. chroococcum*, *B. megaterium* and *B. sporothermodurens* of 1.25×10^{10} cfu g^{-1}, 1.55×10^{10} cfu g^{-1}, 1.61×10^{10} cfu g^{-1} and 1.52×10^{10} cfu g^{-1} respectively after third month of storage, which was significantly superior to treatment T2 which recorded 3.6×10^{9} cfu g^{-1}, 6.3×10^{9} cfu g^{-1}, 6.5×10^{9} cfu g^{-1} and 1.25×10^{10} cfu g^{-1} of *A. lipoferum, A. chroococcum, B. megaterium* and *B. sporothermodurens* respectively (Table 8).

4.2.1.3 Viable count after fourth month of storage

After fourth month of storage, the treatment T1 recorded the maximum significant population of *A. lipoferum*, *A. chroococcum*, *B. megaterium* and *B. sporothermodurens* of 1.12×10^{10} cfu g^{-1}, 1.46×10^{10} cfu g^{-1}, 1.53×10^{10} cfu g^{-1} and 1.42×10^{10} cfu g^{-1} respectively, which was significantly superior to treatment T2 which recorded 3.1×10^{9} cfu g^{-1}, 5.0×10^{9} cfu g^{-1}, 4.7×10^{9} cfu g^{-1} and 9.2×10^{9} cfu g^{-1} of *A. lipoferum, A. chroococcum, B. megaterium* and *B. sporothermodurens* respectively (Table 9).

4.2.1.3 Viable count after fifth month of storage

Treatment T1 recorded the maximum significant population of *A. lipoferum*, *A. chroococcum*, *B. megaterium* and *B. sporothermodurens* of 9.7×10^{8} cfu g^{-1}, 1.33×10^{9} cfu g^{-1}, 1.40×10^{9} cfu g^{-1} and 1.31×10^{9} cfu g^{-1} respectively after fifth month of storage, which was significantly superior to treatment T2 which recorded 2.5×10^{8} cfu g^{-1}, 3.5×10^{8} cfu g^{-1}, 3.8×10^{8} cfu g^{-1} and 8.0×10^{8} cfu g^{-1} of *A. lipoferum, A. chroococcum, B. megaterium* and *B. sporothermodurens* respectively (Table 10).

4.2.1.3 Viable count after sixth month of storage

After sixth month of storage, the treatment T1 recorded the maximum significant population of *A. lipoferum*, *A. chroococcum*, *B. megaterium* and *B. sporothermodurens* of 8.1×10^{8} cfu g^{-1}, 1.15×10^{9} cfu g^{-1}, 1.20×10^{9} cfu g^{-1} and 1.23×10^{9} cfu g^{-1} respectively, which was significantly superior to treatment T2 which recorded 1.9×10^{8} cfu g^{-1}, 2.6×10^{8}

cfu g^{-1}, 2.6×10^8 cfu g^{-1} and 5.9×10^8 cfu g^{-1} of *A. lipoferum, A. chroococcum, B. megaterium* and *B. sporothermodurens* respectively (Table 11).

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Table 6. Population of component organisms of PGPR mix-1 in encapsulated formulation amended with 10% Standard starch after first month of storage at room temperature and refrigerated condition

TREATMENTS	VIABLE COUNT AFTER FIRST MONTH (log cfu g^{-1})			
	Azospirillum lipoferum	*Azotobacter chroococcum*	*Bacillus megaterium*	*Bacillus sporothermodurens*
T1 - 10% STANDARD STARCH (ROOM TEMPERATURE)	10.137	10.227	10.253	10.217
T2 - 10% STANDARD STARCH (REFRIGERATED CONDITION)	9.740	9.870	9.893	10.177
P VALUE	0.00008	0.000005	0.00016	0.03163

Each value represents a mean of 3 replications

Table 7. **Population of component organisms of PGPR mix-1 in encapsulated formulation amended with 10% Standard starch after second month of storage at room temperature and refrigerated condition**

TREATMENTS	VIABLE COUNT AFTER SECOND MONTH (log cfu g^{-1})			
	Azospirillum lipoferum	*Azotobacter chroococcum*	*Bacillus megaterium*	*Bacillus sporothermodurens*
T1 - 10% STANDARD STARCH (ROOM TEMPERATURE)	10.120	10.207	10.217	10.177
T2 - 10% STANDARD STARCH (REFRIGERATED CONDITION)	9.667	9.813	9.877	10.117
P VALUE	0.00018	0.00005	0.00004	0.008581

Each value represents a mean of 3 replications

Table 8. Population of component organisms of PGPR mix-1 in encapsulated formulation amended with 10% Standard starch after third month of storage at room temperature and refrigerated condition

TREATMENTS	VIABLE COUNT AFTER THIRD MONTH (log cfu g^{-1})			
	Azospirillum lipoferum	*Azotobacter chroococcum*	*Bacillus megaterium*	*Bacillus sporothermodurens*
T1 - 10% STANDARD STARCH (ROOM TEMPERATURE)	10.097	10.190	10.207	10.183
T2 - 10% STANDARD STARCH (REFRIGERATED CONDITION)	9.560	9.800	9.813	10.097
P VALUE	0.00002	0.000005	0.00005	0.00005

Each value represents a mean of 3 replications

Table 9. Population of component organisms of PGPR mix-1 in encapsulated formulation amended with 10% Standard starch after fourth month of storage at room temperature and refrigerated condition

TREATMENTS	VIABLE COUNT AFTER FOURTH MONTH (log cfu g^{-1})			
	Azospirillum lipoferum	*Azotobacter chroococcum*	*Bacillus megaterium*	*Bacillus sporothermodurens*
T1 - 10% STANDARD STARCH (ROOM TEMPERATURE)	10.047	10.163	10.183	10.153
T2 - 10% STANDARD STARCH (REFRIGERATED CONDITION)	9.490	9.697	9.663	9.963
P VALUE	0.00003	0.00007	0.00023	0.00004

Each value represents a mean of 3 replications

Table 10. **Population of component organisms of PGPR mix-1 in encapsulated formulation amended with 10% Standard starch after fifth month of storage at room temperature and refrigerated condition**

TREATMENTS	VIABLE COUNT AFTER FIFTH MONTH (log cfu g^{-1})			
	Azospirillum lipoferum	*Azotobacter chroococcum*	*Bacillus megaterium*	*Bacillus sporothermodurens*
T1 - 10% STANDARD STARCH (ROOM TEMPERATURE)	8.987	9.123	9.147	9.117
T2 - 10% STANDARD STARCH (REFRIGERATED CONDITION)	8.393	8.547	8.580	8.903
P VALUE	0.000349	0.00004	0.00002	0.00019

Each value represents a mean of 3 replications

Table 11. Population of component organisms of PGPR mix-1 in encapsulated formulation amended with 10% Standard starch after sixth month of storage at room temperature and refrigerated condition

TREATMENTS	VIABLE COUNT AFTER SIXTH MONTH (log cfu g^{-1})			
	Azospirillum lipoferum	*Azotobacter chroococcum*	*Bacillus megaterium*	*Bacillus sporothermodurens*
T1 - 10% STANDARD STARCH (ROOM TEMPERATURE)	8.910	9.060	9.077	9.090
T2 - 10% STANDARD STARCH (REFRIGERATED CONDITION)	8.273	8.407	8.407	8.773
P VALUE	0.000204	0.000101	0.00009	0.00007

Each value represents a mean of 3 replications

4.2.2 Moisture content of beads during six months of storage at room temperature and refrigerated condition

The moisture content of encapsulated beads of the best treatment selected from experiment 4.1.1 viz., 10% standard starch was monitored for a period of six months stored at monthly intervals at room temperature (Treatment 1) and refrigerated condition (Treatment 2) and it showed a significant variation in each month. A reduction in moisture content of beads was observed from the first month to the end of sixth month and the results are presented in Table 12.

4.2.2.1 Moisture content of beads after first month of storage

After one month of storage, highest moisture content of 12.83% was recorded in beads stored at refrigerated temperature, whereas beads stored under room temperature recorded a moisture content of 12.07% (Table 12).

4.2.2.2 Moisture content of beads after second month of storage

Moisture content recorded after two months of storage showed significantly superior moisture content of 12.28% in alginate beads stored at refrigerated condition. However, a slight decrease in moisture content was recorded in beads stored at room temperature from 12.07% to 11.72% (Table 12).

4.2.2.3 Moisture content of beads after third month of storage

A slight decline in moisture content was observed in beads stored at refrigerated temperature as well as in room temperature. Moisture percentage of 12.24% and 11.45% was recorded in the beads stored at refrigerated condition and room temperature respectively (Table 12).

4.2.2.4 Moisture content of beads after fourth month of storage

Observations on fourth month after storage, revealed a maximum moisture content of 12.25% was recorded in alginate beads stored at refrigerated condition which was statistically superior to the moisture content of beads stored at room temperature (11.30%) (Table 12).

4.2.2.5 Moisture content of beads after fifth month of storage

During the fifth month of storage, significantly higher moisture content of 11.70% was observed in alginate beads stored at refrigerated conditions. However, a reduction in moisture content was recorded in the beads stored under room temperature which recorded a moisture percentage of 11.06% (Table 12).

4.2.2.6 Moisture content of beads after sixth month of storage

Moisture content observed after sixth months of storage showed statistically significant moisture percentage of 11.45% in the beads stored under refrigerated temperature compared to beads stored in room temperature which recorded a moisture percentage of 10.69% (Table 12).

Table 12. Moisture content of encapsulated beads of PGPR mix-I beads stored at room temperature and refrigerated condition at monthly intervals

TREATMENTS	MOISTURE CONTENT (%)					
	FIRST MONTH	SECOND MONTH	THIRD MONTH	FOURTH MONTH	FIFTH MONTH	SIXTH MONTH
T1 - 10% STANDARD STARCH (ROOM TEMPERATURE)	12.070	11.723	11.450	11.297	11.063	10.693
T2 - 10% STANDARD STARCH (REFRIGERATED CONDITION)	12.833	12.283	12.237	12.250	11.703	11.450
P VALUE	0.00223	0.00964	0.00170	0.00211	0.02328	0.00998

Each value represents a mean of 3 replications

4.3 Evaluation of rate of release of immobilized bacteria from encapsulated beads of PGPR mix-I

4.3.1 Rate of release of *Azospirillum lipoferum*, *Azotobacter chroococcum*, *Bacillus megaterium* and *Bacillus sporothermodurens* in encapsulated PGPR mix-I (cfu g^{-1}).

Data taken after 24 hours of incubation in saline solution at 32$^{°}$C (T1) showed adequate release of immobilized bacteria from alginate beads (Plate 17). After 24 hours, population of *A. lipoferum*, *A. chroococcum*, *B. megaterium* and *B. sporothermodurens* recorded were 2.42×10^6 cfu g^{-1}, 2.24×10^6 cfu g^{-1}, 2.47×10^6 cfu g^{-1} and 2.36×10^6 cfu g^{-1} respectively, which was significantly superior to treatments T2 and T3 (Table 13).

After 48 hours of incubation in saline solution at 32$^{°}$C (T2), population of *A. lipoferum, A. chroococcum, B. megaterium* and *B. sporothermodurens* recorded were 1.82×10^6 cfu g^{-1}, 1.68×10^6 cfu g^{-1}, 2.33×10^6 cfu g^{-1} and 2.08×10^6 cfu g^{-1} respectively, which was significantly superior to treatment T3 (Table 13 and Plate 18).

After 30 days of incubation on a thin layer (2cm thickness) of sterile water at 4$^{°}$C (T3), population of *A. lipoferum*, *A. chroococcum*, *B. megaterium* and *B. sporothermodurens* recorded were 1.37×10^6 cfu g^{-1}, 1.32×10^6 cfu g^{-1}, 1.72×10^6 cfu g^{-1} and 1.44×10^6 cfu g^{-1} respectively (Table 13 and Plate 19).

Table 13. Rate of release of immobilized bacteria from encapsulated beads of PGPR mix-I

TREATMENTS	RELEASE OF BACTERIA FROM IMMOBILIZED BEADS (log cfu g^{-1})			
	Azospirillum lipoferum	*Azotobacter chroococcum*	P solubilizer (*Bacillus megaterium*)	K solubilizer (*Bacillus sporothermodurens*)
T1-24 HOURS	6.383^a	6.350^a	6.390^a	6.373^a
T2-48 HOURS	6.260^b	6.223^b	6.367^b	6.317^b
T3-30 DAYS	6.137^c	6.117^c	6.233^c	6.160^c
SEm (±)	0.020	0.018	0.016	0.010
CD (0.05)	0.068	0.062	0.055	0.034

Each value represents a mean of 3 replications

Figures in a column followed by same letters do not differ significantly at p≥0.05
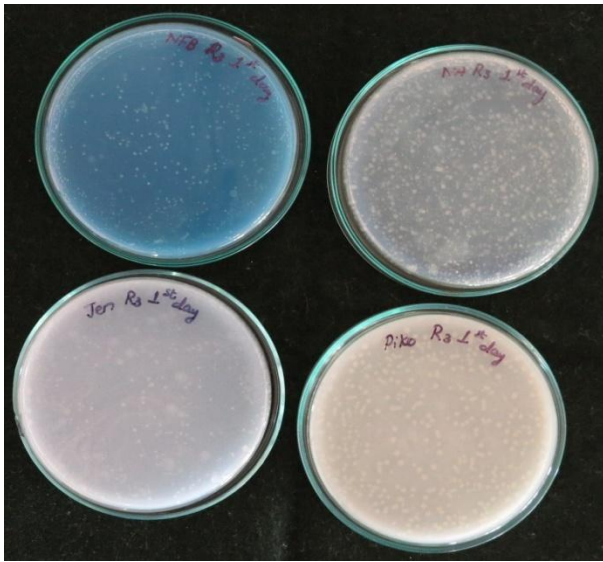
Plate 17. T1 – 24 hours (10^3 dilution)

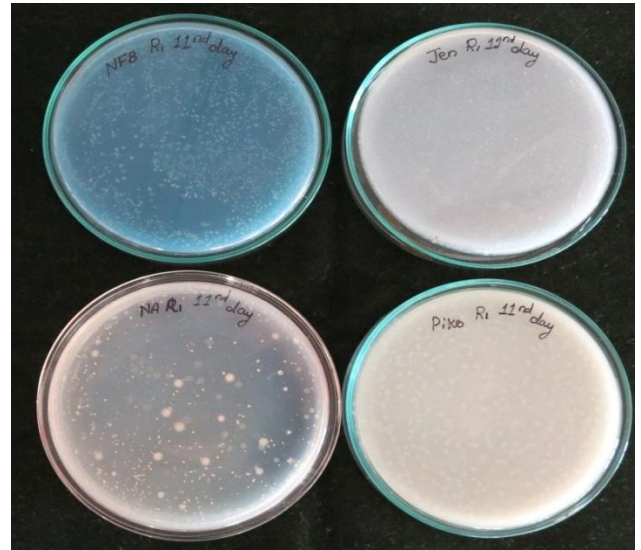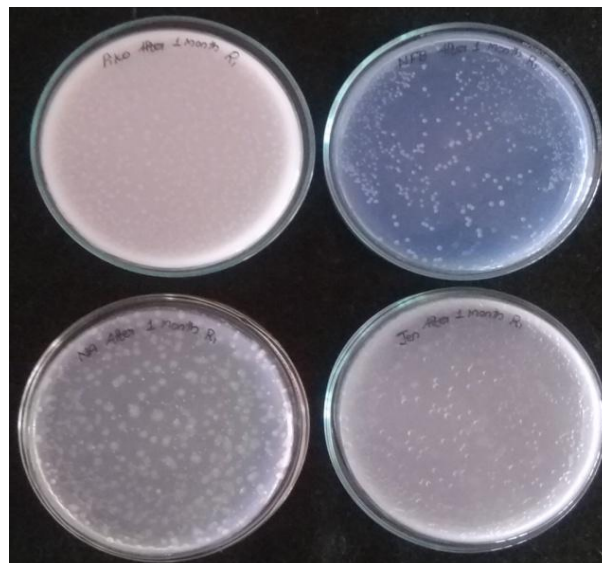**Plate 18. T2 – 48 hours (10^3 dilution)**

Plate 19. T3 – 30 days (10^3 dilution)

Rate of release of component organisms of PGPR mix-1 in encapsulated formulation amended with 10% Standard starch after 24 hours, 48 hours and 30 days

4.4 Evaluation of biodegradation of encapsulated beads of PGPR mix-I

The biodegradation of encapsulated beads of PGPR mix-I with 10% starch as filler material was studied at weekly intervals in sterile and non sterile soil. According to the degree of visible degradation, values were assigned as per biodegradation scale.

4.4.1 Evaluation of biodegradation of encapsulated beads in sterile soil

4.4.1.1 Degradation during first week

During the first week, beads with PGPR mix-I showed onset of degradation and biodegradation value of 0.36 and 0.12 were recorded respectively for each treatment as biodegradation scale in sterile soil (Table 14 and plates 20, 21)

4.4.1.2 Degradation during second week

Beads with PGPR mix-I showed slight visible degradation on bead edges and 0.64 was recorded as biodegradation scale. There was onset of degradation in beads without PGPR mix-I and 0.33 was recorded as biodegradation scale in sterile soil (Table 14 and plates 24, 25).

4.4.1.3 Degradation during third week

In sterile soil, beads with PGPR mix-I showed slight visible degradation on bead edges and 1.04 was recorded as biodegradation scale. Beads without PGPR mix-I showed slight visible degradation on bead edges in sterile soil and 0.59 was recorded as biodegradation scale (Table 14 and plates 28, 29).

4.4.1.4 Degradation during fourth week

During the fourth week, beads with PGPR mix-I showed one-half to three-fourth degradation and 1.28 was recorded as biodegradation scale. Beads without PGPR mix-I showed slight visible degradation on bead edges and 1.04 was recorded as degradation scale in sterile soil (Table 14 and plates 32, 33).

4.4.2 Evaluation of biodegradation of encapsulated beads in non sterile soil

4.4.2.1 Degradation during first week

In non sterile soil, beads with PGPR mix-I showed slight visible degradation on bead edges (plate x) and 0.59 was recorded as biodegradation scale. Beads without PGPR mix-I showed onset of degradation and 0.37 was recorded as biodegradation scale in non sterile soil (Table 14 and plates 22, 23).

4.4.2.2 Degradation during second week

Beads with PGPR mix-I showed one-half to three-fourth degradation and recorded 1.15 as biodegradation scale, while beads without PGPR mix-I showed slight visible degradation on bead edges and 0.71 was recorded in non sterile soil (Table 14 and plates 26, 27).

4.4.2.3 Degradation during third week

During the third week, beads with PGPR mix-I showed one-half to three-fourth degradation on bead edges and 1.48 was recorded as biodegradation scale. Beads without PGPR mix-I showed slight visible degradation on bead edges (plate x) and 1.07 was recorded as biodegradation scale in non sterile soil (Table 14 and plates 30, 31).

4.4.2.4 Degradation during fourth week

90% of beads of become mushy and 2.12 was recorded as biodegradation scale. One-half to three-fourth degradation was observed in beads without PGPR mix-I and 1.41 was recorded as biodegradation scale in non sterile soil (Table 14 and plates 34, 35).

Plate 20. Beads with PGPR mix-I in sterile soil

Plate 21. Beads without PGPR mix-I in sterile soil

Plate 22. Beads with PGPR mix-I in non sterile soil

Plate 23. Beads without PGPR mix-I in non sterile soil

Biodegradation of beads with and without PGPR mix-I in sterile and non sterile soil after one week of soil inoculation

**Plate 24. Beads with PGPR mix-I in
sterile soil**

**Plate 25. Beads without PGPR mix-I in
sterile soil**

**Plate 26. Beads with PGPR mix-I in non
sterile soil**
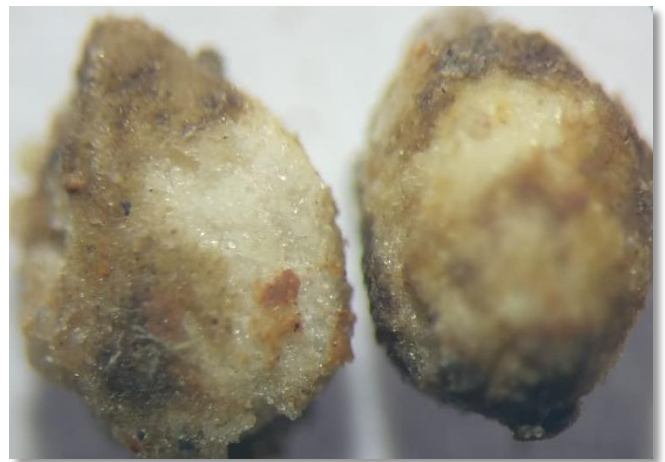
**Plate 27. Beads without PGPR mix-I in
non sterile soil**

**Biodegradation of beads with and without PGPR mix-I in sterile and non
sterile soil after two weeks of soil inoculation**

Plate 28. Beads with PGPR mix-I in sterile soil

Plate 29. Beads without PGPR mix-I in sterile soil

Plate 30. Beads with PGPR mix-I in non sterile soil

Plate 31. Beads without PGPR mix-I in non sterile soil

Biodegradation of beads with and without PGPR mix-I in sterile and non sterile soil after three weeks of soil inoculation

Plate 32. Beads with PGPR mix-I in sterile soil

Plate 33. Beads without PGPR mix-I in sterile soil

Plate 34. Beads with PGPR mix-I in non sterile soil

Plate 35. Beads without PGPR mix-I in non sterile soil

Biodegradation of beads with and without PGPR mix-I in sterile and non sterile soil after four weeks of soil inoculation

Table 14. Biodegradation of encapsulated beads of PGPR mix-I

TREATMENTS	BIODEGRADATION SCALE			
	FIRST WEEK	SECOND WEEK	THIRD WEEK	FOURTH WEEK
BEADS WITH PGPR MIX-I IN NON STERILE SOIL	0.59	1.15	1.48	2.12
BEADS WITH PGPR MIX-I IN STERILE SOIL	0.36	0.64	1.04	1.28
BEADS WITHOUT PGPR MIX-I IN NON STERILE SOIL	0.37	0.71	1.07	1.41
BEADS WITHOUT PGPR MIX-I IN STERILE SOIL	0.12	0.33	0.59	1.04

Each value represents a mean of 3 replications

4.4.3 Week wise evaluation of biodegradation of beads

In Kruskal-Wallis rank sum test done, significance was observed and then it was compared using Dunn's test. Treatments were T1 (Beads with bacteria in non sterile soil), T2 (Beads without bacteria in non sterile soil), T3 (Beads with bacteria in sterile soil), T4 (Beads without bacteria in sterile soil).

4.4.3.1 Biodegradation during first week

Kruskal-Wallis rank sum test gives a chi-squared value of 9.6299 with df = 3 and p-value = 0.02199. There is a significant difference between treatments and so multiple comparison was done using Dunn test.

During first week, treatment T1 recorded the highest biodegradation and T4 recorded the least biodegradation. Treatments T2 and T3 were on par with both the treatments T1 and T4 (Table 15).

4.4.3.2 Biodegradation during second week

Kruskal-Wallis rank sum test gives a chi-squared value of 10.607 with df = 3 and p-value = 0.01405. There is a significant difference between treatments and so multiple comparison was done using Dunn test.

During second week, treatment T1 recorded the highest biodegradation and T4 recorded the least biodegradation. Treatments T2 and T3 were on par with both the treatments T1 and T4 (Table 16).

4.4.3.3 Biodegradation during third week

Kruskal-Wallis rank sum test gives a chi-squared value of 10.25 with df = 3 and p-value = 0.01656. There is a significant difference between treatments and so multiple comparison was done using Dunn test.

During third week, treatment T1 recorded the highest biodegradation and T4 recorded the least biodegradation. Treatments T2 and T3 were on par with both the treatments T1 and T4 (Table 17).

4.4.3.4 Biodegradation during fourth week

Kruskal-Wallis rank sum test gives a chi-squared value of 11 with df = 3 and p-value = 0.01173. There is a significant difference between treatments and so multiple comparison was done using Dunn test.

During fourth week, treatment T1 recorded the highest biodegradation and T4 recorded the least biodegradation. Treatments T2 and T3 were on par with both the treatments T1 and T4 (Table 18).

4.4.4 Treatment wise evaluation of biodegradation of beads

Kruskal-Wallis rank sum test gives a chi-squared value of 46.205 with df = 15 and p-value = 4.932e-05. There is a significant difference between treatments and so multiple comparison was done using Dunn test.

Treatment T4 (beads with bacteria in non sterile soil during fourth week) showed significantly different from treatment T13 (beads without bacteria in sterile soil during first week). Treatments T4, T3, T12, T8, T2, T11, T7, T16, T10, T6, T15 found to be on par with each other. Similarly, treatments T3, T12, T8, T2, T11, T7, T16, T10, T6, T15, T1 found to be on par with each other. T8, T2, T11, T7, T16, T10, T6, T15, T1, T9, T5, T14 found to be on par with each other. T11, T7, T16, T10, T6, T15, T1, T9, T5, T14, T13 found to be on par with each other (Table 19).

where,

T1-Beads with bacteria in non sterile soil during first week

T2- Beads with bacteria in non sterile soil during second week

T3- Beads with bacteria in non sterile soil during third week

T4- Beads with bacteria in non sterile soil during fourth week

T5- Beads with bacteria in sterile soil during first week

T6- Beads with bacteria in sterile soil during second week

T7- Beads with bacteria in sterile soil during third week

T8- Beads with bacteria in sterile soil during fourth week

T9-Beads without bacteria in non sterile soil during first week

T10- Beads without bacteria in non sterile soil during second week

T11- Beads without bacteria in non sterile soil during third week

T12- Beads without bacteria in non sterile soil during fourth week

T13- Beads without bacteria in sterile soil during first week

T14- Beads without bacteria in sterile soil during second week

T15- Beads without bacteria in sterile soil during third week

T16- Beads without bacteria in sterile soil during fourth week

Table 15. Biodegradation of encapsulated beads of PGPR mix-I during first week

Treatment	Dunn's test significance
T1	a
T2	ab
T3	ab
T4	b
Chi-squared value	9.6299
df	3
p-value	0.02199

Treatment with same letters are not significantly different (alpha=0.05)

Table 16. Biodegradation of encapsulated beads of PGPR mix-I during second week

Treatment	Dunn's test significance
T1	a
T2	ab
T3	ab
T4	b
Chi-squared value	10.607
df	3
p-value	0.01405

Treatment with same letters are not significantly different (alpha=0.05)

Table 17. Biodegradation of encapsulated beads of PGPR mix-I during third week

Treatment	Dunn's test significance
T1	a
T2	ab
T3	ab
T4	b
Chi-squared value	10.25
df	3
p-value	0.01656

Treatment with same letters are not significantly different (alpha=0.05)

Table 18. Biodegradation of encapsulated beads of PGPR mix-I during fourth week

Treatment	Dunn's test significance
T1	a
T2	ab
T3	ab
T4	b
Chi-squared value	11
df	3
p-value	0.01173

Treatment with same letters are not significantly different (alpha=0.05)

Table 19. Treatment wise evaluation of biodegradation of encapsulated beads of PGPR mix-I

Treatment	Dunn's test significance
T1	abc
T2	abd
T3	ad
T4	d
T5	bc
T6	abcd
T7	abcd
T8	abd
T9	bc
T10	abcd
T11	abcd
T12	ad
T13	c
T14	bc
T15	abcd
T16	abcd
Chi-squared value	46.205
df	15
p-value	4.932e-05

Treatment with same letters are not significantly different (alpha=0.05)

DISCUSSION

5. DISCUSSION

Plant growth promoting rhizobacteria (PGPR) are beneficial bacteria which have the capability to colonize the roots and either promotes plant growth through direct action or via biological control of plant diseases (Kloepper and Schroth, 1978). PGPR act as a viable alternative for chemical fertilizers that will effectively provide nitrogen, phosphorous, potassium and various phytohormones to plants and prevent the depletion of soil fertility and soil quality. This group of bacteria plays a significant role in the biogeochemical cycle in soil ecosystems, ultimately fortifying plants and sustaining agriculture. Strains in the genera such as *Serratia*, *Pseudomonas*, *Burkholderia*, *Agrobacterium*, *Xanthomonas*, *Azospirillum*, *Bacillus*, *Enterobacter*, *Rhizobium*, *Arthrobacter*, *Acetobacter*, *Acinetobacter*, *Achromobacter*, *Aerobacter*, *Azotobacter*, *Micrococcus* and *Flavobacterium* are reported to have PGPR activity (Rodriguez and Fraga, 1999; Bloemberg and Lugtenberg, 2001; Esitken *et al*., 2003).

PGPR enhance the nutrient availability to host plants by nitrogen fixation, solubilization of phosphorus and potassium in the rhizosphere. According to the studies by Nautiyal *et al*. (2000) phosphate-solubilizing bacteria are common in rhizospheres and improves nutrition to plants by phosphorous solubilization of rhizosphere soil. Banerjee *et al*. (2005) reported that the most potential phosphate solubilizing bacteria belong to the genera *Bacillus*, *Rhizobium* and *Pseudomonas*. Plant growth promoting rhizobacteria can produce organic acids which in turn solubilize potassium rock. PGPR such as *Bacillus edaphicus*, *Bacillus mucilaginosus*, *Burkholderia*, *Paenibacillus* sp., *Pseudomonas* sp. are potassium solubilizers and are able to release potassium in available form. Hu *et al*. (2006) reported that *Bacillus megaterium* and *B. mucilaginosus* were capable of solubilizing both rock phosphate and potassium. Investigations by Sakthidharan (2011) reported that application of K solubilizers developed by KAU have influenced plant biochemical properties such as beta carotene, vitamin C and crude protein content in Amaranthus (Sakthidharan, 2011).

The demerits associated with conventional formulations which causes greater variability in quality could be rectified by the introduction of encapsulated formulations (Albareda *et al.,* 2008). Studies of Kim *et al* (1996) reported that bioencapsulation stabilizes microbial cells by greatly potentially increasing the viability and maintaining stability during production, storage and handling process. It also ensures a further protection in the course of rehydration.

Considering the merits of alginate based encapsulated formulation over carrier and liquid based, the present investigation was designed to standardize the alginate formulation of PGPR mix-I and its evaluation. This investigation was focused to standardize the protocol for the preparation of encapsulated formulation of PGPR mix–I in completely randomized design by using various filler materials such as 10% Standard starch, 15% Standard starch, 10% Wheat, 15% Wheat, 10% Talc and 15% Talc with 2% Sodium alginate and control was maintained with 2% Sodium alginate alone without any filler material.

It was observed that after 24 hours of encapsulation, total viable count of all component cultures of PGPR mix-I was maximum in formulation amended with 10% Standard starch (Figure 1). It was interesting to observe that in the present investigation, throughout the three months period of standardization study, formulation amended with 10% Standard starch exhibited maximum viable count of component cultures consistently (Figure 1 to 4). The results were in agreement with the findings of Bashan *et al*. (2002) who reported that starch is needed for increasing dry matter content of alginate beads which supports high cell count and allows a progressive and gradual release of cells into the soil. Ivanoa *et al.* (2006) has developed alginate plus carrier based formulation for nitrogen fixing bacteria *Azospirillum* and found that the starch performed better than other filler materials mainly since it has increased the dry matter which is needed for stable microbial population during storage. Similar results were also obtained by Schoebitz *et al*. (2012) who stated that immobilized alginate beads amended with starch as filler material makes the stable manufacturing of alginate beads with a high cfu value. In the present study, the matrix formulation containing alginate and starch provided the best results regarding cell survival after the bioencapsulation process. After testing the survival rates with different filler materials, alginate starch (10%) matrix was selected and used for supplementary experiments.

Investigations of Fages (1992) reported that important objectives of a best biofertilizer formulation is the stabilization of the cell viability during a long period of storage to ensure proper protection of bacteria in the soil. The entrapment of microbial cells into a biopolymer such as alginate or xanthan gum helps to protect cells from environmental stresses and ensures slow release of immobilized microbes into the soil (Dommergues *et al*., 1979; Jung *et al*., 1982). This technique has been successfully accomplished in nitrogen fixing bacteria like *Azospirillum* (Bashan 1986).
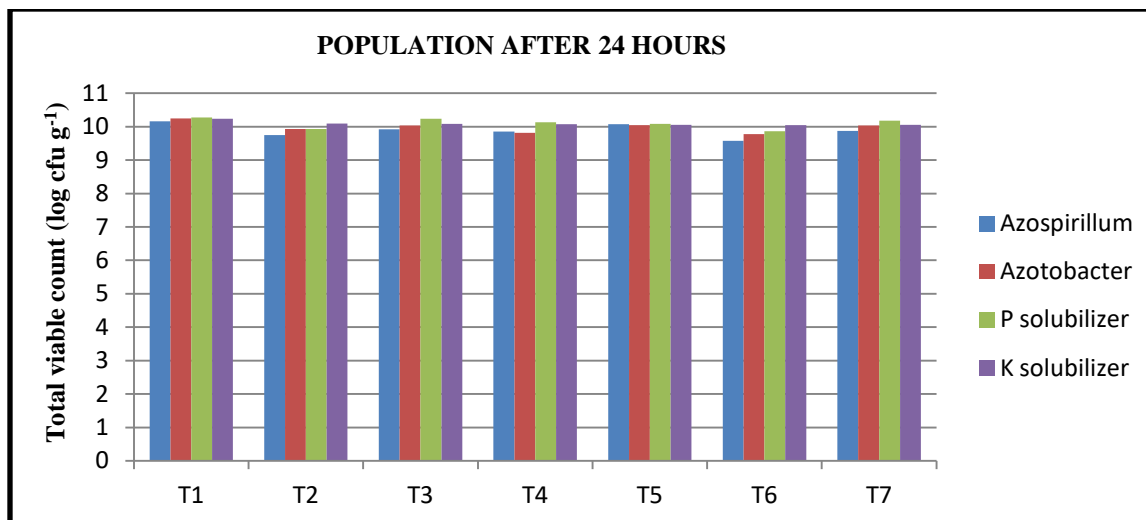
Fig. 1. Population of component organisms of PGPR mix-I in encapsulated formulation with different filler materials after 24 hours
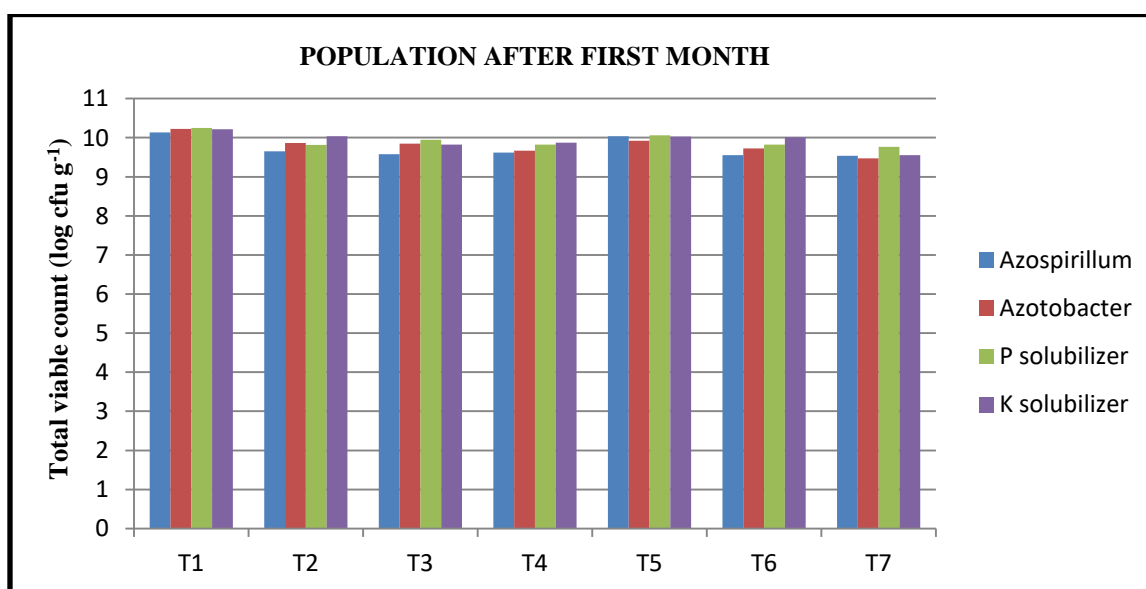
Fig. 2. Population of component organisms of PGPR mix-I in encapsulated formulation with different filler materials after first month of encapsulation

T1- 10 % Starch	T2- 15 % Starch	T3–10% Wheat
T4–15 % Wheat	T5-10% Talc	T6- 15% Talc
T7- 2% Sodium alginate (control)		

Fig. 3. Population of component organisms of PGPR mix-I in encapsulated formulation with different filler materials after second month of encapsulation
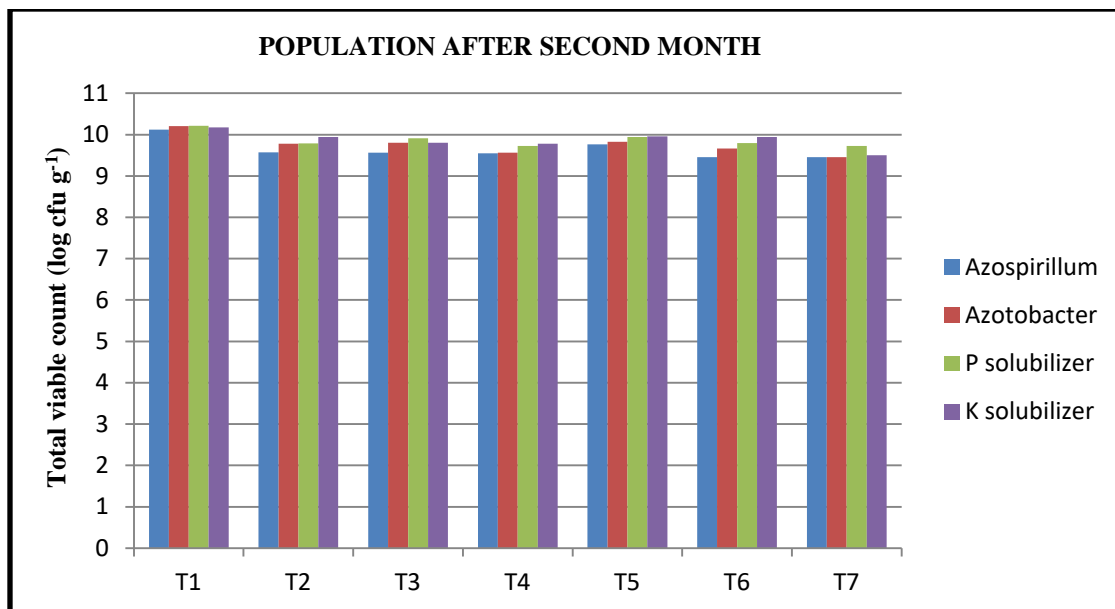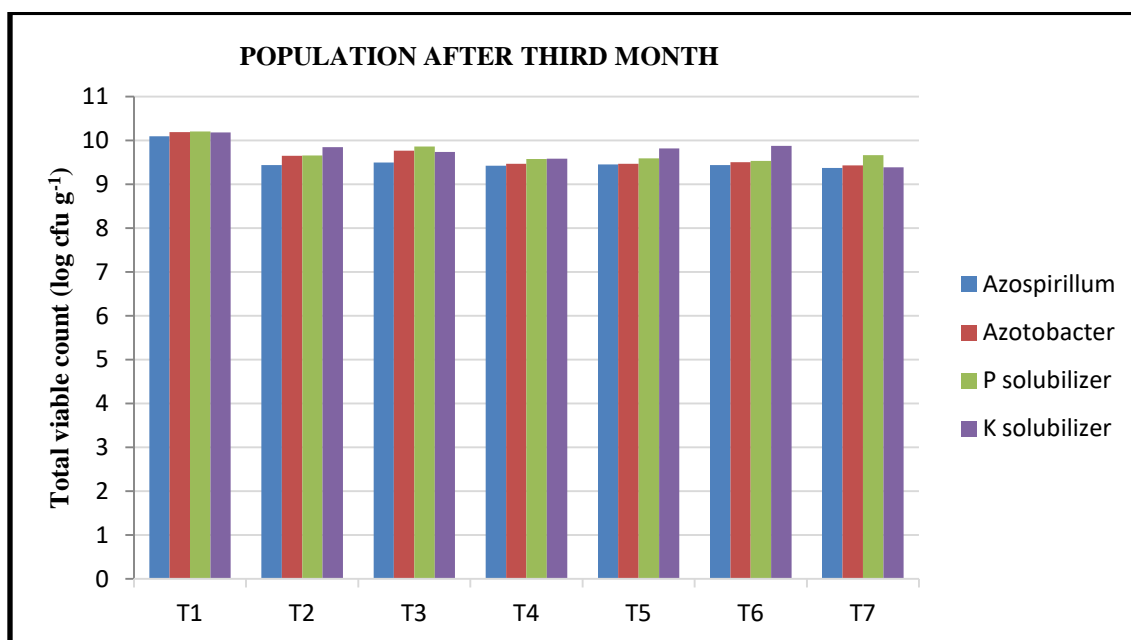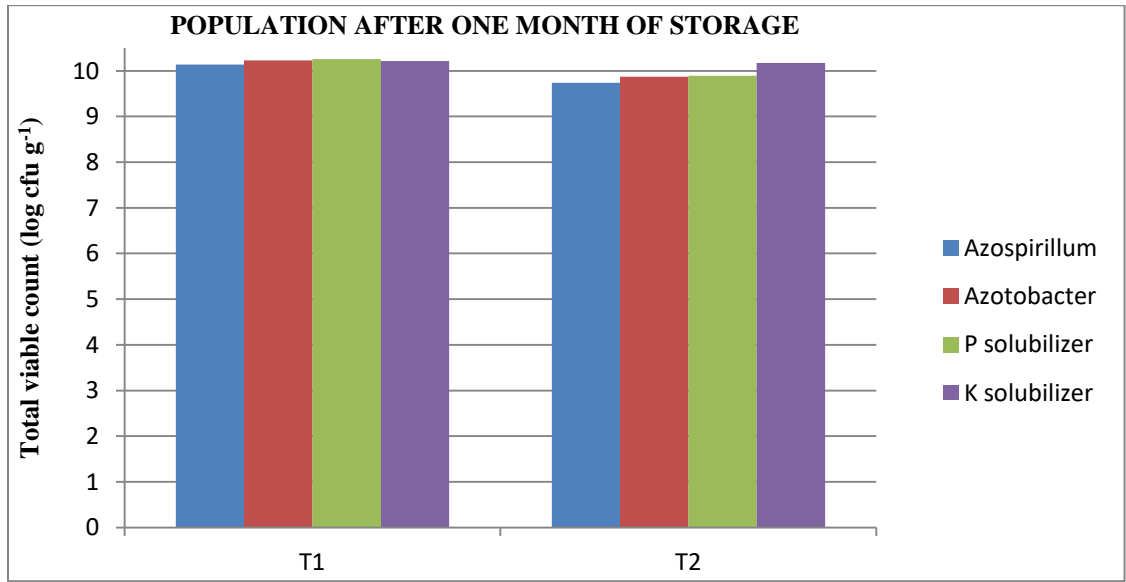

Fig. 4. Population of component organisms of PGPR mix-I in encapsulated formulation with different filler materials after third month of encapsulation

T1- 10 % Starch	**T2- 15 % Starch**	**T3–10% Wheat**
T4–15 % Wheat	**T5-10% Talc**	**T6- 15% Talc**
T7- 2% Sodium alginate (control)		

In the present study, shelf life of alginate starch (10%) matrix entrapped component cultures of PGPR mix-I was determined by storing the beads in room temperature and refrigerated condition for a period of 6 months. By the end of each month, beads from both the stored conditions were checked for population of component cultures of PGPR mix-I. The study revealed that the beads stored at room temperature showed higher viable count of component cultures than the beads kept at refrigerated conditions (Fig 5 to 10). These results were in line with the study of Fages (1992) who reported that alginate beads kept under room temperature conditions for 1 year showed acceptable population of 10^{10} cfu g^{-1} of *Azospirillum lipoferum* compared to the beads stored in refrigerated condition. Study conducted by Bashan and Gonzalez (1999) showed that encapsulated strains of *Azospirillum brasilense* Cd and *Pseudomonas fluorescens* 313 have recorded population in the range of 10^5-10^6 cfu g^{-1} even after 14 years of storage and did not disssipate the capacity to promote growth of *Triticum aestivum*. Here in this investigation it was found that encapsulation of the biofertilizer consortia of PGPR mix-I in calcium alginate matrix presented a population greater in terms of long storage when stored under room temperature conditions. Similar results were also obtained by Ivanoa *et al*. (2006) who reported that *Azospirillum brasilense* entrapped in alginate-starch beads showed stable population in the beads stored under room temperature condition for six months. Encapsulated beads in Sodium alginate matrix presented high survival rates of 71.7% (4ºC) and 77.8% (room temperature) after storage for 180 days as reported by Yanhui *et al*., (2015).

In the present investigation, moisture content of alginate beads was determined which was stored at room temperature for three months during standardization period (Fig 11) and both at room temperature and refrigerated condition for six months during the storage period (Fig 12). It was found that the highest moisture content of 12.83% was recorded in refrigerated condition and 12.07% in room temperature stored alginate beads during the first month. The lowest moisture content of 11.45% under refrigerated condition and 10.70% at room temperature was recorded for the alginate beads after six months of storage. During the prolonged storage, beads kept under refrigerated temperature were found to retain more moisture (12.83% to 11.45%) than the alginate beads kept at room temperature (12.07% to 10.70%). Similar results were obtained by Paul *et al*. (1993) who reported that loss of moisture content was prominent in alginate beads of *Azospirillum lipoferum* stored at room temperature compared to the refrigerated condition during the storage period of five months.

Fig. 5. Population of component organisms of PGPR mix-I in encapsulated formulation amended with 10% Standard starch after one month of storage
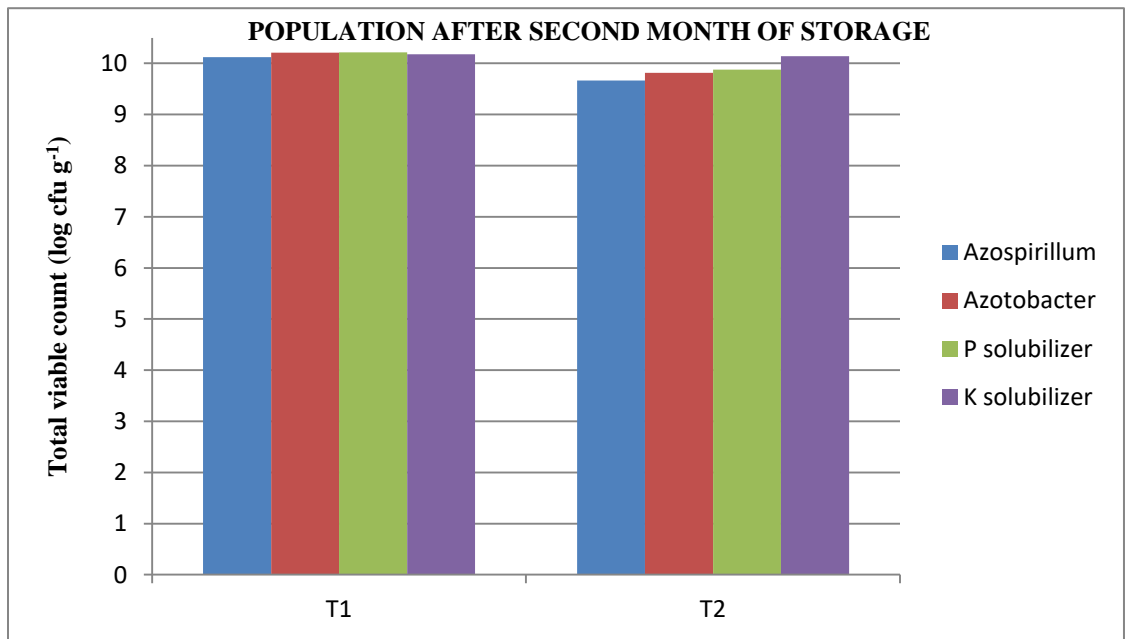
Fig. 6. Population of component organisms of PGPR mix-I in encapsulated formulation amended with 10% Standard starch after second month of storage

> **T1- 10 % Starch (Room temperature)**
>
> **T2- 10 % Starch (Refrigerated condition)**
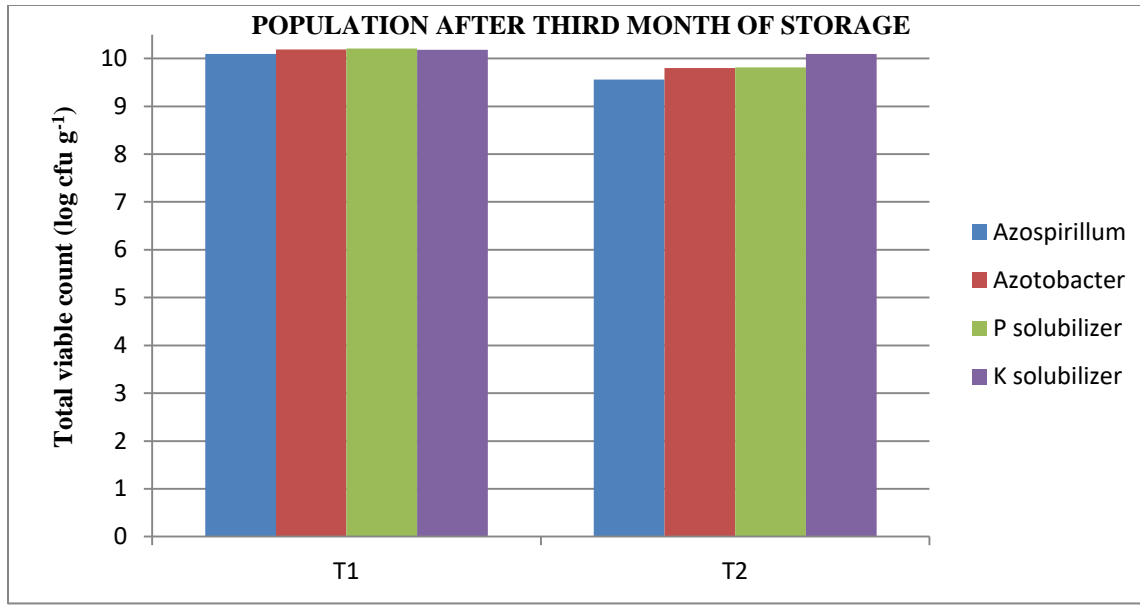
Fig. 7. Population of component organisms of PGPR mix-I in encapsulated formulation amended with 10% Standard starch after third month of storage
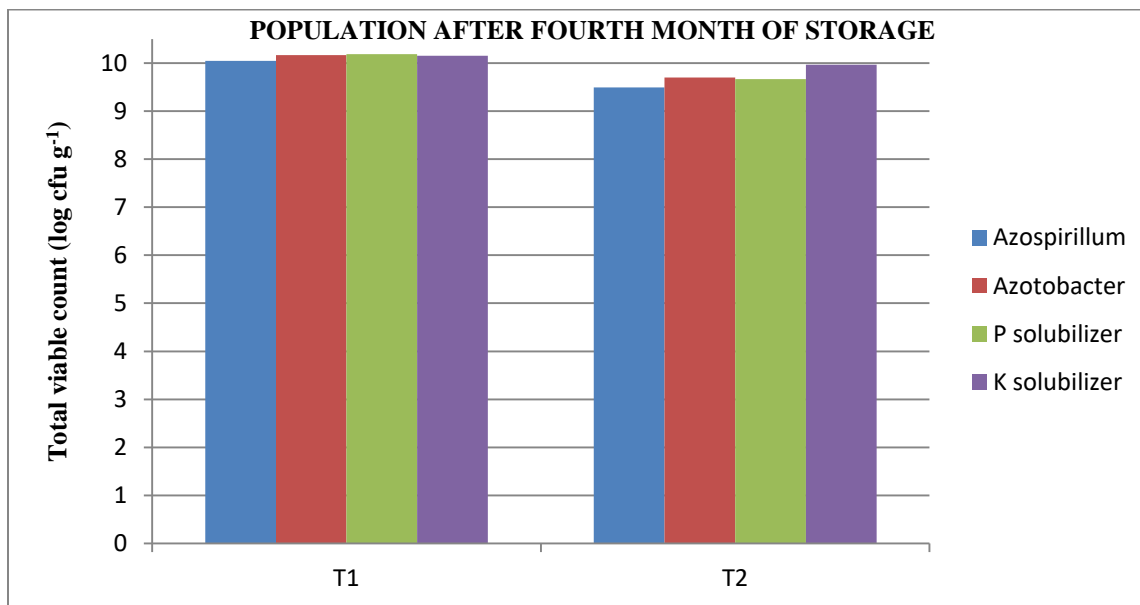
Fig. 8. Population of component organisms of PGPR mix-I in encapsulated formulation amended with 10% Standard starch after fourth month of storage

T1- 10 % Starch (Room temperature)

T2- 10 % Starch (Refrigerated condition)
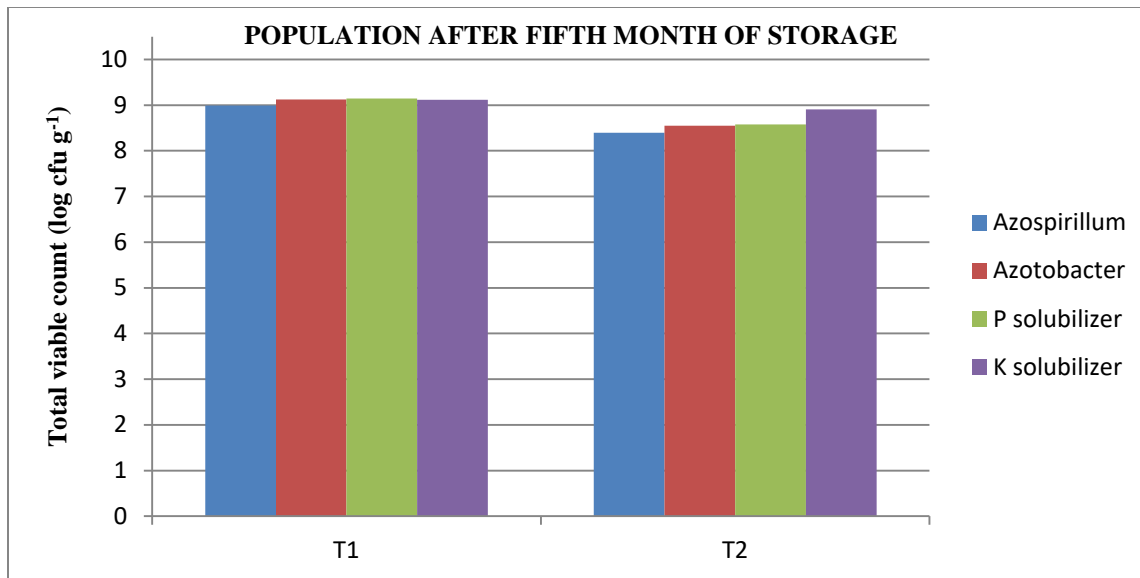
Fig. 9. Population of component organisms of PGPR mix-I in encapsulated formulation amended with 10% Standard starch after fifth month of storage
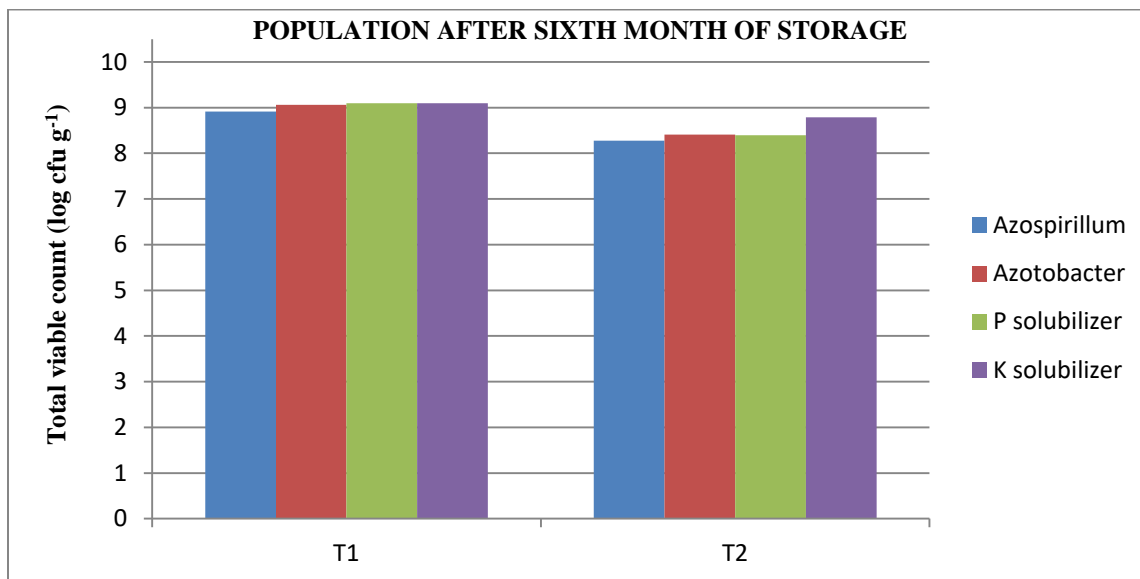
Fig. 10. Population of component organisms of PGPR mix-I in encapsulated formulation amended with 10% Standard starch after sixth month of storage

T1- 10 % Starch (Room temperature)

T2- 10 % Starch (Refrigerated condition)
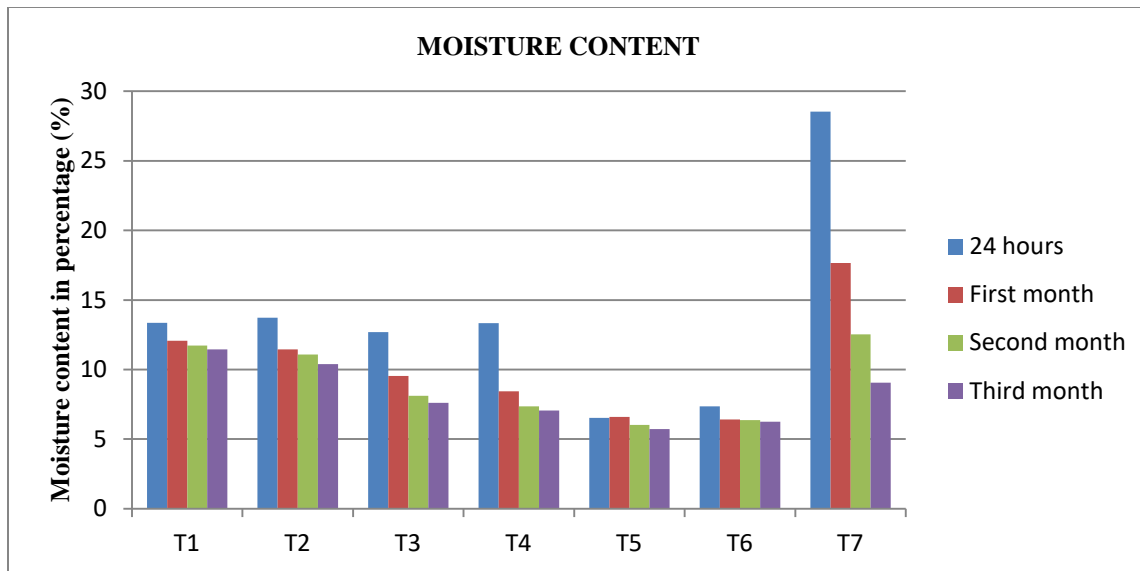
Fig. 11. Moisture content of encapsulated beads of PGPR mix-I at monthly intervals

T1- 10 % Starch	T2- 15 % Starch	T3–10% Wheat
T4–15 % Wheat	T5-10% Talc	T6- 15% Talc
T7- 2% Sodium alginate (control)		
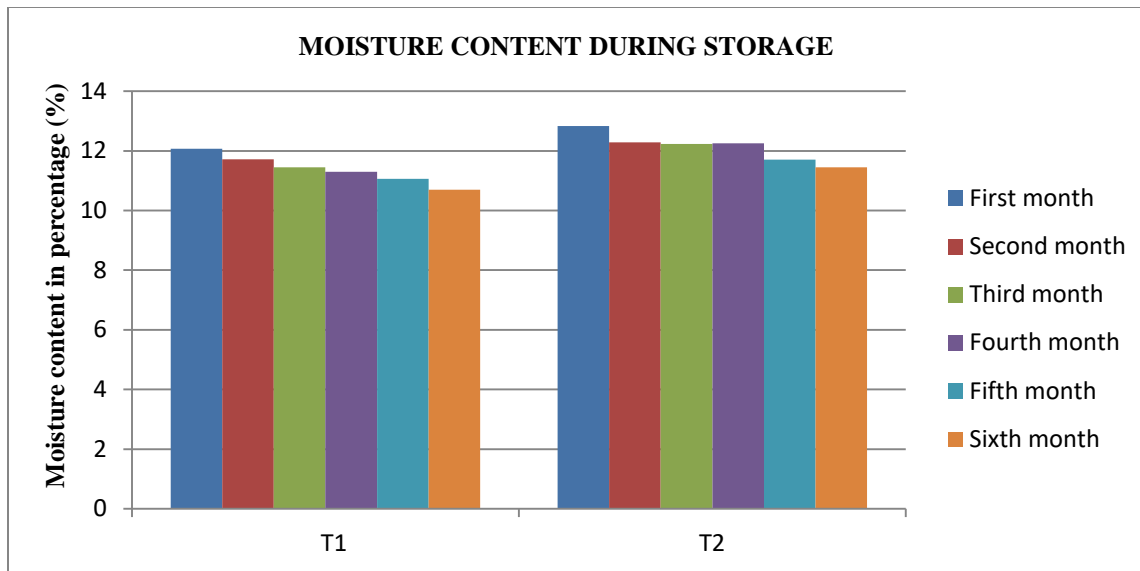
Fig.12 . Moisture content of encapsulated beads of PGPR mix-I at monthly intervals during six months of storage

T1- 10 % Starch (Room temperature)

T2- 10 % Starch (Refrigerated condition)
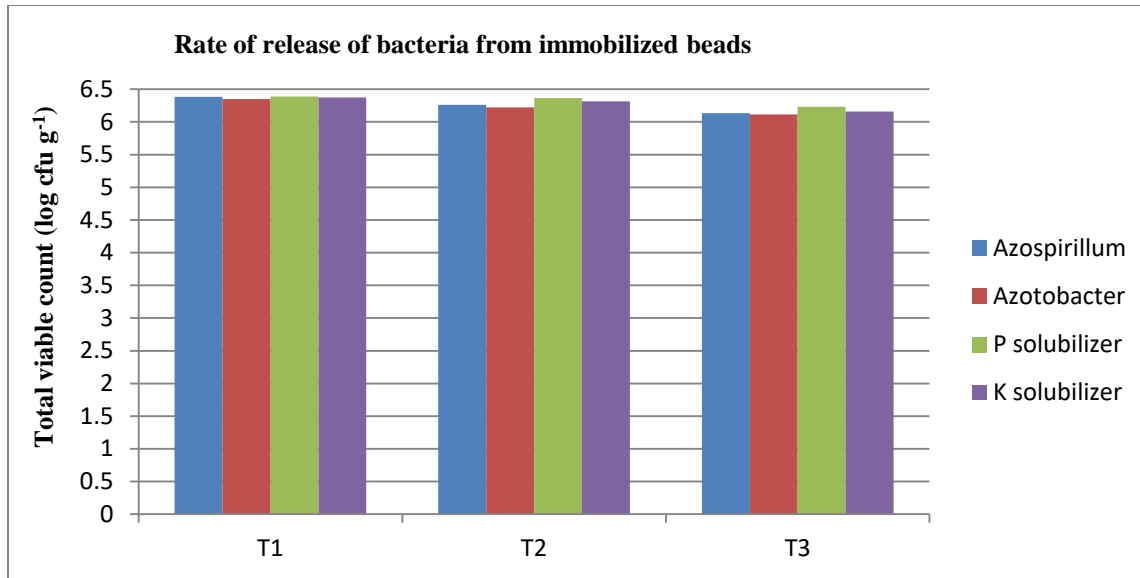
Fig. 13. Rate of release of bacteria from immobilized beads of PGPR mix-I

T1- 24 hours

T2- 48 hours

T3- 30 days

Another experiment done to evaluate the rate of release of immobilized bacteria from beads. According to the studies of *Chan et al*. (2009) immobilized beads provides adequate protection, controlled release and stabilization of bacteria within the beads. The advantages of using alginate as an encapsulation material is their ability to biodegrade in soil, nontoxic nature and gradual release of immobilized bacteria into the soil.

Hence the present study investigated the release of entrapped PGPR mix-I from the alginate beads. The release of bacteria was determined as per the procedure described by Bashan (1986) and the population of component cultures was taken at intervals of 24 hours, 48 hours and 30 days (Fig 13). After 24 hours of incubation in saline solution at 32°C, population of *Azospirillum lipoferum*, *Azotobacter chroococcum*, *Bacillus megaterium and Bacillus sporothermodurans* recorded were 2.42×10^6 cfu ml^{-1}, 2.24×10^6 cfu ml^{-1}, 2.47×10^6 cfu ml^{-1} and 2.36×10^6cfu ml^{-1} respectively. After 48 hours of incubation in saline solution at 32°C, population of *Azospirillum lipoferum, Azotobacter chroococcum, Bacillus megaterium* and *Bacillus sporothermodurans* recorded were 1.82×10^6 cfu ml^{-1}, 1.68×10^6 cfu ml^{-1}, 2.33×10^6 cfu ml^{-1} and 2.08×10^6 cfu ml^{-1} respectively. After 30 days of incubation on a thin layer of sterile water at 4ºC, population of *Azospirillum lipoferum*, *Azotobacter chroococcum*, *Bacillus megaterium and Bacillus sporothermodurans* recorded were 1.37×10^6 cfu ml^{-1}, 1.32×10^6 cfu ml^{-1}, 1.72×10^6 cfu ml^{-1} and 1.44×10^6 cfu ml^{-1} respectively. This study is in agreement with the findings of Bashan (1986) who concluded that population of 10^5 to 10^6 cfu g^{-1} of beads were released from 24 hours till the end of fourth week from an initial population in the beads of 5.0 x 10^7 cfu g^{-1} of beads. Slow release study with beads of *Pseudomonas* sp. has also recorded similar results (Bashan, 1986). The rate of release of bacteria from the capsules was fastest in the first 24-48 hours. After that (between 3 and 30 days), cells were less swiftly released as reported by Yanhui *et al*. (2015). The bacteria in the outermost layer of the encapsulated beads were rapidly released in the initial stage. The quick release of microbial cells can be credited to the bead expansion as a result of water entry into the outermost layer first followed by gradual diffusion into the centre.

The final experiment of this study was to evaluate the biodegradation of encapsulated beads of PGPR mix-I (Fig 14). According to Liakos *et al*. (2014) biodegradation occurred possibly through the breaking of covalently linked (1-4) glycoside bonds of sodium alginate composed of unbranched chains of β-d-mannuronate (M) and α-l-guluronate (G) residues by the enhanced loss of the matrix with extended incubation time in natural soil conditions possibly by
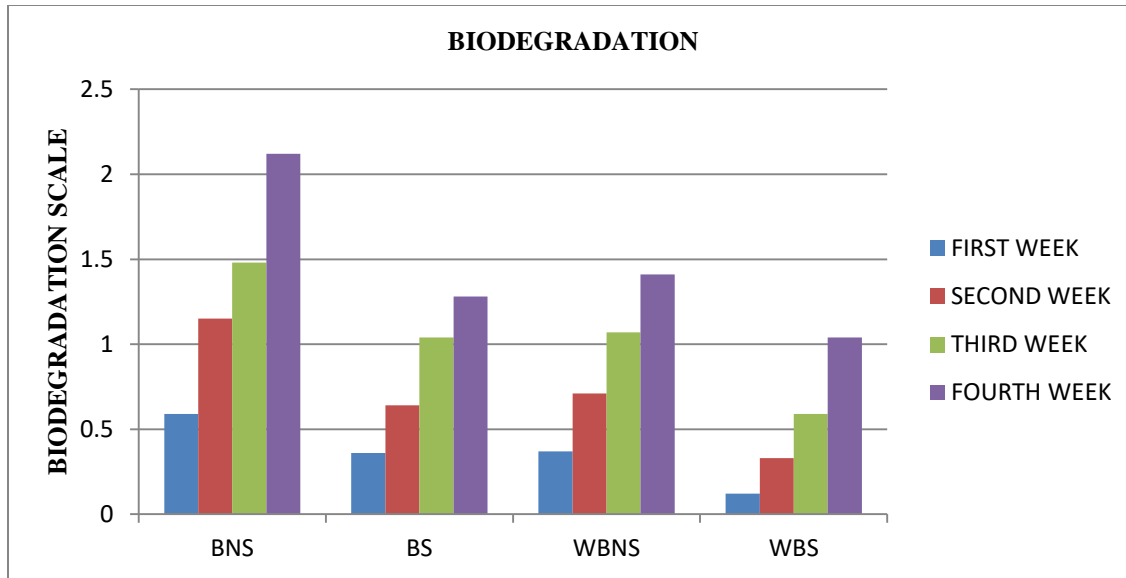
the combined action of soil microflora, soil moisture content, residual enzymatic activity and physical properties of soil.

Beads with and without bacteria were buried in two soil types, sterile soil and non-sterile soil and irrigated for maintaining moisture content level at field capacity. Observations were taken at weekly intervals as per biodegradation scale given by Bashan (1986).

It was observed that the beads with bacteria in non-sterile soil showed highest scale of biodegradation throughout the biodegradation study (mean value 1.34) and beads without bacteria in sterile soil showed the lowest scale (mean value 0.52). As per Bashan (1986), the biodegradation of the beads depends on soil microflora and moisture content of the soil. Biodegradation is faster if the density of the microflora surrounding the beads is higher. Similarly more the moisture content in the soil, higher will be the rate of biodegradation of the beads. Biodegradation is accomplished from the outside layer inward by the combined effect of both native soil microflora and moisture content of the soil, thus consecutive exposing of various layers of the bead to the adjoining soil until complete degradation. In the present investigation, in nonsterile soil the rate of biodegradation of encapsulated PGPR mix-I found to be more as the biodegradation was carried out by the combined effect of both native soil microflora and moisture content of the soil. Achmon *et al.* (2019) found that alginate encapsulated beads biodegraded quickly in non-sterile soil and temperature fluctuations can have a significant effect on the biodegradation rate. While in sterile soil, biodegradation found to be less as the moisture is the only factor influencing biodegradation of beads.

These results were in line with the study of Bashan (1986) who reported that alginate beads of *Azospirillum brasilense* kept in non sterile soil showed the highest degree of biodegradation (mean value 1.43) and beads without bacteria in sterile soil showed the lowest scale (mean value 0.2) at the end of fourth week after inoculation in the soil. In the present study, beads without bacteria in non-sterile soil and with bacteria in sterile soil showed mean values of 0.89 and 0.82 respectively, which was in between of the highest and the lowest values of biodegradation scale. Similar trend was reported by Bashan (1986) with alginate beads of *Azospirillum brasilense* where biodegradation index of 0.76 and 0.38 were observed for beads without bacteria in non-sterile soil and with bacteria in sterile soil respectively, which was in between the highest and the lowest biodegradation scale.

In the present investigation, calcium alginate based encapsulated beads of PGPR mix-I amended with 10% Standard starch exhibited maximum viable count of component cultures of PGPR mix-I throughout the three months period of standardization study. In terms of evaluation of shelf life and moisture retention during storage, beads stored under room temperature condition was found to be better. The rate of release of component cultures of PGPR mix-I from the encapsulated formulation was more during the first 24-48 hours. Biodegradation studies of encapsulated beads of PGPR mix-I revealed that the beads inoculated with PGPR mix-I in non sterile soil showed highest biodegradation throughout the period of investigation.

Fig. 14. Biodegradation of encapsulated beads with and without PGPR mix-I in sterile and non sterile soil

BNS	**- Beads with bacteria in non sterile soil**
WBNS	**- Beads without bacteria in non sterile soil**
BS	**- Beads with bacteria in sterile soil**
WBS	**- Beads without bacteria in sterile soil**

SUMMARY

6. SUMMARY

DEVELOPMENT OF ENCAPSULATED FORMULATION OF

PGPR MIX – I AND ITS EVALUATION

The study entitled "Development of encapsulated formulation of PGPR mix-I and its evaluation" was conducted during 2018-2020, in the Department of Agricultural Microbiology, College of Agriculture, Vellayani, Thiruvananthapuram, with the objective to develop calcium alginate based encapsulated formulation of PGPR mix-I and its evaluation for slow release and biodegradation.

The component cultures of PGPR mix-I were procured from the Department of Agricultural Microbiology, College of Agriculture, Vellayani for standardization of protocol for preparation of calcium alginate based encapsulated bead formulation of PGPR mix-I. Encapsulated bead formulation of PGPR mix-I was prepared by standard procedures.

An experiment was carried out to standardize the protocol for preparation of calcium alginate based encapsulated formulation of PGPR mix–I in completely randomized design with different treatments such as 10% Standard starch, 15% Standard starch, 10% Wheat flour, 15% Wheat flour, 10% Talc, 15% Talc and control treatment as 2% Sodium alginate alone in three replications.

Consistent viable count was recorded in encapsulated formulation amended with 10% Standard starch. It exhibited maximum viable count of each of the component cultures of PGPR mix-I as a result of three month population study. A significant decline of total viable population in control treatment was observed in each month compared to encapsulated formulation amended with 10% Standard starch.

Based on the population study, encapsulated formulation of PGPR mix-I 10% Standard starch amended was adjudged as the best combination of filler material and hence the shelf life studies of the same had to be continued at monthly intervals at room temperature and refrigerated conditions for six months by serial dilution and plate count method. Significant viable count was recorded in encapsulated beads stored at room temperature condition throughout the shelf life study.

The moisture content of beads were also monitored during standardization and shelf life study. During standardization study, moisture content of PGPR mix-I encapsulated beads of each treatment was monitored for a period of three months at monthly intervals at room temperature and it showed a significant variation among treatments in each month. A reduction in moisture content of beads was observed from first month to the end of sixth month in all treatments. Beads amended with 10% Standard starch showed a moisture content of 13.37%, 12.07%, 11.72% and 11.45% after 24 hours of drying, first, second and third month respectively.

During shelf life study, 10% Standard starch combination at refrigerated condition showed moisture content in the range of 12.83% to 11.45% while at room temperature the same has recorded values in the range of 12.07% to 10.70%.

Evaluation of rate of release of immobilized bacteria from encapsulated beads was determined as per the procedure described by Bashan (1986) and the number of released bacteria was determined by the plate count method in respective selective medium. The higher cfu of component cultures of PGPR mix-I was observed after gentle shaking at 32ºC for 24hours (T1) in75ml of sterile saline solution. After 24 hours, population of *Azospirillum lipoferum*, *Azotobacter chroococcum*, *Bacillus megaterium and Bacillus sporothermodurans* recorded were 2.42×10^6 cfu ml^{-1}, 2.24×10^6 cfu ml^{-1}, 2.47×10^6 cfu ml^{-1} and 2.36×10^6cfu ml^{-1} respectively, which was significantly superior to treatments T2 (48 hours) and treatment T3 (30 days).

Evaluation of biodegradation of encapsulated beads was studied at weekly intervals in sterile and non-sterile soil with PGPR mix-I inoculated and non-inoculated beads with three replications each (Bashan, 1986). Both the sets were observed weekly for their rate of biodegradation. As per biodegradation scale values like 0, >0-0.5, >0.5-1, >1-2, >2-2.5 or 3 was assigned according to the degree of visible degradation which indicates no visible degradation,onset of degradation, slight visible degradation on bead edges, one-half to three-fourth of the beads degraded, 90% of beads become mushy, full degradation (beads are disintegrated into small pieces or not found in the nylon bag) respectively (Bashan, 1986).

The PGPR mix-I inoculated beads with bacteria in non-sterile soil showed highest scale of biodegradation throughout the biodegradation study (mean value 1.34) and beads without bacteria in sterile soil showed the lowest scale (mean value 0.52).

Kruskal-Wallis rank sum test was done and there was a significant difference between treatments and so multiple comparison was done using Dunn test. During all the four weeks of biodegradation study, treatment T1(beads with PGPR mix-I in non sterile soil) recorded the highest biodegradation and T4 (beads without PGPR mix-I in sterile soil) recorded the least biodegradation. Treatments T2 (beads with PGPR mix-I in sterile soil) and T3 (beads without PGPR mix-I in non sterile) were on par with both the treatments T1 and T4 in all the four weeks.

Treatment wise evaluation of biodegradation of beads was done with Kruskal-Wallis rank sum test and gives a chi-squared value of 46.205 with df = 15 and p-value = 4.932e-05. There was a significant difference between treatments and so multiple comparison was done using Dunn test. Treatment T4 (beads with bacteria in non sterile soil during fourth week) showed significantly different from treatment T13 (beads without bacteria in sterile soil during first week). Treatments T4, T3, T12, T8, T2, T11, T7, T16, T10, T6, T15 found to be on par with each other. Similarly, treatments T3, T12, T8, T2, T11, T7, T16, T10, T6, T15, T1 found to be on par with each other. T8, T2, T11, T7, T16, T10, T6, T15, T1, T9, T5, T14 found to be on par with each other. T11, T7, T16, T10, T6, T15, T1, T9, T5, T14, T13 found to be on par with each other.

Further studies are required before developing commercial formulations. Hence, the future line of work has to be focused on the following areas.

- *In vivo* studies for evaluation of growth promotion and yield in different crops

- Standardization of dosage for different crops

- Addition of different additives to increase the shelf life and viability

- Development of cost effective technologies for large-scale production of encapsulated formulation

- Assessment of biodegradation in different soil types and climatic zones

- Create awareness on the benefits of encapsulated formulation among farmers

- Need for proper marketing

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7. REFERENCES

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APPENDIX

8. APPENDIX - I

COMPOSITION OF MEDIA USED

1. Nitrogen free bromothymol medium

Malic acid	- 5g
K_2HPO_4	- 0.5g
$MgSO_4.7H_2O$	- 0.2g
NaCl	- 0.1g
$CaCl_2$	- 0.02g
Trace element solution	- 2ml
BTB	- 2ml
$FeSO_4$	- 0.05g
Vitamin solution	- 4ml
KOH	- 4g
Agar-agar	- 20g
Distilled water	- 1000 ml

Malic acid, K_2HPO_4, $MgSO_4.7H_2O$, NaCl, $CaCl_2$, trace element solution, BTB, $FeSO_4$, Vitamin solution and KOH were dissolved in 500 ml distilled water and volume made up to 1000 ml. 20 g agar-agar was added into this mixture and autoclaved at 15 lbs pressure and 121ºC for 15 min.

2. Jenson's medium

Sucrose	- 20g
K_2HPO_4	- 1g

MgSO$_4$	- 0.5g
NaCl	- 0.5g
FeSO$_4$	- 0.1
NaMoO$_4$	- 0.005g
CaCO$_3$	- 2g
Distilled water	- 1000 ml
pH	- 7 to 7.3

Sucrose, K$_2$HPO$_4$, MgSO$_4$, NaCl, FeSO$_4$, NaMoO$_4$ and CaCO$_3$ were dissolved in 500 ml distilled water and volume made up to 1000 ml. 20 g agar-agar was added into this mixture and autoclaved at 15 lbs pressure and 121ºC for 15 min.

3.   Pikovaskaya's medium

Glucose	- 10g
Ca(PO$_4$)$_2$	- 5g
(NH$_4$)$_2$SO$_4$	- 0.5g
KCl	- 0.2g
MgSO$_4$	- trace
FeSO$_4$	- trace
Yeast extract	- 0.5g
Agar-agar	- 15g
Distilled water	- 1000 ml

Glucose, Ca(PO$_4$)$_2$, (NH$_4$)$_2$SO$_4$, KCl, MgSO$_4$, Yeast extract and FeSO$_4$ were dissolved in 500 ml distilled water and volume made up to 1000 ml. 20 g agar-agar was added into this mixture and autoclaved at 15 lbs pressure and 121ºC for 15 min.

4. **Nutrient Agar medium**

Peptone	- 5g
Beef extract	- 3g
NaCl	- 5g
Agar-agar	- 20g
Distilled water	- 1000 ml
pH	- 7

Peptone, Beef extract and NaCl were dissolved in 500 ml distilled water and volume made up to 1000 ml. 20 g agar-agar was added into this mixture and autoclaved at 15 lbs pressure and 121ºC for 15 min.

5. **PGPR mix-I medium**

Malic acid	- 5g
Sucrose	- 10g
KH_2PO_4	- 1g
$MgSO_4$	- 0.4g
NaCl	- 0.2 g
$CaCl_2$	- 0.02g
$CaCO_3$	- 0.75g
$NaMoO_4$	- 5mg
NH_4Cl	- 100mg
Trace element solution	- 2ml
$FeSO_4$	- 0.05g
Vitamin solution	- 4 ml
KOH	- 4g

pH	- 6.8
Agar-agar	- 20g

Malic acid, Sucrose, KH_2PO_4, $MgSO_4$, NaCl, $CaCl_2$, $CaCO_3$, $NaMoO_4$, NH_4Cl, Trace element solution, $FeSO_4$, Vitamin solution, KOH were dissolved in 500 ml distilled water and volume made up to 1000 ml and autoclaved at 15 lbs pressure and 121⁰C for 15 min.

COMPOSITION OF CALCIUM CHLORIDE SOLUTION USED

1. **Calcium chloride solution(0.25 M)**

   Calcium chloride   - 36.755g

   Distilled water   - 1000ml

   Calcium chloride was dissolved in 800 ml of distilled water and mixed well till it dissolved completely. Then volume made up to 1000 ml and autoclaved at 15 lbs pressure and 121⁰C for 15 min.

COMPOSITION OF PHOSPHATE BUFFER USED

1. Phosphate buffer (0.1 M)

Na_2HPO_4 - 20.209g

NaH_2PO_4 - 3.394g

pH   - 5.8 to 7.4

Sodium phosphate dibasic and sodium phosphate monobasic were dissolved in 800ml of distilled water. Adjust the pH in the range of 5.8 - 7.4 and then volume made up to 1000 ml and autoclaved at 15 lbs pressure and 121ºC for 15 min.

APPENDIX – IV

COMPOSITION OF SALINE SOLUTION USED

1. **Saline solution (0.1 M)**

    NaCl   - 8.5g

    Water   - 1000ml

    pH   - 6.5 to 7.5

     Sodium chloride was dissolved in 800ml of water. Adjust the pH in the range of 6.5 - 7.5 and then volume made up to 1000 ml and autoclaved at 15 lbs pressure and 121ºC for 15 min.

ABSTRACT

DEVELOPMENT OF ENCAPSULATED FORMULATION OF PGPR MIX- I AND ITS EVALUATION

by

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ABSTRACT OF THE THESIS

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ABSTRACT

DEVELOPMENT OF ENCAPSULATED FORMULATION
OF PGPR MIX – I AND ITS EVALUATION

The study entitled "Development of encapsulated formulation of PGPR mix-I and its evaluation" was conducted during 2018-2020, in the Department of Agricultural Microbiology, College of Agriculture, Vellayani, Thiruvananthapuram, with the objective to develop calcium alginate based encapsulated formulation of PGPR mix-I and its evaluation for slow release and biodegradation.

The component cultures of PGPR mix-I were procured from the Department of Agricultural Microbiology, College of Agriculture, Vellayani for standardization of protocol for preparation of calcium alginate based encapsulated bead formulation of PGPR mix-I. Encapsulated bead formulation of PGPR mix-I was prepared by standard procedures.

An experiment was carried out to standardize the protocol for preparation of calcium alginate based encapsulated formulation of PGPR mix–I in completely randomized design with different treatments such as 10% Standard starch, 15% Standard starch, 10% Wheat flour, 15% Wheat flour, 10% Talc, 15% Talc and control treatment as 2% Sodium alginate alone in three replications.

Consistent viable count was recorded in encapsulated formulation amended with 10% Standard starch. It exhibited maximum viable count of each of the component cultures of PGPR mix-I as a result of three month population study. A significant decline of total viable population in control treatment was observed in each month compared to encapsulated formulation amended with 10% Standard starch.

Based on the population study, encapsulated formulation of PGPR mix-I 10% Standard starch amended was adjudged as the best combination of filler material and hence the shelf life studies of the same had to be continued at monthly intervals at room temperature and refrigerated conditions for six months by serial dilution and plate count method. Significant viable count was recorded in encapsulated beads stored at room temperature condition throughout the shelf life study.

The moisture content of beads were also monitored during standardization and shelf life study. During standardization study, moisture content of PGPR mix-I encapsulated beads of

each treatment was monitored for a period of three months at monthly intervals at room temperature and it showed a significant variation among treatments in each month. A reduction in moisture content of beads was observed from first month to the end of sixth month in all treatments. Beads amended with 10% Standard starch showed a moisture content of 13.37%, 12.07%, 11.72% and 11.45% after 24 hours of drying, first, second and third month respectively.

During shelf life study, 10% Standard starch combination at refrigerated condition showed moisture content in the range of 12.83% to 11.45% while at room temperature the same has recorded values in the range of 12.07% to 10.70%.

Evaluation of rate of release of immobilized bacteria from encapsulated beads was determined as per the procedure described by Bashan (1986) and the number of released bacteria was determined by the plate count method in respective selective medium. The higher cfu of component cultures of PGPR mix-I was observed after gentle shaking at $32^{\circ}C$ for 24hours (T1) in75ml of sterile saline solution.

Evaluation of biodegradation of encapsulated beads was studied at weekly intervals in sterile and non-sterile soil with PGPR mix-I inoculated and non-inoculated beads with three replications each (Bashan, 1986). Both the sets were observed weekly for their rate of biodegradation. As per biodegradation scale values like 0, >0-0.5, >0.5-1, >1-2, >2-2.5 or 3 was assigned according to the degree of visible degradation which indicates no visible degradation,onset of degradation, slight visible degradation on bead edges, one-half to three-fourth of the beads degraded, 90% of beads become mushy, full degradation (beads are disintegrated into small pieces or not found in the nylon bag) repectively (Bashan, 1986).

The PGPR mix-I inoculated beads with bacteria in non-sterile soil showed highest scale of biodegradation throughout the biodegradation study (mean value 1.34) and beads without bacteria in sterile soil showed the lowest scale (mean value 0.52).

Kruskal-Wallis rank sum test was done and there was a significant difference between treatments and so multiple comparison was done using Dunn test. During all the four weeks of biodegradation study, treatment T1 (beads with PGPR mix-I in non sterile soil) recorded the highest biodegradation and T4 (beads without PGPR mix-I in sterile soil) recorded the least biodegradation. Treatments T2 (beads with PGPR mix-I in sterile soil) and T3 (beads without PGPR mix-I in non sterile) were on par with both the treatments T1 and T4 in all the four weeks.

Treatment wise evaluation of biodegradation of beads   was done with Kruskal-Wallis rank sum test and gives a chi-squared value of 46.205 with df = 15 and p-value = 4.932e-05. There was a significant difference between treatments and so multiple comparison was done using Dunn test. Treatment T4 (beads with bacteria in non sterile soil during fourth week) showed significantly different from treatment T13 (beads without bacteria in sterile soil during first week).

In the present investigation, calcium alginate based encapsulated beads of PGPR mix-I amended with 10% Standard starch exhibited maximum viable count of component cultures of PGPR mix-I throughout the three months period of standardization study. In terms of evaluation of shelf life and moisture retention during storage, beads stored under room temperature condition was found to be better. The rate of release of component cultures of PGPR mix-I from the encapsulated formulation was more during the first 24-48 hours. Biodegradation studies of encapsulated beads of PGPR mix-I revealed that the beads inoculated with PGPR mix-I in non sterile soil showed highest biodegradation throughout the period of investigation.

സംഗ്രഹം

കാൽസ്യം ആൽജിനേറ്റ് അടിസ്ഥാനമാക്കിയുള്ള എൻക്യാപ്സുലേറ്റഡ് ഫോർമുലേഷൻ വികസിപ്പിക്കുകയെന്ന ലക്ഷ്യത്തോടെയും അതിന്റെ മന്ദഗതിയിലുള്ള ബഹിർഗമനവും ജൈവ വിഘടനത്തിന്റെ തോതും വിലയിരുത്തുന്നതിന് വേണ്ടിയും "പിജിപിആർ മിക്സ് -1 എൻക്യാപ്സുലേറ്റഡ് ഫോർമുലേഷന്റെ വികസനവും അതിന്റെ വിലയിരുത്തലും" എന്ന ഗവേഷണ പഠനം 2018-2020 കാലയളവിൽ വെള്ളായണി കാർഷിക കോളേജിലെ കാർഷിക മൈക്രോബയോളജി വിഭാഗം നടത്തി.

കാൽസ്യം ആൽജിനേറ്റ് ഉപയോഗിച്ചുള്ള പിജിപിആർ മിക്സ് -1 എൻക്യാപ്സുലേറ്റഡ് ഫോർമുലേഷൻ അഥവാ ബീഡ് ഫോർമുലേഷൻ തയ്യാറാക്കുന്നത്തിനു വേണ്ടി മാതൃ കൽച്ചറുകൾ വെള്ളായണി കാർഷിക കോളേജിലെ കാർഷിക മൈക്രോബയോളജി വിഭാഗത്തിൽ നിന്നും സംഭരിച്ചു. മുൻ പഠനങ്ങളുടെ അടിസ്ഥാനത്തിൽ പിജിപിആർ മിക്സ് -1 ഇന്റെ എൻക്യാപ്സുലേറ്റഡ് ഫോർമുലേഷൻ തയ്യാറാക്കി.

കാൽസ്യം ആൽജിനേറ്റ് ഉപയോഗിച്ചുള്ള പിജിപിആർ മിക്സ് -1 എൻക്യാപ്സുലേറ്റഡ് ഫോർമുലേഷൻ തയ്യാറാക്കുന്നത്തിനു വേണ്ടിയുള്ള മിശ്രിതങ്ങൾ ചിട്ടപ്പെടുത്തുന്നതിനു വേണ്ടി ഒരു പരീക്ഷണം നടത്തി. ഇതിനായി 10% സ്റ്റാൻഡേർഡ് അന്നജം, 15% സ്റ്റാൻഡേർഡ് അന്നജം, 10% ഗോതമ്പ് മാവ്, 15% ഗോതമ്പ് മാവ് , 10% ടാൽക്ക്, 15% ടാൽക്ക്, കൺട്രോൾ ട്രീറ്റ്മെന്റ് ആയി 2% സോഡിയം ആൽജിനേറ്റ് മാത്രം എന്നിവ മൂന്ന് റെപ്ലിക്കേഷൻ വീതം പരീക്ഷിച്ചു.

10% സ്റ്റാൻഡേർഡ് അന്നജം ഉപയോഗിച്ച് രൂപപ്പെടുത്തിയ എൻക്യാപ്സുലേറ്റഡ് ഫോർമുലേഷനിൽ ബാക്റ്റീരിയകളുടെ എണ്ണം സ്ഥിരമായും ഗണ്യമായും രേഖപ്പെടുത്തി. ബാക്റ്റീരിയകളുടെ എണ്ണം ഇപ്പറഞ്ഞ ഉത്പന്നത്തിൽ പ്രതിമാസ ഇടവേളകളിൽ മൂന്ന് മാസം വരെ പഠന വിധേയമാക്കി. 10% സ്റ്റാൻഡേർഡ് അന്നജം ഉപയോഗിച്ച് രൂപപ്പെടുത്തിയ എൻ ക്യാപ്സുലേറ്റഡ് ഫോർമുലേഷനിൽ ബാക്റ്റീരിയകളുടെ എണ്ണം ഗണ്യമായി വർധിച്ചതായി കണ്ടു. എന്നാൽ ഇവയില്ലാതെയുള്ള കൺട്രോൾ ട്രീട്മെന്റിൽ ബാക്റ്റീരിയകളുടെ എണ്ണം ഓരോ മാസവും ഗണ്യമായി കുറയുന്നത് കണ്ടു.

ബാക്റ്റീരിയകളുടെ എണ്ണത്തിന്റെ അടിസ്ഥനത്തിൽ 10% സ്റ്റാൻഡേർഡ് അന്നജം ഉപയോഗിച്ചു രൂപപ്പെടുത്തിയ പിജിപിആർ മിക്സ് -1 എൻക്യാപ്ലേറ്റഡ് ഫോർമുലേഷൻ ഏറ്റവും മികച്ച ഫില്ലർ മെറ്റീരിയൽ ആയി തിരഞ്ഞെടുക്കപ്പെട്ടു. ആയതിനാൽ അതിന്റെ സൂക്ഷിപ്പു കാലാവധി പഠനങ്ങൾ പ്രതിമാസ ഇടവേളകളിൽ സാധാരണ താപനിലയിലും ശീതീകരിച്ച അവസ്ഥയിലും താരതമ്യ പഠനം നടത്തി. ഈ പഠനം ആറ് മാസം തുടരുകയും ചെയ്തു. ഇപ്രകാരം ഉള്ള പഠനത്തിൽ, സാധാരണ താപനിലയിൽ സൂക്ഷിച്ചിരുന്ന എൻക്യാപ്ലേറ്റഡ് ഫോർമുലേഷനിൽ ബാക്റ്റീരിയകളുടെ ഗണ്യമായ എണ്ണം രേഖപ്പെടുത്തി.

മേൽ പഠനങ്ങളിൽ ഈ ഫോർമുലേഷനിൽ അടങ്ങിയിരിക്കുന്ന ഈർപ്പവും പഠനത്തിന് വിധേയമാക്കി. എല്ലാ പഠനങ്ങളിലും ഇവയുടെ ഈർപ്പം ആദ്യ മാസം മുതൽ ആറാം മാസം വരെ കുറയുന്നതായി കണ്ടു. സാധാരണ മുറി താപനിലയിൽ 10% സ്റ്റാൻഡേർഡ് അന്നജം ഉപയോഗിച്ച് രൂപപ്പെടുത്തിയ ബീഡുകൾ 13.37% (24 മണിക്കൂർ ഉണങ്ങിയതിനുശേഷം), 12.07%(ആദ്യ മാസം), 11.72%(രണ്ടാം മാസം), 11.45%(മൂന്നാം മാസം) എന്നിങ്ങനെ ഈർപ്പം രേഖപ്പെടുത്തി.

സൂക്ഷിപ്പു കാലാവധി പഠനത്തിനിടയിൽ, 10% സ്റ്റാൻഡേർഡ് അന്നജം ഉപയോഗിച്ച് രൂപപ്പെടുത്തിയ ബീഡുകൾ ശീതീകരിച്ച അവസ്ഥയിൽ 12.83% മുതൽ 11.45% ഈർപ്പം കാണിച്ചു. എന്നാൽ സാധാരണ താപനിലയിൽ ഇത് 12.07% മുതൽ 10.70% ഈർപ്പമാണ് രേഖപ്പെടുത്തിയത്.

ഇപ്രകാരം ഉരുത്തിരിച്ചെടുത്ത എൻക്യാപ്ലേറ്റഡ് ഫോർമുലേഷനിൽ നിന്ന് ബാക്റ്റീരിയകളുടെ ബഹിർഗമനം അതത് സെലക്ടീവ് മാധ്യമത്തിൽ നിർണ്ണയിച്ചു. 75 മില്ലി അണുവിമുക്തമായ ഉപ്പുവെള്ള ലായനിയിൽ 24 മണിക്കൂർ 32 ഡിഗ്രി സെൽഷ്യസിൽ സൂക്ഷിച്ച ശേഷം ബാക്റ്റീരിയകളുടെ എണ്ണം നിർണയിക്കുകയും അതിൽ ഗണ്യമായ എണ്ണം കാണപ്പെടുകയും ചെയ്തു..

ഇപ്രകാരം ഉരുത്തിരിച്ചെടുത്ത പിജിപിആർ മിക്സ് -1 എൻക്യാപ്ലേറ്റഡ് ഫോർമുലേഷന്റെ ജൈവ വിഘടന നിരക്ക് പഠിക്കുന്നതിനായി അണുവിമുക്തവും അണുവിമുക്തമല്ലാത്തതുമായ മണ്ണിൽ പ്രതിവാര ഇടവേളകളിൽ പിജിപിആർ മിക്സ് -1 അടങ്ങിയതും അടങ്ങാത്തതുമായ ബീഡുകൾ മൂന്ന് തനിപ്പകർപ്പുകൾ ഉപയോഗിച്ചു വിലയിരുത്തി. ജൈവ

വിഘടന നിരക്ക് കണക്കിലെടുത്ത് രണ്ട് സെറ്റുകളും ആഴ്ചതോറും നിരീക്ഷിച്ചു. ബാഷൻ (1986) ഉരുത്തിരിച്ചെടുത്ത ജൈവ വിഘടന സ്കെയിലിന്റെ അടിസ്ഥാനത്തിൽ ജൈവ വിഘടനം പഠനവിധേയമാക്കി.

അണുവിമുക്തമല്ലാത്ത മണ്ണിൽ ബാക്റ്റീരിയകൾ അടങ്ങിയ പിജിപിആർ മിക്സ് -1 ബീഡുകൾ ജൈവ വിഘടന പഠനത്തിലുടനീളം ഉയർന്ന തോതിലുള്ള വിഘടനം പ്രദർശിപ്പിച്ചു (ശരാശരി മൂല്യം 1.34). അണുവിമുക്തമായ മണ്ണിൽ ബാക്ടീരിയകളില്ലാത്ത ബീഡുകൾ ഏറ്റവും കുറഞ്ഞ തോതിൽ വിഘടനം പ്രദർശിപ്പിച്ചു (ശരാശരി മൂല്യം 0.52).

പിജിപിആർ മിക്സ് -1 എൻക്യാപ്സുലേറ്റഡ് ഫോർമുലേഷന്റെ ജൈവ വിഘടനം വിലയിരുത്തിയത് ക്രൂസ്കൽ-വാലിസ് റാങ്ക് സം ടെസ്റ്റ് ഉപയോഗിച്ചാണ്. ഇത് 46.205 എന്ന കൈ -സ്ക്വയർ മൂല്യവും df മൂല്യം = 15 ഉം p- മൂല്യം = 4.932e-05 ഉം നൽകുന്നു. ട്രീട്മെന്റുകൾ തമ്മിൽ കാര്യമായ വ്യത്യാസമുണ്ടായിരുന്നു. അതിനാൽ ഡൺ ടെസ്റ്റ് ഉപയോഗിച്ച് ഒന്നിലധികം താരതമ്യം ചെയ്തു.

നിലവിലെ ഈ പഠനത്തിൽ, 10% സ്റ്റാൻഡേർഡ് അന്നജം ഏറ്റവും നല്ല ഫില്ലർ മെറ്റീരിയൽ ആയി തിരഞ്ഞെടുത്തു. ഇവ ഉപയോഗിച്ച് രൂപപ്പെടുത്തിയ പിജിപിആർ മിക്സ് -1 ന്റെ കാൽസ്യം ആൽജിനേറ്റ് അടിസ്ഥാനമാക്കിയുള്ള എൻ ക്യാപ്സുലേറ്റഡ് ഫോർമുലേഷന്റെ സൂക്ഷിപ്പു കാലാവധി വിലയിരുത്തൽ, സംഭരണ സമയത്ത് ഈർപ്പം നിലനിർത്തൽ എന്നിവ കണക്കിലെടുക്കുമ്പോൾ, സാധാരണ താപനിലയിൽ സൂക്ഷിച്ചിരുന്ന ബീഡ് ഫോർമുലേഷൻ മികച്ചതായി കണ്ടെത്തി. എൻക്യാപ്സുലേറ്റഡ് ഫോർമുലേഷനിൽ നിന്ന് പിജിപിആർ മിക്സ് -1 ൽ അടങ്ങിയിരിക്കുന്ന ബാക്ടീരിയ ഘടകങ്ങളുടെ ബഹിർഗമന നിരക്ക് ആദ്യ 24-48 മണിക്കൂറുകളിൽ കൂടുതലായിരുന്നു. അണുവിമുക്തമാക്കാത്ത മണ്ണിൽ ബാക്ടീരിയ അടങ്ങിയ ബീഡിൽ നല്ല തോതിൽ ജൈവ വിഘടനം നടന്നതായി ഈ പഠനത്തിൽ നിന്നും ബോധ്യപ്പെട്ടു.