MICROENCAPSULATION OF *Trichoderma viride* FOR MANAGEMENT OF MAJOR SOIL BORNE FUNGAL PATHOGENS

THESIS

Submitted in partial fulfillment of
the requirement for the degree of

Master of Science in Agriculture

by

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(2017-11-026)

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DECLARATION

I, hereby declare that the thesis entitled **"Microencapsulation of *Trichoderma viride* for management of major soil borne fungal pathogens"** is a bonafide record of research done by me during the course of research and that it has not previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title, of any other University or Society.

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CERTIFICATE

Certified that this thesis entitled **"Microencapsulation of *Trichoderma viride* for management of major soil borne fungal pathogens"** is a record of research work done independently by **Mrs. Saleena M. (2017-11-026)** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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We, the undersigned members of the advisory committee of **Ms. Saleena M. (2017-11-026)**, a candidate for the degree of **Master of Science in Agriculture** with major field in Plant Pathology, agree that the thesis entitled **"Microencapsulation of *Trichoderma viride* for management of major soil borne fungal pathogens"** may be submitted by her in partial fulfillment of the requirement for the degree.

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INTRODUCTION

1. INTRODUCTION

The realms of plant microbiota are diverse and fascinating. These unseen majority of the phytobiome embraces both beneficial as well as detrimental entities. The latter thus cause havoc to cultivated lands which are meant to feed the escalating world population. Therefore, their control is one of the major challenges that human race ever face. Constant and meticulous efforts gave rise to an array of chemical pesticides that enriched the global agrochemical market from time to time. However, final destination of the remnants of these chemicals into soil, water, air and ecosystem, created tremendous chaos, thus, they became another most dreadful challenge for humanity.

In this context, the arena of crop disease management is in search of new avenues and horizons. The need of the hour is to develop an eco-friendly approach to combat plant pathogens efficiently, without impairing the mother nature. This call for viable alternatives has led the scientific community to engage in unveiling the potential of biopesticides. Exploiting their benefits as biocontrol agents appears to be a more promising single move tactic, towards the issues of crop loss due to phytopathogens as well as environmental degradation due to synthetic pesticides.

The efforts put forth by diligent scientists all over the world revealed the magnificent world of biocontrol agents. *Trichoderma* spp. are one among them, which are excellent biological means for plant disease management, and was first recognized in the early 1930s (Weindling, 1932). Several species of *Trichoderma* are found to combat soil borne fungal pathogens like *Pythium* sp., *Phytophthora* sp., *Rhizoctonia* sp., *Sclerotium* sp., and *Fusarium* sp. which pose extreme menace to cultivated crops (Cook and Baker,1983). The antagonistic fungi with its diverse mechanisms of action, inhibits the growth and survival of notorious phytopathogens.

Antagonistic feature of *Trichoderma* spp. are attributed principally to the phenomenon of mycoparasitism or hyperparasitism, where the hyphal interaction plays

the dominant role in coiling, penetration, and subsequent dissolution of the host cytoplasm (Weindling, 1932). Antibiosis is another key tool in which antibiotics, toxins, and volatiles are emanated to external premises (De Marco *et al.*, 2003). Competition and rhizosphere competence also contributes to proficient antagonism through highlighted supremacy in competition for space and nutrition. Furthermore, an array of lytic enzymes including chitinases and glucanases indulge in cell wall degradation of pathogenic entity (Viterbo *et al*., 2002). Apart from disease control, *Trichoderma* spp. promote plant growth as well as induce abiotic stress tolerance in plants (Djonovic *et al*., 2007).

However, microbial biomass as such applied in the field has to encounter the competition from well adapted native populations, fluctuating environment and unfavourable edaphic factors. Therefore, on commercial release, the inoculum should be supported by appropriate formulation to evade rapid decline of population and to ensure extended shelf life of the product. Being the key component towards commercial success, a promising formulation helps to stabilize the organism during production, distribution and storage. It also facilitates easy handling and application of the product along with safeguarding the bioagent in hostile environmental conditions (Jones and Burges, 1998).

Till date, several formulations of *Trichoderma* spp. has been developed. Dry, powdered or moist biomass of *Trichoderma* spp. were formulated into a variety of dusts, powders, pellets, granules or gels supplemented with inert carriers or food bases. To date, most marketed *Trichoderma* sp. inoculants are talc (Magnesium tri silicate) based which usually have a reduced shelf life and viability. Also, it is bulky to handle, transport and storage. Moreover, these talc based formulations suffer major setbacks like high rate of contamination and low field performance. Therefore, refinement and revision of previously developed formulations are also equally demanded as the discovery of new potentials strains.

To achieve the same, several trials on polymer based formulations has been conducted. From these scrupulous attempts, some polymers were observed as potential microbial carriers offering substantial practical advantages over talc. Such polymers can be used for microencapsulation, an advanced technology for convenient and efficient immobilization of bioagent in microcapsules or beads. Propagules are gradually released from these capsules upon natural degradation of their matrix, ensuring the protection and survival of bioagents from lethal influences of environment. Microencapsulation imparts prolonged shelf life along with offering consistent superior batch quality of the formulation. Apart from that, it also provides ample opportunity for easy and need based manipulations of formulation (Chen *et al*., 2013).

Like any other factor, carrier material also plays a vital role in farmer acceptance and field performance of a formulation. There are many lacunae in defining best carrier materials in conventional formulations. Sodium alginate, a polysaccharide with excellent gel forming properties is identified as a potential carrier for *Trichoderma*. It is eco-friendly and biodegradable in nature as well as uniform and consistent in properties. The resulting preparation is lighter and compact, renders shipment and storage an easy task, in contrary to most organic matter preparations.

Remarkably, the evolving tendencies in the field of crop protection are strongly aligned towards the ideals of biological control. To deal with the exacerbating requirements, corresponding improvements are demanded in each and every aspect of production technology. The present study therefore, is undertaken to standardize and prepare sodium alginate based bead formulations of *Trichoderma* with the potential to overcome the drawbacks of existing formulations. Major objectives of the study are framed as:

- Standardization and preparation of alginate based bead formulation of *Trichoderma viride*
- Evaluation of shelf life of *T. viride* encapsulated alginate beads
- *In vivo* evaluation of encapsulated *T. viride* formulation for biocontrol efficacy and plant growth promotion
- Assessment of biodegradation of *T. viride* encapsulated alginate beads in soil

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Trichoderma, a genus of fungi that comprise numerous fungal strains belongs to the family Hypocreaceae. It is the most predominant culturable fungi present in almost all soils. (Harman *et al*., 2004). The genus was described as early as 1794 by Persoon. However, it's biocontrol activity was first recognized only in early 1930's (Weindling, 1932). Till date, several strains of the fungus have been isolated and found to excel in biocontrol of many soil borne plant pathogenic fungi under green house and field conditions (Sesan *et al*., 1999; Elad, 2000; Lewis and Lemsden, 2001; Benitez *et al*., 2004). Successful use of *Trichoderma* spp. for the control of soil borne diseases caused by pathogens like *Rhizoctonia*, *Sclerotium*, *Fusarium*, *Pythium* and *Phytophthora* have been reported (Cook and Baker,1983). The broad spectrum anti-fungal activity of the fungus is attributed to combination of several mechanisms including mycoparasitism, antibiosis and deployment of an array of tools called enzymes. Apart from that, many species of this genus are identified as opportunistic avirulent plant symbionts, which boost plant defence mechanisms as well (Harman *et al.*, 2004).

Application of such biocontrol agents gained momentum in the present day agriculture. However, attempts to surpass the bottlenecks associated with the direct field application of microbial inoculum resulted in the development of several bioagent formulations.

2.1 FORMULATION: THE KEY STEP TOWARDS COMMERCIALIZATION

Microorganisms applied in the field encompass many challenges in response to competition from well adapted indigenous microbial population, varying temperature, drought, unfavourable pH, adsorption to soil particles or draining-off by rain or irrigation water (Bashan, 1986; Winder *et al*., 2003; Young *et al*., 2006). Moreover, the germination of conidia or chlamydospores of *Trichoderma* spp. are susceptible to soil

fungistasis (Xu *et al*., 2004; Pan *et al*., 2006). Therefore, the inoculum of bioagent directly applied to the field has restricted gradients of dispersion and reproduction in soils. Consequently, the commercial development of bioagents against soil borne pathogens in the spermosphere or rhizosphere demands potential to overcome obstacles in growth, formulation and delivery. To achieve the same, microbial inoculum should be supported by appropriate formulation on commercial release. The formulation helps to elude rapid decline of the organism population and to extend the shelf life. It also facilitates the stabilization of the organism during production, distribution and storage. Apart from that, a promising formulation enables easy handing and application of the product, ensure protection of bioagent from harmful environmental factors and enhance the activity of the organism (Jones and Burges, 1998). Castillejos *et al*. (2002) acknowledged the formulation as one of the chief priorities of biopesticide exploration. Similarly, Legget *et al*. (2011) considered formulation as a key step towards the successful commercialization of any promising biocontrol agent.

An effective formulation requires thorough knowledge of the biocontrol agent, pathogen to be controlled, environment and interactions of bioagent with other organisms. The development of a commercially viable formulation also requires a better understanding of common application practices and equipments, as well as the desires of the farmers. Ingredients must be ecologically safe, easily available and acceptable to regulatory agencies in areas where the product gets access. These constraints resulted in limited extend of commercialization and detains the field from attaining momentum. To encounter the prevailing situation, researches are still going on in the field of inoculant production to develop advanced formulations.

However, several preparations based on variety of carrier materials were formulated for application in agriculture (Cassidy *et al*., 1996; Bashan, 1998; Schisler *et al*., 2004; John *et al*., 2011; Vemmer and Patel, 2013). Delivery systems of preparations meant for seed treatment and soil drenching for the control of various soil borne plant

pathogens are also available. Remarkably, the potential isolates of *Trichoderma* spp. are also subjected to formulations using versatile organic and inorganic carriers either through solid or liquid fermentation technologies. Kumar *et al*. (2014) pointed out that these formulations are applied though seed treatment, bio-priming, seedling dip, soil application or foliar spray for the proper delivery of biocontrol agent. Till date, a number of formulations of *Trichoderma* spp. based on diverse carrying materials have been developed. Several researches with noticeable progress have been conducted on various formulations of *Trichoderma* spp. and some of the relevant published works are reviewed here.

2.2 SOLID FORMULATIONS

There are systems which describe the growth of biocontrol agents on solid fermentation media consisting of various grains or inert carriers amended with food bases. Dry, powdered or moist biomass can be formulated into a variety of dusts, powders, pellets, granules or gels supplemented with inert carriers or food bases.

2.2.1 Solid organic substances as carrier materials

Mukhopadhyay *et al*. (1986) developed wheat bran-saw dust modified medium based formulation of *Trichoderma* sp. Likewise, Upadhyay and Mukhopadhyay (1986) also proposed a formulation of *Trichoderma* sp. with sorghum grain as the base material. Similarly, Gangadharan and Jeyarajan (1990) used crop residues like tapioca rind and tapioca refuse along with FYM and pressmud for the formulation of *Trichoderma* sp. They also tried substrates like gobar gas slurry, mushroom spent bed, paddy husk, wheat bran and groundnut shell for mass multiplication of *T. harzianum*. Wheat flour based granule formulation of *Trichoderma* sp. was developed by Connick *et al*. (1991) where, 80g wheat flour was mixed with 52 ml fermented biomass of *Trichoderma* sp. in sufficient volume of water, which was dried, powdered and passed through 18 mesh sieve

to get the formulation. Moreover, Taylor *et al*. (1991) developed a powder formulation of *T. harzianum* for dry seed treatment using a solid particulate material Agro-Lig or muck soil.

Sangeetha *et al*. (1993) prepared a formulation of *Trichoderma* sp. using substrates like FYM, wheat bran, rice bran, peat soil and paddy straw. Similarly, Raguchander *et al*. (1993) utilized groundnut shell medium for preparing *Trichoderma* formulation. Spent tea leaf waste and coffee husk were used for formulation of *Trichoderma* sp. by Bhai *et al*. (1994). Likewise, Jagadeesh and Geeta (1994) utilized wheat bran and biogas manure as base material for mass multiplication of *Trichoderma* sp. Jayaraj and Ramabadran (1996) used pigeonpea husk and tapioca waste in combination with pressmud for the formulation of *Trichoderma*. Sawant and Sawant (1996) developed a *Trichoderma* formulation based on coffee husk, which was found very effective in managing *Phytophthora* foot rot of black pepper in Karnataka and Kerala. Moreover, Kumar and Marimuthu (1997) used decomposed coconut coir pith for formulation of *Trichoderma* sp. Jahagirdar *et al*. (1998) appraised 10 different substrates for the mass production of *T. viride* under *in vitro* condition, where the wheat bran promoted the maximum sporulation (2.15×10^6 cfu g^{-1}) followed by farm yard manure (1.93×10^6 cfu g^{-1}) and cow dung (1.15×10^6 cfu g^{-1}) after 21 days of incubation. Among different oilcakes evaluated, pongamia cake performed the best (9.1×10^4 cfu g^{-1}) followed by neem cake and groundnut cake. Wheat flour-kaolin based formulation of *Trichoderma* sp. was developed by Prasad and Rangeswaran (1998) by mixing 80 g wheat flour and 20 g kaolin in 52 ml fermented biomass of *Trichoderma*.

Prakash *et al*. (1999) reported that tea waste was the best media among four different substrates evaluated for the mass production of *T. harzianum* and *T. virens* (*Gliocladium virens*). Biomass multiplied on tea waste was stored for three months without much decline in the population. Singh *et al*. (2001) evaluated 11 semi solid substrates for the mass multiplication of six isolates of *Trichoderma* spp. where the

combination of wheat straw and wheat bran (3:1) was found best for three isolates of *T. harzianum*. Further, sugarcane waste in combination with spent tea leaves supported *Trichoderma reesei*, while, *T. viride* and *T. koningii* showed maximum proliferation on spent tea leaves-wheat bran substrate. Gandhikumar *et al.* (2001) scrutinized the efficacy of different carrier materials like shelled maize cob powder, coir pith, peat and black gram shell powder to sustain the population of *T. viride* and *T. harzianum*. The proliferation of *T. viride* was superior in shelled maize cob powder followed by black gram shell powder. Shelled maize cob powder supported maximum population of *T. harzianum* while, peat recorded a minimum population of both species after 30 days of storage. Similarly, Saju *et al*. (2002) utilised coir pith, FYM and decomposed coffee pulp to evaluate the shelf-life of *T. viride*, where FYM was the best carrier for prolonged storage of *T. viride*. Likewise, spent malt based formulation of *Trichoderma* sp. was prepared by Gopalakrishnan *et al*. (2003).

Balasubramanian *et al*. (2008) proposed the protocol for mass multiplication of *Trichoderma* sp. in banana waste amended with urea and rock phosphate. The substrate was composted with culture of *Bacillus polymixa, Pleurotus sajor caju* and *T. viride* for 45 days and enriched culture was made available for field application. Several researchers developed wettable powder formulations of various *Trichoderma* strains (Elad, 2000; Nagayama *et al*., 2007; Sesan and Oancea, 2010; Sriram *et al*., 2011). Similarly, Singh and Nautiyal (2012) prepared a concentrated formulation of *T. harzianum* MTCC-3841 (NBRI-1055) by simple scrapping method, where spores scrapped from potato dextrose agar plates were used to prepare the concentrated formulation. Moreover, Kumar *et al*. (2013) prepared sorghum grains and charcoal based formulation of *T. viride.* Khan *et al*. (2011) formulated *T. viride* in de-oiled castor cake, vermicompost and well decomposed farmyard manure.

2.2.2 Solid inorganic substances as carrier materials

Gandhikumar *et al.* (2001) utilized carrier materials like gypsum ($CaSO_4.2H_2O$) and talc to sustain the population of *T. viride* and *T. harzianum*. Likewise, Karunanithi *et al*. (2001) used different carrier materials *viz.,* talc, peat, lignite, kaolin, gypsum and fly ash for the mass multiplication of *T. viride*, where talc and gypsum maintained the population of *T. viride* for 150 days on storage. Jayaraj *et al*. (2006) developed formulations of *T.harzianum* strain M1 with base materials like talc, lignite, fly ash, bentonite, polyethylene glycol and gelatin-glycerin gel for seed treatment in tomato to control damping-off caused by *Pythium aphanidermatum*. Similarly, Kumar *et al*. (2013) prepared talc based formulations of *T. viride* and Khan *et al*. (2011) formulated *T. viride* in gypsum and talc powder.

2.3 LIQUID FORMULATIONS

Taylor *et al*. (1991) developed a liquid seed coating formulation of *T. harzianum* strain 1295 - 22 with an aqueous binder (Pelgel or Polyox N-10), where a continuous and uniform coating of < 0.1mm thickness was formed around the seed by spraying this suspension onto seeds in a tumbling drum. Batta (2004) formulated the conidia of *T. harzianum* in coconut oil and soybean oil based water in oil type invert emulsion with 6.0×10^7 conidia ml^{-1} formulation to combat blue mold causing post-harvest decay of apple. Similarly, Kolombet *et al*. (2008) developed a liquid formulation of *T. asperellum* with starch as the base material. They lowered the pH to reduce the metabolic activity of bioagent and to regulate O_2 supply so as to remain the formulation viable for atleast six months at room temperature. Likewise, Sathiyaseelan *et al*. (2009) developed liquid formulations of *T. viride* using five different liquid carriers. Oils like paraffin oil, soya bean oil, combination of paraffin oil and glycerol (1:1), soya bean oil and glycerol (1:1), Paraffin oil and soybean oil (1:1) were used, among which paraffin oil showed higher activity. Solanki *et al*. (2011) prepared liquid formulation of *Trichoderma*

sp. amended with chitin (1% v:v) and CMC (0.5% w:v) for the management of root rot in tomato under greenhouse condition. Moreover, Navaneetha *et al*. (2015) formulated a suspension concentrate (SC) of *T. harzianum* Th4dSC and conidial biomass consortium of *T. harzianum* Th4d SC with *T .asperellum* Tv5 SC.

2.4 BOTTLE NECKS OF SOLID AND LIQUID FORMULATIONS OF *Trichoderma* spp.

Eventhough, a number of *Trichoderma* formulations were developed within last few decades, commercialization was not achieved to that extent. Navaneetha *et al*. (2015) opined that the composition, concentration, shelf life and erratic performance are the major concern in formulation of bioagents in general and *Trichoderma* spp. in particular. In addition to that, Papavizas (1985) stated that the inadequate knowledge regarding the methods of mass production and suitable delivery systems created critical impediments in commercialization. Moreover, many researchers could detect several limitations with respect to shelf life and field performance of already existing formulations. A few noticeable findings are illustrated below.

According to Mukhopadhyay (1994), the drawbacks associated with talc based powder formulations include dehydration, poor shelf life and loss of viability of propagules beyond three months of storage. In addition, Singh *et al*. (2000) observed that these pitfalls may affect the efficiency and marketability of the formulation. Similarly, Sankar and Jeyarajan (1996) observed that viable propagules of *Trichoderma* in talc formulation was reduced by 50 per cent after 120 days of storage. Likewise, Prasad and Rangeshwaran (2000) reported a significant drop in *Trichoderma* population in talc, kaolin and bentonite carrier materials after 120 days of storage. Moreover, Chaube *et al*. (2002) observed 82 per cent conidial viability of *T. virens* in a talc based preparation for more than six months at 5ºC under refrigerated condition while same level of viability

was retained only for three months at room temperature.

Ramanujam *et al*. (2010) remarked the technique of mass multiplication of *Trichoderma* spp. in cheap cereal grains as laborious since the resultant product is bulky and prone to contamination. Further, they noticed that talc, peat, lignite and kaolin based formulations of *Trichoderma* have a shorter shelf life of only three to four months. Jin and Custis (2011) agreed with the promising biocontrol efficacy of wetteble powder formulation of various *Trichoderma* strains on field trials. Nevertheless, they pointed out significant drawbacks related to dust formation, hazards to environment or users and variable water activity resulting in high microbial contamination and reduced shelf life. Meng *et al*. (2015) also made a similar opinion in this regard. Moreover, Sriram *et al*. (2011) reported that the liquid fermentation based formulations of *Trichoderma* spp. were vulnerable to desiccation compared to solid state fermentation based formulations. He also pointed out that a liquid formulation without the addition of glycerol remained viable only four to five months. In addition, John *et al*. (2011) observed quick loss of viability of liquid formulation due to lack of carrier protection.

Kumar *et al*. (2013) recorded 69.04, 70.3, and 72.44 per cent decline in population of *T. viride* in talc, sorghum grains and charcoal based formulations respectively after 120 days of storage. Likewise, Khan *et al*. (2011) evaluated the shelf life of *T. viride* formulated in de-oiled castor cake, gypsum, talc powder, vermicompost and well decomposed farmyard manure at 30ºC. They observed substantial decline in the population of bioagent 80 days after storage, where the vermicompost, de-oiled castor cake and farmyard manure formulations retained shelf life only for 220, 190 and 180 days respectively. Further, they observed that talc and gypsum based formulations had shorter shelf life and solid fermentation method suffer from major setbacks like demand of huge volume of substrates, chances of contamination during fermentation and lengthy fermentation process.

From the above reviewed researches, it is inferred that the prevailing formulations demands a promising carrier material having a steady and sustained release pattern along with superior field performance. Interestingly, the evolving tendencies in the arena of crop protection are strongly aligned towards the principles of biological control. To deal with the exacerbating requirements, proportionate improvements are sought in each and every aspect of production technology. To fulfil the same, refinement of already established formulations are also demanded equally as the discovery of new potential strains.

2.5 MICROENCAPSULATION: AN EVOLVING TOOL FOR BIOCONTROL

Vert *et al*. (2012) defined microcapsules as hollow micro particles composed of a solid shell surrounding a core, forming space available for temporary or permanent entrapment of substances. The technique of encapsulation was considered as a provision for ensured protection and maintenance of the properties of microorganisms (Bashan, 1998; dos Santos *et al*., 2015). Nagpal *et al*. (2012) designated microencapsulation as an excellent technique to modify and delay the drug release pattern. Moreover, Rathore *et al*. (2013) remarked microencapsulation as an efficient and convenient tool for the preservation and application of microorganisms. For the production of microcapsules, the process of ionotropic gelation was employed as a reliable and relatively easy method (Khan and Bajpai, 2011; Rasel and Hasan, 2012). However, Szczech and Maciorowski (2016) adopted the technique of oil emulsification to produce alginate microcapsules with organic additives.

John *et al*. (2011) revealed that most of the currently available solid and liquid formulations showed inconsistent viability of bioagent during storage and field application. Hence, they also suggested the immobilization of bioagents for improved shelf life, controlled microbial release and enhanced field efficacy. According to them, microencapsulation is an adoptable technology with the possibility to overcome the

pitfalls of existing formulations. Furthermore, the performance of a formulation predominantly relies on the carrier material. However, John *et al.* (2011) observed that lack of sufficient knowledge regarding the best carrier materials created lacunae in conventional formulations. Therefore, the discipline of plant protection is in search of a reliable carrier material too.

2.5.1 Sodium alginate: the promising carrier material

Yabur *et al*. (2007) reported that alginate, a biopolymer derived from the macroalgae is widely used among the different carriers. According to Sutherland (1991), sodium alginate (SA) is a water soluble salt of alginic acid, a naturally occurring non-toxic polysaccharide found in brown algae. Chemically, it is a polysaccharide composed of varying proportions of two uronic acids; α-L- guluronic (G) and β-D-mannuronic (M) acids linked by (1-4) glycosidic linkages. Aslani and Kennedy (1996) noticed that, they are arranged in homopolymeric blocks of MM or GG and blocks with an alternating sequence (MG blocks). These are mainly extracted from brown algae such as *Laminaria* spp., *Macrocystis* sp., *Ascophyllum* sp., *Eklonia* spp., *Lessonia* spp., *Durvillea* spp., *Sargassum* spp. etc. Sriamornsak *et al*. (2007) opined that sodium alginate is a promising candidate in drug delivery systems due to assured biological safety. Added advantages of sodium alginate as a carrier material include non-toxic and near sterile nature, consistent quality and compatibility with microorganisms (Pourjavadi *et al*., 2006).

Alginate exhibits a unique gel-forming property in the presence of a multivalent cation such as calcium ions in an aqueous medium. It takes place mainly at junctions in the G-G sequence rich region, known as ‘egg box junctions’ (Xing *et al*., 2003; Rees, 1981). Deasy (1984) suggested that the hydrogel forming property enabled the alginate to encapsulate macromolecular entities as well as low molecular weight therapeutic agents within the polymer matrix. It also facilitates pH dependent controlled release of the encapsulated drug from sodium alginate preparations. The gelling property of alginate

and cross linking nature of divalent metals contributes to alginate beads formation in drug delivery systems. According to the reports of Thu *et al*. (1997), divalent cations of metals like calcium, barium, tin *etc*. come in contact with alginate solution which resulted in rapid ion binding and the formation of a polymeric network. This in turn produced a centripetally moving gelling zone where alginate moved from the gel core towards this gelling zone and resulted in the deletion of alginate within the core. Tavakol *et al*. (2013) described that the diffusion of cross-linker ion into polymer droplet resulted in the consolidation of alginate-polymer chains into an egg-box structure. Consequently, water soluble sodium alginate on reaction with $CaCl_2$ produces water insoluble calcium alginate matrix and Lin and Ayres (1992) observed that these matrices were highly permeable in nature.

Kumar *et al*. (2001) defined beads as discrete spherical microcapsules that serve as the solid substrate on which the drugs are coated or encapsulated. Sustained release properties and uniform distribution of drugs from alginate beads were recognized by Xing *et al*. (2003). According to Sugawara *et al*. (1994) and Ostberg *et al*. (1994), the bead formulation was achieved by extruding the drug amended sodium alginate solution into calcium chloride bath. Chan *et al*. (2011) observed that alginate dissolved in water or in liquid microbial culture in contact with solution of di or trivalent cations formed thermally stable and water insoluble hydrogel globules called ‘beads’.

In the past decades, several alginate based formulations of microbial inoculum have been evaluated on experimental basis in agriculture, including for the encapsulation of the arbuscular mycorrhizal fungi (Ganry *et al.,* 1982), ectomycorrhizal fungi (Le Tacon *et al*., 1985), plant growth promoting bacteria (Trevors *et al*., 1992), and fungi (De Lucca *et al*., 1990) and bacteria (Aino *et al*., 1997) used as biocontrol agents against soil-borne pathogens. They also have wide applications in waste water treatment (Cassidy *et al*., 1996), entrapping microbial cells in food processing (Krasaekoopt *et al.,* 2003; Onwulata 2012; Tripathi and Giri 2014), or to utilize in the field of medicine and

pharmaceuticals (Cook *et al*., 2012).

2.5.1.1 Effect of concentrations of sodium alginate and $CaCl_2$ on bead properties

Yotsuyanagi *et al*. (1987) observed that sodium alginate at higher concentrations provided more calcium binding sites which brought about higher degree of crosslinking. Similarly, Aslani and Kennedy (1996) reported that at higher concentrations of sodium alginate, the process of cross linking proceeded at a lower rate. This resulted in complete diffusion of calcium ions into the droplet and thus, better cross-linking of interiorly placed polymer chains were achieved. Likewise, Blandino *et al*. (1999) recognized that high concentrations of sodium alginate and $CaCl_2$ enhanced the drug loading capacity and was found to impart controlled release characteristics to beads. Al-Musa *et al.* (1999) reported that Ca^{2+} in the cross linking solution rapidly cross linked with the outer polymer chains at low concentrations of sodium alginate droplet. It prevented further diffusion of calcium ions into the droplet and thus, the inner polymer chains left un-crosslinked.

El-Kamel *et al*. (2003) also obtained highly cross-linked beads with more rigid gel network and sustained release characteristics at three per cent $CaCl_2$ (w/v). The results were further confirmed by particle size studies, in which bead size was found smaller, owing to the increased concentration of crosslinking agent. From the particle size study of sodium alginate beads, Rajinikanth *et al*. (2003) concluded that small sized beads with more rigid and compact matrix were formed at high alginate concentrations. They also recorded a reduction in diffusion of drug from alginate matrix at higher concentrations of $CaCl_2$ solution due to increased degree of cross linking. Similarly, El-Zatahry *et al.* (2006) observed that the concentration of crosslinking agent was inversely proportional to drug release. Presence of adequate divalent cations at high concentrations of crosslinkers leads to compact gel formation and little structural rearrangements of beads to cause shrinkage.

Khazaeli *et al*. (2008) noticed that increase in alginate concentration resulted in high viscosity of solution, which in turn produced larger beads with limited diffusivity. Mandal *et al*. (2010) reported that the concentration of sodium alginate was directly proportional to the rigidity and compactness of calcium alginate matrix and inversely proportional to particle size and drug release rate. However, Sherina *et al*. (2012) recorded that the average particle size of micro beads increased on increasing the concentration of sodium alginate. Huang and Lin (2017) reported that the beads which gelled in high concentrations of cross linkers rarely shrank. While studying the impact of calcium ion concentration on the viability and sporulation of *T. viride* in sodium alginate microbeads, Juric *et al*. (2019) derived an inverse relationship between rate of release of bioagent and calcium ion concentration. It revealed the impact of calcium ions on enstrengthening the alginate network structure.

2.5.1.2 Effect of different cations on bead property

Haug and Smidsrod (1970) reported that the affinity of copper ions towards the alginates was ten times more than that of calcium ions. However, they recorded a higher weight for Cu-alginate beads than Ca-alginate beads. Likewise, Morch *et al.* (2006) evaluated the affinity of alginate towards Ba^{2+} and Ca^{2+} where they found that the affinity was more towards Ba^{2+} than Ca^{2+}. Hence, it was inferred that the beads incubated in Ba^{2+} solution were bound more tightly with minimum degree of shrinkage. Similarly, it was found that the beads incubated in Ca^{2+} solution were bigger compared to beads kept in Ba^{2+} solution, hence concluded that the calcium alginate beads were not sufficiently bound due to reduced affinity between sodium alginate and $CaCl_2$ (Loh *et al.*, 2012; Chuang *et al.*, 2017). Darrabie *et al*. (2006) studied the impact of cross-linking cations on the swelling behaviour of microbeads and concluded that calcium-alginate beads were more susceptible to swelling than the corresponding barium-alginate beads. The influence of Ca^{2+} concentration on the process of gelation and shrinkage was more pronounced than that of Ba^{2+} concentration.

Moreover, Morch *et al*. (2006) reported that alginate beads were sensitive to anti-gelling cations like sodium, magnesium and chelating agents like EDTA, citrate, phosphate, lactate *etc*., which implies that these cations and chelating agents can be used to dissolve the beads. Harper *et al.* (2014) studied the effect of different cations on the physical properties of alginate films and recognized that Ba^{2+} produced strong alginate films compared to Ca^{2+} ions. Similarly, Kaygusuz *et al.* (2014) conducted a detailed study on the effects of cations on the rate of release of encapsulated entity from alginate beads. Eventhough significantly higher encapsulation efficiency of model dye was recorded in barium-alginate beads compared to calcium-alginate beads, the rate of release of dye from Ca-alginate beads was more compared to the counterpart.

2.5.1.3 Concentration of inoculum of bioagent in the formulation

According to Knudsen *et al*. (1991), proliferation of the propagules of *Trichoderma* sp. was not observed in alginate formulations. Moreover, Bashan *et al.* (2002) pointed out that the process of cross linking and encapsulation killed a part of entrapped bacteria in the formulation. Therefore, the initial inoculum should contain sufficient number of bioagent propagules so as to compensate the losses during processing. While preparing sodium alginate bead formulation of *T. harzianum*, De Jaeger *et al*. (2011) standardised the concentration of conidia as 150 number ml^{-1} of sodium alginate solution. Further, they recorded a mean bead yield of 30 beads ml^{-1} alginate solution. Hence, each bead contained approximately five conidia of *T. harzianum*.

Adzmi *et al.* (2012) suspended the conidial pellets of *T. harzianum* UPM40 in 250 ml of alginate-montmorillonite (MMT) mixture, where the final spore concentration was estimated to 1.97×10^{10} cfu ml^{-1} mixture. Similarly, Thilagavathi *et al*. (2015) harvested seven day old mycelial mat of *T. asperellum* strain TTH1 grown in yeast molasses broth and the conidia were resuspended in sufficient volume of sterile deionised water so as to

obtain a final spore concentration of 10^8 conidia ml^{-1} suspension. Likewise, Szczech and Maciorowski (2016) harvested the conidia of *Trichoderma* TRS106 from a 10 day old culture on malt agar medium and were suspended in 10 ml of 0.85 per cent sterile sodium chloride (NaCl) solution. The aliquot was further made upto 100 ml using sterile NaCl solution to achieve a final spore concentration of 10^8 conidia ml^{-1}.

2.5.2 Novel alginate bead based formulations

A number of sodium alginate based formulations of *Trichoderma* sp. have been developed in the recent past. Fravel *et al*. (1985) prepared alginate prills by mixing 25g sodium alginate and 50g wheat flour with 200 ml fermented biomass of *Trichoderma*, where the mixture was added drop wise into $CaCl_2$ solution to form spherical beads. Similarly, Lewis and Papavizas (1987) prepared alginate pellet formulations of 11 isolates of *Trichoderma* spp. and *G. virens* in which pellets containing *T. harzianum* Th-58 and *T. hamatum* TRI-4 performed superior. Likewise, Marois *et al*. (1989) prepared the pellets of *T. viride* biotype T-1R9 by dropwise addition of bioagent amended sodium alginate solution into $CaCl_2$ or calcium gluconate solution. The resultant gel pellets were dried and applied to manage soil borne plant diseases. Similarly, Knudsen *et al*. (1991) formulated the mycelial biomass of *T. harzianum* into alginate pellets. Moreover, Shaban and El-Komy (2001) also prepared conidial formulations of *T. harzianum* and *T. pseudokoningii* (Rifai) in alginate pellets. De Jaeger *et al*. (2011) immobilized the conidia of *T. harzianum* along with the spores of *Glomus* sp. in sodium alginate (2%) where noticeable growth of both organisms outside the calcium alginate coating indicated their mutual compatibility. Likewise, Adzmi *et al*. (2012) encapsulated *T. harziaum* UPM40 isolated from healthy roots of groundnut in calcium alginate-MMT clay.

In an experiment conducted by Anis *et al*. (2012), sunflower seeds coated with *T. viride* using 2 per cent sodium alginate showed significantly higher vigor index, plant length and biomass compared to non treated control. Thilagavathi *et al*. (2015) entrapped

T. asperellum strain TTH1 in sodium alginate (2% w/v) beads where the formulation was achieved by dropwise addition of sodium alginate solution to 0.1 M $CaCl_2$ using a micropipette. Similarly, Szczech and Maciorowski (2016) employed emulsification technique to immobilize *T. virens* TRS106 in calcium alginate (2% sodium alginate and 0.1 M $CaCl_2$) microbeads. Vincekovic *et al*. (2016) prepared chitosan amended alginate beads of *T. viride* loaded with copper cations so as to investigate the compatibility of the chemical with the bioagent. The results revealed that the proximity of copper ions did not inhibit the activity of bioagent. Locatelli *et al*. (2018) also developed sodium alginate granule formulations of *Trichoderma* sp. by ionic gelation method. Likewise, Juric *et al*. (2019) encapsulated spores of *T. viride* in calcium alginate microspheres to achieve simultaneous delivery of chemical as well as biologically active agents to plants. dos Santos *et al*. (2015) employed a factorial design tool to determine the independent variable like proportion of the sodium alginate, sodium polyphosphate and glycerol so as to increase the water activity (aW) of the beads. Results indicated that higher proportion of polyphosphate in the bead was more significant for a larger aW.

Apart from *Trichoderma* spp., numerous other fungal as well as bacterial biocontrol agents were also encapsulated in sodium alginate and employed successfully in plant disease management. Bashan and Gonzalez (1999) immobilized two plant growth promoting bacteria, *Azospirillum brasilense* Cd and *Pseudomonas fluorescens* 313 in alginate beads with and without skim milk supplement. Subsequently, the beads were dried and stored at ambient temperature. Similarly, Walker and Connick (1983) suggested the incorporation of myco herbicides into sodium alginate as a promising approach to exploit the potential of biocontrol fungi. Marois *et al.* (1989) prepared alginate pellets of *G. virens* G13, *Penicillium oxalicum*, and *Talaromyces flavus* Tf-1. Knudsen *et al*. (1991) formulated the mycelial biomass of *Beauveria bassiana* in wheat bran amended alginate pellets where rapid conidial production was noticed in treated pellets than in non-treated pellets. Similarly, Fravel *et al*. (1995) prepared alginate prill

formulation of *T. flavus* with organic carriers to combat *Verticillium dahliae*.

Saha and Pan (1995) immobilized *G. virens* in bentonite clay amended sodium alginate (1%) prills using 0.2 M $CaCl_2$ solution. These prills were subjected to air drying in laminar flow cabinet for 24 h and later stored in polypacks at room temperature. Moreover, Shah *et al.* (1998) devised a protocol to immobilize the aphid pathogenic fungus *Erynia neoaphidis* in alginate matrix. Bashan *et al.* (2002) formulated alginate microbeads as a carrier of plant growth promoting bacteria (PGPB) *Azospirillum brasilense* meant for seed treatment. Likewise, Trivedi *et al.* (2005) formulated five different carrier based preparations of plant growth-promoting rhizobacteria (PGPR) *viz*. *Bacillus subtilis* and *Pseudomonas corrugate*. Similarly, Chen *et al.* (2013) encapsulated *Bacillus cereus* strain C1L in sodium alginate to enhance its stability to combat lily leaf blight. Likewise, Szczech and Maciorowski (2016) entrapped bacterial biocontrol agents *viz.*, *Burkholderia cepacia* strain CAT5, *Bacillus* spp. strains PZ9 and SZ61 separately in calcium alginate beads. Moreover, Zommere and Nikolajeva (2017) immobilized a natural bacterial association of seven strains isolated from acid tar-contaminated soil in sodium alginate gel (1.5%) to identify the ideal conditions for entrapment and prolonged storage of a bacterial association.

2.6 PROPERTIES AND DIFFERENT CHARACTERISTICS OF ENCAPSULATED BEADS OF BIOCONTROL AGENTS

According to Tal *et al.* (1997), defects like distorted shapes, uneven size, poor mechanical strength or high porosity of microbeads may impair the stability and viability of encapsulated cell during the course of storage. However, a study conducted by Locatelli *et al*. (2018) revealed the existence of chemical interactions between the constituents, which provided stability to beads. Apart from that, a detailed investigation on important physicochemical properties of microbeads lead by Juric *et al*. (2019)

highlighted the existence of intermolecular interactions including hydrogen bonds and electrostatic interactions and its complex nature. However, the evidences of spore germination inside the matrix and germ tube penetration out of the microspheres indicated the presence of a conducive environment for the growth of *T. viride*.

2.6.1 Gelation and curing of calcium alginate beads

Yotsuyanagi *et al.* (1991) remarked that the gelation of alginate is an instantaneous process with rapid expulsion of water from the gel matrix in acidic conditions. Tateshita *et al*. (1993) assessed the gelling rate during bead formation from weight loss of the beads in calcium chloride solution. Rajinikanth *et al*. (2003) attributed the mechanism of gelation or cross-linking of sodium alginate with $CaCl_2$ to the tight junctions between the guluronic acid residues in sodium alginate and they noticed that the number of cross linked points increased with increase in alginate concentration in the microbeads. Das and Senapati (2007) also observed the gelation of drug loaded polymer solution drops immediately after being in contact with $CaCl_2$ solution. Tous *et al.* (2014) estimated the time period required for the complete curing of microbeads and they considered the time taken to stabilize the bead weight as the time of gelation or curing.

However, the time of gelation or curing of the microbeads depends on prevailing experimental conditions. Declerck *et al.* (1996) maintained *Glomus versiforme* entrapped sodium alginate (2% w/v) beads in 0.1 M $CaCl_2$ solution under agitation for 30 min. Similarly, Bashan *et al.* (2002) cured *A. brasilense* entrapped sodium alginate (2% w/v) beads for 30 min in 0.1 M $CaCl_2$ solution. During the course of experiments for developing slow release therapeutics, Das and Senapati (2007) immersed the drug loaded alginate beads in $CaCl_2$ bath for 15 min to enhance the rigidity of the beads. Likewise, Mandal *et al.* (2010) cured the drug entrapped sodium alginate beads for 60 min. Sherina *et al.* (2012) maintained the drug loaded alginate beads on a stirrer for 60 min and further rigidization was achieved by addition of one ml glutraldehyde (25%) solution. Adzmi *et*

al. (2012) allotted 180 - 360 min for hardening *T. harzianum* UPM40 entrapped sodium alginate (3% w/v) microbeads in 0.5M $CaCl_2$ at room temperature.

Similarly, Thilagavathi *et al*. (2015) hardened *T. asperellum* occupied sodium alginate (2% w/v) beads for 60 - 180 min in 0.1 M $CaCl_2$ solution to harvest regular beads. Likewise, Szczech and Maciorowski (2016) retained sodium alginate (2% w/v) microbeads of fungal and bacterial bioagents in 0.1 M $CaCl_2$ solution for 60 min at room temperature under constant agitation on a magnetic stirrer. Mattam and Sailaja (2016) maintained the drug incorporated microbeads in $CaCl_2$ solution for 30 min under continuous agitation at 100 rpm. Moreover, Zommere and Nikolajeva (2017) maintained the sodium alginate (3% w/v) beads in 0.2 M $CaCl_2$ bath for 60 min to complete gelation at room temperature. In addition to that, Huang and Lin (2017) stated that the cured alginate beads required a minimum additional time span of 120 min after ionic gelation process to acquire stability.

2.6.2 Moisture content and storage of microbeads

Das and Senapati (2007) recovered the beads by filtering through Whatman no. 42 filter paper spread on Petri plates and stored in air-tight containers after 48 h of drying at room temperature. After the process of gelation, De Jaeger *et al*. (2011) collected *T. harzianum-Glomus* sp. co-entrapped sodium alginate beads in sterile nylon mesh (100 μ) and stored at 4^0C in Petri plates after washing with sterile distilled water. Likewise, Adzmi *et al.* (2012) collected the microbeads by sieving and subjected to drying at 28 - 32^0C for 24 h after washing several times in sterile water. Further, the beads were stored in bottles which maintained at 5 and 30^0C and the moisture content was estimated to 5.39 per cent after 24 h of drying at 30^0C. Similarly, Thilagavathi *et al*. (2015) stored the microbeads at 4^0C after washing twice in sterile water. In addition, Tous *et al.* (2014) estimated the water content of drug loaded sodium alginate beads as 90 - 93 per cent. Patel *et al.* (2016) estimated the water content of calcium alginate beads through

gravimetric analysis immediately after preparation and after drying, where the mean water loss ranged from 76.7 ± 0.7 to 89.1 ± 1.5 per cent.

2.6.3 Size and shape of beads

The size of microbead is an important parameter in determining the bead quality. Several researchers tried to interpret the relationship between the size of microbeads and O_2 penetration into it. Sun *et al.* (1989) detected that 80 per cent of the bead volume remained unoccupied by aerobic bioagents due to the limited O_2 diffusion. In one of the studies, Ogbonna *et al*. (1991) confirmed that 100-300 µm was the optimum bead size for the efficient diffusion of oxygen in different polymeric beads. Bashan *et al.* (2002) also recognized the limited O_2 supply as the major drawback of microbeads. Owing to the efficient penetration of oxygen, nutrients, and metabolites, Szczech and Maciorowski (2016) observed the enhanced cell concentration and viability of bioagent in beads with reduced size. Further, Vincekovic *et al*. (2016) observed the influence of the size of microbeads and entrapped bioagent on the entrapment efficiency, swelling behaviour and release pattern.

Findings of many researchers evidenced that the size of a bead is determined by several factors. Morch *et al.* (2006) stated that the affinity of alginate towards divalent ions plays a crucial role in determining the bead size. Further it was confirmed by Liu *et al.* (2013) where they found that alginate beads gelled in Ca^{2+} solutions were smaller in diameter than that cured in Ba^{2+} solutions. Morch *et al.* (2006) also made similar observations with 10 - 20 mM Ba^{2+} solution and 50 mM Ca^{2+} solution. In addition, Woo *et al.* (2007) identified the role of temperature on the size of microbeads as they observed eight per cent fall in bead size after gelation at 90^0C. However, Smrdel *et al.* (2006) could not detect any significant effect of incubation temperature on the bead size at 5^0C and 40^0C. Vaithilingam *et al*. (2011) revealed that a prolonged gelling period did not influence the bead size in Ba^{2+} solution even after 20 min of incubation. However,

Smrdel *et al.* (2006) observed a gradual reduction of the size of alginate beads upon incubation in Ca^{2+} solution over a period of 30 min of gelation. Szczech and Maciorowski (2016) found that the nature of encapsulated microorganism has an impact on capsule size and productivity. Moreover, Lee *et al.* (2017) emphasized the strong influence of the properties of sodium alginate and the method of bead production on the size of beads.

Chan *et al*. (2011) defined the shape of beads in terms of sphericity factor (SF), which ranged from zero for a perfect sphere shaped bead to unity for an elongated bead. Accordingly, Adzmi *et al*. (2012) estimated the sphericity factor of calcium-alginate-montmorillonite beads and plain calcium alginate beads of *T. harzianum* UPM40 as 0.065 and 0.123 respectively.

Eventhough, the bead surface looks smoother, scanning electron microscopic study of bead morphology conducted by Sherina *et al*. (2012) evidenced that the beads have spherical shape with rough surface. In a similar study, Tous *et al*. (2014) also confirmed that the surface of spherical microbead is rough and less porous. Further, they detected the presence of small cracks and fissures on bead surface at high magnification (1000X). Similarly, Bajpai and Kirar (2016) also spotted the presence of cracks, pits and unreacted $CaCl_2$ residues on the surface of the bead at 1000X magnification. They concluded that these cracks and fissures were developed as a result of the process of drying of cross linked matrix.

While encapsulating PGPB *A. brasilense* in sodium alginate, Bashan *et al.* (2002) obtained microbeads with mean diameter ranging from 100 to 200 µm. Similarly, Adzmi *et al*. (2012) estimated the average diameter of *T. harzianum* UPM40 encapsulated beads as 1.68 mm. Patel *et al*. (2016) determined the particle size of beads with the help of ocular and stage micrometer (0.01 mm accuracy) fitted to an optical microscope, where the mean diameter ranged from 1.04 ± 0.20 to 2.15 ± 0.356 mm. Szczech and

Maciorowski (2016) obtained peat and chitosan amended microcapsules with an average diameter of 50 µm. Further, Zommere and Nikolajeva (2017) reported that the mean diameter of bacterial consortia entrapped microbead was about 2 mm.

2.6.4 Bead weight and per cent yield

Adzmi *et al*. (2012) estimated the average weight of *T. harzianum* UPM40 encapsulated beads as 5.64 mg. Similarly, Sherina *et al*. (2012) quantified the yield of drug loaded sodium alginate bead within the range of 74.5 ± 3.01 to 92.2 ± 2.55. Likewise, Patel *et al*. (2016) recorded 24.90 to 93.60 per cent yield in drug loaded alginate beads.

2.6.5 Recovery of population from encapsulated beads

Lewis and Papavizas (1987) reported that propagules entrapped in alginate pellet formulation of 11 isolates of *Trichoderma* spp. and *Gliocladium* proliferated to 10^6-10^{11} colony forming units (cfu) g^{-1} pellet in soil. Declerck *et al.* (1996) devised the concept of per cent potentially infective bead (per cent PIB) where the beads were considered potentially infective, if atleast one conidia germinated out of the bead. Accordingly, De Jaeger *et al.* (2011) recorded 72.00 ± 4.5 per cent PIB for *T. harzianum* entrapped microbeads on second day of experiment and the maximum population of 96.00 ± 1.9 per cent was observed on the sixth day and thereafter remained unchanged. A study on the release of *T. virens* TRS106 from wet alginate beads in soil conducted by Szczech and Maciorowski (2016) revealed that 5.5 x 10^5 cfu g^{-1} soil was released after four days of incubation.

2.7 SHELF LIFE AND QUALITY OF ENCAPSULATED MICROBEADS

Enhanced shelf life is one of the prime motives as well as an inextricable factor in

biocontrol research. Consequently, there are many reports on diverse approaches adopted by researchers to achieve the same. According to several literatures, the storage temperature and additive substances are the two main factors that influence the shelf life of biocontrol formulation significantly. While formulating the conidia of *T. harzianum* and *T. pseudokoningii* Rifai in alginate pellets, Shaban and El-Komy (2001) observed the better survival of coated conidia compared to directly added naked conidia after three months of application. Szczech and Maciorowski (2016) lyophilised the of *T. virens* TRS106 immobilized microbeads to escalate the stability and viability of bioagent, however, the viability was found to reduce under storage as well as in soil after the application. Moreover, they pointed out the influence of the type of microorganism on the shelf life of calcium alginate microbeads. Further, they noticed the first symptoms of spoilage in wet microbeads after three months of storage.

2.7.1 Effect of storage temperature

Lewis and Papavizas (1987) reported that the efficacy of *Trichoderma* encapsulated pellets attenuated after a storage period of six weeks at 5 or 25^{0}C against *Rhizoctonia solani*. Connick *et al.* (1997) considered the storage temperature as the major abiotic factor affecting the shelf life of bioagent formulations. However, Bashan and Gonzales (1999) detected significant number of viable cells of *A. brasilense* and *P. fluorescens* in alginate beads even after 14 years of storage at ambient temperature. Abuelgasim *et al*. (2004) opined that the storage of biocontol agent formulations at low temperature favoured the reduced metabolic activity of microorganism and subsequently enhanced the shelf life of the formulation. Similarly, Cigdem and Merih (2005) attributed the loss of viability of biocontol agent formulations at elevated temperature to the accelerated microbial activity and toxic waste production resulted from desiccation.

De Jaeger *et al*. (2011) maintained sodium alginate beads of *T. harzianum* and

Glomus sp. at 4^0C in Petri dishes. Adzmi (2012) revealed the significant loss of viability and cell release of encapsulated *T. harzianum* UPM40 at room temperature in three months of storage compared 5^0C over six months of storage. Similarly, Thilagavathi *et al*. (2015) observed that *T. asperellum* strain TTH1 entrapped sodium alginate beads maintained at 4^0C remained without much decline in population upto 12 months. Moreover, Locatelli *et al*. (2018) reported that the starch amended sodium alginate granular formulation of *Trichoderma* retained the conidial viability for 14 months at room temperature.

2.7.2 Role of additives

Many researchers conducted experiments on various additives to improve the shelf life of sodium alginate formulations. Krasaekoopt *et al*. (2004) suggested that amalgamation of additional constituents into the alginate matrix or coating of capsules with them enhanced the cell viability, capsule stability and shelf life of encapsulated microorganisms. The composition of microbeads significantly influenced the degree of contamination and shelf life quality, particularly for wet product.

Lewis and Papavizas (1987) observed the enhanced viability of *Trichoderma* spp. and *Gliocladium virens* entrapped alginate pellets amended with wheat bran and kaolin clay during the course of storage. Similarly, Knudsen *et al*. (1991) recorded higher proliferation of hyphae of *T. harzianum* in PEG (polyethlene glycol) amended alginate pellets compared to that of untreated pellets. Apart from that, many researchers reported that the incorporation of components like clay, skim milk, chitosan, sugars, gum acacia or maize starch improved the quality of microbeads (Bashan, 1998; Krasaekoopt *et al.*, 2004). Shaban and El-Komy (2001) observed the increased survival of the conidia of *T. harzianum* and *T. pseudokoningii* (Rifai) in cellulose (10%) amended formulation than in unamended formulation. Szczech and Maciorowski (2016) supplemented sodium alginate bead formulations of bacterial bioagents *viz*., *Burkholderia cepacia* strain CAT5, *Bacillus* spp. strains PZ9 and SZ61, and fungal bioagent *Trichoderma virens* TRS106 with

chitosan, peat powder or skim milk. They observed that skim milk deteriorated the quality of microbeads and contradictory to the above reported literature, additives had little role in the viability of entrapped bioagent and their release in soil.

Further, Vincekovic *et al*. (2016) observed that the proximity of copper ions did not inhibit the activity of bioagent in chitosan amended alginate beads of *T. viride* loaded with copper cations. Similarly, Locatelli *et al*. (2018) reported that starch incorporated in the alginate formulation interacted with alginate and imparted more conidial viability during drying. Moreover, dos Santos *et al*. (2015) observed the enhanced viability of spores of *Trichoderma* sp. formulated with alginate, polyphosphate and glycerol for 120 days under storage at room temperature.

2.8 OTHER IMPACTS OF ADDITIVES ON ENCAPSULATED BEADS

Bashan (1986) remarked that beads amended with 0.75 per cent skim milk were more biodegradable in nature. Knudsen *et al.* (1991) observed that PEG treated alginate pellets did not lose much weight even after 0 - 4 h drying in comparison to water-treated or untreated pellets. Several researchers reported that chemical composition of additives are significant in determining the bead yield. Further, it was noticed that highly heterogeneous polymers with elaborately branched molecular structures and more or less similar monosaccharide compositions resulted in reduced bead yield. It was also assumed that a linear polymer like sodium alginate is unable to accommodate another highly branched polymer within them (Islam *et al*., 1997; Street and Anderson,1983).

Bashan *et al.* (2002) observed that beads amended with skim milk tended to release a higher number of entrapped bacteria into soil. Szczech and Maciorowski (2016) also made similar observations on skim milk, whereas the addition of peat and chitosan into alginate matrix did not modify the releasing pattern of bioagent. Krasaekoopt *et al.* (2004) reported that the blending of additives along with alginate matrix during encapsulation had substantial effect on bead yield and particle size. Similarly, Young *et*

al. (2006) observed that humic acid enriched sodium alginate beads were regular and spherical in shape with an average diameter ranging between 2 - 3 mm. Likewise, Lin *et al*. (2008) reported that chitosan enriched alginate beads were highly stable and mechanically resistant. Adzmi *et al*. (2012) reported that the properties like sphericity, flowability, density, visual quality and rigidity of microbeads were found to improve upon enrichment with montmorillonite clay. Further, thermogravimetric analysis evidenced improved thermal stability of Ca-alginate-MMT beads in comparison with the pure alginate beads.

Similarly, Patel *et al*. (2016) reported that the addition of chitosan in bead formulation significantly improved the particle size after drying. Likewise, Szczech and Maciorowski (2016) observed that additives amalgamated in alginate matrix during microencapsulation had noticeable influence on the yield and size of microbeads. They recorded 60 per cent increase in the yield of peat amended beads and 42 per cent in chitosan enriched beads. In addition to that, peat augmented the bead quality while, both the additives reduced the incidence of contamination. Likewise, Zommere and Nikolajeva (2017) noticed 16 per cent increase in weight and specific gravity of clay amended sodium alginate beads compared to that without clay.

2.9 BIOCONTROL EFFICIENCY AND PLANT GROWTH PROMOTION OF ENCAPSULATED BEADS OF *Trichoderma* spp.

Various soil borne and foliar pathogenic fungi attacks agricultural crop plants resulting in immense crop losses (Khan *et al*., 2011). According to Oerke (2006), 16 per cent of global crop loss is due to microbes, in which 70-80 per cent is exclusively due to fungal pathogens. The major soil borne fungal diseases include pre and post emergent damping-off caused by *Pythium* spp., *Rhizhoctonia* spp, foot and root rots caused by *Phytophthora* spp, vascular wilts caused by *Fusarium* spp., *Verticillium* spp. *etc*.

Since the global agricultural community urges transition from chemical control to biological control, the role of fungal bioagents like *Trichoderma* spp. became more prominent. The accounts of successful applications of biocontrol agents in the management of many fungal pathogens are documented by many researchers. Owing to mycophilic nature, *Trichoderma* are the most frequently used antagonists. *Trichoderma* species retained good antagonistic abilities against plant pathogenic fungi like *Rhizoctonia* (Sivan and Chet, 1986), *Fusarium* (Lewis and Papavizas, 1987), *Macrophomina phaseolina* (Khan and Gupta 1998). *Pythium* (Naseby *et al*., 2000). Harman and Taylor (1990) unveiled the potential of some strains of *Trichoderma* spp. to protect seedlings from damping-off and root infections along with ensuring plant growth promotion. Moreover, Malik and Dawar (2003) reported that *T. harzianum* protected the root systems of many crops from the attack of *Fusarium solani, R. solani* and *M. phaseolina*.

Lewis and Papavizas (1987) observed that alginate pellets with eight different isolates of *Trichoderma* reduced 34 - 78 per cent survival of *R. solani* in infested beet seeds in soil. They also observed that pellets of four isolates prevented damping-off of cotton and three isolates controlled damping-off of sugar beet under greenhouse conditions. De Jaeger *et al*. (2011) observed that *T. harzianum* neither affect the establishment nor symbiotic potential of co-entrapped AM fungi, rather it stimulated the spore production and fitness of AM fungi. Likewise, Anis *et al*. (2012) recorded high germination per cent in sunflower seeds coated with sodium alginate formulation of *T. viride* or *Trichoderma. resei* compared to uncoated seeds. Further, seeds coated with *T. viride* in two per cent sodium alginate showed maximum vigour index, plant height and biomass followed by that with one per cent sodium alginate.

Similarly, Thilagavathi *et al*. (2015) evaluated the efficacy of sodium alginate bead formulation of *T. asperellum* strain TTH1 against most virulent isolate of *Sclerotium rolfsii* (SrSB3) causing sugarbeet root rot disease both under pot culture and

field condition. They recorded 35 per cent reduction in disease incidence under greenhouse condition and five per cent under field condition. Szczech and Maciorowski (2016) evaluated the release of entrapped *T. viride* TRS106 from alginate beads in soil and their effectiveness in control of *Fusarium* wilt of tomato plants. However, the results revealed that the control of disease or the reduction of inoculum density in the soil was not achieved by the application of these encapsulated *Trichoderma* sp.

2.10 BIODEGRADATION OF ENCAPSULATED BEADS IN SOIL

Deasy (1984) opined that the biodegradable nature of sodium alginate has been extensively exploited for developing controlled release excipients. Disbursal of encapsulated active agents from these excipients takes place in a gradual and controlled fashion by virtue of matrix degradation. It assures the protection of entrapped propagules against lethal environmental factors than freely inoculated microorganisms (Young *et al*., 2006; Vemmer and Patel, 2013). However, Bashan *et al*. (2002) suggested that the degradability of microbeads in soil was immensely influenced by the type of polymers and beads and thus, the release of bioagent occurs at different rates.

2.10.1 Estimation and mechanism of biodegradation

Bashan *et al.* (2002) conducted an experiment to assess the biodegradation of *A. brasilense* immobilized sodium alginate beads. They observed higher level of biodegradation in beads containing bacteria and skim milk compared to that without any additives and bioagent. Further, they concluded that the degree of degradation depended on type of polymers or beads which in turn affected the rate of release of entrapped bioagent. Similarly, Bajpai and Kirar (2016) conducted a detailed study on the water uptake of calcium alginate beads with various composition of concentration of alginate, ionic strength of cross-linker Ca^{2+} ions and the degree of cross-linking. Results from gravimetric analysis revealed that calcium alginate beads were stable for more than 48 h at pH 7.4.

Kikuchi *et al*. (1999) explained that the counter ions present in the swelling medium replaced Ca^{2+} attached to –COO− groups of M blocks in calcium alginate beads. This resulted in the relaxation of M chains and consequent swelling of the beads. Thus, the complete hydration of the polymer resulted in the loosening of structural integrity causing disruption of egg-box cavities and subsequent disintegration and dissolution of alginate beads. George and Nikolaos (2006) described that the loss of Ca^{2+} from –COO- groups of alginate resulted in increased repulsive forces between the negatively charged –COO- residues. This lead to weakening of the cross-linking and resulted in loosening of polymer matrix. It enhanced the permeability of beads to take up more water until disintegration and bursting. According to Bajpai and Kirar (2016), degradation of alginate beads was achieved by exchange of cross-linking Ca^{2+} ions present within the so called 'egg-box' cavity by counter ions in the swelling medium.

2.10.2 Swelling, erosion and shrinking of microbeads

With regard to swelling, experimental parameters like swelling percentage, swelling ratio and swelling index are defined by many scientists. Turkoglu *et al*. (1997) reported that the process of ion exchange as well as the formation of the solute, alginate resulted in erosion of the beads. However, Ouwerx *et al.* (1998) reported that alginate beads tends to shrink under high acidic conditions or at pH values less than four, the –COO- groups of alginate are protonized and hence, the inter radical repulsive forces decreased. This resulted in the expulsion of entrapped water and subsequent shrinking and weight loss of beads. Further, El-Gindy (2002) attributed the rapid release of bioagent in the phosphate buffer to the phenomena of swelling and erosion. He observed the formation of highly porous alginate skins which facilitated water inflow into beads. Al-Kassas *et al*. (2007) illustrated that maximum swelling of beads was achieved in 1.5 h in phosphate buffer (pH 7.4) after which the erosion and breaking down of beads were noticed. Crcarevska *et al.* (2008) stated that the volume of ionically cross-linked, dried beads increased within a few minutes after immersing in water or buffers. The

phenomenon was attributed to matrix rehydration that strongly relied on the degree of cross-linking.

Sherina *et al.* (2012) observed that the swelling behaviour of micro beads were directly proportional to the concentration of sodium alginate, probably due to increased water absorption at high polymer concentrations. They also added that an increased concentration of coating polymer diminished the swelling behaviour. Santhi *et al.* (2013) also carried out similar experiments to describe the swelling behaviour of micro beads by quantifying the per cent water uptake by the beads. The ratio of increase in weight after swelling to dry bead weight was referred as swelling ratio. According to the reports of Tous *et al*. (2014), the release of entrapped drug from the bead was highly influenced by the swelling and erosion properties of the beads. Further, they observed less shrinkage in beads with high concentrations of sodium alginate. They also pointed out that the additives without swelling or shrinkage property may hamper the water sorption capacity of beads.

Patel *et al*. (2016) assessed the swelling index of microbeads by determining the water sorption and resultant weight changes of beads. Likewise, Huang and Lin (2017) conducted a detailed investigation on the role of two common ion crosslinkers *viz.,* Ca^{2+} and Ba^{2+}on active shrinkage of gelled alginate beads at different concentrations and temperature. Subsequently, they observed that the beads incubated in Ca^{2+} solution shrank more actively than that in Ba^{2+} solution.

2.10.2.1 Role of pH in swelling and degradation

While developing a polymer based slow release protein delivery system, Polk *et al*. (1994) observed the significant influence of the pH of extra capsular environment on the release of protein, where 15 per cent release occurred at pH 3.0 while 73 per cent at pH 8.0 over 24 h. Turkoglu *et al*. (1997) reported that alginate beads exhibited highest rate of swelling at pH 7.4 while the lowest rate was noticed at pH 1.0. It was assumed

that swelling of beads at higher pH was due to the exchange of Ca^{2+} with anti-gelling ions like Na^{+} or K^{+} in the buffer.

Sherina *et al*. (2012) also reported that in acidic environment the alginate beads shrunk and encapsulated drug was not released, whereas in alkaline environment they easily swell and the drug was released. They recorded a slowest swelling ratio at pH 1.2 and the highest at an elevated pH. According to the report of Tous *et al*. (2014), swelling and erosion of the bead was strongly depended on the pH of the surrounding medium. They recorded a complete drug release in phosphate buffer (pH 7.4) within 2 h of immersion, whereas the release profile was poor in 0.1 M HCl (pH 1.0). Moreover, the beads retained the intact form upto 4 h in acidic pH without noticeable erosion and change in sphericity, whereas it was only upto 1.5 h in pH 7.4.

MATERIALS &METHODS

3. MATERIALS AND METHODS

The present investigation on 'Microencapsulation of *Trichoderma viride* for management of major soil borne fungal pathogens' was conducted in the Department of Plant Pathology, College of Horticulture, Vellanikkara, Thrissur during 2017 - 2020. The materials used and methods employed during the course of study is described in detail in this chapter.

3.1 PRESERVATION OF FUNGAL CULTURE

The fungal reference culture of KAU, the *Trichoderma viride* was used for the experiment. The culture was collected from the Department of Plant Pathology, COH, Vellanikkara, and was sub cultured into potato dextrose agar (PDA) (Appendix I) slants and stored under refrigerated condition for further studies.

3.2 STANDARDIZATION AND PREPARATION OF ALGINATE BEAD BASED FORMULATION OF *Trichoderma viride*

3.2.1 Standardisation of media with additives to study its effect on shelf life of *T. viride*

To study the effect of different additives on the shelf life of sodium alginate bead based formulation of *T. viride*, potato dextrose (PD) broth (Appendix II) was used as the growing medium. Different additives were amended in 50 ml PD broth in 100 ml conical flask at specific concentrations and thus, eight different treatment combinations were laid out (Table 3.1 and Table 3.2). Subsequently, sterilization of broth was carried out at 121^0C and 15 psi pressure for 20 min. After cooling, each flask was inoculated with seven day old well sporulated mycelial disc (8 mm) of *T. viride.* It was then incubated at room temperature for further shelf life studies.

3.2.1.1 Evaluation of additives on shelf life of T. viride

The effect of additives on shelf life of *T. viride* was evaluated at monthly intervals upto seven months by serial dilution and plating technique on potato dextrose agar medium. The green mycelial mat formed in the additive amended potato dextrose broth (PDB) was agitated on a rotary shaker after 7 days, 1, 2, 3, 4, 5, 6 and 7 months at 100 rpm for 30 min so as to disperse the conidia.

One ml of conidial suspension in PDB was pipetted out into nine ml sterile water blank to achieve a concentration of 10^{-1}. Likewise, the process continued till a concentration of 10^{-8} was attained. Each one ml aliquot from 10^{-6} and 10^{-8} dilutions were poured into sterile Petri plates. Thereafter, molten potato dextrose agar (PDA) medium was poured in plates over the conidial suspension and rotated in both clockwise and anti-clockwise direction to achieve uniform spore distribution. After solidification of the medium, plates were kept for incubation at room temperature. A control without additives was also maintained for comparison and observations were recorded after 72 h of incubation. After seven months of storage, the most promising additive combination was selected based on the maximum colony count developed on dilution plates and the results were expressed in number of colony forming units (cfu) per ml of the suspension which was calculated using the following formula:

$$\text{Number of colony forming units (cfu)} = \frac{\text{Mean colony count x Dilution factor}}{\text{Volume of spore suspension}}$$

3.2.2 Standardization of different parameters for bead preparation

Different experimental variables like concentration of sodium alginate, concentration of calcium chloride ($CaCl_2$), volume of spore suspension of *T. viride* and parameters like height of air column were standardized.

Table 3.1 List of additives amended in growing medium of *T. viride*

Treatment	Combination of additives
T_1	Mannitol, PVP, CMC, Tween 80
T_2	Mannitol, PEG, CMC, Tween 80
T_3	Mannitol, PVP, Liquid paraffin , Tween 80
T_4	Mannitol, PEG, Liquid paraffin, Tween 80
T_5	Trehalose, PVP, CMC, Tween 80
T_6	Trehalose, PEG, CMC, Tween 80
T_7	Trehalose, PVP, Liquid paraffin, Tween 80
T_8	Trehalose, PEG, Liquid paraffin, Tween 80
T_9	Control

Table 3.2 Different treatment combinations of growing medium of *T. viride*

Class of additives	Compound used	Concentration
Sugar	Mannitol	2%
	Trehalose	15mM
Wetting agent	Polyvinyl pyrrolidone (PVP)	1%
	Polyethylene glycol (PEG)	0.25%
Adhesive	Carboxy methyl cellulose (CMC)	0.5%
	Liquid paraffin	1%
Surfactant	Tween 80	0.5%

3.2.2.1 Concentration of sodium alginate and calcium chloride

To standardise the concentration of sodium alginate in the formulation, 2.5, 3, and 3.5 g of food grade sodium alginate powder (91%) was weighed and each of them was separately dissolved in 100 ml luke warm distilled water. Similarly, to optimise the concentration of calcium chloride ($CaCl_2.2H_2O$) which was used as the cross linking agent, 2.5, 3 and 3.5 g of $CaCl_2$ was weighed and each of them was also separately dissolved in 100 ml distilled water taken in 250 ml conical flasks. Subsequently, sterilization of $CaCl_2$ solution was carried out at 121^0C temperature and 15 psi pressure for 20 min. The alginate solution was transferred to a 500 ml separating funnel and $CaCl_2$ solution was kept below the funnel inside a conical flask, and the beads were prepared by following the technique of ionotropic gelation and cross linking method (Sherina *et al*., 2012; Tous *et al*., 2014). For this, sodium alginate solution was added dropwise into $CaCl_2$ solution and the fully matured beads were harvested by sieving after 60 min of curing.

Likewise, the beads were prepared in all the possible combinations of respective concentrations of sodium alginate and $CaCl_2$ solutions. However, the concentration of sodium alginate was reduced to 0.75 and 1.5 per cent when the solution was supplemented with additives so as to reduce its viscosity and based on the shape of the beads formed, the concentration of sodium alginate and calcium chloride was standardized. Care was taken to use freshly prepared solutions of sodium alginate and $CaCl_2$. Beads without additives (control) and without the biocontrol agent (absolute control) were also prepared from the respective sodium alginate solutions.

3.2.2.2 Preparation of spore suspension of T. viride

Eight mm mycelial disc of *T. viride* from actively growing one week old culture on PDA medium was cut using a sterile cork borer. These bits were inoculated into 50 ml adjuent amended, sterilized potato dextrose broth as in 3.2.1 in 100 ml conical flask and incubated for seven days. After incubation, the fully matured green mycelial mat was

harvested and macerated using an electric blender. The green conidial suspension was then sieved out from the ruptured mycelial bits.

3.2.2.3 Standardization of spore suspension of T. viride in sodium alginate solution

The standardized sodium alginate solution prepared as in 3.2.2.1 was amended with spore suspension of *T. viride* in all the given combinations as depicted in Table 3.3 [Thilagavathi *et al.* (2015)]. Further, beads prepared out of each treatment employing ionotropic gelation technique were eluted from $CaCl_2$ solution using an ordinary sieve, washed twice in sterile distilled water and subjected to air drying for 48 h. After drying, the beads were dissolved in phosphate buffer (2 M, pH 7) and serially diluted to 10^8 dilution and plated on PDA medium. Plates were maintained at room temperature and observations were recorded 72 h after incubation.

3.2.2.4 Estimation of optimum height of air column

Optimum height of air column is the distance between the orifice of the separating funnel where sodium alginate solution is taken for bead preparation and the level of calcium chloride maintained in the beaker kept below the funnel. Optimum height is determined from the analysis of shape of the bead formed from standard sodium alginate solution by adjusting the column height at 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 30 and 35 cm.

3.2.3 Preparation and characterization of microbeads

The microbeads were prepared out of 30 different combinations of sodium alginate and $CaCl_2$ solution amended with additives (Table 3.4). A control was also maintained with 2.5 per cent each of sodium alginate and $CaCl_2$ solution without any additives. The beads thus prepared were subjected to characterization with respect to various properties.

Table 3.3 Standardization of spore suspension of *T. viride* in sodium alginate solution

Treatment	Volume of sodium alginate (ml)	Volume of *T. viride* spore suspension (ml)
T_1	99	1
T_2	98	2
T_3	97	3
T_4	96	4
T_5	95	5
T_6	94	6
T_7	93	7
T_8	92	8
T_9	91	9
T_{10}	90	10
Control	100	0

3.2.3.1 Preparation of microbeads

Microbeads were prepared by ionotropic gelation and cross linking technique as per the protocol of Sherina *et al*. (2012) and Tous *et al*. (2014). Sodium alginate solution amended with *T. viride* spore suspension was filled in a 500 ml separating funnel (4 mm bore diameter) fitted on a stand. Calcium chloride solution was kept below the funnel. Thereafter, the valve of the separating funnel was opened to facilitate the uninterrupted dripping of the sodium alginate solution amended with the spore suspension of the bioagent into the calcium chloride solution without any air bubbles. Care was also taken to prevent the formation of air bubbles in the alginate mixture in the separating funnel. At the same time, the beaker was shaken intermittently to avoid the agglomeration of beads. Rate of bead formation was adjusted to 14 - 17 beads per min. The beads were retained in the $CaCl_2$ solution for 60 min for curing. Excess calcium chloride solution was drained off using a strainer and the beads formed were washed twice in sterile distilled water.

3.2.3.2 Drying and storage of microbeads

After washing the prepared beads in sterile distilled water, excess moisture was removed by blotting with tissue paper. The beads were spread over open plastic trays surface sterilized with 70 per cent ethyl alcohol. It was then kept for drying for a time span of 48 h at room temperature. After drying, beads were transferred to sterilized empty jam bottles, which was properly labelled. The bottles were thereafter sealed air tight using cling film strips.

3.2.3.3 Bead weight

The weight of 10 randomly selected beads was estimated from each treatment. Mean bead weight was calculated and expressed in mg.

3.2.3.4 Bead yield

The yield of production of micro beads from different treatments was estimated

Table 3.4 Different combinations of sodium alginate, calcium chloride and additives for preparation of beads

Treatment	Additives	Concentration of sodium alginate (%)	Concentration of $CaCl_2$ (%)
T_1	Mannitol, PVP, CMC, Tween 80	1.5	2.5
T_2	Mannitol, PVP, CMC, Tween 80	1.5	3
T_3	Mannitol, PVP, CMC, Tween 80	1.5	3.5
T_4	Mannitol, PVP, CMC, Tween 80	0.75	2.5
T_5	Mannitol, PVP, CMC, Tween 80	0.75	3
T_6	Mannitol, PVP, CMC, Tween 80	0.75	3.5
T_7	Mannitol, PVP, Liquid paraffin, Tween80	1.5	2.5
T_8	Mannitol, PVP, Liquid paraffin, Tween80	1.5	3
T_9	Mannitol, PVP, Liquid paraffin, Tween80	1.5	3.5
T_{10}	Mannitol, PVP, Liquid paraffin, Tween80	0.75	2.5
T_{11}	Mannitol, PVP, Liquid paraffin, Tween80	0.75	2.5
T_{12}	Mannitol, PVP, Liquid paraffin, Tween80	0.75	3.5
T_{13}	Mannitol, PEG, Liquid paraffin, Tween80	1.5	2.5
T_{14}	Mannitol, PEG, Liquid paraffin, Tween80	1.5	3
T_{15}	Mannitol, PEG, Liquid paraffin, Tween80	1.5	3.5
T_{16}	Mannitol, PEG, Liquid paraffin, Tween80	0.75	2.5
T_{17}	Mannitol, PEG, Liquid paraffin, Tween80	0.75	3
T_{18}	Mannitol, PEG, Liquid paraffin, Tween80	0.75	3.5
T_{19}	Trehalose, PVP, CMC, Tween 80	1.5	2.5
T_{20}	Trehalose, PVP, CMC, Tween 80	1.5	3
T_{21}	Trehalose, PVP, CMC, Tween 80	1.5	3.5
T_{22}	Trehalose, PVP, CMC, Tween 80	0.75	2.5
T_{23}	Trehalose, PVP, CMC, Tween 80	0.75	3
T_{24}	Trehalose, PVP, CMC, Tween 80	0.75	3.5
T_{25}	Trehalose, PEG, CMC, Tween 80	1.5	2.5
T_{26}	Trehalose, PEG, CMC, Tween 80	1.5	3
T_{27}	Trehalose, PEG, CMC, Tween 80	1.5	3.5
T_{28}	Trehalose, PEG, CMC, Tween 80	0.75	2.5
T_{29}	Trehalose, PEG, CMC, Tween 80	0.75	3
T_{30}	Trehalose, PEG, CMC, Tween 80	0.75	3.5
T_{31}	Control	3	3

from the final weight of dried product with respect to the sum of weight of ingredients used for bead preparation **(**Sabry**,** 2018; Sherina *et al*., 2012). The per cent production yield was calculated using the formula:

$$\text{Percentage yield} = \frac{\text{Practical yield}}{\text{Theoretical yield}} \times 100$$

where, Practical yield - Total weight of beads produced
Theoretical yield - Sum of weights of ingredients used

3.2.3.5 Number of beads per ml of sodium alginate solution

Sodium alginate beads after drying for 48 h was evenly spread on a clean tray. These beads were then divided into four equal quadrats and total number of beads from one quadrant was counted. The number of beads obtained was multiplied by four to get total number of beads from each treatment. The total bead number was thereafter divided with total volume of sodium alginate solution used for bead preparation. Thus, the number of beads produced out of each ml sodium alginate solution and was expressed in number ml^{-1}.

3.2.3.6 Bead size

Bead size was measured using digital microscopy software motic image plus 2.0 and a stereo microscope. Ten randomly selected beads of each treatment were measured, where the average diameter was calculated and expressed in mm.

3.2.4 Evaluation of shelf life of *T. viride* encapsulated alginate beads

Shelf life of *T. viride* encapsulated alginate beads were evaluated at monthly intervals upto six months from the date of preparation. For this, microbeads were prepared well in advance and stored in air tight jam bottles with proper labeling. For estimating the shelf life of microbeads, standard serial dilution and plating technique was employed. Bead sample weighing 10 g from each treatment was suspended in 90 ml potassium phosphate buffer (0.2 M, pH 7) taken in a 250 ml conical flask. To attain

complete dissolution, it was agitated using a rotary shaker at 100 rpm for 24 h (Bashan *et al*., 2002).

One ml of the aliquot was added to 9 ml sterile water blank to make a concentration of 10^{-1}. Further it was serially diluted upto 10^{-8} concentration. Each one ml aliquot from 10^{-6} dilution and 10^{-8} dilution were poured into nine cm Petri plates with 20 ml potato dextrose agar (PDA) medium. Plates were rotated clockwise and anticlockwise to ensure uniform distribution of spores and were incubated at room temperature. Three replications were maintained and observations were recorded after 72 h of incubation. After third month of shelf life study, the treatments which rendered less number of colonies were discarded and a promising treatment was selected after six months.

3.2.5 Assessment of degree of contamination in microbeads

During the course of storage, the beads of all treatments (T_1-T_{31}) were inspected for the degree of contamination as per the protocol of Szczech and Maciorowski (2016). For the estimation, a 0 - 4 scoring index was employed as shown below.

0 – Product without discolouration and having the characteristic smell of fresh beads
1 – Product with slight discolouration and devoid of typical smell
2 – Unpleasant smell, distinct discolouration
3 – Strong unpleasant smell, discolouration of product, visible coatings
4 – Product with coatings and moulds, completely contaminated

3.2.6 Other parameters for standardization of microbeads

Some of the additional parameters like per cent potentially infective beads, moisture content in the microbeads, swelling behaviour of microbeads at different pH, per cent shrinking, sphericity factor, time of gelation and population of *T. viride* in soil at different pH was estimated.

3.2.6.1 Per cent potentially infective beads

Five randomly selected beads from each treatment was embedded on 20 ml solidified potato dextrose agar (PDA) medium in a symmetrical fashion with one at centre and the remaining four at four corners of the Petri plate (Declerck *et al*., 1996). Observations were taken under a microscope after 24, 48 and 72 h of incubation at room temperature. Twenty five beads were evaluated in each treatment. The number of beads with atleast one germinated conidium was recorded and the results were expressed in percentage.

3.2.6.2 Moisture content of beads

Moisture content of beads was estimated using an instant moisture analyser. Exactly three grams of beads were weighed and placed in the tray of the analyser and observations were recorded after 15 - 20 min. Analysis was carried out after 0, 24, 48, and 72 h after drying.

3.2.6.3 Swelling behaviour of microbeads at different pH

Swelling property of beads was recorded as per the protocol of Sherina *et al*. **(**2012) and Tous *et al*. (2014**).** Swelling ratio of beads was estimated at two different pH. One gram of bead was weighed and incubated in 10 ml potassium phosphate buffer (pH 7.4) and in 10 ml of 0.1 M HCl (pH 1). After a time interval of 10, 20, 30, 45, 60, 90, 120 and 240 min, the beads were taken out of the solution, blotted in a tissue paper to remove excess moisture and weighed. The time taken to attain maximum weight or swelling was accounted as the time required for bead disintegration.

Swelling ratio was estimated using the formula:

$$\text{Swelling ratio} = \frac{\text{Swollen weight - Initial weight}}{\text{Initial weight}}$$

Swelling percentage was calculated from the formula:

$$\text{Swelling per cent} = \frac{\text{Swollen weight - Initial weight}}{\text{Initial weight}} \times 100$$

3.2.6.4 Shrinking percentage

Shrinking percentage of microbeads was estimated by adopting the procedure described by Tous *et al*. (2014) with a slight modification. Diameter of three randomly selected beads were measured at three different positions under a stereo microscope, immediately after preparation and after being subjected to 48 h of open drying. Shrinking percentage was calculated from the formula:

$$\text{Shrinking per cent} = \frac{\text{Diameter before drying - Diameter after drying}}{\text{Diameter before drying}} \times 100$$

3.2.6.5 Sphericity factor

Sphericity factor was estimated from the shape of the bead as per the protocol of Chan *et al*. (2011). Diameter of five randomly selected beads was estimated in three different directions using a stereo microscope. From the data obtained, sphericity factor was estimated using the formula:

$$\text{Sphericity Factor (SF)} = \frac{(d_{max} - d_{min})}{(d_{max} + d_{min})} \times 100$$

where d_{min} is the minimum diameter and d_{max} is the maximum diameter of the bead.

3.2.6.6 Estimation of time of gelation

The time of gelation is the time at which bead attains a stable weight in $CaCl_2$ solution or it is the time required for bead curing. Time required to complete the process of gelation was estimated as per the protocol of Tous *et al*. (2014). It is calculated from the change in weight of bead during gelation in $CaCl_2$ solution.

For this, 10 randomly selected microbeads from the respective treatments were eluted from $CaCl_2$ solution at 0, 15, 30, 45, 60, 75 and 90 min of formation. The weight of each bead was recorded after the removal of surface moisture using a blotting paper.

3.2.6.7 Effect of pH on the selected combination of microbead in soil under in vitro condition

The release of encapsulated bioagent in soil was estimated as per Szczech and Maciorowski (2016) and the role of pH in erosion and degradation of microbeads were established as per Sherina *et al*. (2012) and Tous *et al*. (2014).

For this, the release of encapsulated *Trichoderma* in the soil was carried out at different pH levels. The pH of sterile soil sample was estimated using a digital pH meter. Further, addition of one gram lime ($CaCO_3$) rendered a higher pH. The sterilized soil sample 100g each was taken from these two lots in separate beakers. Thereafter, *Trichoderma* encapsulated microbeads were added @ 500 mg per 100g soil. Another 100 gram sample without the addition of microbeads served as control. Sterile water (10 ml) was added in all the beakers to moisten the soil.

After a time interval of 1, 2 and 7 days, 10 g soil was withdrawn from the treatments and enumeration of *T. viride* was carried out using standard serial dilution and plating technique. One ml aliquot from 10^{-6} concentration was plated on *Trichoderma* Selective Medium (Appendix III) (Elad and Chet, 1983) and the plates were incubated at room temperature. Experiment was carried out in triplicate and observations were recorded after 72 h of incubation.

3.3 *In vivo* EVALUATION OF BIOCONTROL EFFICACY AND PLANT GROWTH PROMOTION OF ENCAPSULATED *Trichoderma viride*

Three different pot culture experiments were laid out to evaluate the *in vivo* efficacy and plant growth promoting potential of sodium alginate encapsulated *T. viride.* Experiments were carried out on bush cowpea variety Bhagyalakshmi against soil borne fungal pathogens *viz*., *Pythium aphanidermatum*, *Rhizoctonia solani* and *Fusarium oxysporum* as test pathogens. The effects of three doses of bead formulation were compared with the recommended dose of already established talc based and liquid formulation along with an untreated control.

The experiments were carried out during September - December 2019 in the poly house of Department of Plant Pathology, College of Horticulture, Vellanikkara. The details of pot culture experiments are as follows:

Design	: CRD
Treatment	: 6
Replications	: 4
Number of plants per replication	: 5
Crop	: Cowpea
Variety	: Bhagyalakshmi

The treatment details are as follows:

T1: Alginate based *T. viride* (1.0 g plant^{-1})

T2: Alginate based *T. viride* (3.0 g plant^{-1})

T3: Alginate based *T. viride* (5.0 g plant^{-1})

T4: Talc based *T. viride* (20 g l^{-1})

T5: Liquid based *T. viride* (5 ml l^{-1})

T6: Control

3.3.1 Preparation of potting mixture and raising of crop

Potting mixture composed of sand, soil and cow dung in the ratio 1:1:1 was filled in grow bags of size 60 x 30 x 30 cm. Formalin (40%) diluted thrice with water was used for chemical sterilization of potting mixture. Treated grow bags were covered with thick polythene sheets to ensure the complete sterilization without any volatility losses. The polythene sheets were retained for one week and the bags thereafter were kept exposed for another 10 days to eliminate the risk of phytotoxicity. After sufficient watering, three seeds of bush cowpea var. Bhagyalakshmi were dibbled in each bag. Treatments were applied immediately after sowing and twice at 20 days interval after symptom appearance. All cultural operations excluding the fungicide application were followed (KAU, 2011).

3.3.2 Challenge inoculation with test pathogens

Evaluation of biocontrol efficacy of *T. viride* encapsulated sodium alginate beads were accomplished by artificial inoculation of test pathogens 30 days after sowing.

3.3.2.1 Pythium aphanidermatum

Eight mm mycelial disc of actively growing culture of *Pythium aphanidermatum* was inoculated on carrot agar in nine cm Petri dishes. The plates were kept for incubation at room temperature and after seven days, the mycelium was scraped from the medium and was uniformly suspended in 500 ml sterile water to adjust the spore concentration to 5.0×10^6 cfu ml^{-1}. From this, 10 ml of the aliquot was inoculated to the root zone of the crop (Sivan *et al*., 1984).

3.3.2.2 Rhizoctonia solani

A plug of PDA medium with actively growing mycelia of *Rhizoctonia solani* was inoculated on PDA medium in Petri plates and was incubated at room temperature. Mycelia from fully grown culture was harvested by fine scraping and was uniformly

suspended in 500 ml sterile water to adjust the spore concentration to 1.0 x 10^6 cfu ml^{-1}. From this, five ml of the suspension was inoculated to root zone of the crop (Baskar *et al*., 2018).

3.3.2.3 Fusarium oxysporum

Eight mm mycelia disc of *F. oxysporum* was cultured on PDA medium in nine cm Petri plates in the dark at room temperature for 10 days. The conidia were aseptically scrapped from the medium using an inoculation needle and uniformly suspended in sterile distilled water to attain a spore concentration of 1.0 x 10^4 cfu ml^{-1} and 20 ml of the suspension was drenched in the root zone of the crop (Szczech and Maciorowski, 2016).

3.3.3 Observations

Plant biometry, yield and disease incidence were documented at definite intervals.

3.3.3.1 Plant biometry

Biometric parameters like plant height, number of leaves and number of days taken to flower were recorded.

3.3.3.1a Height of the plant

Height of each plant was measured and noted at 30, 45 and 60 days after sowing.

3.3.3.1b Number of leaves

Number of leaves from each plant were counted and documented at 30, 45 and 60 days after sowing.

3.3.3.1c Number of days to first flowering

Number of days taken to first flowering were observed in each treatment and recorded.

3.3.3.2 Crop yield

Crop yield was estimated in terms of weight of pods for each treatment and the weight was denoted in g per plant.

3.3.3.3 Estimation of per cent disease incidence

After challenge inoculation, the plants were regularly monitored for symptom appearance. Per cent disease incidence was estimated using the formula given by Wheeler (1969).

$$\text{Per cent Disease Incidence (PDI)} = \frac{\text{Number of plants infected}}{\text{Total number of plants observed}} \times 100$$

Per cent Disease Severity (PDS) was also estimated as per the disease rating scale given in Table 3.5

3.3.4 Estimation of population of *T. viride* in different treatments

Population of *T. viride* in the potting mixture of different treatments was estimated prior to the experiment after fumigation. Thereafter, estimation of the population was carried out at monthly interval after treatment application. For this, soil samples were drawn from each replication of all the treatments. After thorough mixing, sample size was reduced by quadrant method. Enumeration was carried out by adopting serial dilution and plating technique on *Trichoderma* specific medium (TSM).Ten gram soil from each sample was added to 90 ml sterile water blank to get 10^{-2} dilution. One ml of aliquot from this dilution was pipetted into nine ml sterile water blank to make a dilution of 10^{-3}. Likewise, dilution continued upto 10^{6} and one ml from 10^{-6} was pipetted out into a sterile Petri plate where cooled, molten media was poured over the suspension and the plates were rotated clockwise and anticlockwise to ensure uniform mixing. Observations were taken 72 h after incubation of plates. Similarly, the experiment was

Table 3.5 Disease Rating Scale for estimation of Per cent Disease Severity (PDS)

Disease incidence (%)	Description	Rating of bioefficacy of formulation
0	Germination > 90%, no seed rotting, seedlings healthy, root and shoot portions well developed	Highly Efficient (HE)
1-15	Germination 80 - 90%, infection on main as well as lateral roots, seedlings are well developed	Efficient (E)
15-30	Germination 70 - 80%, development of roots restricted and growth is less compared to Score 1. Infection occurred on roots. Shoot portions developed but growth retarded compared to Score 1	Moderately Efficient (ME)
30-45	Germination 60 - 70%, length of roots and shoots short compared to Score 1. Germination of seeds inhibited.50% of root area infected. Shoot portions also showed infection.	Moderately Inefficient (MI)
45-60	Seed germination 50 to 60%. Development of roots and shoots greatly retarded. Shoot portions showed more infection.	Inefficient (I)
Above 60	Less than 50% germination and seed rotting	Highly Inefficient (HI)

3.4 ASSESSMENT OF BIODEGRADATION OF *T. viride* ENCAPSULATED ALGINATE BEADS IN SOIL

The method adopted by Bashan *et al*. (2002) was employed for estimating the biodegradation of *T. viride* encapsulated alginate beads of T_1 - T_6 in soil. Exactly 40 beads were bundled in properly labeled nylon bags. Subsequently, they were buried five cm deep in garden soil filled in earthen pots. The pots were watered upto saturation and maintained for 12 days. The bags were pulled out in every three days interval to estimate the degree of biodegradation. The experiment was carried out in triplicate and the number of partially, completely and non-degraded beads were estimated.

3.5 BENEFIT - COST RATIO ANALYSIS

Benefit cost ratio for the production of 1 Kg alginate bead based formulation of *Trichoderma viride* was calculated. For calculation, only recurring cost was incorporated which includes the cost of raw materials and chemicals.

3.6 STATISTICAL ANALYSIS

Data were subjected to analysis of variance (ANOVA) and were analysed using the statistical package Web Agri Stat Package (WASP 2.0). Level of significance, mean and standard error were estimated for various sets of data. Duncan's Multiple Range Test (DMRT) was employed for the multiple comparison between the treatment means and the data wherever needed was subjected to appropriate transformation as suggested by Gomez and Gomez (1984).

RESULTS

4. RESULTS

The studies on "Microencapsulation of *Trichoderma viride* for management of major soil borne fungal pathogens" was carried out in the Department of Plant Pathology, College of Horticulture, Vellanikkara during the period 2017-2020. The results of the experiment are detailed below.

4.1 PRESERVATION OF FUNGAL CULTURE

The fungal reference culture of *Trichoderma viride* was sub cultured and successfully maintained throughout the course of research programme under refrigerated conditions for further studies. Colonies of *T. viride* are wooly in texture and initially they are white in colour. As the conidia are formed, they turn green or yellowish green. Under microscope, septate hyaline hyphae, conidiophores, phialides, conidia and chlamydospores are observed.

4.2 STANDARDIZATION AND PREPARATION OF ALGINATE BEAD BASED FORMULATION OF *Trichoderma viride*

4.2.1 Standardisation of media with additives and its effect on shelf life of *T. viride*

The effect of seven different additives belonging to four different classes *viz*., sugar, wetting agent, adhesive and surfactant on the shelf life of *T. viride* was examined. These additives in eight different possible combinations were amended to 50 ml potato dextrose (PD) broth taken in 100 ml conical flasks, sterilized and inoculated with *T. viride*. A control was also maintained without amending any additives and thus, a total of nine treatments were laid out. Thereafter, the flasks were kept for incubation and shelf life study was conducted at monthly intervals employing serial dilution and plating technique. The population of *T. viride* was enumerated at 10^{-6} and 10^{-8} dilutions at seven days after inoculation (7 DAI) and also at 1, 2, 3, 4, 5, 6 and 7 months after inoculation (MAI) upto seven months (Table 4.1a and Table 4.1b). The data presented in the table

clearly indicates that in general, with increase in dilution from 10^{-6} to 10^{-8}, the count of fungi gradually decreased where less number of colonies were formed on PDA medium in all treatments. It was also observed that seven days after inoculation (7 DAI), both at 10^{-6} and 10^{-8}, the count was comparatively higher compared to the rest of the months and the least was observed at 210 DAI.

At 7 DAI in 10^{-6} dilution (Table 4.1a), a significant difference was noticed among various treatments with the colony count ranging from 12.6 to 28.0 x 10^6 cfu ml^{-1} with the highest population in treatment T_5 (trehalose, PVP, CMC, tween 80) (28.0 x 10^6 cfu ml^{-1}) closely followed by T_4 (mannitol, PEG, liquid paraffin, tween 80) (25.0 x 10^6 cfu ml^{-1}) and the least population of 12.6 x 10^6 cfu ml^{-1} was observed in T_2 (mannitol, PEG, CMC, tween 80). At the same time interval in 10^{-8} dilution (Table 4.1b) also, the highest population was enumerated in T_5 (18.0 x 10^8 cfu ml^{-1}) followed by T_1 (mannitol, PVP, CMC, tween 80) (5.3 x 10^8 cfu ml^{-1}) and the least was recorded in control (T_9) (0.33x10^8 cfu ml^{-1}). At 1 MAI, in 10^{-6} dilution, T_5 outperformed the other treatments again with 23.0 x 10^6 cfu ml^{-1} followed by T_4 (20.0 x 10^6 cfu ml^{-1}) and the minimum colony count was recorded in T_8 (trehalose, PEG, liquid paraffin, tween 80) (0.6 x 10^6 cfu ml^{-1}). Similarly, at 10^{-8} dilution also, maximum number of colonies were recorded in T_5 (9.3 x 10^8 cfu ml^{-1}) followed by T_4 (1.6 x 10^8 cfu ml^{-1}) and the colony count was found nil in T_2, T_3, T_7, T_8 and T_9. At 2 MAI too, the same trend was noticed with the maximum colony forming units in T_5 (24.0 x 10^6 cfu ml^{-1}) and the minimum in control (0.3 x 10^6 cfu ml^{-1}) at 10^{-6} dilution. Likewise, at the higher dilution of 10^{-8}, T_5 recorded the maximum colony count (15.0 x 10^8 cfu ml^{-1}) followed by T_1 (1.3 x 10^8 cfu ml^{-1}) while the colony count was nil in T_2, T_4, T_7, T_8 and control. After two months, the treatments T_2, T_7 and T_8 were discarded due to reduced colony count. The remaining treatments were carried forward for further studies.

At 3 MAI, maximum colony count was recorded in T_5 (trehalose, PVP, CMC, tween 80) (28.0 x 10^6 cfu ml^{-1}) followed by T_6 (trehalose, PEG, CMC, tween 80) (23.0 x 10^6 cfu ml^{-1}) whereas, minimum colony count was documented in control (T_9) (1.0x10^6 cfu ml^{-1}) at 10^{-6} dilution. Similarly, a maximum of 21.0 x 10^8 cfu ml^{-1} was documented again in T_5 followed by T_1 (2.0 x 10^8 cfu ml^{-1}) while the colony count was nil in control

Table 4.1a Population of *T. viride* at 10^{-6} dilution at monthly intervals

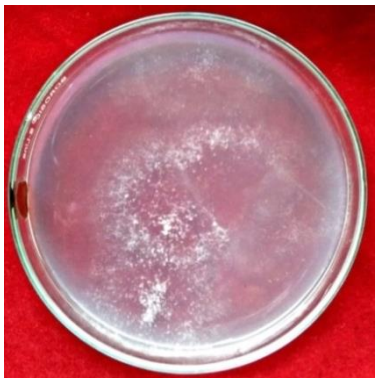
Sl. No.	Treatment	*Population of *T. viride* (x10^6 cfu ml^{-1})							
		7 DAI	1 MAI	2 MAI	3 MAI	4 MAI	5 MAI	6 MAI	7 MAI
1.	T_1- Mannitol, PVP, CMC, Tween 80	20.0 (4.54)^{b}	18.0 (4.26)^{ab}	18.0 (4.29)^{b}	1.6 (1.46)^{c}	4.6 (2.26)^{c}	4.0 (1.8)^{c}	18.6 (4.32)^{b}	12.0 (3.45)^{c}
2.	T_2- Mannitol, PEG, CMC, Tween 80	12.6 (4.32)^{b}	2.3 (1.64)^{de}	2.6 (1.77)^{de}	-	-	-	-	-
3.	T_3- Mannitol, PVP, Liquid paraffin , Tween 80	19.0 (4.43)^{b}	15.0 (3.90)^{bc}	16.0 (4.06)^{bc}	1.3 (1.34)^{c}	10.0 (3.23)^{b}	3.6 (1.60)^{c}	10.3 (3.20)^{c}	8.6 (2.92)^{c}
4.	T_4- Mannitol, PEG, Liquid paraffin, Tween 80	25.0 (4.99)^{a}	20.0 (4.52)^{ab}	13.0 (3.65)^{c}	19.0 (4.44)^{b}	30.0 (5.52)^{a}	15.0 (4.02)^{b}	39.0 (6.24)^{a}	42.0 (6.47)^{a}
5.	T_5- Trehalose, PVP, CMC, Tween 80	28.0 (5.29)^{a}	23.0 (4.84)^{a}	24.0 (4.94)^{a}	28.0 (5.33)^{a}	31.0 (5.59)^{a}	45.0 (6.54)^{a}	40.0 (6.31)^{a}	43.0 (6.55)^{a}
6.	T_6- Trehalose, PEG, CMC, Tween 80	21.0 (4.27)^{b}	10.0 (3.21)^{c}	3.6 (2.03)^{d}	23.0 (4.84)^{b}	12.3 (3.57)^{b}	19.0 (4.40)^{b}	21.6 (4.65)^{b}	30.0 (5.46)^{b}
7.	T_7- Trehalose, PVP, Liquid paraffin, Tween 80	13.0 (3.64)^{c}	4.3 (2.192)^{d}	0.6 (1.46)^{ef}	-	-	-	-	-
8.	T_8- Trehalose, PEG, Liquid paraffin, Tween 80	15.0 (4.85)^{c}	0.6 (1.05)^{e}	0.6 (1.38)^{ef}	-	-	-	-	-
9.	T_9- Control	13.0 (3.60)^{c}	1.3 (1.34)^{e}	0.3 (1.22)^{f}	1.0 (1.17)^{c}	2.0 (1.28)^{d}	2.6 (1.64)^{c}	8.0 (2.81)^{c}	3.3 (1.80)^{d}
	CD(0.01)	0.46	1.10	0.56	0.68	0.88	1.86	0.67	0.81

DAI - Days after inoculation. MAI - Months after inoculation. * Mean of three replications. In each column figures followed by the same letter do not differ significantly according to DMRT. Square root transformed values are given in parenthesis.

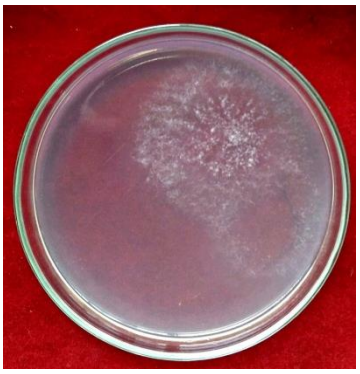
Table 4.1b Population of *T. viride* at 10^{-8} dilution at monthly intervals

Sl. No.	Treatment	*Population of *T. viride* (x10^8 cfu ml^{-1})							
		7 DAI	1 MAI	2 MAI	3 MAI	4 MAI	5 MAI	6 MAI	7 MAI
1.	T_1- Mannitol, PVP, CMC, Tween 80	5.3 (2.83)^{b}	1.3 (1.34)^{b}	1.3 (1.26)^{b}	2.0 (1.55)^{b}	3.0 (1.58)^{bc}	2.3 (5.04)^{b}	8.6 (2.97)^{b}	8.3 (2.96)^{b}
2.	T_2- Mannitol, PEG, CMC, Tween 80	3.0 (1.85)^{bcd}	0.0 (0.70)^{c}	0.0 (0.70)^{c}	-	-	-	-	-
3.	T_3- Mannitol, PVP, Liquid paraffin , Tween 80	2.3 (1.67)^{cd}	0.0 (0.70)^{c}	0.6 (1.05)^{bc}	0.6 (1.05)^{bc}	0.3 (0.87)^{c}	0.0 (0.70)^{e}	6.6 (2.66)^{b}	7.6 (2.84)^{b}
4.	T_4- Mannitol, PEG, Liquid paraffin, Tween 80	4.3 (2.12)^{bc}	1.6 (1.55)^{b}	0.0 (0.70)^{c}	1.6 (1.44)^{b}	0.3 (0.87)^{c}	2.3 (1.67)^{d}	15.3 (3.96)^{a}	9..3 (3.13)^{b}
5.	T_5- Trehalose, PVP, CMC, Tween 80	18 (4.29)^{a}	9.3 (3.12)^{a}	15 (3.92)^{a}	21 (4.62)^{a}	15 (3.95)^{a}	6.0 (5.66)^{a}	15.6 (4.02)^{a}	13.3 (3.71)^{a}
6.	T_6- Trehalose, PEG, CMC, Tween 80	1.0 (1.67)^{cd}	1.3 (1.34)^{b}	0.6 (1.05)^{bc}	1.3 (1.26)^{bc}	3.0 (1.78)^{b}	3.1 (2.51)^{c}	8.6 (2.97)^{b}	10.0 (3.23)^{b}
7.	T_7- Trehalose, PVP, Liquid paraffin, Tween 80	1.6 (1.46)^{de}	0.0 (0.70)^{c}	0.0 (0.70)^{c}	-	-	-	-	-
8.	T_8- Trehalose, PEG, Liquid paraffin, Tween 80	0.6 (0.99)^{e}	0.0 (0.70)^{c}	0.0 (0.70)^{c}	-	-	-	-	-
9.	T_9- Control	0.3 (0.87)^{e}	0.0 (0.70)^{c}	0.0 (0.70)^{c}	0.0 (0.71)^{c}	1.3 (1.55)^{bc}	0.0 (0.70)^{e}	0.0 (0.70)^{c}	0.3 (0.87)^{c}
	CD(0.01)	0.88	0.88	0.63	0.92	1.05	0.69	0.85	0.55

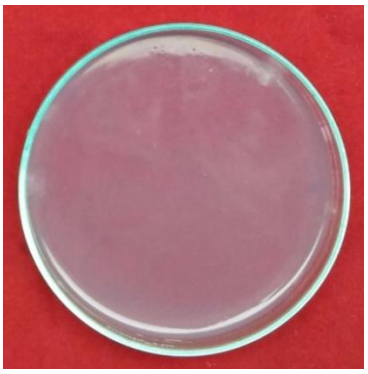
DAI - Days after inoculation. MAI - Months after inoculation. * Mean of three replications. In each column figures followed by the same letter do not differ significantly according to DMRT. Square root transformed values are given in parenthesis.



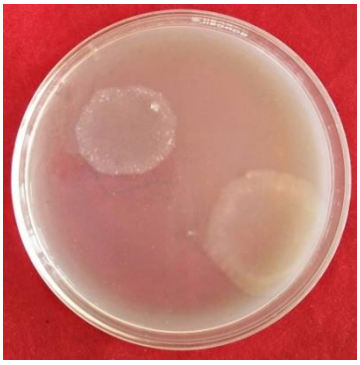
7 DAI



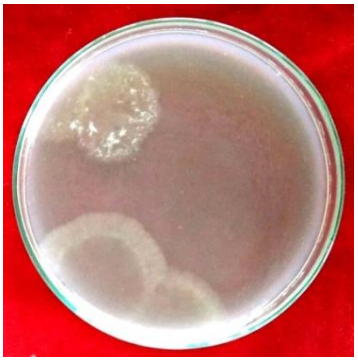
30 DAI



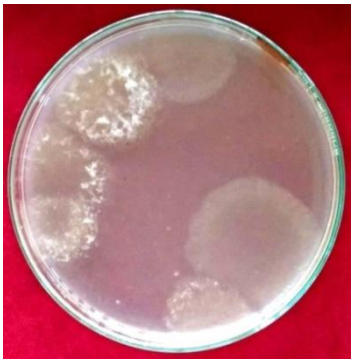
60 DAI



90 DAI



120 DAI



150 DAI

180 DAI

210 DAI

Plate 4.1 Population of *T. viride* from adjuvant amended culture broth (x10^8 cfu ml^{-1}) in T_5

(T_9) at 10^{-8} dilution. At 4 MAI, maximum colony count was recorded in T_5 (31.0 x 10^6 cfu ml^{-1} itself closely followed by T_4 (mannitol, PEG, liquid paraffin, tween 80) (30.0 x 10^6 cfu ml^{-1}) and minimum number was noted in control (2.0 x 10^6 cfu ml^{-1}) at 10^{-6} dilution whereas, the maximum colony count at 10^{-8} dilution was documented in T_5 (15.0 x 10^8 cfu ml^{-1}) followed by 3.0 x 10^8 cfu ml^{-1} in T_1 and T_6 and a minimum of 0.3 x 10^8 cfu ml^{-1} was recorded in T_3 and T_4. At 5 MAI, the highest colony count was recorded in T_5 (45.0 x 10^6 cfu ml^{-1}) followed by T_6 (19 x 10^6 cfu ml^{-1}) while, the least was documented in control (2.6 x 10^6 cfu ml^{-1}) at 10^{-6} dilution. The same trend was noticed in 10^{-8} dilution with maximum in T_5 and minimum in control. At 6 MAI, again T_5 recorded a maximum of 40.0 x 10^6 cfu ml^{-1} and 15.6 x 10^8 cfu ml^{-1} in both the dilutions, while the minimum colony count was documented in control (8.0 x 10^6 cfu ml^{-1} in 10^{-6} dilution and zero in 10^{-8} dilution). Further, at 7 MAI, maximum colony count was recorded in T_5 (43.0 x 10^6 cfu ml^{-1}) closely followed by T_4 (42.0 x 10^6 cfu ml^{-1}) and the minimum number of colonies were enumerated in control (3.3 x 10^6 cfu ml^{-1}) at 10^{-6} dilution. In the same time interval at 10^{-8} dilution, a maximum of 13.3 x 10^8 cfu ml^{-1} was recorded in T_5 followed by T_6 (10.0 x 10^8 cfu ml^{-1}) and the minimum count of 0.3 x 10^8 cfu ml^{-1} was recorded in control.

It is pertinent to note that, after seven months of shelf life studies, the treatment T_5, a combination of additives comprising of trehalose (15 mM), PVP (1%), CMC (0.5%) and tween 80 (0.5%) was found to be superior compared to other treatments. Since the performance of T_5 remained consistent throughout the course of experiment, it was selected as the best additive combination.

4.2.2 Standardization of different parameters for bead preparation

Different experimental parameters like concentration of sodium alginate and calcium chloride, volume of *T. viride* spore suspension required per 100 ml of sodium alginate solution and optimum vertical distance between the orifice of the funnel to surface of $CaCl_2$ bath were standardized as follows:

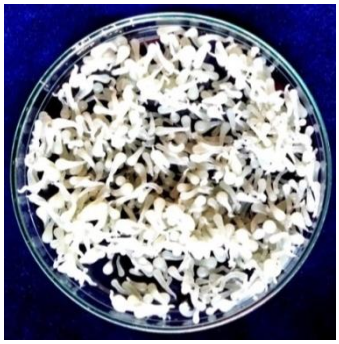
Table 4.2 Standardization of sodium alginate and $CaCl_2$ solution for bead formation

Sl. No.	Concentration (%)		Status of bead formation			
			Without additives		With additives	
	Sodium alginate	$CaCl_2$	Formation of bead	Type of bead	Formation of bead	Type of bead
1.	0.75	2.5	-	Alginate worms and long tailed beads	+	Spherical and smooth textured beads
2.	0.75	3.0	-	Alginate worms and long tailed beads	+	Spherical and smooth textured beads
3.	0.75	3.5	-	Alginate worms and long tailed beads	+	Spherical and smooth textured beads
4.	1.5	2.5	-	Alginate worms and long tailed beads	+	Spherical and smooth textured beads
5.	1.5	3.0	-	Alginate worms and long tailed beads	+	Spherical and smooth textured beads
6.	1.5	3.5	-	Alginate worms and long tailed beads	+	Spherical and smooth textured beads
7.	2.5	2.5	+	Spherical and smooth textured beads	-	Alginate worms and long tailed beads
8.	2.5	3.0	+	Spherical and smooth textured beads	-	Alginate worms and long tailed beads
9.	2.5	3.5	+	Spherical and smooth textured beads	-	Alginate worms and long tailed beads
10.	3.0	2.5	+	Spherical and smooth textured beads	-	Alginate worms and long tailed beads
11.	3.0	3.0	+	Spherical and smooth textured beads	-	Alginate worms and long tailed beads
12.	3.0	3.5	+	Spherical and smooth textured beads	-	Alginate worms and long tailed beads
13.	3.5	2.5	+	Spherical and smooth textured beads	-	Alginate worms and long tailed beads
14.	3.5	3.0	+	Spherical and smooth textured beads	-	Alginate worms and long tailed beads
15.	3.5	3.5	+	Spherical and smooth textured beads	-	Alginate worms and long tailed beads

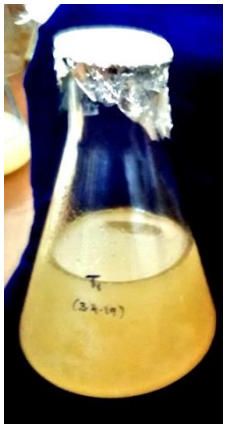
'+'Beads formed

'-' Beads not formed

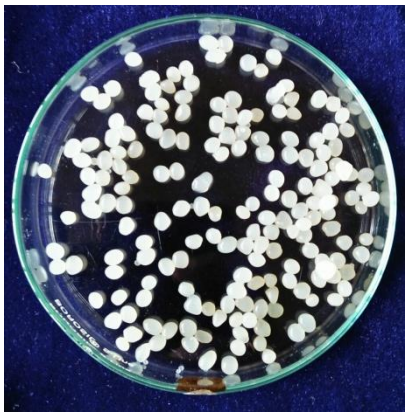
3.5 % sodium alginate solution + additives



Beads prepared from 3.5 % sodium alginate solution with additives



1.5 % sodium alginate solution + additives



Beads prepared from 1.5 % sodium alginate solution with additives

Plate 4.2 Effect of concentration of sodium alginate on bead shape

4.2.2.1 Concentration of sodium alginate and calcium chloride

Concentration of sodium alginate for bead preparation was standardized by selecting three random concentrations *viz.*, 2.5, 3.0 and 3.5 per cent. Similarly, 2.5, 3.0 and 3.5 per cent calcium chloride ($CaCl_2.2H_2O$) solution was also prepared and the freshly prepared $CaCl_2$ solution immediately after sterilization (121^0C temperature and 15 psi pressure for 20 min) was cooled and used for bead preparation. Based on the shape of the beads formed, an optimum concentration of sodium alginate and $CaCl_2$ was selected for further preparation of beads. The data are furnished in Table 4.2.

It was observed that sodium alginate at 2.5, 3.0 and 3.5 per cent concentration without the additives yielded high quality spherical beads with ivory colour. However, when additives were added in their respective concentrations as in Table 3.2 the alginate solution, beads were not formed; instead it extruded as continuous stream which resulted in the formation of the so called alginate worms. Moreover, there were numerous bubbles entrapped in the solution which yielded highly porous long tailed beads. At these concentrations, melting and mixing of ingredients were also found tedious. Subsequently, the concentration of sodium alginate solution was reduced to 0.75 and 1.5 per cent and at this concentration, along with the specified combination of additives (Table 3.1) yielded spherical beads with smooth texture. However, $CaCl_2$ solution at all the three specific concentrations along with 2.5, 3.0 and 3.5 per cent sodium alginate without additives or with 0.75 and 1.5 per cent sodium alginate with additives yielded good quality spherical beads.

Hence, 0.75 and 1.5 per cent sodium alginate and 2.5, 3.0 and 3.5 per cent $CaCl_2$ solution was selected to prepare the microbeads in 31 different combinations which are listed as in Table 3.4 of 3.2.3.

4.2.2.2 Preparation of spore suspension of T. viride

Well sporulated seven days old mycelial mat of *T. viride* was macerated to harvest the conidia for the preparation of conidial suspension. The process rendered dark green coloured conidial suspension. It was further sieved to separate out the mycelial bits and

was used for bead preparation.

4.2.2.3 Standardization of spore suspension of T. viride in sodium alginate solution

Volume of the conidial suspension of *T. viride* required for the preparation of beads was estimated. For this, microbeads were prepared using different combinations of volume of conidial suspension of *T. viride* and sodium alginate solution (Table 4.3), where a total of 10 treatments along with control were delineated for the experiment. Subsequently, these beads were dissolved in potassium phosphate buffer (2 M, pH 7) to enumerate the population of *T. viride* at 10^{-8} dilution adopting serial dilution and plating technique. From the table, it is obvious that as the volume of conidial suspension increased, population of entrapped *T. viride* also increased proportionately.

In general, the population ranged from zero to 6.0 x 10^8 cfu g^{-1} bead, where the maximum colony forming units were recorded in T_{10} (10 ml conidial suspension with 90 ml sodium alginate solution) and the minimum in T_1 (one ml conidial suspension with 99 ml sodium alginate solution). Among the treatments, T_5 (five ml conidial suspension with 95 ml sodium alginate solution) yielded a population of 2.3 x 10^8 cfu g^{-1} bead, which was selected for further studies as this combination was capable of compensating any cell mortality during the process of cross linking to achieve minimum of a single colony in 10^{-8} dilution.

4.2.2.4 Estimation of optimum height of air column

The optimum vertical distance between orifice of the separating funnel and level of $CaCl_2$ solution in the beaker was determined principally based on the shape of the microbeads. The influence of this column height on the shape of the beads is clearly depicted in Table 4.4. The column height was maintained as 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 30 and 35 cm where 2 cm rendered long tailed beads and 4 cm yielded short tailed beads. Further, it was noticed that when the distance was adjusted to 6 - 35 cm, exact spherical beads were formed. The formation of very minute beads was also observed when the height was maintained between 6 - 35 cm, but not at two and four cm. Giving

Table 4.3 Standardization of spore suspension of *T. viride* for preparation of microbeads

Treatment	Volume of sodium alginate (ml)	Volume of *T. viride* spore suspension (ml)	*Population of *T. viride* ($x10^8$ cfu ml^{-1})
T_1	99	1	0.00
T_2	98	2	0.33
T_3	97	3	0.66
T_4	96	4	1.00
T_5	95	5	2.33
T_6	94	6	3.00
T_7	93	7	3.30
T_8	92	8	4.66
T_9	91	9	5.30
T_{10}	90	10	6.00
Control	100	0	0.00

*Mean of three replications

Table 4.4 Effect of height of air column on shape of alginate beads

Sl. No.	Height of air column (cm)	*Bead shape
1.	2	Long tailed beads
2.	4	Short tailed beads
3.	6	Spherical beads
4.	8	Spherical beads
5.	10	Spherical beads
6.	12	Spherical beads
7.	14	Spherical beads
8.	16	Spherical beads
9.	18	Spherical beads
10.	20	Spherical beads
11.	30	Spherical beads
12.	35	Spherical beads

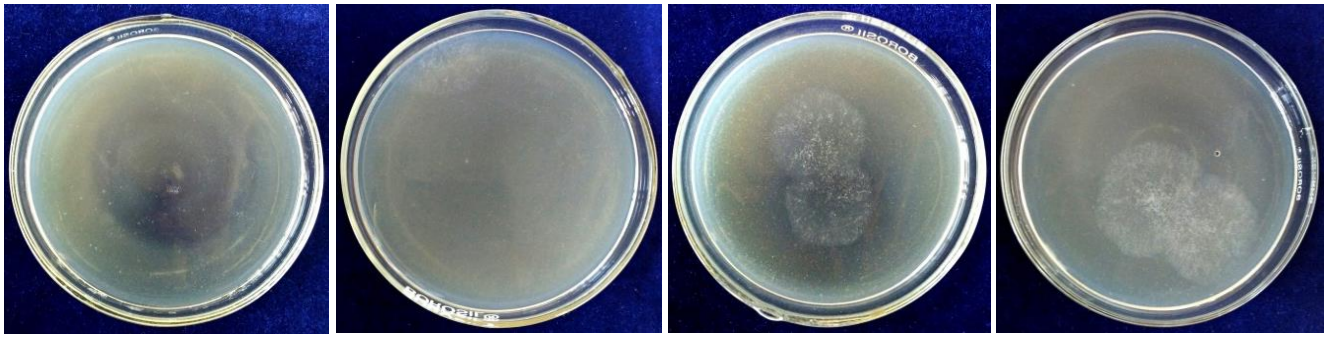
*Mean of three replications

T1 T2 T3 T4

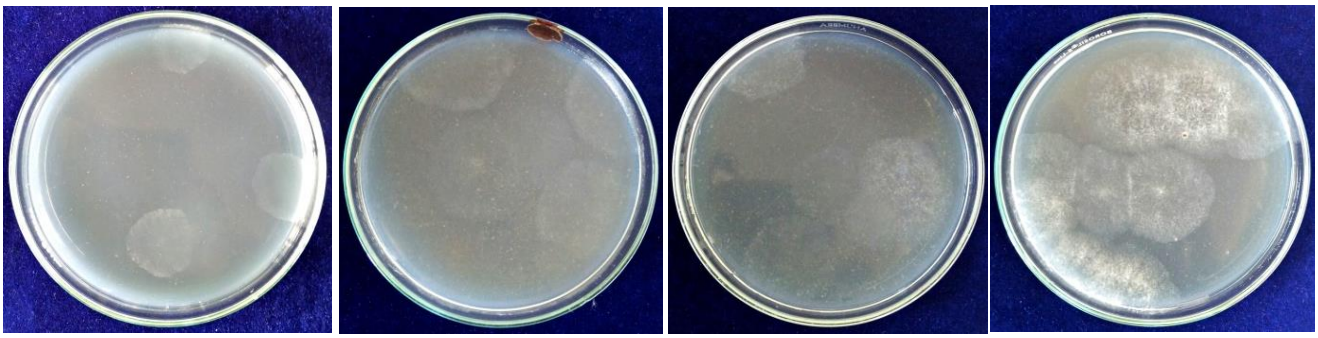
T5 T6 T7 T8

T9 T10 T11

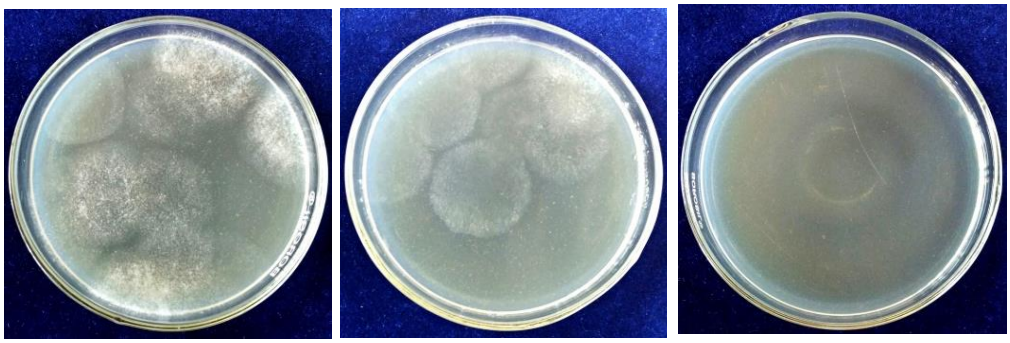
Plate 4.3 Microbeads prepared using different volumes of *T. viride* spore suspension and sodium alginate



T1 T2 T3 T4



T5 T6 T7 T8



T9 T10 T11

Plate 4.4 Population of *T. viride* from beads prepared using different volumes of *T. viride* spore suspension (x10^8 cfu ml^{-1})

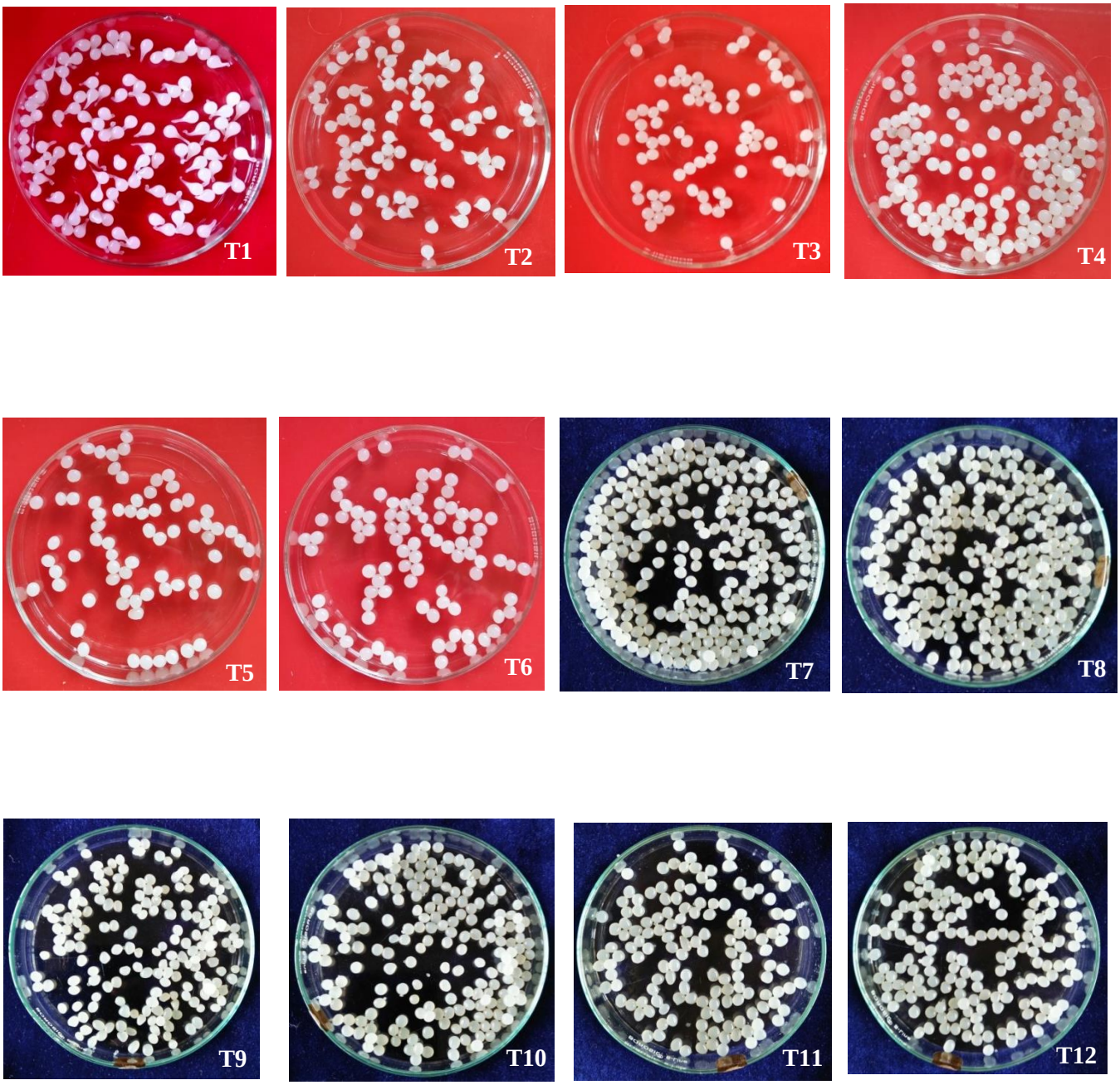


Plate 4.5 Shape of the beads at different vertical column heights

primary emphasis to the shape of the bead and also in the convenience of handling the apparatus, a vertical height range of 8 - 12 cm was selected for bead preparation.

4.2.3 Preparation and characterization of microbeads

Beads of 31 treatments as mentioned in 3.2.3 were prepared by using standardized sodium alginate solution amended with various adjuvants in their respective concentration along with standardized $CaCl_2$ solution. Similarly, control beads were prepared without amending any additives. The beads thus prepared were subjected to characterization with respect to properties like bead weight, bead size, swelling behaviour at different pH, shrinking percentage and sphericity factor. Moreover, per cent bead yield, number of beads obtained from each ml of sodium alginate solution and moisture content of the beads were also estimated apart from percentage of potentially infective beads.

4.2.3.1 Preparation of microbeads

The additives described in the treatments T_1, T_3, T_4, T_5 and T_6, selected after two months of shelf life study in 4.2.1, along with sodium alginate and $CaCl_2$ in standardized concentrations (Table 3.4 of 3.2.3.) was used for preparation of beads. Thus, a total of 31 treatments including control were laid out and the treatments are listed in Table 3.4 of 3.2.3.

The microbeads were prepared by employing ionotropic gelation and cross linking technique. For this, sodium alginate powder and additives were dissolved in luke warm water, into which the conidial suspension of *T. viride* was added. After thorough mixing, numerous bubbles were observed in the mixture, which resulted in low quality and bubble entrapped beads. However, when a waiting period of 10 - 15 min was allotted between transfer of the solution into the separating funnel and bead preparation, no bubbles were observed in the mixture. Further, the mixture was extruded dropwise into sterile $CaCl_2$ solution kept in a beaker below the separating funnel.

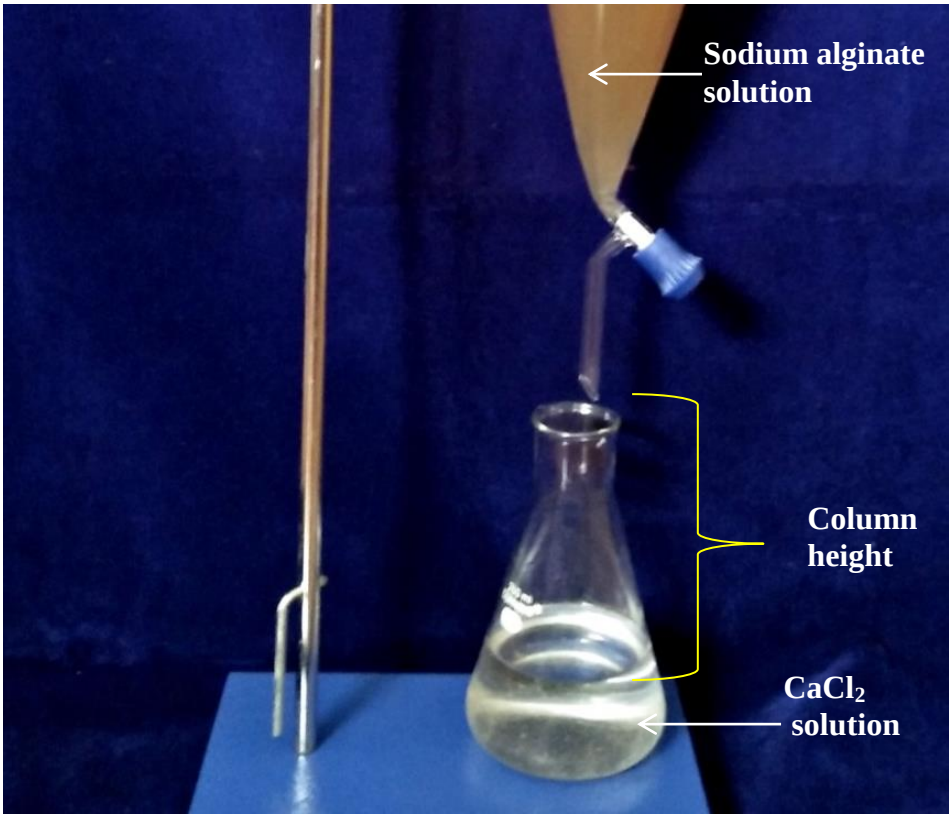


Plate 4.6 Bead production apparatus

Bioagent amended sodium alginate solution taken in a separating funnel

Dropwise addition of the solution into sterilized $CaCl_2$ solution

Washing in sterile distilled water

Drain the excess water

Spread over sterile tissue paper

Open drying for 48 h

Store in dry sterile containers

Plate 4.7 Steps involved in the preparation of mirobeads

The clockwise or anti-clockwise rotation of stopper valve of the separating funnel was found to regulate the rate of dripping of sodium alginate solution. When water soluble sodium alginate came in contact with $CaCl_2$ solution, immediately water insoluble calcium alginate beads were formed and at the time of formation, the beads were transparent and were floating in $CaCl_2$ bath. However, as the time progressed, an inwardly moving opaque zone became visible and finally, beads began to sink in the $CaCl_2$ bath and completely became opaque. For the preparation of solutions as well as for washing of the beads, sterile distilled water was used and freshly prepared sodium alginate and $CaCl_2$ solutions were used for the bead preparation.

4.2.3.2 Drying and storage of microbeads

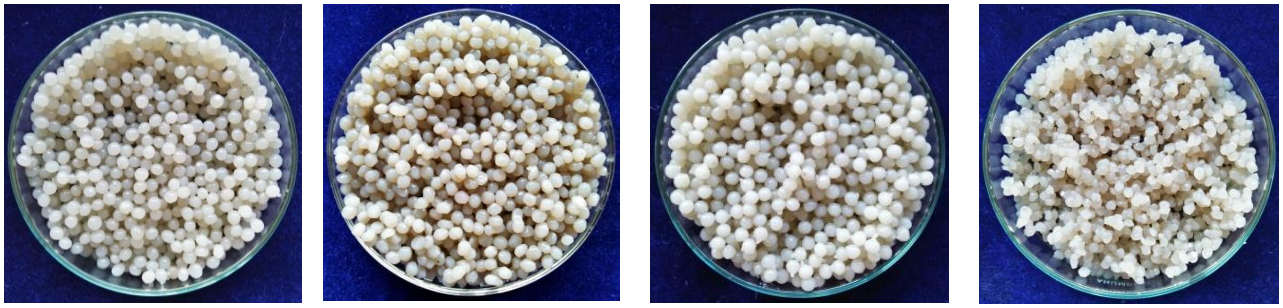
The prepared beads were dried open at room temperature for a period of 48 h and it was observed that in all treatments, the beads turned ivory colour, coherent and were found rough and less glossy.

4.2.3.3 Bead weight

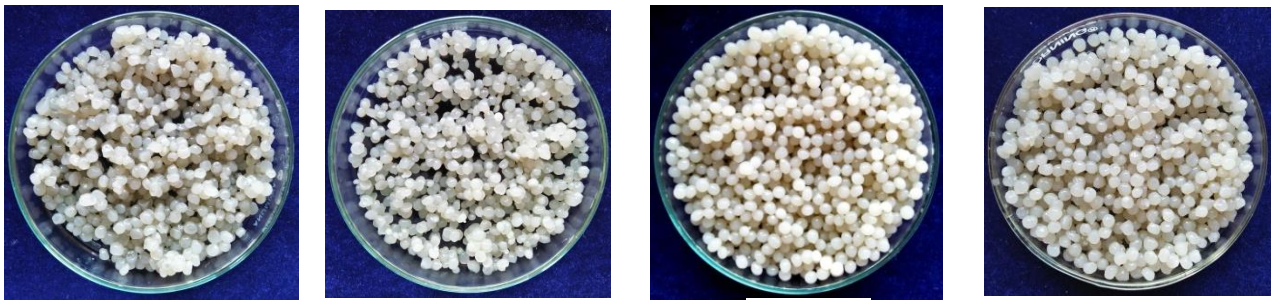
The individual weight of 10 randomly selected beads of all the 31 treatments including control was estimated and the results are depicted in Table 4.5. The average bead weight ranged from 22.84 ± 4.1 to 96.20 ± 1.8 mg, where the maximum bead weight was noticed in control (T_{31}) followed by T_{15} (mannitol, PEG, liquid paraffin, tween 80, sodium alginate 1.5%, $CaCl_2$ 3.5%) (47.73 ± 1.6), T_{30} (trehalose, PEG, CMC, tween 80, sodium alginate 0.75%, $CaCl_2$ 3.5%) (40.76 ± 6.2) and the least in T_4 (mannitol, PVP, CMC, tween 80, sodium alginate 0.75% $CaCl_2$ 2.5%). From the data, it was inferred that the weight of individual beads were directly proportional to the concentration of sodium alginate.

4.2.3.4 Bead yield

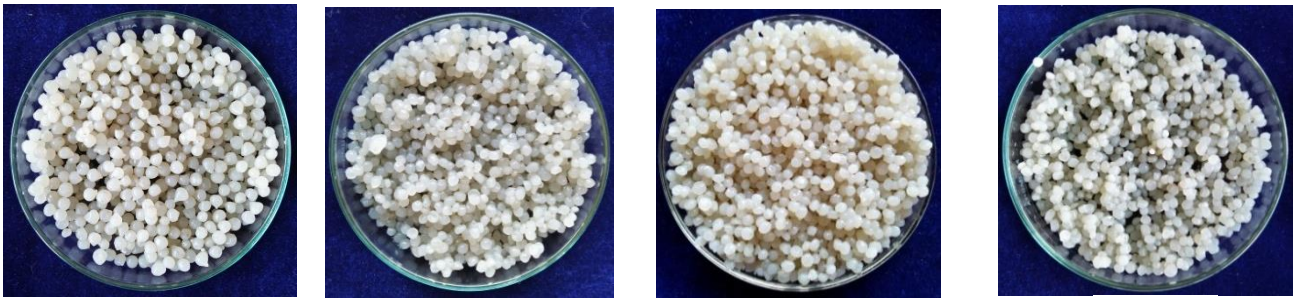
The weight of final product formed to the weight of ingredients used in terms of percentage is referred as per cent bead yield and the yield was estimated for all the 31 treatments including control. The data furnished in Table 4.5 revealed that the bead yield ranged from 24.51 to 69.09 per cent with the maximum yield in T_{19} (trehalose, PVP,



T1 T2 T3 T4



T5 T6 T7 T8



T9 T10 T11 T12

Plate 4.8 (i) Microbeads prepared from different combinations of sodium alginate, $CaCl_2$ and additives.

T13

T14

T15

T16

T17

T18

T19

T20

T21

T22

T23

T24

Plate 4.8 (ii) Microbeads prepared from different combinations of sodium alginate, $CaCl_2$ and additives.

T25

T26

T27

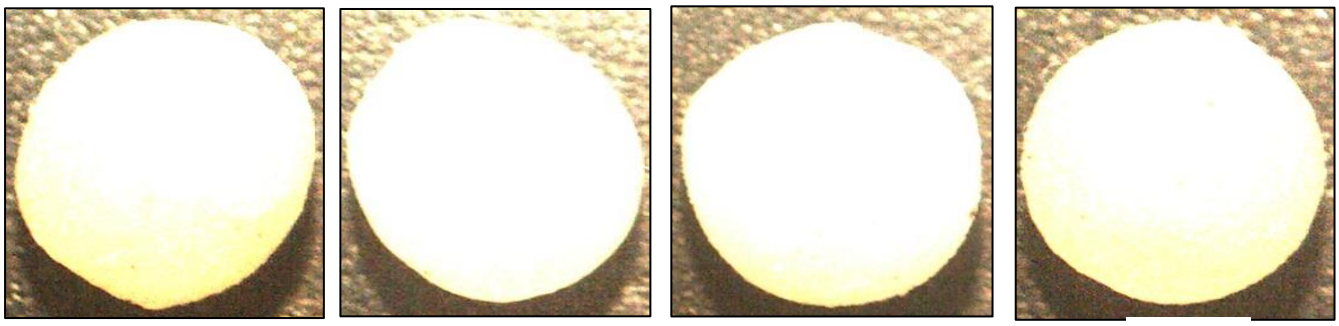
T28

T29

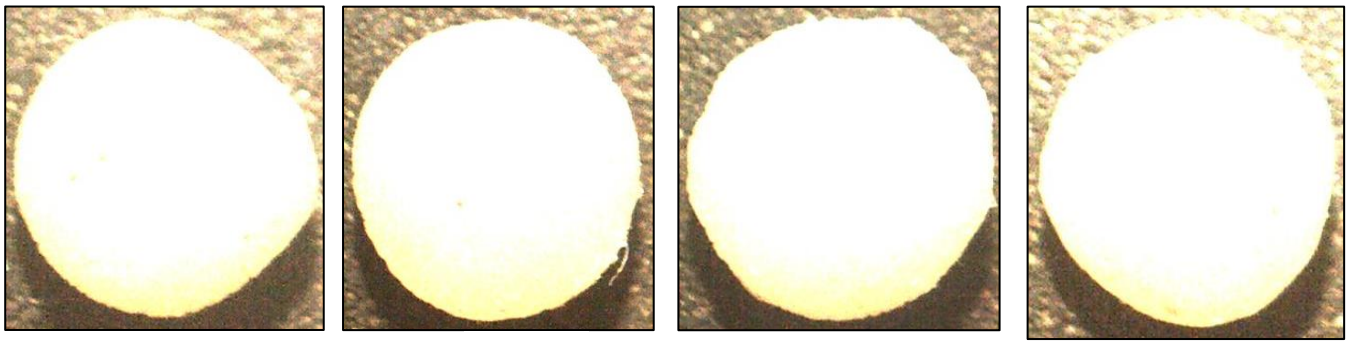
T30

T31

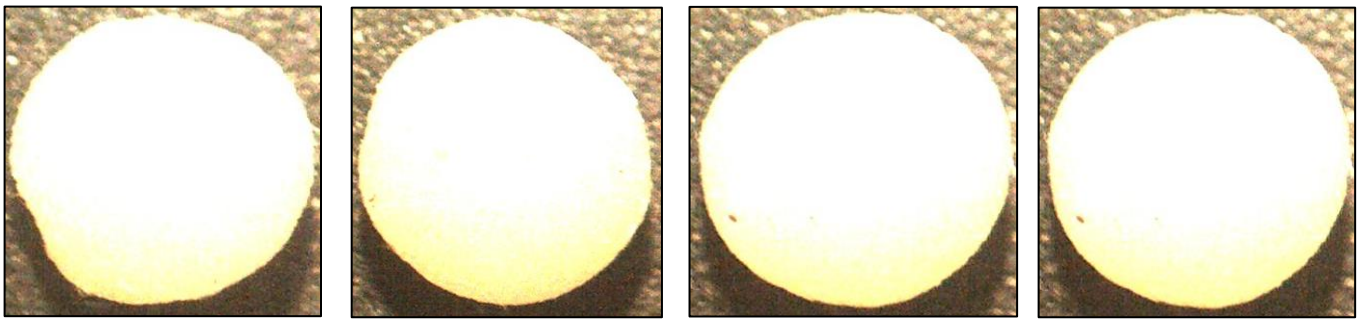
Plate 4.8 (iii) Microbeads prepared from different combinations of sodium alginate, $CaCl_2$ and additives.



T1 T2 T3 T4



T5 T6 T7 T8



T9 T10 T11 T12

Plate 4.9 (i) Image of microbeads under stereomicroscope (100X)

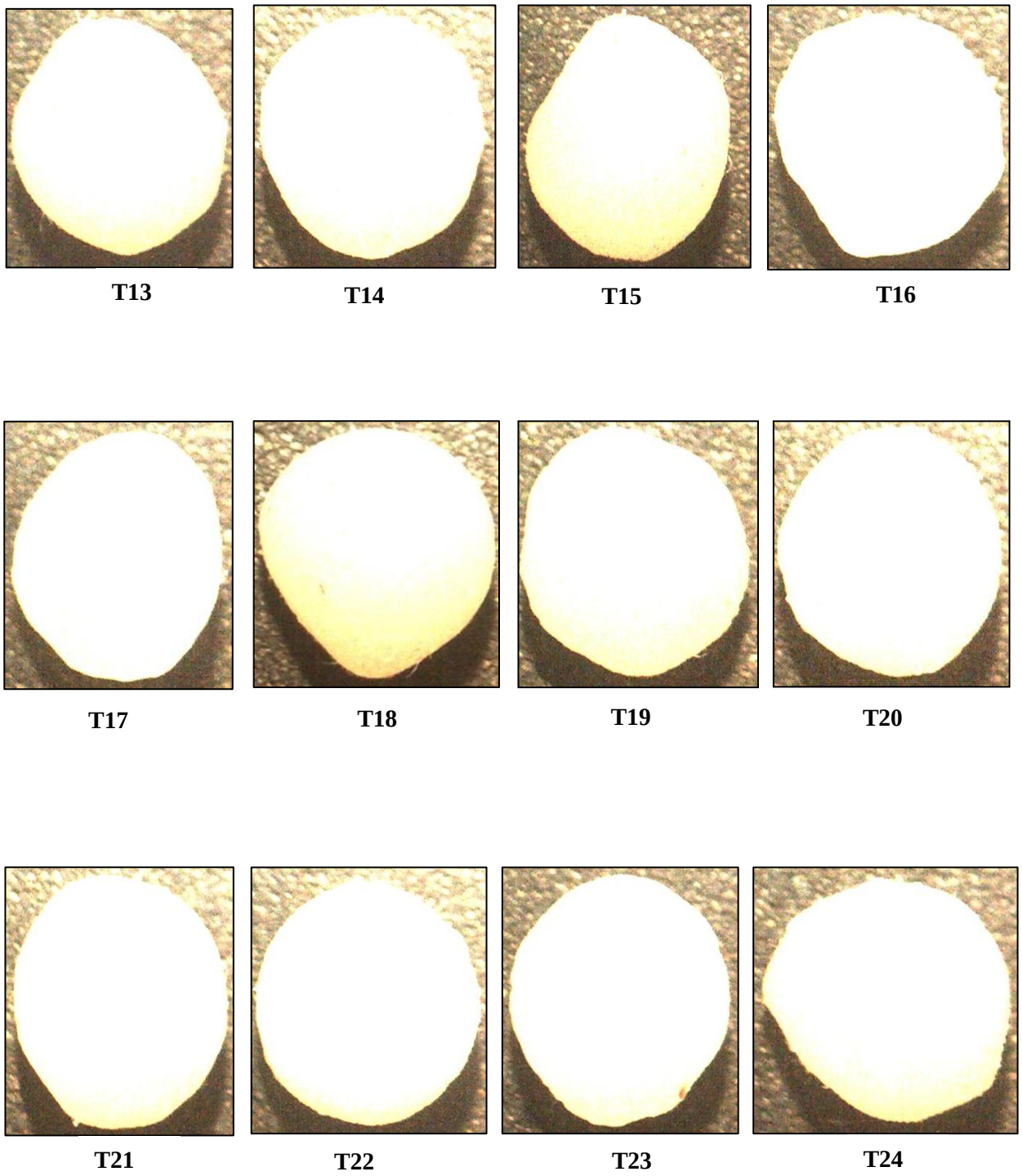
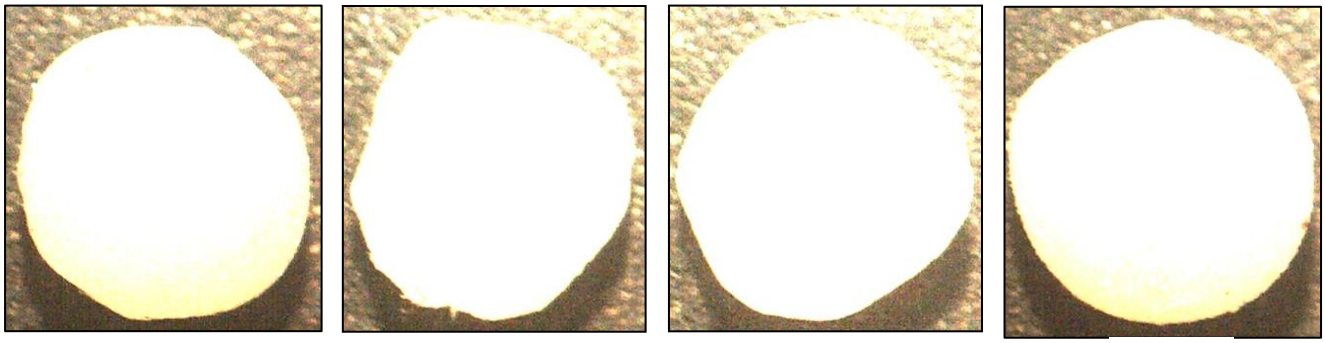
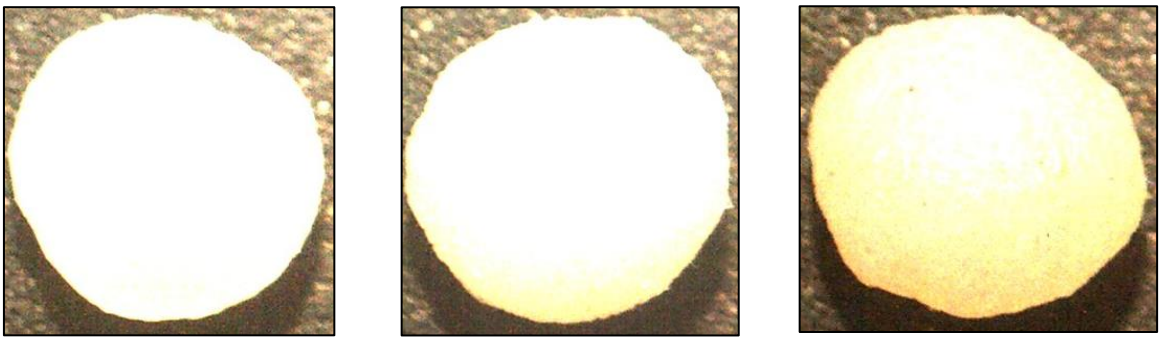


Plate 4.9 (iii) Image of microbeads under stereomicroscope (100X)



T25 T26 T27 T28



T29 T30 T31

Plate 4.9 (iii) Image of microbeads under stereomicroscope (100X)

CMC, tween 80, sodium alginate 1.5%, $CaCl_2$ 2.5%), closely followed by T_9 (mannitol, PVP, liquid paraffin, tween 80, sodium alginate 1.5%, $CaCl_2$ 3.5%) (65.50), T_{10} (mannitol, PVP, liquid paraffin, tween 80 sodium alginate 0.75%, $CaCl_2$ 2.5%) (62.76) and the least in T_4 (mannitol, PVP, CMC, tween 80, sodium alginate 0.75% $CaCl_2$ 2.5%). Further, the control with 2.5 per cent sodium alginate without the additives recorded 43.95 per cent yield.

4.2.3.5 Number of beads per ml sodium alginate solution

Maximum number of beads formed per ml of sodium alginate solution was 26.91 (T_9), which was 200 per cent more than that of control, followed by 22.42 (T_{10}) and 20.86 (T_{19}) (trehalose, PVP, CMC, tween 80, sodium alginate 1.5%, $CaCl_2$ 2.5%). In control (T_{31}), only eight beads were formed from one ml of alginate solution, which is the minimum. From the Table 4.5, in general it was observed that incorporation of any additive improved the bead yield compared to control. Also, it was noticed that both T_9 and T_{10} contained the additive combination of mannitol, PVP, liquid paraffin and tween 80.

4.2.3.6 Bead size

The size of alginate beads was estimated for all the 31 treatments including control by measuring the bead diameter with the aid of a stereo microscope. From the Table 4.5, it was noticed that the bead diameter ranged from 1.31 ± 0.01 to 2.42 ± 0.10 mm with the maximum in control (T_{31}) followed by T_9 (mannitol, PVP, liquid paraffin, tween 80 sodium alginate 1.5%, $CaCl_2$ 3.5%) (1.92 ± 0.098) and minimum in T_{22} (trehalose, PVP, CMC, tween 80, sodium alginate 0.75%, $CaCl_2$ 2.5%). Bead diameter was found to increase as the concentration of sodium alginate and $CaCl_2$ increased.

4.2.4 Evaluation of shelf life of *T. viride* encapsulated alginate beads

Evaluation of shelf life of *T. viride* encapsulated alginate beads for all the 31 treatments including control was carried out by dissolving microbeads in potassium phosphate buffer (2 M, pH 7) followed by serial dilution and plating on potato dextrose agar (PDA) medium. The study was conducted at monthly intervals upto six months

Table 4.5 Characterization of alginate microbeads

Sl. No.	Treatment	*Bead weight (mg)	**Bead diameter (mm)	Per cent bead yield	No. of beads ml^{-1} alginate solution
1.	T_1-Mannitol, PVP, CMC, Tween 80, SA 1.5%, $CaCl_2$ 2.5%	25.01 ± 1.7	1.70 ± 0.09	43.23	16.52
2.	T_2-Mannitol, PVP, CMC, Tween 80, SA 1.5%, $CaCl_2$ 3.0%	26.34 ± 1.1	1.73 ± 0.02	24.95	14.86
3.	T_3-Mannitol, PVP, CMC, Tween 80, SA 1.5%, $CaCl_2$ 3.5%	28.60 ± 1.6	1.86 ± 0.11	35.79	17.01
4.	T_4-Mannitol, PVP, CMC, Tween 80, SA 0.75% $CaCl_2$ 2.5%	22.84 ± 4.1	1.38 ± 0.07	24.51	10.93
5.	T_5-Mannitol, PVP, CMC, Tween 80, SA 0.75%, $CaCl_2$ 3.0%	23.70 ± 1.9	1.45 ± 0.02	39.37	10.41
6.	T_6-Mannitol, PVP, CMC, Tween 80, SA 0.75%, $CaCl_2$ 3.5%	25.07 ± 2.7	1.70 ± 0.08	30.37	13.03
7.	T_7-Mannitol, PVP, Liquid paraffin, Tween 80, SA 1.5%, $CaCl_2$ 2.5%	28.30 ± 1.2	1.79 ± 0.05	36.22	16.00
8.	T_8-Mannitol, PVP, Liquid paraffin, Tween 80 SA 1.5%, $CaCl_2$ 3.0%	32.19 ± 3.8	1.83 ± 0.04	29.80	15.53
9.	T_9-Mannitol, PVP, Liquid paraffin, Tween 80 SA 1.5%, $CaCl_2$ 3.5%	29.81 ± 5.5	1.92 ± 0.09	65.50	26.91
10.	T_{10}-Mannitol, PVP, Liquid paraffin, Tween 80 SA 0.75%, $CaCl_2$ 2.5%	25.96 ± 2.2	1.56 ± 0.03	62.76	22.42
11.	T_{11}-Mannitol, PVP, Liquid paraffin, Tween 80 SA 0.75%, $CaCl_2$ 3.0%	26.57 ± 3.3	1.62 ± 0.16	60.04	16.00
12.	T_{12}-Mannitol, PVP, Liquid paraffin, Tween 80 SA 0.75%, $CaCl_2$ 3.5%	27.70 ± 8.1	1.76 ± 0.04	50.02	15.60
13.	T_{13}-Mannitol, PEG, Liquid paraffin, Tween 80, SA 1.5%, $CaCl_2$ 2.5%	37.00 ± 2.9	1.79 ± 0.06	43.35	17.75
14.	T_{14}-Mannitol, PEG, Liquid paraffin, Tween 80, SA 1.5%, $CaCl_2$ 3.0%	38.63 ± 4.0	1.81 ± 0.08	43.76	15.90
15.	T_{15}-Mannitol, PEG, Liquid paraffin, Tween 80, SA 1.5%, $CaCl_2$ 3.5%	47.73 ± 1.6	1.82 ± 0.08	51.56	14.53

*Mean weight of 10 beads ± standard deviation

** Mean diameter of 10 beads ± standard deviation

SA - sodium alginate

Contd. Table 4.5 Characterization of alginate microbeads

Sl. No.	Treatment	*Bead weight (mg)	**Bead diameter (mm)	Per cent bead yield	No. of beads ml^{-1} alginate solution
16.	T_{16}-Mannitol, PEG, Liquid paraffin, Tween 80, SA 0.75%, $CaCl_2$ 2.5%	26.28 ± 1.2	1.63 ± 0.04	52.26	11.83
17.	T_{17}-Mannitol, PEG, Liquid paraffin, Tween 80, SA 0.75%, $CaCl_2$ 3.0%	29.76 ± 1.5	1.64 ± 0.11	54.32	15.27
18.	T_{18}-Mannitol, PEG, Liquid paraffin, Tween 80, SA 0.75%, $CaCl_2$ 3.5%	38.54 ± 3.8	1.70 ± 0.05	58.62	15.81
19.	T_{19}-Trehalose, PVP, CMC, Tween 80, SA 1.5%, $CaCl_2$ 2.5%	24.72 ± 2.8	1.54 ± 0.08	69.09	20.86
20.	T_{20}-Trehalose, PVP, CMC, Tween 80, SA 1.5%, $CaCl_2$ 3.0%	26.43 ± 1.7	1.64 ± 0.18	37.80	13.57
21.	T_{21}-Trehalose, PVP, CMC, Tween 80, SA 1.5%, $CaCl_2$ 3.5%	28.30 ± 1.5	1.76 ± 0.06	40.95	12.94
22.	T_{22}-Trehalose, PVP, CMC, Tween 80, SA 0.75%, $CaCl_2$ 2.5%	24.24 ± 8.0	1.31 ± 0.01	29.89	17.53
23.	T_{23}-Trehalose, PVP, CMC, Tween 80, SA 0.75%, $CaCl_2$ 3.0%	26.84 ± 6.1	1.36 ± 0.08	29.27	15.55
24.	T_{24}-Trehalose, PVP, CMC, Tween 80, SA 0.75%, $CaCl_2$ 3.5%	34.50 ± 3.1	1.48 ± 0.12	28.46	13.58
25.	T_{25}-Trehalose, PEG, CMC, Tween 80, SA 1.5%, $CaCl_2$ 2.5%	30.25 ± 6.3	1.67 ± 0.05	54.57	12.70
26.	T_{26}-Trehalose, PEG, CMC, Tween 80, SA 1.5%, $CaCl_2$ 3.0%	26.63 ± 1.3	1.68 ± 0.04	39.34	10.30
27.	T_{27}-Trehalose, PEG, CMC, Tween 80, SA 1.5%, $CaCl_2$ 3.5%	25.93 ± 2.2	1.88 ± 0.12	38.15	12.60
28.	T_{28}-Trehalose, PEG, CMC, Tween 80, SA 0.75%, $CaCl_2$ 2.5%	33.09 ± 1.0	1.52 ± 0.03	29.70	10.35
29.	T_{29}-Trehalose, PEG, CMC, Tween 80, SA 0.75%, $CaCl_2$ 3.0%	38.54 ± 4.3	1.63 ± 0.08	28.29	18.03
30.	T_{30}-Trehalose, PEG, CMC, Tween 80, SA 0.75%, $CaCl_2$ 3.5%	40.76 ± 6.2	1.66 ± 0.04	28.95	12.55
31.	T_{31}-(Control) SA 2.5%, $CaCl_2$ 2.5%	96.20 ± 1.8	2.42 ± 0.13	43.95	8.00

*Mean weight of 10 beads ± standard deviation

** Mean diameter of 10 beads ± standard deviation

SA - sodium alginate

where the population of *T. viride* was enumerated at 10^{-6} and 10^{-8} dilutions and the data are furnished in Table 4.6a and Table 4.6b. Furthermore, the population of *T. viride* entrapped in a single bead was also estimated for all the 31 treatments including control and the data are presented in Table 4.6c.

In general, it was observed that the colony forming units were more at 10^{-6} dilution compared to that of 10^{-8} in all the treatments. Further, it was observed that the population at 1 month after storage (1 MAS) was higher compared to subsequent months at both the dilutions. At 1 MAS in 10^{-6} dilution (Table 4.6a), maximum population of *T. viride* was observed in T_{21} (34.6 x 10^6) closely followed by T_{20} (trehalose, PVP, CMC, tween 80, sodium alginate 1.5%, $CaCl_2$ 3.0%) (33.6 x 10^6) and T_{19} (33.3 x 10^6) whereas, the minimum population was recorded in control (T_{31}) (13.0 x 10^6cfu g^{-1} bead). At 10^{-8} dilution (Table 4.6b), maximum colony count was observed in T_{21} (6.0 x 10^8) followed by T_{23} (5.0 x 10^8) and the minimum colony count of 1.0 x 10^8 was noticed in T_7, T_8, T_{15} and T_{17}. At 2 MAS, maximum population of 34.0 x 10^6 was recorded in T_{21} followed by T_{19} (32.6 x 10^6) while minimum colony count of 10.3 x 10^6 was observed in T_{15} and T_{17} at 10^{-6} dilution. Further at 10^{-8} dilution, a maximum population of 3.6 x 10^8 was observed in T_{21} followed by 3.0 x 10^8 in T_{23} and T_{25} and the minimum population of 0.3 x 10^8 was recorded in T_7, T_8, T_9, T_{15} and T_{17}.

At 3 MAS, at 10^{-6} dilution, 12.0 x 10^6 cfu g^{-1} bead was recorded in T_{21} closely followed by T_{22} (11.0 x 10^6) and the colony count was nil in T_{17}. Moreover, at 10^{-8} dilution, a maximum of 2.3 x 10^8 cfu g^{-1} bead was recorded in T_{21} closely followed by T_{22} (2.0 x 10^8) whereas, the colony count was nil in T_1, T_4, T_5, T_8, T_{14}, T_{15}, T_{16}, T_{17}, T_{25}, T_{26} and control. After three months of shelf life evaluation, treatments T_7 to T_{18} were discarded as the count was found nil and the remaining treatments were thus carried forward for further shelf life studies. At 4 MAS, maximum population was documented in T_{28} (14.6 x 10^6) followed by T_{22} (13.6 x 10^6) and the minimum population was recorded in T_4 (2.6 x 10^6) at 10^{-6} dilution, whereas at 10^{-8} dilution, maximum population was noticed in T_{21} (4.6 x 10^8) followed by T_{29} (3.6 x 10^8) and the colony count was nil in T_1 and T_2. At 5 MAS, at 10^{-6} dilution, maximum population was recorded in T_{22} (15.0 x

Table 4.6a Population of *T. viride* at monthly intervals at 10^{-6} dilution

Treatments	*Population of *T. viride* (x 10^6cfu g^{-1} bead)					
	1 MAS	2 MAS	3 MAS	4 MAS	5 MAS	6 MAS
T_1	27.3 (5.27)^{bcd}	23.3 (4.87)^{efg}	6.6 (2.67)^{hij}	4.3 (2.07)^{g}	6.6 (2.58)^{fgh}	5.3 (2.29)^{h}
T_2	24.6 (5.01)^{bcdef}	21.3 (4.67)^{ghi}	8.6 (3.02)^{def}	3.0 (1.71)^{g}	5.3 (2.29)^{hi}	5.0 (2.22)^{h}
T_3	23.0 (4.88)^{def}	22.6 (4.80)^{efgh}	6.6 (2.67)^{hij}	4.0 (1.98)^{g}	8.3(2.88)^{def}	7.6 (2.75)^{fg}
T_4	22.3 (4.77)^{ef}	20.3 (4.56)^{hi}	5.6 (2.48)^{ij}	2.6 (1.62)^{g}	4.6 (2.14)^{i}	5.3 (2.29)^{h}
T_5	28.3 (5.36)^{b}	26.0 (5.14)^{cd}	6.3 (2.61)^{hij}	4.6 (2.09)^{g}	7.3 (2.70)^{efg}	6.0 (2.44)^{gh}
T_6	22.6 (4.81)^{ef}	19.0 (4.41)^{i}	5.3 (2.41)^{j}	4.3 (2.07)^{g}	5.6 (2.37)^{hi}	5.0 (2.22)^{h}
T_7	15.6 (4.01)^{gh}	11.3 (3.43)^{lm}	10.0(3.23)^{bcd}	-	-	-
T_8	16.0 (4.06)^{gh}	11.3 (3.42)^{im}	7.3 (2.79)^{fgh}	-	-	-
T_9	14.6 (3.89)^{gh}	12.0 (3.53)^{klm}	7.0 (2.73)^{ghi}	-	-	-
T_{10}	17.3 (4.22)^{g}	13.6 (3.76)^{jk}	6.0 (2.54)^{hij}	-	-	-
T_{11}	24.6 (5.01)^{bcdef}	21.3 (4.63)^{ghi}	10.0(3.23)^{bcd}	-	-	-
T_{12}	27.3 (5.27)^{gh}	24.6 (5.01)^{de}	8.3 (2.97)^{efg}	-	-	-
T_{13}	16.0 (4.06)^{gh}	11.0 (3.38)^{lm}	6.6 (2.67)^{hij}	-	-	-
T_{14}	15.6 (4.02)^{gh}	12.3 (3.57)^{jkl}	6.6 (2.67)^{hij}	-	-	-
T_{15}	13.3 (3.71)^{h}	10.3 (3.29)^{m}	2.6 (1.77)^{kl}	-	-	-

* Mean of three replications

In each column figures followed by same letter do not differ significantly according to DMRT

Square root transformed values are given in parenthesis

MAS - Months after storage

Contd. Table 4.6a Population of *T. viride* at monthly intervals at 10^{-6} dilution

Treatments	*Population of *T. viride* (x 10^6cfu g^{-1} bead)					
	1 MAS	2 MAS	3 MAS	4 MAS	5 MAS	6 MAS
T_{16}	17.6 (4.25)^{g}	14.0 (3.80)^{j}	9.3 (3.13)^{bcde}	-	-	-
T_{17}	15.3 (3.97)^{gh}	10.3 (3.29)^{m}	0.0 (0.7)^{n}	-	-	-
T_{18}	23.3 (4.87)^{def}	19.6 (3.17)^{i}	1.6 (1.46)^{m}	-	-	-
T_{19}	33.3 (5.81)^{a}	32.6 (5.75)^{a}	10.3 (3.28)^{abcd}	11.3(3.36)^{abcde}	12.6 (3.55)^{b}	12.0 (3.45)^{bcde}
T_{20}	33.6 (5.84)^{a}	13.3 (3.71)^{jk}	9.0 (3.07)^{cde}	8.3 (2.88)^{ef}	11.0 (3.31)^{bc}	11.0 (3.30)^{cde}
T_{21}	34.6 (5.92)^{a}	34.0 (5.87)^{a}	12 (3.53)^{a}	11.3(3.33)^{abcde}	13.0 (3.60)^{ab}	14.0 (3.74)^{b}
T_{22}	27.3 (5.25)^{bcd}	28.3 (5.36)^{bc}	11.0(3.38)^{ab}	13.6 (3.69)^{ab}	15.0 (3.86)^{a}	18.0 (4.23)^{a}
T_{23}	26.3 (5.17)^{bcde}	29.3 (5.46)^{b}	10.3(3.29)^{abc}	10.0(3.15)^{bcdef}	11.0 (3.31)^{bc}	12.0 (3.45)^{bcde}
T_{24}	22.0 (4.74)^{f}	23.0 (4.84)^{efg}	6.6 (2.67)^{hij}	9.6 (3.10)^{cdef}	10.0 (3.15)^{cd}	10.0 (3.16)^{def}
T_{25}	27.0 (5.27)^{bc}	22.0 (4.74)^{fgh}	1.3 (1.34)^{m}	12.0 (3.44)^{abcd}	12.0 (3.46)^{bc}	11.3 (3.36)^{bcde}
T_{26}	25.3(5.07)^{bcdef}	23.6 (4.91)^{def}	2.6 (1.77)^{kl}	12.6 (3.55)^{abc}	12.0 (3.45)^{bc}	13.0 (3.59)^{bc}
T_{27}	28.3(5.36)^{b}	26.0 (5.14)^{cd}	3.6 (2.03)^{k}	11.3(3.33)^{abcde}	12.6 (3.55)^{b}	13.0 (3.60)^{bc}
T_{28}	24.3(4.97)^{cdef}	22.0 (4.74)^{fgh}	10.3 (3.29)^{abc}	14.6 (3.81)^{a}	13.0 (3.59)^{ab}	12.6 (3.55)^{bcd}
T_{29}	26.6(5.21)^{bcd}	23.00 (4.84)^{efg}	7.3 (2.79)^{fgh}	8.6 (2.93)^{def}	9.0 (2.99)^{de}	11.3 (3.36)^{bcde}
T_{30}	25.6(5.10)^{bcdef}	22.33(4.77)^{efgh}	8.6 (3.02)^{def}	7.6 (2.75)^{f}	8.3 (2.88)^{def}	9.6 (3.09)^{ef}
T_{31}	13.0 (3.66)^{h}	13.66 (3.76)^{jk}	2.0(1.55)^{lm}	8.0 (2.82)^{ef}	6.0 (2.44)^{ghi}	8.3(2.87)^{fg}
CD (0.01)	0.513	0.892	0.334	0.737	0.406	0.576

* Mean of three replications

In each column figures followed by same letter do not differ significantly according to DMRT

Square root transformed values are given in parenthesis

MAS - Months after storage

Table 4.6b Population of *T. viride* at monthly intervals at 10^{-8} dilution

Treatment	*Population of *T. viride* (x10^{8} cfu g^{-1} bead)					
	1 MAS	2 MAS	3 MAS	4 MAS	5 MAS	6 MAS
T_1	2.0 (1.41)^{fghi}	1.3(1.34)^{cd}	0.0 (0.7)^{e}	0.0 (0.70)^{e}	0.0 (0.70)^{g}	0.0 (0.70)^{f}
T_2	2.0 (1.38)^{fghi}	1.0 (1.22)^{cde}	0.6 (1.05)^{cde}	0.0 (0.70)^{e}	0.0 (0.70)^{g}	0.0 (0.70)^{f}
T_3	3.0 (1.73)^{def}	1.3 (1.34)^{cd}	0.3 (0.87)^{de}	0.3 (0.87)^{de}	0.3 (0.87)^{fg}	0.3 (0.87)^{ef}
T_4	2.3 (1.52)^{cde}	1.3 (1.34)^{cd}	0.0 (0.7)^{e}	1.0 (1.22)^{cde}	0.3 (0.87)^{fg}	0.6 (1.05)^{def}
T_5	2.0 (1.38)^{ghi}	1.6 (1.44)^{c}	0.0 (0.7)^{e}	2.0 (1.55)^{abcd}	1.6 (1.46)^{cde}	2.0 (1.22)^{de}
T_6	1.3 (1.13)^{ij}	0.6 (1.05)^{de}	0.3 (0.87)^{de}	1.3 (1.34)^{bcde}	0.6 (1.05)^{efg}	1.3 (1.34)^{d}
T_7	1.0 (1.0)^{j}	0.3 (0.87)^{e}	0.6 (1.05)^{cde}	-	-	-
T_8	1.0 (1.0)^{j}	0.3 (0.87)^{e}	0.0 (0.7)^{e}	-	-	-
T_9	1.3 (1.13)^{ij}	0.3 (0.87)^{e}	0.6 (1.05)^{cde}	-	-	-
T_{10}	1.6 (1.27)^{hij}	0.6 (1.05)^{de}	1.0 (1.22)^{bcd}	-	-	-
T_{11}	2.0 (1.41)^{fghi}	1.0 (1.22)^{cde}	0.6 (1.05)^{cde}	-	-	-
T_{12}	2.3 (1.52)^{efgh}	1.3 (1.34)^{cd}	0.6 (1.05)^{cde}	-	-	-
T_{13}	2.3 (1.52)^{efgh}	0.6 (1.05)^{de}	0.3 (0.87)^{de}	-	-	-
T_{14}	2.6 (1.52)^{efg}	1.3 (1.34)^{cd}	0.0 (0.7)^{e}	-	-	-
T_{15}	1.0 (1.0)^{j}	0.3 (0.87)^{e}	0.0 (0.7)^{e}	-	-	-

* Mean of three replications

In each column figures followed by same letter do not differ significantly according to DMRT

Square root transformed values are given in parenthesis

MAS - Months after storage

Contd. Table 4.6b Population of *T. viride* at monthly intervals at 10^{-8} dilution

Treatment	*Population of *T. viride* (x10^{8} cfu g^{-1} bead)					
	1 MAS	**2 MAS**	**3 MAS**	**4 MAS**	**5 MAS**	**6 MAS**
T_{16}	2.0 (1.41)^{fghi}	1.0 (1.22)^{cde}	0.0 (0.7)^{e}	-	-	-
T_{17}	1.0 (1.0)^{j}	0.3 (0.87)^{e}	0.0 (0.7)^{e}	-	-	-
T_{18}	3.3 (1.82)^{cde}	2.3 (0.87)^{e}	1.0 (1.17)^{bcd}	-	-	-
T_{19}	3.0 (1.73)^{def}	1.3 (1.34)^{cd}	1.0 (1.17)^{bcd}	1.0 (1.22)^{cde}	1.0 (1.22)^{ef}	1.0 (1.17)^{de}
T_{20}	2.6 (1.62)^{efg}	0.6 (1.05)^{de}	0.3 (0.87)^{de}	0.3 (0.87)^{de}	0.6 (1.05)^{efg}	1.3 (1.34)^{d}
T_{21}	6.0 (2.44)^{a}	3.6 (2.03)^{a}	2.3 (1.58)^{a}	4.6 (2.17)^{a}	4.3 (2.19)^{a}	5.0 (2.34)^{a}
T_{22}	2.0 (1.41)^{fghi}	1.0 (1.22)^{cde}	2.0 (1.55)^{ab}	2.6 (1.73)^{abc}	2.6 (1.77)^{abc}	4.0 (2.11)^{ab}
T_{23}	5.0 (2.22)^{ab}	3.0 (1.87)^{ab}	0.6 (1.05)^{cde}	2.0 (1.55)^{abcd}	1.6 (1.46)^{cde}	3.0 (1.85)^{bc}
T_{24}	2.6 (1.60)^{efgh}	1.0 (1.22)^{cde}	0.6 (1.05)^{cde}	1.3 (1.26)^{cde}	1.3 (1.34)^{de}	1.0 (1.22)^{de}
T_{25}	4.6 (2.15)^{abc}	3.0 (1.85)^{ab}	0.0 (0.7)^{e}	1.3 (1.26)^{cde}	1.0 (1.17)^{ef}	1.0 (1.17)^{de}
T_{26}	2.0 (1.38)^{ghi}	2.0 (1.58)^{bc}	0.0 (0.7)^{e}	1.0 (1.17)^{cde}	1.0 (1.22)^{ef}	1.3 (1.34)^{d}
T_{27}	3.0 (1.71)^{defg}	1.3 (1.34)^{cd}	0.6 (0.7)^{cde}	2.3 (1.55)^{abcd}	2.6 (1.73)^{bcd}	3.0 (1.87)^{bc}
T_{28}	4.3 (2.07)^{bc}	2.0 (1.55)^{bc}	1.3 (1.34)^{abc}	1.3 (1.34)^{bcde}	1.3 (1.28)^{ef}	1.6 (1.46)^{cd}
T_{29}	2.0 (1.38)^{ghi}	1.3 (1.34)^{cd}	1.6 (1.46)^{ab}	3.6 (1.96)^{ab}	3.3 (1.95)^{ab}	3.0 (1.85)^{bc}
T_{30}	4.0 (1.98)^{bcd}	2.0 (1.58)^{bc}	1.6 (1.44)^{abc}	1.6 (1.46)^{bcd}	1.3 (1.34)^{de}	1.3 (1.28)^{de}
T_{31}	2.0 (1.41)^{fghi}	1.6 (1.46)^{c}	0.0 (0.7)^{e}	0.3 (0.87)^{de}	0.0 (0.70)^{g}	0.0 (0.70)^{f}
CD (0.01)	0.446	0.497	0.528	0.914	0.568	0.554

* Mean of three replications

In each column figures followed by same letter do not differ significantly according to DMRT

Square root transformed values are given in parenthesis

MAS - Months after storage

Table 4.6c Population of *T. viride* entrapped in a single bead at monthly intervals at 10^{-5} dilution

Treatments	*Population of *T. viride* (x10^5 cfu per bead)					
	1 MAS	2 MAS	3 MAS	4 MAS	5 MAS	6 MAS
T_1	7.01 (0.843)^{fg}	5.98 (0.772)^{h}	1.69 (0.430)^{hij}	1.10 (0.044)^{i}	1.69 (0.230)^{gh}	1.36 (0.128)^{i}
T_2	4.63 (0.668)^{ijk}	4.01 (0.607)^{kl}	1.62 (0.411)^{hij}	0.56 (0.265)^{k}	0.99 (0.002)^{i}	0.94 (0.039)^{j}
T_3	5.29 (0.720)^{hi}	5.19 (0.712)^{i}	1.51 (0.402)^{ij}	0.92 (0.045)^{ij}	1.90 (0.288)^{g}	1.74 (0.242)^{i}
T_4	5.57 (0.747)^{h}	5.07 (0.703)^{i}	1.40 (0.386)j	0.65 (0.186)^{jk}	1.15 (0.056)^{i}	1.32 (0.113)^{i}
T_5	11.79 (1.078)^{ab}	10.83 (1.034)^{bc}	2.62 (0.561)^{cde}	1.91 (0.237)^{h}	3.04 (0.484)^{ef}	2.50 (0.392)^{h}
T_6	5.95 (0.770)^{gh}	5.00 (0.693)^{ij}	1.39 (0.376)^{jk}	1.13 (0.052)^{i}	1.47 (0.175)^{h}	1.31 (0.113)^{i}
T_7	4.41 (0.649)^{jkl}	3.20 (0.504)^{m}	2.83 (0.580)^{bcde}	-	-	-
T_8	4.43 (0.645)^{jkl}	3.13 (0.498)^{m}	2.02 (0.480)^{fgh}	-	-	-
T_9	3.87 (0.586)^{l}	3.18 (0.504)^{m}	1.86 (0.453)^{hi}	-	-	-
T_{10}	5.26 (0.724)^{hi}	4.13 (0.611)^{kl}	1.82 (0.446)^{hi}	-	-	-
T_{11}	7.92 (0.893)^{def}	6.85 (0.823)^{gh}	3.21 (0.627)^{b}	-	-	-
T_{12}	8.13 (0.907) ^{def}	7.33 (0.867)^{fg}	2.47 (0.542)^{def}	-	-	-
T_{13}	4.20 (0.626)^{kl}	2.89 (0.451)^{m}	1.73 (0.438)^{hij}	-	-	-
T_{14}	4.64 (0.661)^{ijk}	3.66 (0.565)^{l}	1.96 (0.473)^{gh}	-	-	-
T_{15}	5.12 (0.709)^{hij}	3.97 (0.594)^{kl}	1.00 (0.302)^{l}	-	-	-

* Mean of three replications

In each column figures followed by same letter do not differ significantly according to DMRT

Log transformed values are given in parenthesis

MAS - Months after storage

Contd. Table 4.6c Population of *T. viride* entrapped in a single bead at monthly intervals at 10^{-5} dilution

Treatments	*Population of *T. viride* (x10^5 cfu per bead)					
	1 MAS	2 MAS	3 MAS	4 MAS	5 MAS	6 MAS
T_{16}	8.40 (0.926)^{de}	6.68 (0.827)^{gh}	4.43 (0.730)^{a}	-	-	-
T_{17}	5.91 (0.771)^{gh}	3.97 (0.605)^{kl}	0.00 (0.00)^{n}	-	-	-
T_{18}	8.62 (0.939)^{de}	7.25 (0.860)^{fg}	0.59 (0.209)^{m}	-	-	-
T_{19}	8.93 (0.953)^{cd}	8.75 (0.946)^{de}	2.76 (0.573)^{bcde}	3.03 (0.484)^{cdefg}	3.38 (0.533)^{de}	3.22 (0.503)^{fgh}
T_{20}	11.11 (1.044)^{ab}	4.40 (0.641)^{jk}	2.97 (0.597)^{bcd}	2.74 (0.433)^{efg}	3.63 (0.550)^{cde}	3.63 (0.553)^{ef}
T_{21}	11.93 (1.073)^{ab}	11.73 (1.060)^{ab}	1.24 (0.710)^{a}	3.89 (0.574)^{bcdef}	4.48 (0.657)^{bc}	4.83 (0.689)^{bcde}
T_{22}	7.72 (0.874)^{def}	8.01 (0.905)^{ef}	3.11 (0.611)^{bc}	3.84 (0.586)^{bcde}	4.24 (0.627)^{bc}	5.09 (0.708)^{bc}
T_{23}	7.40 (0.862)^{ef}	8.24 (0.914)^{ef}	2.89 (0.591)^{bcd}	2.81 (0.442)^{defg}	3.09 (0.487)^{ef}	3.37 (0.529)^{fg}
T_{24}	5.80 (0.764)^{h}	6.06 (0.782)^{h}	1.74 (0.434)^{hij}	2.53 (0.405)^{fgh}	2.63 (0.410)^{f}	2.63 (0.419)^{gh}
T_{25}	8.93 (0.951)^{cd}	7.27 (0.864)^{fg}	0.43 (0.159)^{m}	3.70 (0.588)^{bcde}	3.97 (0.593)^{bcd}	3.73 (0.576)^{ef}
T_{26}	10.31 (1.013)^{bc}	9.62 (0.986)^{cd}	1.05 (0.317)^{kl}	5.13 (0.712)^{ab}	4.89 (0.685)^{ab}	5.29 (0.722)^{b}
T_{27}	10.90 (1.035)^{ab}	10.02 (1.005)^{cd}	4.62 (0.387)^{j}	4.35 (0.621)^{bcd}	4.85 (0.682)^{ab}	5.01 (0.696)^{bcd}
T_{28}	7.35 (0.869)^{ef}	6.65 (0.825)^{gh}	3.11 (0.616)^{bc}	4.41 (0.646)^{bc}	3.93 (0.595)^{cd}	3.81 (0.586)^{cdef}
T_{29}	8.82 (0.942)^{cd}	7.63 (0.882)^{fg}	2.42 (0.532)^{efg}	2.85 (0.454)^{defg}	2.98 (0.474)^{ef}	3.75 (0.571)^{def}
T_{30}	7.81 (0.890)^{def}	6.81 (0.835)^{gh}	2.62 (0.561)^{cde}	2.31 (0.365)^{gh}	2.53 (0.401)^{f}	2.92 (0.468)^{fgh}
T_{31}	12.51 (1.095)^{a}	13.14 (1.116)^{a}	1.92 (0.440)^{hi}	7.69 (0.888)^{a}	5.77 (0.751)^{a}	7.98 (0.899)^{a}
CD (0.01)	0.095	0.077	0.098	0.236	0.125	0.179

* Mean of three replications

In each column figures followed by same letter do not differ significantly according to DMRT

Log transformed values are given in parenthesis

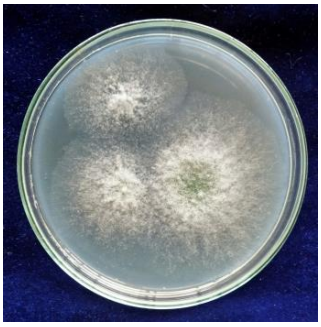
MAS - Months after storage

30 DAS

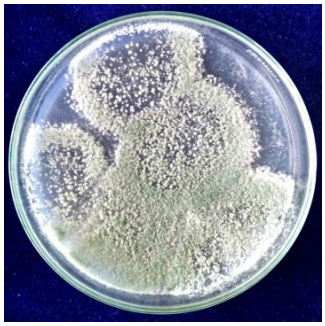
60 DAS

90 DAS

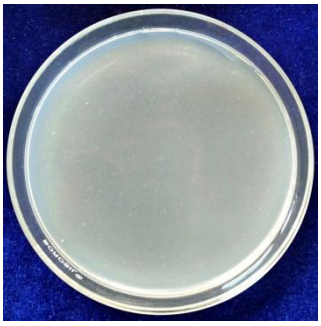
120 DAS



150 DAS



180 DAS



Control

Plate 4.10 Population of *T.viride* (x10^8 cfu g^{-1}) from encapsulated beads (on PDA medium)

10^6) closely followed by T_{21} and T_{28} (13.0 x 10^6) and the minimum colony count was recorded in T_4 (4.6 x 10^6). Similarly at 10^{-8} dilution, highest population was enumerated in T_{21} (4.3 x 10^8) followed by T_{29} (3.3 x 10^8) and the population was nil in T_1, T_2 and control. At 6 MAS, maximum population of *T. viride* was enumerated in T_{22} (18.0 x 10^6) followed by T_{21} (14.0 x 10^6) and the minimum of 5.0 x 10^6 cfu g^{-1} bead was noticed in T_2 and T_6 at 10^{-6} dilution. However, at 10^{-8} dilution, T_{21} recorded a maximum of 5.0 x 10^8 cfu g^{-1} bead closely followed by T_{22} (4.0 x 10^8) and the colony count was nil in T_1, T_2 and control.

In addition to that, Table 4.6c evidenced that, the population of *T. viride* ranged from 3.87 x 10^5 to 12.51 x 10^5 cfu $bead^{-1}$ at 1 MAS, where maximum population was recorded in control (T_{31}), while minimum was reported in treatment T_9. At 2 MAS, a maximum of 13.14 x 10^5 cfu $bead^{-1}$ was noticed in control, while the minimum population was observed in T_{13} (2.89 x 10^5 cfu $bead^{-1}$). At 3 MAS, treatment T_{16} recorded the highest population of 4.43 x 10^5 cfu $bead^{-1}$, whereas the population was nil in T_{17}. In the subsequent months (4, 5 and 6 MAS) also, control recorded the maximum population (7.69 x 10^5, 5.77 x 10^5 and 7.98 x 10^5 cfu $bead^{-1}$). After six months of shelf life study, it was concluded that the beads of all the six treatments (T_{19} - T_{24} in Table 3.4 of 3.2.3) with the additive combination of trehalose (15 mM), PVP (1%), CMC (0.5%), and tween 80 (0.5%) were found superior compared to the treatments with other additive combinations and hence, these six treatments were carried forward for further experiments.

4.2.5 Assessment of degree of contamination in microbeads

The degree of contamination of microbeads from all the 31 treatments including control was estimated using a 0 - 4 scoring index (Szczech and Maciorowski, 2016) and the data are presented in Table 4.7. Results revealed that, one month after preparation (1 MAP), all the treatments were free of contamination and were assigned with score 0 which indicates the product without discolouration and having the characteristic smell of fresh beads. However, at 3 MAP, T_{13}, T_{14}, T_{15}, T_{16}, T_{17}, T_{18} (with mannitol, PEG, liquid paraffin and tween 80 as additives) and control beads (without additives) showed some sort of contamination. These treatments were denoted with score 1 indicating product

Table 4.7 Assessment of degree of contamination in microbeads

Sl No.	Additives used	Treatments	Score		
			1MAP	3 MAP	6 MAP
1.	Mannitol, PVP, CMC, Tween 80	$T_1, T_2, T_3, T_4, T_5, T_6$	0	0	0
2.	Mannitol, PVP, Liquid paraffin, Tween 80	$T_7, T_8, T_9, T_{10}, T_{11}, T_{12}$	0	0	1
3.	Mannitol, PEG, Liquid paraffin, Tween 80	$T_{13}, T_{14}, T_{15}, T_{16}, T_{17}, T_{18}$	0	1	2
4.	Trehalose, PVP, CMC, Tween 80	$T_{19}, T_{20}, T_{21}, T_{22}, T_{23}, T_{24}$	0	0	0
5.	Trehalose, PEG, CMC, Tween 80	$T_{25}, T_{26}, T_{27}, T_{28}, T_{29}, T_{30}$	0	0	0
6.	Nil (Control)	T_{31}	0	1	1

MAP- Months after preparation
0-product without discolouration and having the characteristic smell of fresh beads
1-product with slight discolouration and devoid of typical smell
2-unpleasant smell, distinct discolouration
3-strong unpleasant smell, discolouration of product, visible coatings
4-product with coatings and moulds, completely contaminated

with slight discolouration and devoid of typical smell, while the remaining treatments were assigned with 0, since they were devoid of contamination. Likewise, 6 MAP, T_7, T_8, T_9, $T_{10,}$ $T_{11,}$ T_{12} (with mannitol, PVP, liquid paraffin and tween 80 as additives) and control beads were assigned with score 1 while T_{13}, T_{14}, $T_{15,}$ T_{16}, T_{17} and T_{18} were allotted with score 2 as there was an unpleasant smell with distinct discolouration. Moreover, the remaining treatments were assigned with 0 as they remained free of contamination. The control beads devoid of any additives and initially ivory coloured turned light brown at 2 MAP while, the remaining treatments retained the ivory colour even 6 MAP.

4.2.6 Other parameters for standardization of the prepared microbeads

From 4.2.4 and 4.2.5, six treatments *viz*., T_{19} -T_{24} (Table 3.4 of 3.2.3) were selected for the further studies. These treatments were renamed as T_1, $T_{2,}$ $T_{3,}$ $T_{4,}$ T_5 and T_6 respectively. Further, a control was also maintained for comparison, which was denoted as $T_{7.}$ Some of the additional parameters like per cent potentially infective beads, moisture content in microbeads, swelling behaviour of microbeads at different pH, per cent shrinking, sphericity factor, time of gelation and population of *T. viride* in soil at different pH was estimated for the seven treatments.

4.2.6.1 Per cent potentially infective beads

A bead is considered to be potentially infective, if at least a single spore is germinated out of the bead. To evaluate this, five randomly selected beads from treatments T_1 - T_7 were embedded on potato dextrose agar plates. Data illustrated in Table 4.8 revealed that after 24 h of incubation, none of the beads germinated in any treatments. However, After 48 h, 84.22 ± 8.0 per cent bead germination was recorded in T_1, T_2, T_4, and T_5 while in T_3 and T_6, 79.03 ± 9.8 per cent germination was observed. After 72 h of incubation, cent per cent germination was obtained from all the six treatments except control. Analysis of result from the table thus indicated that beads from the treatments T_1, T_2, T_3, T_4, T_5 and T_6 are potentially infective and hence, fit for field application.

4.2.6.2 Moisture content of beads

Moisture per cent of beads for treatments T_1 - T_7 including control was estimated using an instant moisture analyser and the data are presented in Table 4.9. Immediately after preparation of beads, the moisture per cent ranged from 90.2 ± 0.86 (T_4) to 94.0 ± 1.63 (T_1) per cent. Thereafter, the moisture per cent decreased gradually upto 72 h. It was observed that 24 h after bead preparation, the highest moisture per cent was noticed with control (T_7) (84.5 ± 1.14) which was closely followed by T_3 (84.4 ± 0.43), and the minimum moisture per cent was recorded in T_5 (80.0 ± 3.74) and T_2 (80.0 ± 2.44). After 48 h of drying, a moisture per cent ranging from 59.9 ± 1.95 (T_3) to 75.0 ± 1.63 (T_5) was observed. Further, the moisture per cent was reduced drastically to 20.9 ± 0.63 (T_6) to 28.7 ± 1.28 (T_2) after 72 h of open drying, where the beads became hard, shriveled and turned dark brown.

4.2.6.3 Swelling behaviour of microbeads at different pH

Swelling behaviour of microbeads was estimated at two different pH, 7.4 and 1. For this, 10 randomly selected beads from treatments T_1 - T_7 including control were immersed in solutions with two different pH (pH 1 and 7.4) and the beads were weighed at specific time intervals as illustrated in Table 4.10a and Table 4.10b. Further, the swelling ratio and the swelling per cent were calculated and the results are depicted in Table 4.10c. For beads incubated in phosphate buffer (pH 7.4), it was observed that the weight increased gradually from 0 - 240 min of incubation. The control bead (T_7) with maximum weight (88.5 mg) at 0 min recorded 92.0 mg weight at the end of 240 min whereas, T_5 with minimum initial weight (25.4 mg) was noticed with 43.3 mg weight at the end of 240 min. Consequently, at pH 7.4 maximum swelling ratio was exhibited by T_5 (0.70) followed by T_6 (0.60) and minimum swelling ratio was recorded in control (0.03). From the observations, it is obvious that higher swelling ratio was exhibited in beads with lower concentration (0.75%) of sodium alginate (T_4, T_5 and T_6) and control beads which contained 2.5 per cent sodium alginate were highly resistant to swelling.

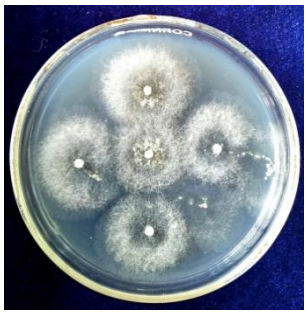
However, when the beads were immersed in HCl (pH 1), a gradual reduction in

Table 4.8 Estimation of per cent potentially infective beads

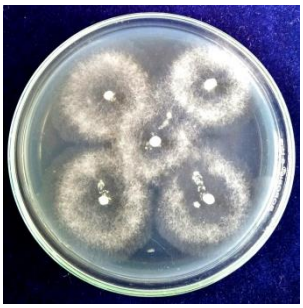
Sl. No.	Treatments	*Per cent germination of beads		
		24 h	48 h	72 h
1.	T_1 - Trehalose, PVP, CMC, Tween 80, SA - 1.5%, $CaCl_2$ - 2.5%	0.579 ± 0.0	84.22 ± 8.0[a]	100 ± 0.0
2.	T_2 - Trehalose, PVP, CMC, Tween 80, SA - 1.5%, $CaCl_2$ - 3%	0.579 ± 0.0	84.22 ± 8.0[a]	100 ± 0.0
3.	T_3 - Trehalose, PVP, CMC, Tween 80, SA - 1.5%, $CaCl_2$ - 3.5%	0.579 ± 0.0	79.03 ± 9.8[a]	100 ± 0.0
4.	T_4 - Trehalose, PVP, CMC, Tween 80, SA - 0.75%, $CaCl_2$ - 2.5%	0.579 ± 0.0	84.22 ± 8.0[a]	100 ± 0.0
5.	T_5 - Trehalose, PVP, CMC, Tween 80, SA -0.75%, $CaCl_2$ - 3%	0.579 ± 0.0	84.22 ± 8.0[a]	100 ± 0.0
6.	T_6 - Trehalose, PVP, CMC, Tween 80, SA- 0.75%, $CaCl_2$ - 3.5%	0.579 ± 0.0	79.03 ± 9.8[a]	100 ± 0.0
7.	Control	0.579 ± 0.0	0.579 ± 0.0[b]	0.0 ± 0.0
CD (0.01)		NS	20.31	NS

*****Mean of 25 beads ± standard deviation
NS - non significant

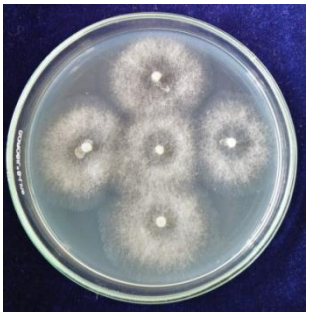
T1



T2

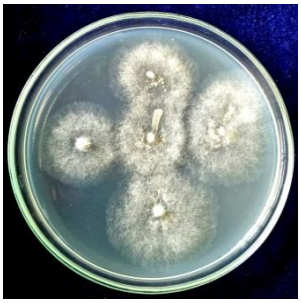


T3

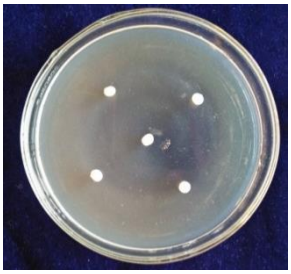


T4

T5



T6



Control

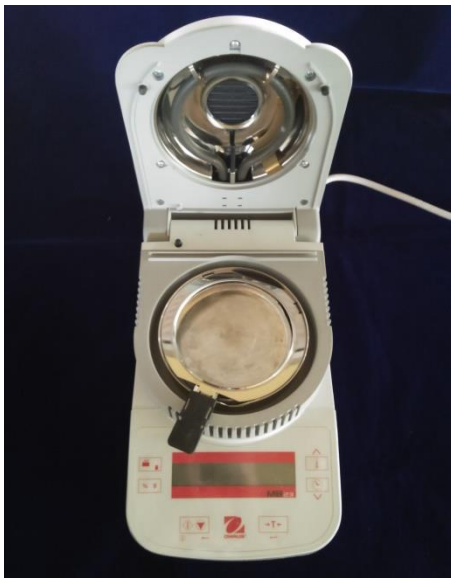
Plate 4.11 Potentially infective beads (after 72 h incubation)

Table 4.9 Estimation of moisture content of beads

Sl. No.	Treatment	*Moisture per cent			
		0 h	**24 h**	**48 h**	**72 h**
1.	T_1 - Trehalose, PVP, CMC, Tween 80, SA - 1.5%, $CaCl_2$ - 2.5%	94.0 ± 1.63	82.0 ± 1.63	61.2 ± 2.01^{c}	23.3 ± 0.60^{bc}
2.	T_2 - Trehalose, PVP, CMC, Tween 80, SA - 1.5%, $CaCl_2$ - 3%	93.4 ± 2.90	80.0 ± 2.44	61.4 ± 0.75^{c}	28.7 ± 1.20^{a}
3.	T_3 - Trehalose, PVP, CMC, Tween 80, SA - 1.5%, $CaCl_2$ - 3.5%	92.0 ± 1.63	84.4 ± 0.43	59.9 ± 1.95^{c}	26.2 ± 0.62^{a}
4.	T_4 - Trehalose, PVP, CMC, Tween 80, SA - 0.75%, $CaCl_2$ - 2.5%	90.2 ± 0.86	82.1 ± 1.67	70.1 ± 3.60^{ab}	23.2 ± 2.35^{bc}
5.	T_5 - Trehalose, PVP, CMC, Tween 80, SA -0.75%, $CaCl_2$ - 3%	91.0 ± 4.08	80.0 ± 3.74	75.0 ± 1.63^{a}	21.8 ± 1.31^{bc}
6.	T_6 - Trehalose, PVP, CMC, Tween 80, SA- 0.75%, $CaCl_2$ - 3.5%	90.9 ± 1.71	81.2 ± 1.49	73.8 ± 2.59^{a}	20.9 ± 0.63^{c}
7.	Control	91.3 ± 1.56	84.5 ± 1.14	67.4 ± 2.50^{b}	23.5 ± 0.41^{b}
CD (0.01)		NS	NS	6.90	3.60

***** Mean of three replications±standard deviation

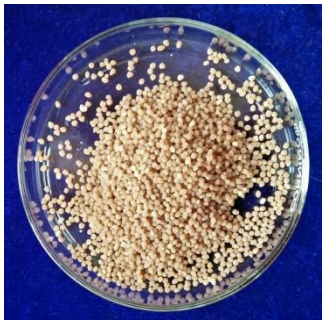
NS - non significant



a) Instant moisture analyser

b) Beads immediately after preparation (T_{21})

c) After 48 h drying (T_{21})



d) After 72 h drying (T_{21})

Plate 4.12 Estimation of moisture content of microbead

Table 4.10a Estimation of swelling behaviour of beads at pH 7.4 in phosphate buffer

Sl. No.	Treatment	*Mean bead weight (mg)								
		0 min	10 min	20min	30 min	45 min	60 min	90 min	120 min	240 min
1.	T_1 - Trehalose, PVP, CMC, Tween 80, SA - 1.5%, $CaCl_2$ - 2.5%	34.6	37.3	41.6	42.0	42.8	43.4	45.0	45.5	47.0
2.	T_2 - Trehalose, PVP, CMC, Tween 80, SA - 1.5%, $CaCl_2$ - 3%	29.6	29.8	30. 2	31. 2	32.0	32. 2	33.9	34.4	36.4
3.	T_3 - Trehalose, PVP, CMC, Tween 80, SA - 1.5%, $CaCl_2$ - 3.5%	33.6	29.6	30.6	31.8	34. 2	36.6	42.6	41.6	45.4
4.	T_4 - Trehalose, PVP, CMC, Tween 80, SA - 0.75%, $CaCl_2$ - 2.5%	30.8	29.4	27.8	29. 2	30.8	32.0	37.8	39	48. 2
5.	T_5 - Trehalose, PVP, CMC, Tween 80, SA - 0.75%, $CaCl_2$ - 3%	25.4	26.6	29.6	30.6	34.2	39.0	40.1	42.0	43.3
6.	T_6 - Trehalose, PVP, CMC, Tween 80, SA- 0.75%, $CaCl_2$ - 3.5%	26.8	28.2	29.0	31.2	33.0	33.9	41.3	42.0	43.0
7.	Control	88.5	88.5	88.9	90.0	90.2	90.6	90.8	91.1	92.0

*****Mean weight of 10 beads

Table 4.10b Estimation of swelling behaviour of beads at pH 1 in 0.1M HCl

Sl. No.	Treatment	*Mean bead weight (mg)								
		0 min	10 min	20min	30 min	45 min	60 min	90 min	120 min	240 min
1.	T_1 - Trehalose, PVP, CMC, Tween 80, SA - 1.5%, $CaCl_2$ - 2.5%	32.1	27.8	25.8	24.5	24.4	22.9	22.4	22.0	20.0
2.	T_2 - Trehalose, PVP, CMC, Tween 80, SA - 1.5%, $CaCl_2$ - 3%	26.6	25.8	25.6	24.6	23.6	22.8	21.8	21.0	19.6
3.	T_3 - Trehalose, PVP, CMC, Tween 80, SA - 1.5%, $CaCl_2$ - 3.5%	32.8	29. 2	28. 2	28. 2	27.6	26.8	26.3	25.7	23.0
4.	T_4 - Trehalose, PVP, CMC, Tween 80, SA - 0.75%, $CaCl_2$ - 2.5%	29.6	27.0	24. 6	24. 2	24.2	24. 2	24.0	23.8	20.3
5.	T_5 - Trehalose, PVP, CMC, Tween 80, SA -0.75%, $CaCl_2$ - 3%	29.0	28.9	28.0	28.0	26.9	26.0	25.0	25.1	23.0
6.	T_6 - Trehalose, PVP, CMC, Tween 80, SA- 0.75%, $CaCl_2$ - 3.5%	27.5	27.1	27.0	26.4	26.0	23.9	23.1	23.1	23.0
7.	Control	90.2	90.2	90.2	90.1	90.0	90.0	89.8	89.7	89.7

*****Mean weight of 10 beads

Table 4.10c Estimation of swelling ratio and swelling per cent of beads at pH 7.4 and pH 1

Sl. No.	Treatment	pH 7.4 (in phosphate buffer)				pH 1 (in 0.1M HCl)			
		Weight at		Swelling ratio	Swelling per cent	Weight at		Swelling ratio	Swelling per cent
		0 min	240 min			0 min	240 min		
1.	T_1 - Trehalose, PVP, CMC, Tween 80, SA - 1.5%, $CaCl_2$ - 2.5%	34.6	47.0	0.35	35.83	32.1	20.0	-0.37	-37.69
2.	T_2 - Trehalose, PVP, CMC, Tween 80, SA - 1.5%, $CaCl_2$ - 3%	29.6	36.4	0.22	22.97	26.6	19.6	-0.26	-26.31
3.	T_3 - Trehalose, PVP, CMC, Tween 80, SA - 1.5%, $CaCl_2$ - 3.5%	33.6	45.4	0.35	35.11	32.8	23.0	-0.29	-29.87
4.	T_4 - Trehalose, PVP, CMC, Tween 80, SA - 0.75%, $CaCl_2$ - 2.5%	30.8	48. 2	0.56	56.49	29.6	20.3	-0.31	-31.41
5.	T_5 - Trehalose, PVP, CMC, Tween 80, SA -0.75%, $CaCl_2$ - 3%	25.4	43.3	0.70	70.47	29.0	23.0	-0.20	-20.68
6.	T_6 - Trehalose, PVP, CMC, Tween 80, SA- 0.75%, $CaCl_2$ - 3.5%	26.8	43.0	0.60	60.44	27.5	23.0	-0.16	-16.36
7.	Control	88.5	92.0	0.03	3.95	90.2	89.7	-0.005	-0.55

bead weight was noticed in all the treatments. Further, it was observed that the control beads with maximum weight (90.2 mg) at 0 min reduced its weight to 89.7 mg at the end of 240 min. Similarly, the treatment T_2 with minimum initial weight (26.6 mg) was reduced to 19.6 mg at the end of 240 min.

From the observations, it is clear that for beads kept in HCl, swelling ratio and swelling per cent are negative, which implies the shrinking nature of beads at a lower pH. Moreover, it was observed that higher degree of shrinking occurred in treatments where sodium alginate concentration was less (0.75%) and control beads with 2.5 per cent sodium alginate were found highly resistant to shrinking.

4.2.6.4 Shrinking percentage

Per cent shrinking of microbeads was estimated by measuring the diameter of beads immediately after formation and after 48 h of drying under a stereo microscope and the data are presented in Table 4.11. Data from the table evidenced that beads in control (T_7) recorded maximum diameter (2.33 mm) before drying followed by T_2 (1.91 mm), whereas, the minimum diameter was shown by T_1 (1.50 mm). After drying for 48 h, maximum diameter was recorded again in control (2.20 mm) followed by T_2 (1.68 mm) and minimum in T_5 (0.88 mm). Consequently, the maximum per cent shrinking was observed in T_5 (47.05%) followed by T_4 (46.98%) and the minimum in control (5.57%). Hence, it is concluded that the degree of shrinking is inversely proportional to the concentration of sodium alginate in the bead.

4.2.6.5 Sphericity factor

Sphericity factor defines the shape of the bead and it assumes significance while the shape of the bead is described. It's value ranges from zero for a perfectly spherical bead to unity for an oblong or elongated bead. It was estimated by measuring the diameter of the bead in three different directions with the help of a stereo microscope and the resultant data are illustrated in Table 4.12. From the table, it is clear that the highest

Table 4.11 Per cent shrinking of beads

Sl. No.	Treatment	*Mean diameter of bead (mm)		Per cent shrinking
		Before drying	After 48 h drying	
1.	T_1 - Trehalose, PVP, CMC, Tween 80, SA - 1.5%, $CaCl_2$ - 2.5%	1.50	1.28	14.66
2.	T_2 - Trehalose, PVP, CMC, Tween 80, SA - 1.5%, $CaCl_2$ - 3%	1.91	1.68	12.04
3.	T_3 - Trehalose, PVP, CMC, Tween 80, SA - 1.5%, $CaCl_2$ - 3.5%	1.67	1.52	8.98
4.	T_4 - Trehalose, PVP, CMC, Tween 80, SA - 0.75%, $CaCl_2$ - 2.5%	1.70	0.90	47.05
5.	T_5 - Trehalose, PVP, CMC, Tween 80, SA -0.75%, $CaCl_2$ - 3%	1.66	0.88	46.98
6.	T_6 - Trehalose, PVP, CMC, Tween 80, SA- 0.75%, $CaCl_2$ - 3.5%	1.87	1.08	42.24
7.	Control	2.33	2.20	5.57

*Average of three beads

Table 4.12 Estimation of sphericity factor of beads

Sl. No.	Treatment	*Mean diameter (mm) after drying		Sphericity factor
		Maximum	Minimum	
1.	T_1 - Trehalose, PVP, CMC, Tween 80, SA - 1.5%, $CaCl_2$ - 2.5%	1.60	1.45	0.049
2.	T_2 - Trehalose, PVP, CMC, Tween 80, SA - 1.5%, $CaCl_2$ - 3%	1.09	0.95	0.068
3.	T_3 - Trehalose, PVP, CMC, Tween 80, SA - 1.5%, $CaCl_2$ - 3.5%	1.42	1.30	0.044
4.	T_4 - Trehalose, PVP, CMC, Tween 80, SA - 0.75%, $CaCl_2$ - 2.5%	0.84	0.68	0.105
5.	T_5 - Trehalose, PVP, CMC, Tween 80, SA -0.75%, $CaCl_2$ - 3%	0.99	0.80	0.106
6.	T_6 - Trehalose, PVP, CMC, Tween 80, SA- 0.75%, $CaCl_2$ - 3.5%	1.28	1.01	0.117
7.	Control	2.10	1.96	0.034

*Mean diameter of three beads

value for maximum diameter was shown by control (T_7) (2.10 mm) followed by T_1 (1.60 mm) and the lowest value for the maximum diameter was recorded in T_4 (0.84 mm). The highest value for minimum diameter was documented in control (1.96 mm) followed by T_1 (1.45 mm) whereas, the lowest value was noticed in T_4 (0.68 mm). Accordingly, the maximum value of sphericity factor was noticed in T_6 (0.117) followed by T_5 (0.106) and T_4 (0.105) which indicates that those beads are not exact spheres. However, control (T_7) (0.034) followed by T_3 (0.044) are having minimum value of sphericity and hence, almost spherical in shape. The results from the table evidenced that the beads with high concentration of sodium alginate (1.5%) assumed an almost spherical shape with least value for sphericity factor and as the concentration is reduced to 0.75 per cent, beads turned disproportionately shrunken and sphericity factor approached unity.

4.2.6.6 Estimation of time of gelation or curing

Gelation is the process of cross linking where calcium chloride when comes in contact with sodium alginate, results in the expulsion of water out of the microbeads which is indicated by the gradual decline in bead weight. For estimation of time of gelation or curing, 10 individual beads immediately after being formed were weighed at specific intervals of time until the bead weight becomes stable or no more reduction in bead weight was noticed. The data obtained from the experiment are tabulated in Table 4.13.

In general, it was observed that the weight of beads gradually declined from initial 15 min and continued to fall until 60 min from the point of formation and slightly increased from 60 - 90 min. The initial bead weight soon after the formation in various treatments ranged from 50.2 to 78.4 mg, with the maximum bead weight in T_1 followed by T_2 (63.9 mg) and the least in T_4. Later, as the time of incubation was increased from 15 min to 90 min, the bead weight gradually decreased till 60 min of incubation with the maximum bead weight of 64.6 mg in T_1 followed by 52.2 and 50.4 in T_3 and T_2 respectively. The least bead weight of 40.3 was observed in T_4. However, it was noticed

Table 4.13 Estimation of time of gelation of alginate beads

Treatment	Mean weight of beads (mg)						
	0 min	**15 min**	**30 min**	**45 min**	**60 min**	**75 min**	**90 min**
T_1 - Trehalose, PVP, CMC, Tween 80, SA - 1.5%, $CaCl_2$ - 2.5%	78.4	71.0	68.9	67.0	**64.6**	65.3	65.4
T_2 - Trehalose, PVP, CMC, Tween 80, SA - 1.5%, $CaCl_2$ - 3%	63.9	57.1	53.3	53.2	**50.4**	51.2	51.5
T_3 - Trehalose, PVP, CMC, Tween 80, SA - 1.5%, $CaCl_2$ - 3.5%	57.1	55.2	54.7	52.9	**52.2**	53.5	54.2
T_4 - Trehalose, PVP, CMC, Tween 80, SA - 0.75%, $CaCl_2$ - 2.5%	50.2	48.3	45.6	42.3	**40.3**	41.1	41.3
T_5 - Trehalose, PVP, CMC, Tween 80, SA -0.75%, $CaCl_2$ - 3%	50.8	49.2	46.4	43.9	**41.1**	42.0	42.9
T_6 - Trehalose, PVP, CMC, Tween 80, SA- 0.75%, $CaCl_2$ - 3.5%	51.1	50.3	48.7	44.6	**42.6**	42.6	42.9
Control	85.2	83.8	82.4	80.0	**79.2**	79.5	79.6

*Mean weight of 10 beads
SA - sodium alginate

that after 60 min, the weight of beads was slightly increased in all the treatments which indicated the termination of the process of cross linking or gelation.

From the colony count characterization of microbeads and other parameters studied, the best treatment combination selected for the *in vivo* evaluation was T_3, which was prepared out of trehalose (15 mM), PVP (1%), CMC (0.5%), tween 80 (0.5%), sodium alginate (1.5%) and $CaCl_2$ (3.5%).

4.2.6.7 Effect of pH on the selected combination of microbead in soil under in vitro condition

The process of swelling and degradation of microbeads decides release of bioagent from the bead into the soil which is determined by the pH of the external medium. The effect of pH on the release of bioagent from microbeads was studied for T_3 (sodium alginate 1.5%, $CaCl_2$ 3.5%, trehalose 15 mM, PVP 1%, CMC 0.5%, and tween 80 - 0.5%), which exhibited a consistent performance in terms of colony count as well as in other bead parameters .The data are furnished in Table 4.14. At pH 5.09, it was noticed that a population of 14.6 x 10^6 cfu g^{-1} soil was released 24 h after incubation and after 48 h, it was increased to 30.6 x 10^6 cfu g^{-1} soil. However, after one week incubation period, the colony count was reduced to 12.3 x 10^6 cfu g^{-1} soil. On the contrary, at pH 8.91, the population of *T. viride* was comparatively less (6.6 x 10^6 cfu g^{-1} soil) than that of pH 5.9 at 24 h incubation. However, the colony count increased to 66.0 x 10^6 cfu g^{-1} after 48 h incubation whereas, the number of released cfu was drastically reduced to 1.0 x 10^6 cfu g^{-1} after seven days of incubation. From the results, it was concluded that the release of entrapped bioagent depends on the pH of the external medium.

4.3 *In vivo* EVALUATION OF BIOCONTROL EFFICACY AND PLANT GROWTH PROMOTION OF ENCAPSULATED *Trichoderma viride*

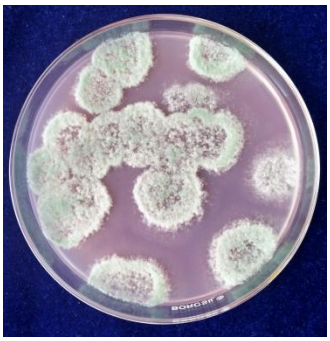
The biocontrol efficacy and growth promotion activity of sodium alginate based bead formulation of *T. viride* was studied and the treatment T_3 (sodium alginate 1.5%,

Table 4.14 Effect of pH on microbeads of selected treatment in soil under *in vitro* conditions

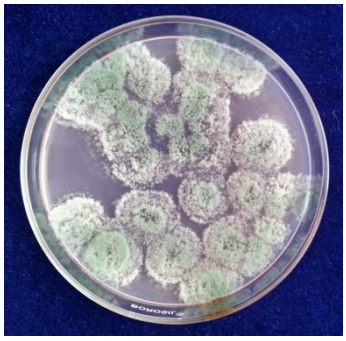
Sl. No.	Time interval	*Population of *T.viride* (x10^6 cfu g^{-1})	
		at pH 5.09	at pH 8.91
1.	24 h	14.6 (7.164)[a]	6.6 (6.819)[b]
2.	48 h	30.6 (7.485)[b]	66.0 (7.819)[a]
3.	7 days	12.3 (7.089)[a]	1.0 (6.000)[b]

* Mean of three replications.
In each row figures followed by the same letter do not differ significantly according to DMRT.
Log transformed values are given in parenthesis

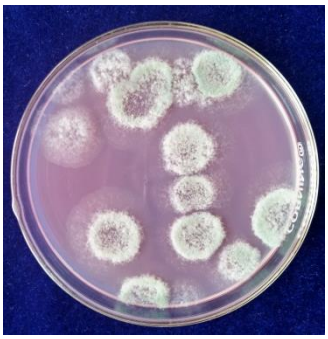
At pH 5.09



After 24 h

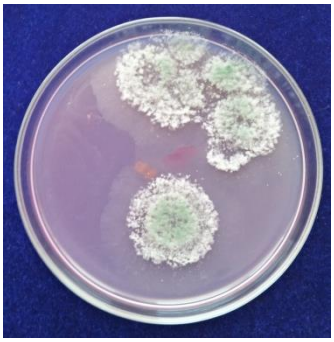


After 48 h

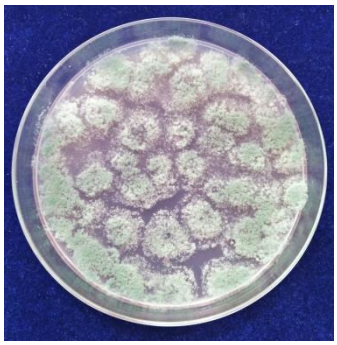


After seven days

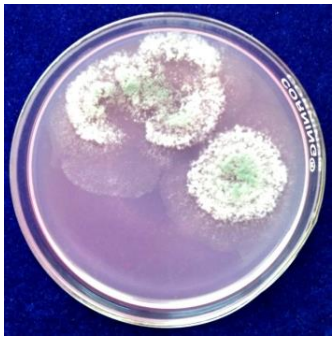
At pH 8.91



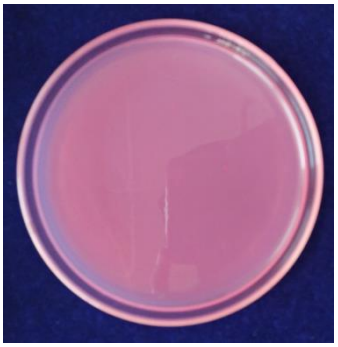
After 24 h



After 48 h



After seven days



Control

Plate 4.13 Effect of pH on microbeads of selected treatment in soil under *in vitro* condition (on *Trichoderma* Selective Medium)

$CaCl_2$ 3.5%, trehalose 15 mM, PVP 1%, CMC 0.5%, and tween 80 - 0.5%), which outperformed the other treatments in all its characteristics was evaluated in the pot culture experiment. The formulation at three different doses *viz*., 1g plant^{-1}, 3g plant^{-1} and 5g plant^{-1} along with the talc based formulation @ 20 g l^{-1} and liquid formulation @ 5ml l^{-1} were compared for their biocontrol efficacy in three different pot culture experiments. A total of six treatments were laid out as described in 3.4. Bush cowpea var. Bhagyalakshmi was used as the test crop and plant pathogenic fungi *viz*., *Pythium aphanidermatum, Rhizoctonia solani* and *Fusarium oxysporum* were employed as test pathogens and they were challenge inoculated.

The pot culture experiments were carried out during December 2019 at College of Horticulture, Vellanikkara. Treatments were applied at the time of sowing, symptom appearance and 20 days after symptom appearance. Estimation of plant biometric characters like plant height and number of leaves were carried out at suitable intervals along with the assessment of number of days required for first flowering and per cent disease incidence.

4.3.1 Experiment 1 - *In vivo* evaluation of microbeads against soil borne pathogen *Pythium aphanidermatum*

4.3.1.1 Plant height

Height of cowpea plants were measured at 30, 45 and 60 days after sowing (DAS) and the derived data are presented in Table 4.15. It was noticed that there was a significant difference in plant height among the treatments at 30, 45 and 60 DAS. At 30 DAS, T_3 (alginate based *T. viride* @ 5.0 g plant^{-1}) recorded the maximum height **(**158.60 cm) followed by T_5 (liquid based *T. viride* @ 5 ml l^{-1}) (156.00 cm) and the minimum height was noticed with control (T_6) (147.25 cm). Relative increase of plant height was significantly different among treatments at 30 - 45 DAS and 45 - 60 DAS. A maximum increase of height of 15.35 per cent was recorded in T_5 followed by 15.25 per cent in T_3 at 30-45 DAS, whereas it was 14.77 and 14.74 per cent respectively at 45 - 60 DAS. Control plants (T_6) recorded only 8.05 and 8.74 per cent increase in height at 30 - 45 DAS and 45 - 60 DAS.


a) View of the pot culture experiment




Two leaf stage **Vegetative growth** **Flowering** **Fruiting**

b) Various stages of crop growth


c) Symptom appearence

d) Application of formulation

e) Release of *T.viride* from beads in soil

Plate 4.14 *In vivo* evaluation of biocontrol efficacy and plant growth promotion of encapsulated *T. viride*

4.3.1.2 Number of leaves

Number of leaves was recorded at 30, 45 and 60 DAS and the data is furnished in Table 4.16. It was observed that there was a significant difference among the treatments at all time intervals. At 30 DAS, T_5 (liquid based *T. viride* @ 5 ml l^{-1}) developed maximum number of leaves among the treatments (13.30) followed by T_4 (talc based *T. viride* @ 20 g l^{-1}) (12.90). At 45 DAS, T_3 (alginate based *T. viride* @ 5.0 g $plant^{-1}$) developed maximum number of leaves **(**24.10) followed by T_5 (24.06). Minimum number was observed in control for both 30 DAS (10.55) and 45 DAS (18.9). After 60 days, the number of leaves developed ranged from 26.20 to 33.95, where the minimum number of leaves was recorded in control (T_6) and maximum in T_3.

4.3.1.3 Number of days for first flowering

The number of days required to first flowering ranged from 37.80 to 45.00 (Table 4.17). It was noticed that T_3 (alginate based *T. viride* @ 5.0 g $plant^{-1}$) took minimum number of days (37.80 DAS) followed by T_2 (alginate based *T. viride* @ 3.0 g $plant^{-1}$) (39.40 DAS) and T_5 (40.00 DAS). Further, T_4 (talc based *T. viride* @ 20 g l^{-1}) took 41.75 days for first flowering while, control plants (T_6) were delayed upto 45 days for first blossom.

4.3.1.4 Yield

Yield of cowpea in all the treatments was recorded and the data is illustrated in Table 4.18. After all the pickings, the maximum yield of 88.95 g was recorded in T_3 (alginate based *T. viride* @ 5.0 g $plant^{-1}$) followed by T_5 (liquid based *T. viride* @ 5 ml l^{-1}) (87.25 g). Further, 85.70 g yield was recorded in T_4 (talc based *T. viride* @ 20 g l^{-1}) and the minimum yield was documented in plants maintained as control (T_6) (68.95 g).

4.3.1.5 Per cent disease incidence

The plants were challenge inoculated with spore suspension of *P. aphanidermatum* @ 5.0 x 10^6 cfu ml^{-1} sterile water 45 DAS. After six days of inoculation, symptoms like water soaked lesions and rotting of collar region was

Table 4.15 Effect of treatments on plant height at 30, 30 - 45, 45 - 60 DAS

Sl. No.	Treatment	30 DAS	*Relative per cent increase in height	
			30 - 45 DAS	45 - 60 DAS
1.	T_1- Alginate based *T. viride* (1.0 g plant^{-1})	151.80^{c}	11.09^{bc}	9.88^{bc}
2.	T_2- Alginate based *T. viride* (3.0 g plant^{-1})	153.35^{bc}	12.17^{ab}	12.35^{ab}
3.	T_3- Alginate based *T. viride* (5.0 g plant^{-1})	158.60^{a}	15. 25^{a}	14.74^{a}
4.	T_4- Talc based *T. viride* (20 g l^{-1})	153.40^{bc}	14.41^{a}	13.97^{a}
5.	T_5- Liquid based *T. viride* (5 ml l^{-1})	156.00^{ab}	15.35^{a}	14.77^{a}
6.	T_6-Control	147.25^{d}	8.05^{c}	8.74^{c}
CD(0.01)		5.41	4.46	4.32

*Mean of 20 replications
In each column figures followed by the same letter do not differ significantly according to DMRT
DAS- days after sowing

Table 4.16 Effect of treatments on number of leaves at 30, 30 - 45, 45 - 60 DAS

Sl. No.	Treatments	*Number of leaves		
		30 DAS	30 - 45 DAS	45 - 60 DAS
1.	T_1- Alginate based *T. viride* (1.0 g plant^{-1})	10.65^{d}	21.55^{c}	31.05^{c}
2.	T_2- Alginate based *T. viride* (3.0 g plant^{-1})	12.10^{c}	22.75^{b}	32.05^{bc}
3.	T_3- Alginate based *T. viride* (5.0 g plant^{-1})	12.65^{b}	24.10^{a}	33.95^{a}
4.	T_4- Talc based *T. viride* (20 g l^{-1})	12.90^{b}	22.65^{b}	32.00^{bc}
5.	T_5- Liquid based *T. viride* (5 ml l^{-1})	13.30^{a}	24.06^{a}	32.95^{ab}
6.	T_6-Control	10.55^{d}	18.90^{d}	26.20^{d}
	CD(0.01)	0.518	1.073	1.416

*Mean of 20 replications
In each column figures followed by the same letter do not differ significantly according to DMRT
DAS - days after sowing

Table 4.17 Effect of treatments on number of days to first flowering

Sl. No.	Treatments	*Days to first flowering
1.	T_1- Alginate based *T. viride* (1.0 g plant^{-1})	41.90^{b}
2.	T_2- Alginate based *T. viride* (3.0 g plant^{-1})	39.40^{bc}
3.	T_3- Alginate based *T. viride* (5.0 g plant^{-1})	37.80^{c}
4.	T_4- Talc based *T. viride* (20 g l^{-1})	41.75^{b}
5.	T_5- Liquid based *T. viride* (5 ml l^{-1})	40.00^{bc}
6.	T_6-Control	45.00^{a}
	CD(0.01)	3.435

*Mean of 20 replications

In each column figures followed by the same letter do not differ significantly according to DMRT

Table 4.18 Effect of treatments on yield

Sl. No.	Treatments	*Yield plant^{-1} (g)
1.	T_1- Alginate based *T. viride* (1.0 g plant^{-1})	75.10^{d}
2.	T_2- Alginate based *T. viride* (3.0 g plant^{-1})	81.85^{c}
3.	T_3- Alginate based *T. viride* (5.0 g plant^{-1})	88.95^{a}
4.	T_4- Talc based *T. viride* (20 g l^{-1})	85.70^{b}
5.	T_5- Liquid based *T. viride* (5 ml l^{-1})	87.25^{ab}
6.	T_6-Control	68.95^{e}
	CD (0.01)	4.15

*Mean of 20 replications

In each column figures followed by the same letter do not differ significantly according to DMRT

Table 4.19 Effect of treatments after the incidence of damping-off caused by *P. aphanidermatum*

Sl. No.	Treatments	Per cent disease incidence (PDI)
1.	T_1- Alginate based *T. viride* (1.0 g plant^{-1})	80^{c}
2.	T_2- Alginate based *T. viride* (3.0 g plant^{-1})	80^{c}
3.	T_3- Alginate based *T. viride* (5.0 g plant^{-1})	70^{d}
4.	T_4- Talc based *T. viride* (20 g l^{-1})	70^{d}
5.	T_5- Liquid based *T. viride* (5 ml l^{-1})	85^{b}
6.	T_6-Control	100^{a}
	CD (0.01)	2.74

*Mean of 20 replications

In each column figures followed by the same letter do not differ significantly according to DMRT

observed. From Table 4.19, it is obvious that the minimum per cent disease incidence of 70 per cent was noticed with T_3 (alginate based *T. viride* @ 5.0 g plant^{-1}) and T_4 (talc based *T. viride* @ 20 g l^{-1}) while, the maximum of cent per cent disease incidence was recorded in T_6 (control). However, T_5 (liquid based *T. viride* @ 5 ml l^{-1}) showed comparatively higher disease incidence of 85 per cent while T_1 (alginate based *T. viride* @1.0 g plant^{-1}) and T_2 (alginate based *T. viride* @ 3.0 g plant^{-1}) showed 80 per cent disease incidence.

4.3.2 Experiment 2 - *In vivo* evaluation of microbeads against soil borne pathogen *Rhizoctonia solani*

4.3.2.1 Plant height

Estimation of plant height was carried out at three different intervals *viz*., 30, 45 and 60 DAS and resulting data are provided in Table 4.20. It was observed that at 30 DAS, T_3 (alginate based *T. viride* @ 5.0 g plant^{-1}) was on par with T_5 (liquid based *T. viride* @ 5 ml l^{-1}), whereas T_1, T_2, T_4 and T_6 differed significantly, where the maximum height was recorded in T_5 (159.40 cm) followed by T_3 (158.80 cm) and minimum height was noted in control plants (T_6) (147.75 cm). At 45 DAS, maximum per cent increase was noticed in T_3 (14.74%) followed by T_5 (14.34 %) and minimum per cent increase in height was recorded in (9.55%). After 60 days, maximum increase in height was noted in T_3 (13.69%) followed by T_4 (talc based *T. viride* @ 20 g l^{-1}) (13.32%), while minimum per cent increase was recorded in control plants (T_6) (10.28%).

4.3.2.2 Number of leaves

Enumeration of number of completely opened leaves was performed at 30, 45 and 60 DAS and the corresponding data are furnished in Table 4.21. At 30 DAS, maximum number of leaves was observed in T_5 (liquid based *T. viride* @ 5 ml l^{-1}) (13.25) followed by T_3 (alginate based *T. viride* @ 5.0 g plant^{-1}) and T_4 (talc based *T. viride* @ 20 g l^{-1}) (12.80 each) whereas, minimum number of leaves was recorded in control plants (10.65). At 45 DAS, maximum number of leaves was counted in T_5 (24.35) followed by T_3

(23.40) and minimum number of leaves was recorded in plants kept as control (T_6) (19.65). At 60 DAS, maximum number of leaves was recorded in T_5 (31.40) followed by T_3 and T_4 (30.95 each) whereas, minimum number of leaves was observed in control plants (25.25).

4.3.2.3 Number of days to first flowering

The number of days needed to first flowering ranged from 40.15 to 44.40 which is tabulated in Table 4.22. Minimum number of days was taken by T_5 (liquid based *T. viride* @ 5 ml l^{-1}), where it flowered 40.15 DAS while, the flowering was delayed upto 44.40 days in control (T_6).

4.3.2.4 Yield

Yield from all the pickings was calculated and the data are presented in Table 4.23. In general, the average yield ranged from 88.10 - 67.15 g $plant^{-1}$. Maximum yield was documented in T_3 (alginate based *T. viride* @ 5.0 g $plant^{-1}$) (88.10 g $plant^{-1}$) followed by T_4 (talc based *T. viride* @ 20 g l^{-1}) (85.15 g $plant^{-1}$) while, minimum yield was recorded in control (T_6) (67.15 g $plant^{-1}$).

4.3.2.5 Per cent disease incidence

Spore suspension of *R. solani* @ 1.0 x 10^6 cfu ml^{-1} sterile water was drenched in the root zone of cowpea plants 45 DAS. Development of symptoms like water soaked lesions, rotting and subsequent shredding of collar region was noticed seven days after challenge inoculation and the data is recorded in Table. 4.24. The minimum per cent disease incidence of 80 per cent was documented in T_3 (alginate based *T. viride* @ 5.0 g $plant^{-1}$) and T_5 (liquid based *T. viride* @ 5 ml l^{-1}) whereas, the maximum of cent per cent was recorded in T_6 (control). Moreover, T_2 (alginate based *T. viride* @ 3.0 g $plant^{-1}$), T_1 (alginate based *T. viride* @ 1.0 g $plant^{-1}$) and T_4 (talc based *T. viride* @ 20 g l^{-1}) showed comparatively higher disease incidence of 90, 95 and 95 per cent respectively.

Table 4.20 Effect of treatments on plant height at 30, 30 - 45, 45 - 60 DAS

Sl. No.	Treatment	30 DAS	*Relative per cent increase in height	
			30 - 45 DAS	45 - 60 DAS
1.	T_1- Alginate based *T. viride* (1.0 g plant^{-1})	150.50^{cd}	9.93^{b}	11.56^{bc}
2.	T_2- Alginate based *T. viride* (3.0 g plant^{-1})	156.75^{ab}	10.57^{b}	12.19^{ab}
3.	T_3- Alginate based *T. viride* (5.0 g plant^{-1})	158.80^{a}	14.74^{a}	13.69^{a}
4.	T_4- Talc based *T. viride* (20 g l^{-1})	152.90^{bc}	11.78^{ab}	13.32^{a}
5.	T_5- Liquid based *T. viride* (5 ml l^{-1})	159.40^{a}	14.34^{a}	12.53^{ab}
6.	T_6-Control	147.75^{d}	9.55^{b}	10.28^{c}
CD(0.01)		5.303	4.09	2.22

*Mean of 20 replications

In each column figures followed by the same letter do not differ significantly according to DMRT

DAS - days after sowing

Table 4.21 Effect of treatments on number of leaves at 30, 30 - 45, 45 - 60 DAS

Sl. No.	Treatments	*Number of leaves		
		30 DAS	30 - 45 DAS	45 - 60 DAS
1.	T_1- Alginate based *T. viride* (1.0 g plant^{-1})	10.85^{d}	21.65^{b}	30.10^{b}
2.	T_2- Alginate based *T. viride* (3.0 g plant^{-1})	11.80^{c}	21.85^{b}	30.00^{b}
3.	T_3- Alginate based *T. viride* (5.0 g plant^{-1})	12.80^{b}	23.40^{a}	30.95^{a}
4.	T_4- Talc based *T. viride* (20 g l^{-1})	12.80^{b}	22.10^{b}	30.95^{a}
5.	T_5- Liquid based *T. viride* (5 ml l^{-1})	13.25^{a}	24.35^{a}	31.40^{a}
6.	T_6-Control	10.65^{d}	19.65^{c}	25.25^{c}
	CD(0.01)	0.575	1.20	1.15

*Mean of 20 replications

In each column figures followed by the same letter do not differ significantly according to DMRT

DAS - days after sowing

Table 4.22 Effect of treatments on number of days to first flowering

Sl. No.	Treatments	*Days to first flowering
1.	T_1- Alginate based *T. viride* (1.0 g plant^{-1})	42.20^{b}
2.	T_2- Alginate based *T. viride* (3.0 g plant^{-1})	41.40^{bc}
3.	T_3- Alginate based *T. viride* (5.0 g plant^{-1})	40.60^{cd}
4.	T_4- Talc based *T. viride* (20 g l^{-1})	40.60^{cd}
5.	T_5- Liquid based *T. viride* (5 ml l^{-1})	40.15^{d}
6.	T_6-Control	44.40^{a}
	CD(0.01)	1.50

*Mean of 20 replications
In each column figures followed by the same letter do not differ significantly according to DMRT.

Table 4.23 Effect of treatments on yield

Sl. No.	Treatments	*Yield plant^{-1} (g)
1.	T_1- Alginate based *T. viride* (1.0 g plant^{-1})	76.80^{d}
2.	T_2- Alginate based *T. viride* (3.0 g plant^{-1})	80.45^{c}
3.	T_3- Alginate based *T. viride* (5.0 g plant^{-1})	88.10^{a}
4.	T_4- Talc based *T. viride* (20 g l^{-1})	85.15^{ab}
5.	T_5- Liquid based *T. viride* (5 ml l^{-1})	84.05^{b}
6.	T_6-Control	67.15^{e}
	CD(0.01)	4.29

*Mean of 20 replications
In each column figures followed by the same letter do not differ significantly according to DMRT.

Table 4.24 Effect of treatments after the incidence of root rot caused by *R. solani*

Sl. No.	Treatments	*Per cent disease incidence (PDI)
1.	T_1- Alginate based *T. viride* (1.0 g plant^{-1})	95^{b}
2.	T_2- Alginate based *T. viride* (3.0 g plant^{-1})	90^{c}
3.	T_3- Alginate based *T. viride* (5.0 g plant^{-1})	80^{d}
4.	T_4- Talc based *T. viride* (20 g l^{-1})	95^{b}
5.	T_5- Liquid based *T. viride* (5 ml l^{-1})	80^{d}
6.	T_6-Control	100^{a}
	CD(0.01)	2.74

*Mean of 20 replications

In each column figures followed by the same letter do not differ significantly according to DMRT.

4.3.3 Experiment 3 - *In vivo* evaluation of microbeads against soil borne pathogen *Fusarium oxysporum*

4.3.3.1 Plant height

Mean height of plants under different treatments were assessed at 30, 45 and 60 DAS and data is furnished in Table 4.25. After 30 days, maximum height was documented in T_3 (alginate based *T. viride* @ 5.0 g plant^{-1}) (160.02 cm) followed by T_5 (liquid based *T. viride* @ 5 ml l^{-1}) (157.35 cm). Further, T_2 (alginate based *T. viride* @ 3.0 g plant^{-1}) (153.70 cm) was on par with T_4 (talc based *T. viride* @ 20 g l^{-1}) (154.90 cm) and T_1 (alginate based *T. viride* @ 1.0 g plant^{-1}) recorded 153.20 cm while, minimum height was recorded in control (T_6) (147.12 cm). At 45 DAS, per cent increase in plant height differed significantly among different treatments. Maximum per cent increase in height was recorded in T_3 (14.60%) while, minimum increase was noted in control plants (9.34%). At 60 DAS, T_3 recorded the maximum per cent increase in height (14.32%) followed by T_4 (12.03%), which is on par with T_5 (liquid based *T. viride* @ 5 ml l^{-1}) (11.99%). Further, T_2 (alginate based *T. viride* @ 3.0 g plant^{-1}) documented 10.78 and T_1 recorded 9.29 per cent increase whereas, minimum increase was noted in control (T_6) (8.71%).

4.3.3.2 Number of leaves

Number of fully expanded leaves was computed at an interval of 30, 45 and 60 DAS and representative data are given in Table 4.26. At 30 DAS, maximum number of leaves was recorded in T_3 (alginate based *T. viride* @ 5.0 g plant^{-1}) (13.30) followed by T_5 (liquid based *T. viride* @ 5 ml l^{-1}) (12.90) and T_4 (talc based *T. viride* @ 20 g l^{-1}) (12.85) whereas, minimum number of leaves was documented in control plants (T_6) (10.50). At 45 DAS, maximum number of leaves was recorded in T_3 (24.70) which was on par with T_5 (24.25) and minimum number of leaves was found in control plants (20.35). Likewise, at 60 DAS, maximum number of leaves was recorded in T_3 and T_5 (31.75 each) while, minimum number was noted in control plants (25.35).

Table 4.25 Effect of treatments on plant height at 30, 30 - 45, 45 - 60 DAS

Sl. No.	Treatment	30 DAS	*Relative per cent increase in height	
			30 - 45 DAS	45 - 60 DAS
1.	T_1- Alginate based *T. viride* (1.0 g plant^{-1})	153.20^{c}	10.61^{de}	9.29^{cd}
2.	T_2- Alginate based *T. viride* (3.0 g plant^{-1})	153,70^{bc}	11.86^{cd}	10.78^{bc}
3.	T_3- Alginate based *T. viride* (5.0 g plant^{-1})	160.02^{a}	14.60^{a}	14.32^{a}
4.	T_4- Talc based *T. viride* (20 g l^{-1})	154.90^{bc}	13.01^{bc}	12.03^{b}
5.	T_5- Liquid based *T. viride* (5 ml l^{-1})	157.35^{ab}	14.32^{ab}	11.99^{b}
6.	T_6-Control	147.12^{d}	9.34^{e}	8.71^{d}
CD(0.01)		5.00	2.05	2.47

*Mean of 20 replications
In each column figures followed by same the letter do not differ significantly according to DMRT
DAS - days after sowing

Table 4.26 Effect of treatments on number of leaves at 30, 30 - 45, 45 - 60 DAS

Sl. No.	Treatments	*Number of leaves		
		30 DAS	30 - 45 DAS	45 - 60 DAS
1.	T_1- Alginate based *T. viride* (1.0 g plant^{-1})	10.90^{d}	21.55^{c}	29.55^{c}
2.	T_2- Alginate based *T. viride* (3.0 g plant^{-1})	12.20^{c}	22.90^{bc}	30.05^{c}
3.	T_3- Alginate based *T. viride* (5.0 g plant^{-1})	13.30^{a}	24.70^{a}	31.75^{a}
4.	T_4- Talc based *T. viride* (20 g l^{-1})	12.85^{b}	22.30^{b}	31.10^{b}
5.	T_5- Liquid based *T. viride* (5 ml l^{-1})	12.90^{b}	24.25^{a}	31.75^{a}
6.	T_6-Control	10.50^{e}	20.35^{d}	25.35^{d}
	CD(0.01)	2.47	0.89	0.78

*Mean of 20 replications
In each column figures followed by same letter do not differ significantly according to DMRT
DAS - days after sowing

Table 4.27 Effect of treatments on days to first flowering

Sl. No.	Treatments	*Days to first flowering
1.	T_1- Alginate based *T. viride* (1.0 g plant^{-1})	42.45^{b}
2.	T_2- Alginate based *T. viride* (3.0 g plant^{-1})	41.55^{c}
3.	T_3- Alginate based *T. viride* (5.0 g plant^{-1})	40.20^{d}
4.	T_4- Talc based *T. viride* (20 g l^{-1})	40.80^{cd}
5.	T_5- Liquid based *T. viride* (5 ml l^{-1})	40.10^{d}
6.	T_6-Control	44.30^{a}
CD(0.01)		1.18

*Mean of 20 replications
In each column figures followed by same letter do not differ significantly according to DMRT.

Table 4.28 Effect of treatments on yield

Sl. No.	Treatments	*Yield plant^{-1} (g)
1.	T_1- Alginate based *T. viride* (1.0 g plant^{-1})	77.65^{c}
2.	T_2- Alginate based *T. viride* (3.0 g plant^{-1})	80.05^{c}
3.	T_3- Alginate based *T. viride* (5.0 g plant^{-1})	90.60^{a}
4.	T_4- Talc based *T. viride* (20 g l^{-1})	88.65^{ab}
5.	T_5- Liquid based *T. viride* (5 ml l^{-1})	86.50^{b}
6.	T_6-Control	67.00^{d}
	CD(0.01)	5.52

*Mean of 20 replications
In each column figures followed by same letter do not differ significantly according to DMRT.

4.3.3.3 Number of days to first flowering

The number of days required to first flowering in various treatments was estimated and the related data are presented in Table 4.27. From the table, it is clear that the number of days required for first blossom varied from 40.10 to 44.30. Minimum number of days for flowering was recorded in T_5 (liquid based *T. viride* @ 5 ml l^{-1}) (40.10) which was on par with T_3 (alginate based *T. viride* @ 5.0 g $plant^{-1}$) (40.20). Flowering in control plants (T_6) were delayed upto 44.30 days.

4.3.3.4 Yield

After all the pickings, the total yield and the average yield was calculated and the data are given in Table 4.28. Maximum yield was recorded in T_3 (alginate based *T. viride* @ 5.0 g $plant^{-1}$) (90.60 g $plant^{-1}$) followed by T_4 (talc based *T. viride* @ 20 g l^{-1}) (88.65 g $plant^{-1}$) and minimum yield was recorded from control plants (T_6) (67.00 g $plant^{-1}$). Further, T_5 (liquid based *T. viride* @ 5 ml l^{-1}), T_2 (alginate based *T. viride* @ 3.0 g $plant^{-1}$) and T_1 (alginate based *T. viride* @ 1.0 g $plant^{-1}$) recorded 86.50, 80.05 and 77.65 g $plant^{-1}$ respectively.

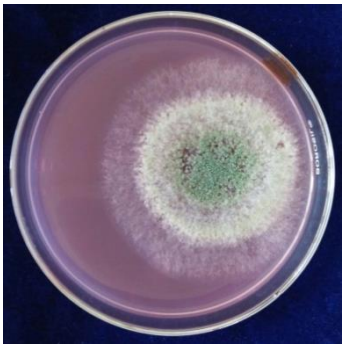
4.3.4 Estimation of population of *T. viride* in different treatments

Population of *T. viride* present in the potting mixture was estimated prior to the application of treatment and at monthly intervals after the application. The method of serial dilution and plating technique was employed to enumerate the population at 10^{-6} dilution and the related data are provided in Table 4.29. Owing to the process of fumigation, population was nil in all the six treatments before its application. One month after application, the maximum population of 3.0 x 10^6 cfu g^{-1} soil was noticed in T_3 (alginate based *T. viride* @ 5.0 g $plant^{-1}$) followed by T_5 (liquid based *T. viride* @ 5 ml l^{-1}) (2.6 x 10^6) and T_4 (talc based *T. viride* @ 20 g l^{-1}) (2.3 x 10^6), whereas the population was found nil in control. During second month, population in T_3 gradually increased to 3.6 x 10^6 cfu g^{-1} soil while, population decreased drastically in T_5 (1.3 x 10^6) and T_4 (0.3 x 10^6) and the population was nil in control. Further, a population of 4.0 x 10^6 cfu g^{-1} soil

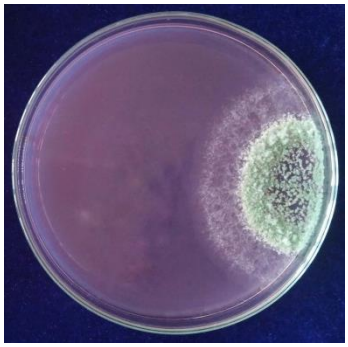
Table 4.29 Estimation of population of *T. viride* in different treatments

Sl. No.	Treatment	*Population of *T. viride* (x10^6 cfu g^{-1})			
		Prior to application	1 MAA	2 MAA	3 MAA
1.	T_1- Alginate based *T. viride* (1.0 g $plant^{-1}$)	0.00	1.3 (1.34)^{c}	1.6 (1.46)^{bc}	2.0 (1.55)^{b}
2.	T_2- Alginate based *T. viride* (3.0 g $plant^{-1}$)	0.00	1.6 (1.44)^{bc}	2.6 (1.76)^{ab}	3.3 (1.95)^{ab}
3.	T_3- Alginate based *T. viride* (5.0 g $plant^{-1}$)	0.00	3.0 (1.85)^{a}	3.6 (2.03)^{a}	4.0 (2.11)^{a}
4.	T_4- Talc based *T. viride* (20 g l^{-1})	0.00	2.3(1.67)^{abc}	0.3 (0.87)^{d}	0.3 (0.87)^{c}
5.	T_5- Liquid based *T. viride* (5 ml l^{-1})	0.00	2.6(1.77)^{ab}	1.3 (1.34)^{c}	0.6 (1.05)^{c}
6.	T_6-Control	0.00	0.0 (0.70)^{d}	0.0 (0.70)^{d}	0.0 (0.70)^{c}
CD(0.01)			0.56	0.55	0.61

* Mean of three replications
In each column figures followed by same letter do not differ significantly according to DMRT
Log transformed values are given in parenthesis
MAA - Months after application

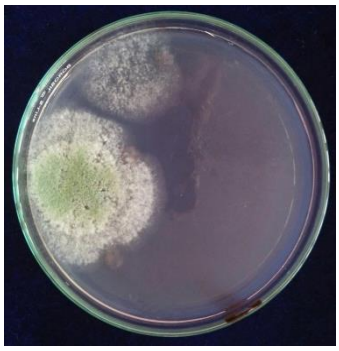


T1



T2

T3



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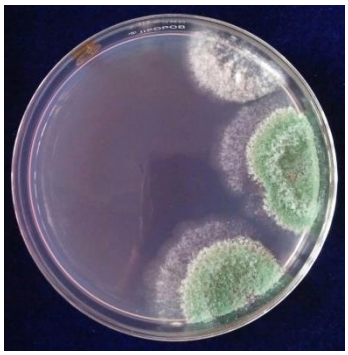

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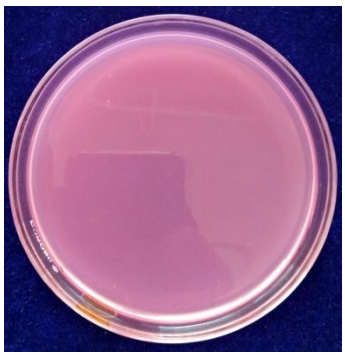
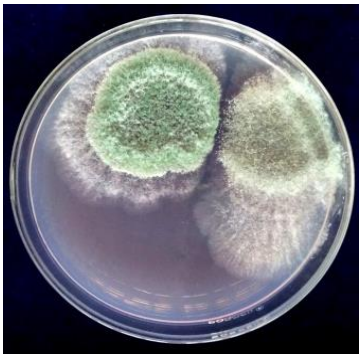
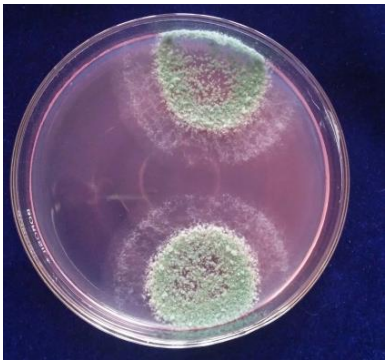


Control

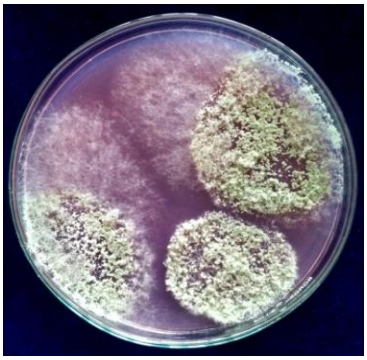
Plate 4.15a Population of *T. viride* from treated pots during first month



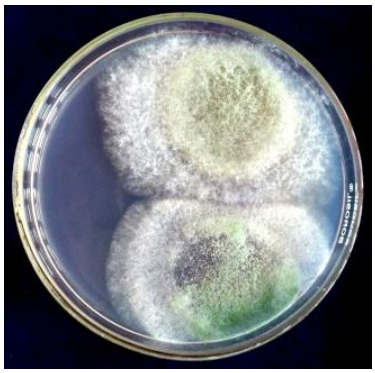
T1



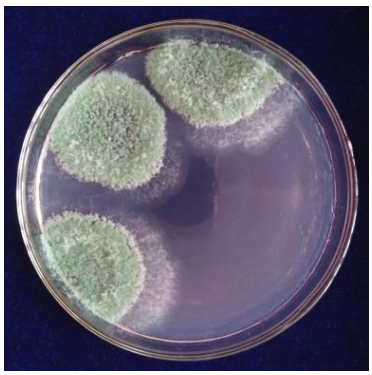
T2



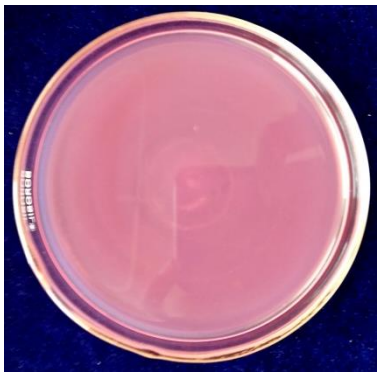
T3



T4

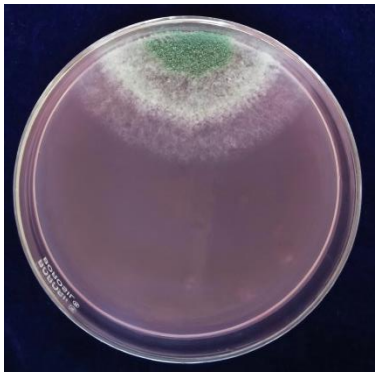


T5

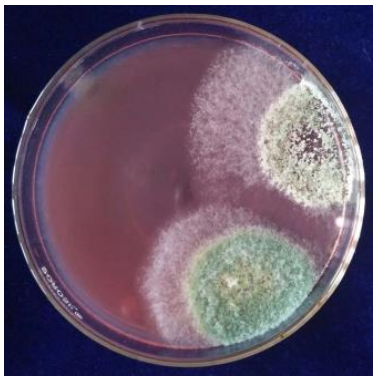


Control

Plate 4.15b Population of *T. viride* from treated pots during second month

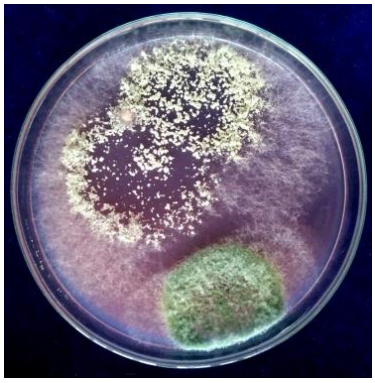


T1



T2

T3



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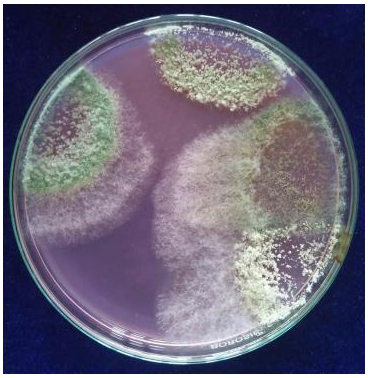
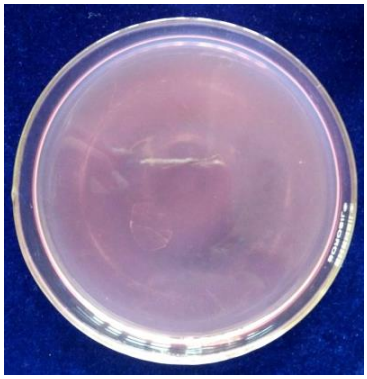


T5



Control

Plate 4.15c Population of *T. viride* from treated pots during third month

was recorded in T_3, whereas the population was further reduced to 0.6 x 10^6 and 0.3 x 10^6 cfu g^{-1} soil in T_5 and T_4 respectively at the end of third month. From the observations, it is clear that sodium alginate bead based formulation showed a gradual increase in the population of *T. viride* compared to talc and liquid based formulations.

4.4 ASSESSMENT OF BIODEGRADATION OF *Trichoderma viride* ENCAPSULATED ALGINATE BEADS IN SOIL

Evaluation of biodegradation of *T. viride* encapsulated microbeads was carried out for the six treatments T_1-T_6 and the observations were recorded at three days interval upto 12 days on completely, partially and non degraded beads and the per cent degradation of microbeads were also calculated as shown in Table 4.30 and Table 4.31.

In general, it was observed that as number of days increased from 3 to12, number of partially degraded beads decreased in number while the number of completely degraded beads increased in number. On third day, no beads were observed without erosion in any of the treatments. Further, a maximum of 37.33 partially degraded beads were observed in T_2 (trehalose, PVP, CMC, tween 80, sodium alginate - 1.5%, $CaCl_2$ - 3%) and control followed by T_1 (trehalose, PVP, CMC, tween 80, sodium alginate - 1.5%, $CaCl_2$ - 2.5%) (33.66) and the minimum number was recorded in T_4 (trehalose, PVP, CMC, tween 80, sodium alginate - 0.75%, $CaCl_2$ - 2.5%). However, maximum number of completely degraded beads was documented in T_4 (15.66) closely followed by T_5 (trehalose, PVP, CMC, tween 80, sodium alginate - 0.75%, $CaCl_2$ - 3%) (14.33) and the minimum of 2.66 was observed in T_2 and control. On sixth day, maximum number of partially degraded beads was recorded in control (T_6) (30.00) followed by T_2 (25.00) and the minimum number was in T_5 (7.66). The maximum number of completely degraded beads was observed in T_5 (32.33) closely followed by T_4 (32.00) while, the minimum number was recorded in control (10.00). Likewise on ninth day, maximum number of partially degraded beads was noticed in control (27.66) followed by T_2 (19.00) and minimum in control (0.00). However, all the 40 beads were completely degraded in T_5 and 39.66 beads were completely degraded in T_4, whereas, only 12.33 beads were completely degraded in control. Similarly, on twelfth day, a maximum of 16 partially degraded beads were recorded in control followed by 10.33 in T_2 and partially degraded beads was nil in T_5. On the same day, maximum number of

completely degraded beads were observed in T_5 (40.00) closely followed by T_4 (39.66) and the minimum number of fully degraded beads were noticed in control (24.00).

Moreover, from the Table 4.31, it is clear that on the third day, maximum per cent degradation of microbeads were recorded in T_4 (39.16%) followed by T_5 (35.83%) and the minimum per cent degradation of 6.66 per cent was noticed in T_2 and control. Likewise, on sixth day, maximum per cent degradation was documented in T_5 (80.83%) closely followed by T_4 (80.00%) and the minimum degradation was noticed in control (25.00). Further, on ninth day, cent per cent biodegradation was observed in T_5 followed by 99.16 per cent in T_4 and the minimum degradation of 30.83 per cent was observed in control. On twelfth day, cent per cent biodegradation was recorded in T_5 followed by T_4 (99.16%) and the minimum per cent degradation was noticed in control (60.00%). From the observations, it was concluded that the rate of biodegradation diminished as the concentration of sodium alginate increased.

4.5 BENEFIT - COST RATIO ANALYSIS

Cost incurred for the production of 1 Kg bead, which can be applied to 66.66 cowpea plants in three split doses @ 5.0 g $plant^{-1}$ was calculated. A total of 1450 ml sodium alginate - additive mixture was required to produce one kilogram bead. For the same, trehalose (7.42 g), PVP (14.5 g), CMC (7.25 g), tween 80 (7.25 ml), sodium alginate (21.75 g) and $CaCl_2$ (50.24 g) was used. The cost of ingredients include trehalose (INR 666.00 Kg^{-1}), PVP (INR 782.00 Kg^{-1}), CMC (INR 1600.00 Kg^{-1}), tween 80 (INR1850.00 mL^{-1}), sodium alginate (INR 1470.00 Kg^{-1}) and $CaCl_2$ (INR463.68 Kg^{-1}). Further, the bottles used for packing amounts to INR 14.16 per piece where four bottles of 300 mL capacity were required. In addition to that, a production cost of INR 46.58Kg^{-1} bead was also accounted and thus, the total cost of production was estimated to be INR 200 Kg^{-1}bead. The cost of talc based formulation required to treat 66.66 cowpea plants in three split doses @ 20.0 g $plant^{-1}$was approximately INR 419.95. Thus, the benefit cost ratio was around 2.09:1.

Table 4.30 Estimation of biodegradation of microbeads

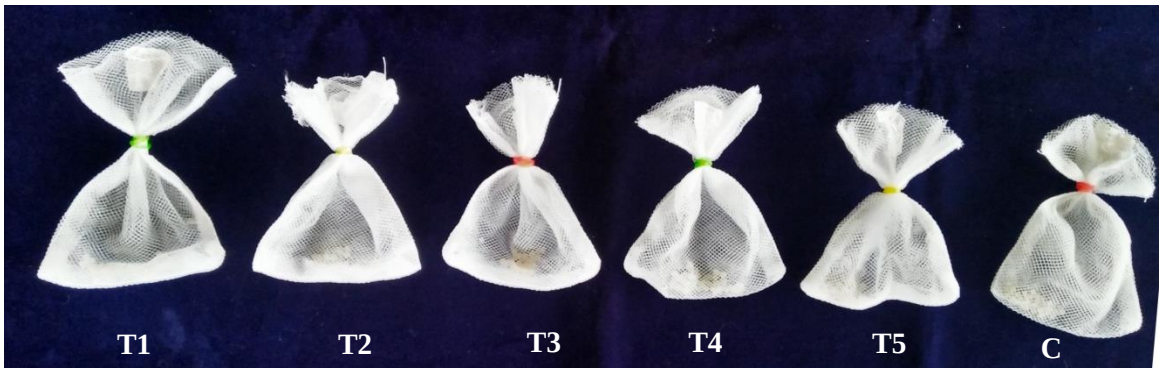
Sl. No.	Treatment	No. of degraded beads							
		Day 3		Day 6		Day 9		Day 12	
		Partially	Completely	Partially	Completely	Partially	Completely	Partially	Completely
1.	T_1	33.66^{ab}	6.33^{bc}	19.66^{c}	20.33^{b}	17.00^{b}	23.00^{b}	9.00^{b}	31.00^{b}
2.	T_2	37.33^{a}	2.66^{c}	25.00^{b}	15.00^{c}	19.00^{b}	21.00^{b}	10.33^{b}	29.66^{b}
3.	T_3	31.33^{b}	8.66^{b}	22.33^{bc}	17.66^{bc}	17.66^{b}	22.33^{b}	9.33^{b}	31.33^{b}
4.	T_4	24.33^{c}	15.66^{a}	8.00^{d}	32.00^{a}	0.33^{c}	39.66^{a}	0.33^{c}	39.66^{a}
5.	T_5	25.66^{c}	14.33^{a}	7.66^{d}	32.33^{a}	0.00^{c}	40.00^{a}	0.00^{c}	40.00^{a}
6.	T_6 (control)	37.33^{a}	2.66^{c}	30.00^{a}	10.00^{d}	27.66^{a}	12.33^{c}	16.00^{a}	24.00^{c}
CD (0.01)		5.33	5.33	5.19	5.19	4.00	4.00	4.07	4.07

* Mean of three replications. In each column figures followed by the same letter do not differ significantly according to DMRT.

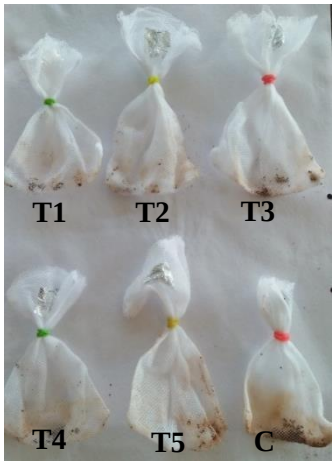
Table 4.31 Per cent degradation of microbeads

Sl. No	Treatment	*Per cent biodegradation			
		Day 3	Day 6	Day 9	Day 12
1.	T_1	15.83	50.83	57.50	77.50
2.	T_2	6.66	37.5	52.50	74.16
3.	T_3	21.66	44.16	55.83	78.33
4.	T_4	39.16	80.00	99.16	99.16
5.	T_5	35.83	80.83	100.00	100.00
6.	T_6 (control)	6.66	25.00	30.83	60.00

* Mean of three replications



Beads in nylon bags buried for degradation study



Beads in nylon bags after pulling out from the pots

Beads after being pulled out from pots (T_{21})

Plate 4.16 Estimation of biodegradation of microbeads

DISCUSSION

5. DISCUSSION

The application of agriculturally important microorganisms are extensively promoted in crop health management. Among the diverse groups of organisms, *Trichoderma* spp. are well known for its potential biocontrol activity along with induction of stress tolerance and promotion of plant growth. The antagonistic potential of these bioagents are principally attributed to phenomena like hyperparasitism (Weindling, 1932), antibiosis (De Marco *et al.*, 2003), rhizosphere competence and potential tools like enzymes (Viterbo *et al*., 2002).

However, the unpredictable and drastic variations in global climate turned even the minor crop diseases into major, causing deficit food supply for the growing world population. In this scenario, the increase in food production is demanded as the only viable solution. But the era of artificial plant protection chemicals diminished gradually as their hazardous face became more visible and the topic assumed top concern even in day to day discussions. Hence, the principles of biocontrol attained relevance and acceptance among the farming community. However, for the successful establishment of a green agriculture strategy in practical, adequate and uninterrupted inflow of inputs should be ensured. Being an important component, the biocontrol agents should be adequately formulated to facilitate the effective application in the field. But a formulation usually come across several hurdles during the course of development and suffers from many setbacks even after commercial release. Presently marketed talc based powder formulation is suffering from drawbacks like dehydration, poor shelf life and loss of viability of propagules beyond three months of storage (Mukhopadhyay, 1994). Further, Ramanujam *et al*. (2010) remarked the technique of mass multiplication of *Trichoderma* spp. *via* solid state fermentation with cheap cereal grains as laborious as the resultant product is bulky and prone to contamination. Jin and Custis (2011) pointed out the significant drawbacks like dust formation, hazards to environment or users and variable water activity resulting in high microbial contamination and reduced shelf life of wetteble powder formulation of various *Trichoderma* strains. Moreover, Sriram *et al*. (2011)

reported the vulnerability of liquid fermentation based formulations of *Trichoderma* spp. to desiccation, while John *et al*. (2011) recorded quick loss of viability of liquid formulation due to lack of carrier protection. Further, an ecofriendly and cheaply available carrier material is one of the key factors in determining the success of a formulation. Considering all these factors, the present investigation was proposed to encapsulate *T. viride* in sodium alginate, a biodegradable polymer for the management of major soil borne fungal pathogens.

5.1 PRESERVATION OF FUNGAL CULTURE

The reference culture of *Trichoderma viride* was sub cultured and successfully maintained throughout the course of research programme under refrigerated conditions. This is in accordance with the study conducted by Stocco *et al*. (2010), where they reported that the storage of fungal cultures at 4^0C limited the O_2 supply, thereby slowed down the metabolic activities of microorganism and thus, increased the storage period. Similarly, Iqbal *et al*. (2017) documented the techniques of fresh culture and slant preservation, where they observed cent per cent viability of *T. viride* in fresh culture and slant culture even after three months of storage under refrigeration.

5.2 STANDARDIZATION AND PREPARATION OF ALGINATE BEAD BASED FORMULATION OF *Trichoderma viride*

The investigation was initiated with the random selection of seven different additive substances with an aim to improve the shelf life of final product. These seven additive substances belonging to four different classes were grouped in eight combinations and was designated as T_1 - T_8. The selection of a best combination was carried out by growing *Trichoderma* in PD broth amended with these additives and subsequent enumeration through serial dilution and plating technique for seven months. After the selection of additives, an optimum concentration of sodium alginate and $CaCl_2$ was determined after attempting several trials with various concentrations. Other parameters like the volume of conidial suspension of *Trichoderma*, time required for gelation and optimum vertical height between orifice of the separating funnel and level of $CaCl_2$ solution was also standardized accordingly. Subsequently microbeads were prepared in 30 different combinations of sodium alginate, $CaCl_2$, and additives, besides a

control without any additives. Further, these beads were subjected to characterization with respect to various physical properties.

5.2.1 Standardisation of media with additives and its effect on shelf life of *T. viride*

Enhanced shelf life is one of the prime objective as well as the challenge in the biocontrol research. Accordingly, additives like mannitol (2%), trehalose (15mM), polyvinylpyrrolidone (1%), polyethylene glycol (0.25%), carboxymethyl cellulose (0.5%), liquid paraffin (1%) and tween 80 (0.5%) were evaluated in the present investigation to study their impact on shelf life of the formulation. Among the eight different combinations, trehalose combined with PVP, CMC and tween 80 was found to yield the maximum colony count even at seventh months of evaluation. The colony count ranged from 23.0 x 10^{6} - 45.0 x 10^{6} cfu ml^{-1}. Viability of the organism was found to increase upon the amendment of given additives.

According to Poosapati *et al*. (2014), *T. asperellum* TaDOR673 showed higher rate of germination and production of biomass upon accumulation of trehalose resulted from prolonged exposure to 37^{0}C, where trehalose served as a heat stress protectant. Moreover, Cliquet and Jackson (1999) reported that the spores of *T. harzianum* accumulated more trehalose when grown at a low water potential. Lee *et al*. (2017) observed that *T. harzianum* KUC1716 was morphologically modified to produce more enzymes by PEG, PVP and tween 80, thus facilitated accessibility to variety of nutrients. They recorded 142 and 177 per cent cellulase production with 0.5 per cent PVP and 1 per cent tween 80 respectively. Surfactants increased the surface area for nutrient absorption by dispersing the mycelia. Reese and Maguire (1969) also reported that secretion of exoprotein and cellulase by microorganism was stimulated by non-ionic surfactants.

According to several literature, integration of some extra components *viz.*, clay, skim milk, chitosan, sugars, gum acacia, or maize starch into the alginate matrix were shown to improve the properties of microcapsules (Bashan, 1998; Krasaekoopt *et al*., 2004). Similarly, the spore viability enhancing property of starch was identified by Locatelli *et al*. (2018). According to Adzmi *et al*. (2012), the filler materials like montmorillonite reinforced the alginate hydrogel network and filled the interstitial voids,

Fig 5.1a Population of *T. viride* (x10^{6}cfu ml^{-1}) at monthly intervals

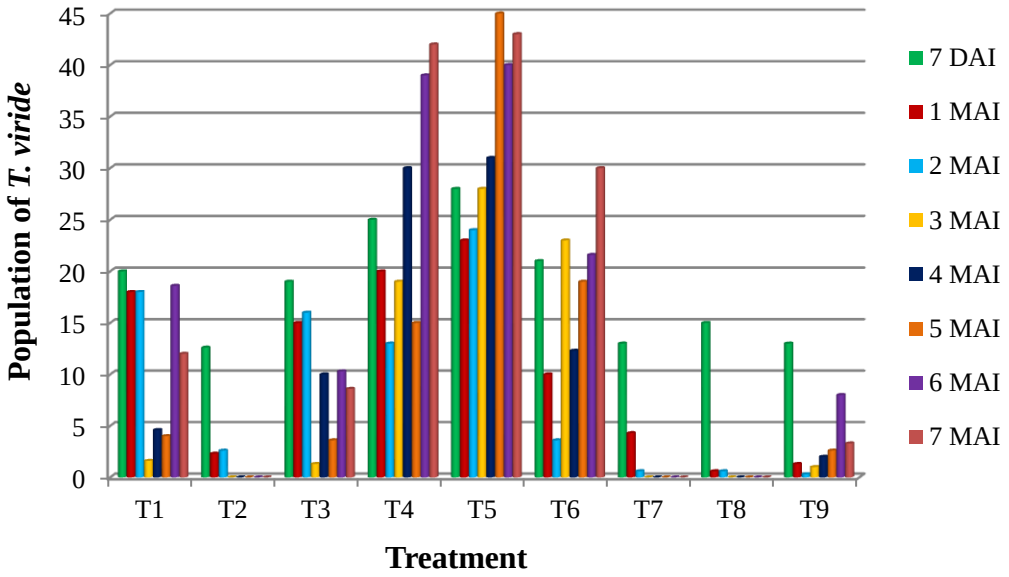
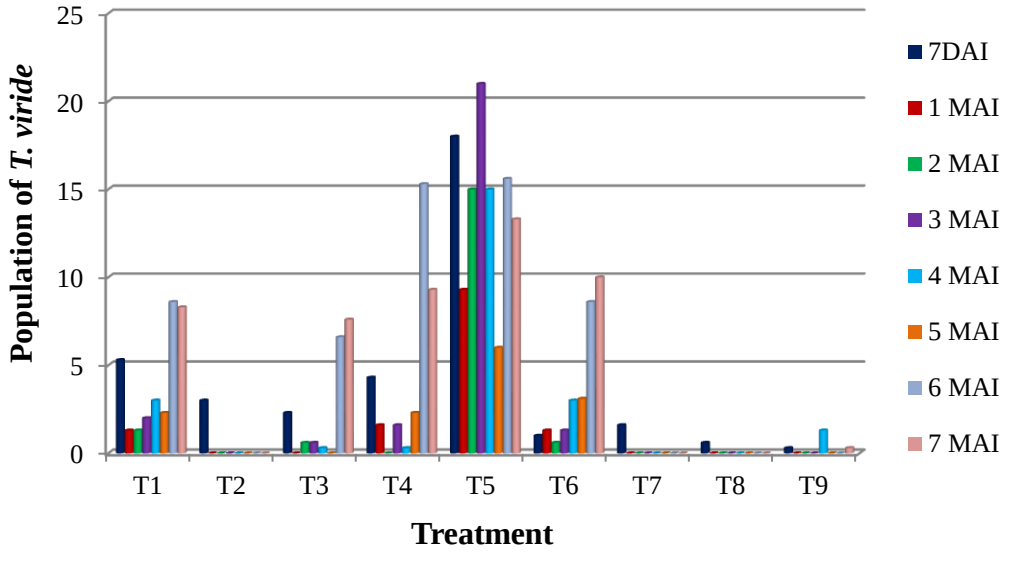


Fig 5.1b Population of *T. viride* (x10^{8} cfu ml^{-1}) at monthly intervals



resulted in improved of sphericity, flowability, density, visual quality and rigidity of the beads. However, while working with additives like chitosan, peat powder and skim milk, Szczech and Maciorowski (2016) observed that the skim milk had deteriorating effect on microbeads. They concluded that the composition of wet microcapsules had prominent impact on the shelf life and the degree of contamination.

The research conducted by Metz *et al.* (2009) detected the presence of a gene that encodes D-Mannitol dehydrogenase in *Trichoderma* sp. While dealing with a liquid formulation of *T. viride,* Sathyaseelan *et al*. (2009) observed the enhanced shelf life with paraffin oil. In the present investigation, trehalose in combination with liquid paraffin, PVP and tween 80, colony count reduced from 13.0 x 10^6 cfu ml^{-1}at seven days after inoculation (DAI) to 0.6 x 10^6 (60 DAI). Knudsen *et al*. (1991) observed that PEG (polyethylene glycol) amended alginate pellet formulation of *T. harzianum* promoted hyphal proliferation in soil but not the conidial multiplication. In the present investigation, when trehalose combined with PEG, CMC, and tween 80, colony count ranged from 3.6 x 10^6 - 30.0 x 10^6 ml^{-1} in potato dextrose broth.

5.2.2 Standardization of different parameters for bead preparation

Type and quality of microbeads highly depends on experimental conditions. Smrdel *et al*. (2008) also emphasized the influence of formulation and processing parameters on the properties of beads prepared through inotropic gelation. Moreover, Huang and Lin (2017) revealed that properties like shrinkage, tightness, release behaviour and swelling of alginate beads are inter-related. In addition, Chan *et al.* (2011) observed that the conidial viability of encapsulated *T. harzianum* UPM40 was closely associated with the bead properties.

Therefore, to obtain elite quality beads, experimental parameters *viz.*, concentration of sodium alginate and calcium chloride, volume of *T. viride* spore suspension required per unit volume of sodium alginate solution and the optimum vertical distance between the orifice of the funnel to surface of $CaCl_2$ bath were standardized.

5.2.2.1 Concentration of sodium alginate and calcium chloride

Concentration of sodium alginate is one of the important factors in determining bead quality. In the present investigation, good quality, spherical beads were formed at 2.5, 3.0 and 3.5 per cent concentrations of sodium alginate without any additives and no beads were formed with additives. As additives were added, viscosity of the solution increased and the resultant solution extruded as continuous stream, resulting in the formation of tailed beads or alginate worms. Therefore, sodium alginate at 0.75 and 1.5 per cent concentration was selected for bead preparation with additives. Sodium alginate solution at these two concentrations along with the specified quantity of additives yielded spherical beads with smooth texture.

Similar line of work was recorded by De Jaeger *et al*. (2011), where they carried out the co entrapment of *T. harzianum* and *Glomus* sp. in two per cent sodium alginate polymer. Similarly, while encapsulating *T. asperellum* strain TTH1, Thilagavathi *et al*. (2015) also used two per cent (w/v) sodium alginate solution. Apart from that, for the microencapsulation of *T. virens* TRS106, Szczech and Maciorowski (2016) selected two per cent sodium alginate solution. However, according to Tal *et al.* (1997), defects like uneven sizes, distorted shapes, high porosity or poor mechanical strength of microbeads may ruin the viability and stability of entrapped bioagent during storage. From the present experiments, it was noted that a reduced concentration of sodium alginate (0.75%) in the beads caused higher degree of shrinkage which gradually resulted in beads with uneven size, distorted shape and poor mechanical strength. On the other hand, Khazaeli *et al*. (2008) observed that the increased alginate concentration resulted in high viscous solution, which in turn produced larger beads with limited diffusivity. Moreover, an increased concentration may result in high viscous polymer mixture with numerous bubble forming highly porous beads. These air pockets in the bead reduce the efficiency of encapsulation. Considering all these aspects, treatment T_{21}, with trehalose (15 mM), PVP (1%), CMC (0.5%), tween 80 (0.5%), $CaCl_2$ (3.5%) and sodium alginate (1.5%) was selected.

Sufficient strength of cross linking agent is inevitable to ensure mechanical

strength and stability of microbeads. In the present study, calcium chloride ($CaCl_2.2H_2O$) solution was prepared in three randomly selected concentrations *viz.,* 2.5, 3.0 and 3.5 per cent. The freshly prepared solution, immediately after being sterilized (121^0C temperature and 15 psi pressure for 20 min) and cooled was used for bead preparation. Solution at all the three specified concentrations yielded good quality spherical beads. Among the three concentrations, best bioagent release profile was recorded in 3.5 per cent. Hence $T_{21,}$ the bead with 3.5 per cent $CaCl_2$ was selected.

El-Zatahry *et al.* (2006) stated that the adequate concentrations of divalent cations in the crosslinker lead to compact beads and little structural rearrangements to cause shrinkage. Similarly, El-Kamel *et al*. (2003) also recorded highly cross-linked beads with rigid gel network and sustained release characteristics at 3 per cent $CaCl_2$ (w/v). Using 3 per cent (w/v) $CaCl_2$ solution, Mandal *et al*. (2010) also recorded highly cross linked beads with rigid network and sustained drug release.

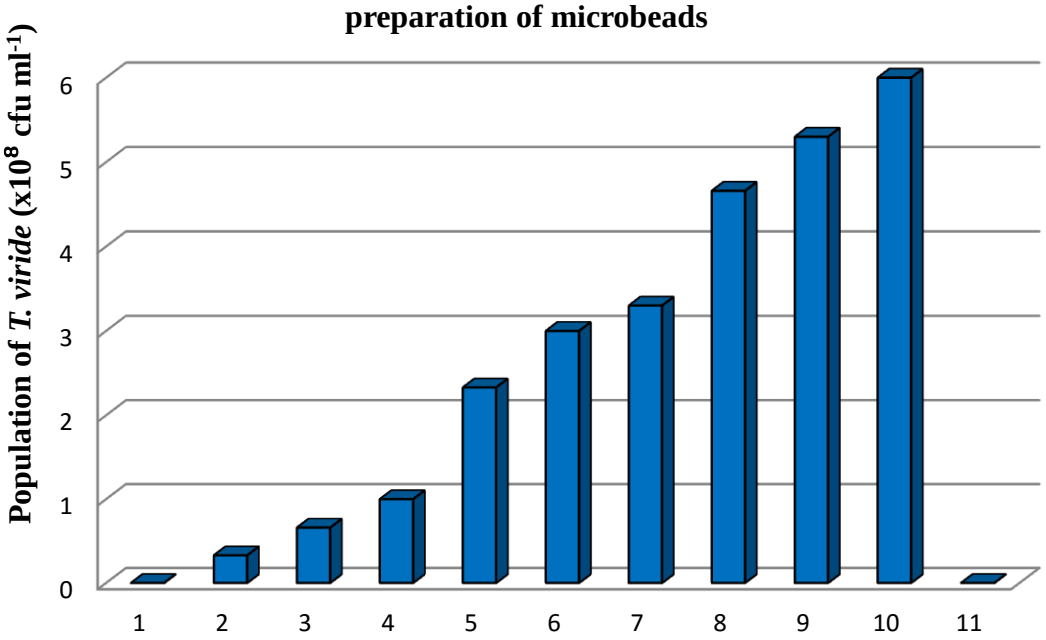
5.2.2.2 Preparation of spore suspension of T. viride

In the present study, conidial suspension was prepared by macerating the well sporulated, one week old mycelial mat of *T. viride*. The suspension was further sieved to separate out the mycelial bits. Similar line of work was recorded by Navaneetha *et al*. (2015), where the conidia of *T. harzianum* Th4d and *T. asperellum* Tv5 was collected from one week old culture by gentle scraping with a sterile transfer loop and subsequent filteration through a double layered, sterile muslin cloth.

5.2.2.3 Standardization of spore suspension of T. viride in sodium alginate solution

To determine the optimum volume of conidial suspension required for the bead preparation, a total of 10 treatments with 1 - 10 ml conidial suspensions were laid out and they were designated as T_1 - T_{10}. Subsequently, microbeads were prepared and these beads were dissolved in phosphate buffer (2M, pH 7) and subjected to serial dilution and plating. At 10^8 dilution, no colony forming units were observed in T_1 at 10^8 dilution, whereas 0.3 x 10^8 and 0.6 x 10^8 cfu g^{-1} bead was noted in T_2 and T_3 respectively. Further, 1.0 x10^8 cfu g^{-1} bead was obtained in T_4 and 2.3 x 10^8 cfu g^{-1} bead was recorded in $T_{5.}$ Further, a population ranged from 3.0 - 6.0 x 10^8 cfu g^{-1} bead was recorded in treatments T_6 - T_{10}.

Fig. 5.2 Standardization of spore suspension of *T. viride* for preparation of microbeads



According to Thilagavathi *et al*. (2015), 10^8 conidia ml^{-1} suspension is the optimum conidial count for bead preparation. Szczech and maciorowski (2016) also made similar observations, where they recorded the conidial count as 10^8 conidia ml^{-1} suspension. Further, Bashan *et al.* (2002) revealed that a portion of bioagent population got killed by the process of crosslinking. Moreover, Knudsen *et al*. (1991) reported that the *Trichoderma* population did not multiply in alginate formulations. Therefore, the initial inoculum should contain sufficient number of bioagent propagules to tackle any unforeseen losses.

Considering the above mentioned literatures, T_5 with an optimum colony count of 2.3×10^8 cfu g^{-1} bead was selected for bead preparation.

5.2.2.4 Estimation of optimum height of air column

The vertical distance between orifice of the separating funnel and level of $CaCl_2$ solution in the beaker was significant in determining the shape of the beads. According to NASA (2012), a raindrop assumes spherical shape at the point of fall due to increased surface tension and subsequently flattens as it falls down. Moreover, depending on the speed of fall, drop assumes classical tear shape. Owing to friction and air turbulence, the tail portion of drops gets separated off and resulting droplets becomes spherical due to increased surface tension. In effect, the phenomena of fluid mechanics rule the bead shape.

In the present investigation, exact spherical beads were recorded at 6 - 35 cm air column height, through which the polymer drop fall. Along with that, very minute beads were also formed. This may be due to the splitting off of the tail portion of pyriform shaped sodium alginate droplets subjected to air turbulence and friction. However, when the distance was adjusted to 2 cm or 4 cm, tailed or pyriform beads were formed without any minute beads. Eventhough the formation of these minute beads caused yield losses, it was inevitable to maintain the spherical shape of the bead. Considering the factors such as bead shape and the convenience of handling of the apparatus, a vertical height range of 8 - 12 cm was selected for bead preparation.

Similar observations were made by Smrdel *et al*. (2008). They reported that the

shape of wet microbeads were significantly influenced by the distance between the top of polymer dispensing needle and the level of hardening solution along with the stirring speed of the hardening solution. It was also noticed that, flattened beads were formed at a column height more than 6 cm. This may be due to the combined effect of vertical distance and stirring speed. Therefore, the height of 6 cm was selected along with 600 rpm stirring speed of the hardening solution.

5.2.3 Preparation and characterization of microbeads

Trichoderma entrapped sodium alginate beads were prepared for 31 treatments including control and the beads were subjected to characterization with respect to physical properties like bead weight and bead size along with bead yield.

5.2.3.1 Preparation of microbeads

Deasy (1984) suggested that the gelling property of alginate and cross linking nature of divalent metals contribute to alginate bead formation in drug delivery systems. In the present investigation, sterile water was used for the preparation of sodium alginate solution. Further, the $CaCl_2$ solution was subjected to sterilization immediately after preparation. Moreover, sterile distilled water was used for washing the beads after being cured.

The results of the study are in congruence with the findings of Thilagavathi *et al*. (2015), where they also provided similar conditions for the sterilization of sodium alginate and $CaCl_2$ solution. Morch *et al*. (2006) reported that the alginate beads were sensitive to anti-gelling cations like sodium and magnesium and chelating agents like EDTA, citrate, phosphate, lactate *etc* and since tap water may contain these cations, distilled water was used for washing the beads, preparation of sodium alginate and $CaCl_2$ solutions. For preparing 4 per cent sodium alginate solution, Smrdel *et al*. (2008) and Das and Senapathi (2008) also used distilled water. In the present investigation, the sodium alginate was melted by keeping on low flame and while melting the ingredients, the solution was continuously stirred to avoid charring. Similarly, Das and Senapathi (2008) reported that the polymer mixture was stirred magnetically while heating gently.

Thorough mixing of sodium alginate solution with *T. viride* conidial suspension left behind numerous bubbles in the mixture. This produced low quality, bubble entrapped beads. Hence, a waiting period of 10 - 15 min was given between mixing of the solution and bead preparation which helped to get rid of bubbles in the mixture. This waiting period should be assigned after transferring the solution into the separating funnel. To meet the similar objective, Patel *et al*. (2016) sonicated the sodium alginate solution for 15 min in an ultrasonicating water bath. While working with the loading of therapeutic agent furosemide in sodium alginate, Das and Senapathi (2008) also sonicated the sodium alginate suspension for 30 min to remove bubbles formed during stirring.

When water soluble sodium alginate comes in contact with $CaCl_2$ solution, immediately water insoluble calcium alginate beads were formed. At the time of formation, the beads were transparent and were found floating in $CaCl_2$ bath. Velings and Mestdagh (1995) also observed the same phenomenon, where they reported that beads were formed immediately when sodium alginate comes in contact with $CaCl_2$ or CuCl solution. They observed that the beads floated on the top of the solution few minutes after formation, and gradually sank due to an increase in the density. They found that this phenomenon depends on the type and concentration of cation. According to them, these gel systems evolved slowly with time, most probably because crosslinking reactions takes place during the gel formation and proceed progressively from the surface to the interior of the bead. Simultaneously, a loss of water *ie*, syneresis, occurs and hence these systems are therefore not in true equilibrium.

As the time progressed, an inwardly moving opaque zone became visible, probably due to the progress in gelation. Finally, beads began to sink in the $CaCl_2$ bath and completely became opaque. Thu *et al*. (1997) also reported the formation of a polymer network on binding of divalent cation with alginate. Further, they observed a centripetally moving gelling zone, where they inferred that it was the alginate which moved from gel core towards gelling zone. Tous *et al*. (2014) also described that the process of gelation is visually detectable on the appearance of translucent circular boundary which gradually shrank with the progression of curing and finally disappeared.

5.2.3.2 Drying and storage of microbeads

Drying technique could influence several bead characteristics such as size (Gal and Nussinovitch, 2007; George and Abraham, 2007; Fundueanu *et al*. 1999), shape, mechanical properties (Gal and Nussinovitch, 2007) and swelling properties (Gal and Nussinovitch, 2007; George and Abraham, 2007). Smrdel *et al*. (2008) also stated that the mode of drying may influence the size, shape and morphology of beads.

In the present investigation, the prepared beads were dried open at room temperature for a period of 48 h and it was observed that in all treatments, the beads turned ivory colour and coherent. The reason can be attributed to the evaporation of water from the beads. These findings are in congruence with the observations of Smrdel *et al*. (2008), where they observed significant shrinking and change in shape of microbeads during air drying at room temperature and fluidized-bed-drying. On the other hand, the freeze-dried beads became more porous and highly resistant to shrinking and the change in shape was probably due to faster sublimation. Das and Senapathi (2008) reported that the open dried beads were bigger due to partial evaporation than oven dried beads. However, complete evaporation caused cracking of the beads. In the present study, the visual observation evidenced that the surface of bead is rough and was found less glossy after 48 h of open drying. This is in accordance with the study conducted by many researchers, where they reported that the bead surface is rough with cracks and fissures and even traces of unreacted $CaCl_2$ residues were noticed (Sherina *et al*., 2012; Bajpai and Kirar, 2016; Tous *et al*., 2014). Moreover, Bajpai and Kirar (2016) also substantiated that the drying of any cross linked matrix resulted in the development of cracks and fissures.

In the current study, dried microbeads were stored at room temperature in sterile, empty jam bottles. Further, De Jaeger *et al*. (2011) maintained *T. harzianum* and *Glomus* sp. entrapped microbeads at 4^0C in a Petri plate. Adzmi *et al.* (2012) dried the microcapsules containing *T. harzianum* UPM40 at 28 - 32^0C for 24 h, which was then stored in bottles and was maintained at 5^0C and 30^0C. Thilagavathi *et al*. (2015) stored *T.*

asperellum strain TTH1 encapsulated microbeads at 4^0C. Likewise, Szczech and Maciorowski, (2016) stored the wet microcapsules of *T. virens* TRS106 in sterile, closed jars at 4 °C. Similar line of work was recorded by Locatelli *et al.* (2018), where the conidial viability of *Trichoderma* was retained for 14 months, even when starch amended sodium alginate granular formulation of *Trichoderma* was maintained at room temperature.

5.2.3.3 Bead weight

The individual weights of 10 randomly selected beads were estimated to obtain mean bead weight. The average bead weight ranged between 22.84 ± 4.1 mg (T_4) to 96.20 ± 1.8 mg (control). It was noticed that weights of beads were directly proportional to concentration of sodium alginate and $CaCl_2$. Adzmi *et al*. (2012) estimated the average weight of *T. harzianum* UPM40 encapsulated sodium alginate (3%) beads as 5.64 mg.

5.2.3.4 Bead yield

Bead yield is one of the most important aspects from commercial perspective. According to Islam *et al*. (1997), chemical compositions of additives play an important role in bead yield. They stated that an additive polymer with high degree of branching, heterogeneity and elaborate molecular structure reduce the bead yield. It is expected that a linear polymer like sodium alginate is unable to accommodate another highly branched polymer within them. Similar findings were reported by Street and Anderson (1983). Krasaekoopt *et al.* (2004) reported that the amalgamation of additives along with alginate matrix during encapsulation had substantial effect on bead yield and particle size. Similarly, El-Kamel *et al*. (2003) also emphasized significant impact of additional components incorporated with alginate matrix on the yield and size of resulting capsules.

In the present work, bead yield was estimated in terms of percentage of ingredients that converted into the final product. The obtained yield ranged from 24.51 (T_4) to 69.09 per cent (T_{19}). Maximum number of beads formed per ml of sodium alginate solution was 26.91 (T_9), which was 200 per cent more than that of control. In

control, only eight beads were formed from each ml solution. From the observation, it is clear that the incorporation of additives improved the bead yield substantially.

The results are in line with the findings of Sherina *et al*. (2012), where they quantified the yield of drug loaded sodium alginate bead within the range of 74.5 ± 3.01 to 92.2 ± 2.55. Similar findings were also documented by Patel *et al*. (2016), where they recorded 24.9 to 93.6 per cent yield from drug loaded alginate beads. Szczech and Maciorowski (2016) observed the noticeable influence of additives blended in alginate matrix on the yield and size of beads. They recorded 60 per cent increase in yield of beads with peat and 42 per cent with chitosan. They also reported that the type of bioagent to be encapsulated also had an impact on the bead size and productivity.

5.2.3.5 Bead size

The size or diameter of the bead is a key factor in determining bead quality. Sun *et al.* (1989) detected that 80 per cent of the bead volume remained unoccupied by aerobic bioagents due to the limited O_2 diffusion. Bashan *et al.* (2002) also recognized the limited O_2 supply as the major disadvantage of microbeads. However, Szczech and Maciorowski (2016) reported that the reduction in bead size increased the surface area and favoured efficient penetration of oxygen, nutrients and metabolites. This in turn enhanced the cell viability. Further, Ogbonna *et al*. (1991) confirmed 0.1 - 0.3 mm as the optimum bead size for efficient diffusion of oxygen in different polymeric beads.

Bead diameter is greatly influenced by several factors. El-Kamel *et al*. (2003) found that the components incorporated into alginate matrix during encapsulation had a significant effect on the size of the capsules. They also found that the bead size reduced, as the concentration of crosslinking agent increased. Obviously diameter of the nozzle used in the bead preparation affect the bead diameter (Smrdel *et al*., 2008). Moreover, Szczech and Maciorowski (2016) pointed out that the type of encapsulated bioagent had an impact on the capsule size. Another factor found to affect the bead diameter was the method of drying. Smrdel *et al*. (2008) identified the influence of the method of drying on bead size. They documented that air drying at room temperature and fluidized-bed-

drying resulted in significant shrinking of microbeads. On the other hand, freeze-dried beads became more porous and highly resistant to shrinking due to sublimation rather than evaporation. In contrary, Das and Senapathi (2008) documented that air drying lead to partial evaporation of water, resulting in bigger beads compared to oven dried beads.

In the present investigation, bead diameter was measured using digital microscopy software motic image plus 2.0 and a stereo microscope. The maximum size of microbeads (2.42 ± 0.102 mm) was recorded in control, where the concentration of sodium alginate was 2.5 per cent. At 1.5 per cent sodium alginate solution, bead size ranged from 1.54 ± 0.079 (T_{19}) to 1.92 ± 0.098 (T_9) mm whereas it ranged from 1.31 ± 0.010 (T_{22}) to 1.76 ± 0.039 mm (T_{12}) at 0.75 per cent of sodium alginate. Since the concentration of sodium alginate in all the treatments (T_1-T_{30}) were lower (0.75 - 1.5%) compared to control (2.5%), the bead diameter was also less in treatments T_1 - T_{30} compared to control. It was concluded that the bead diameter was directly proportional to concentration of sodium alginate.

The present findings are also in line with the report of Sherina *et al*. (2012), where they observed the average bead diameter ranging from 1.10 ± 3.3 - 1.34 ± 1.6 mm and inferred that the particle size of micro beads were directly proportional to alginate concentration. Similar reports were also putforth by Patel *et al*. (2016), where they recorded various formulations of drug loaded alginate beads with an average particle size ranging from 1.04 ± 0.20 to 2.15 ± 0.356 mm. Das and Senapathi (2008) recorded larger drops at higher relative viscosity of polymer solution. Khazaeli *et al.* (2008) also observed that high viscous solutions resulted from increased alginate concentrations, which produced larger beads with limited diffusivity. The findings of the present investigation are also comparable with the studies of Adzmi *et al*. (2012), where they documented the average diameter of *T. harzianum* UPM40 encapsulated beads as 1.68 mm. Szczech and Maciorowski (2016) estimated the mean diameter of peat and chitosan amended microbeads as 0.05 mm. They also emphasized the impact of the nature of encapsulated microorganism on bead size. Zommere and Nikolajeva (2017) reported the mean diameter of bacterial consortia entrapped microbead as 2.00 mm. Likewise, Bashan *et al*. (2002) recorded the mean diameter of *A. brasilense* as 0.001-1.00 mm.

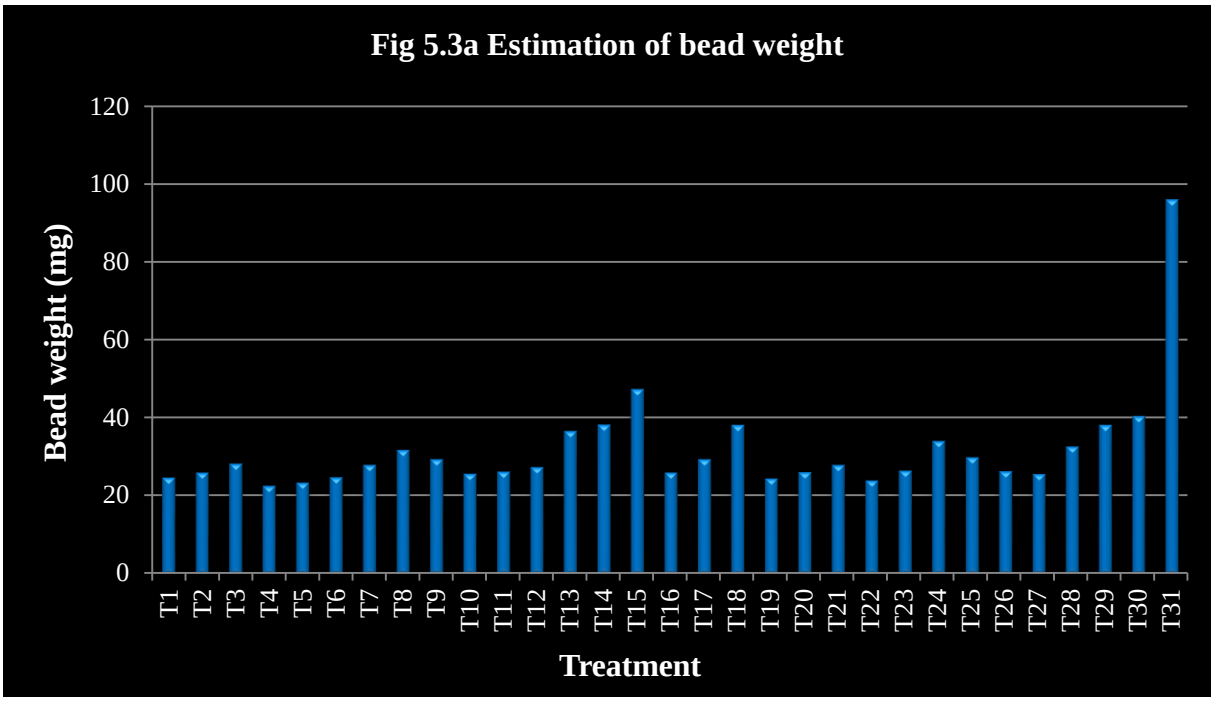


Fig 5.3a Estimation of bead weight
Bead weight (mg)
120
100
80
60
40
20
0
T1 T2 T3 T4 T5 T6 T7 T8 T9 T10 T11 T12 T13 T14 T15 T16 T17 T18 T19 T20 T21 T22 T23 T24 T25 T26 T27 T28 T29 T30 T31
Treatment

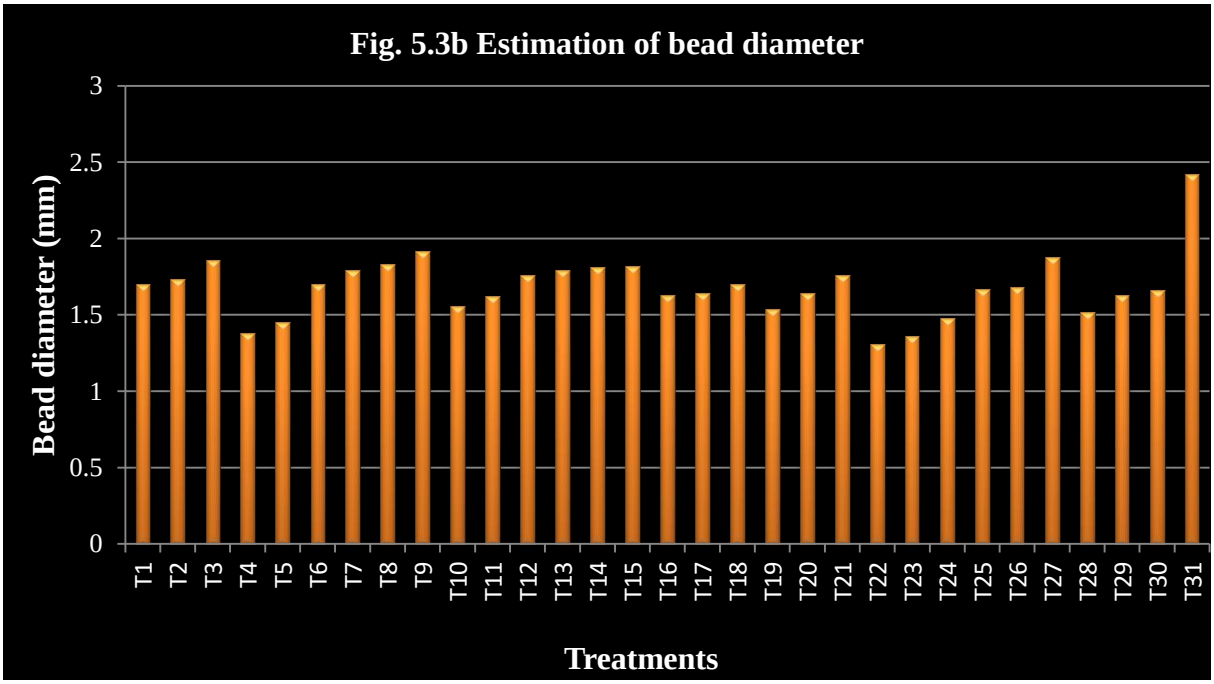


Fig. 5.3b Estimation of bead diameter
Bead diameter (mm)
3
2.5
2
1.5
1
0.5
0
T1 T2 T3 T4 T5 T6 T7 T8 T9 T10 T11 T12 T13 T14 T15 T16 T17 T18 T19 T20 T21 T22 T23 T24 T25 T26 T27 T28 T29 T30 T31
Treatments

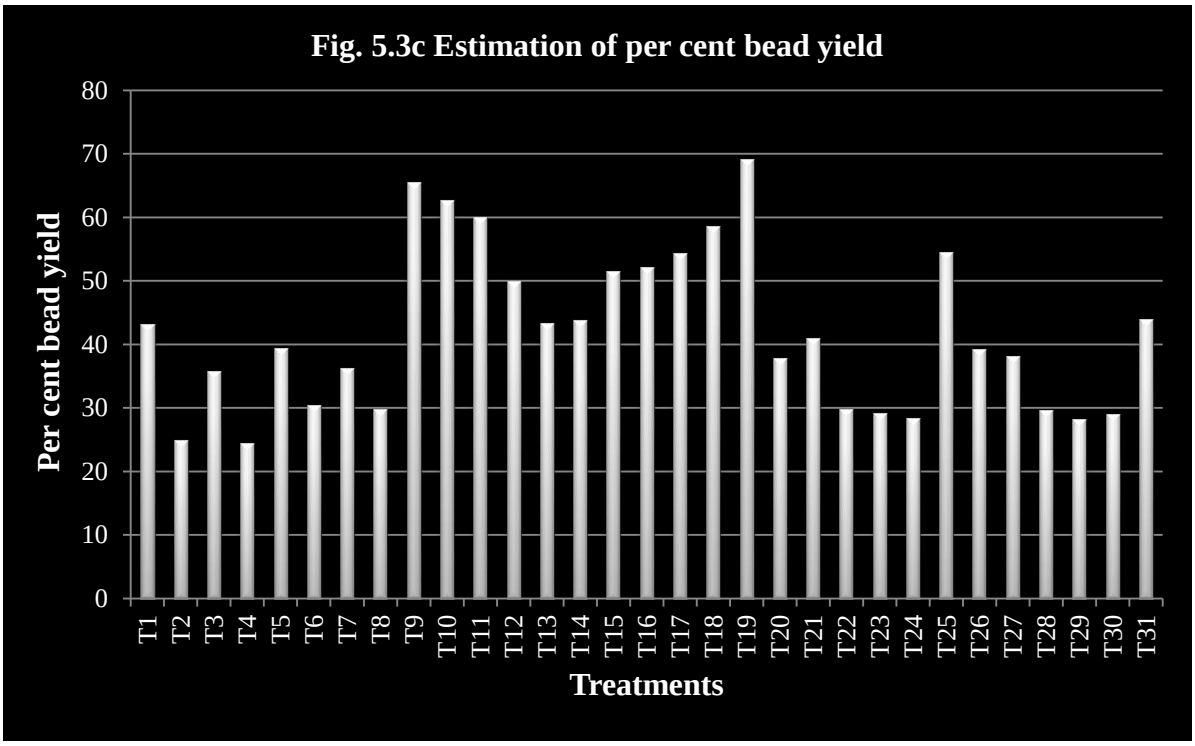


Fig. 5.3c Estimation of per cent bead yield
Per cent bead yield
80
70
60
50
40
30
20
10
0
T1 T2 T3 T4 T5 T6 T7 T8 T9 T10 T11 T12 T13 T14 T15 T16 T17 T18 T19 T20 T21 T22 T23 T24 T25 T26 T27 T28 T29 T30 T31
Treatments

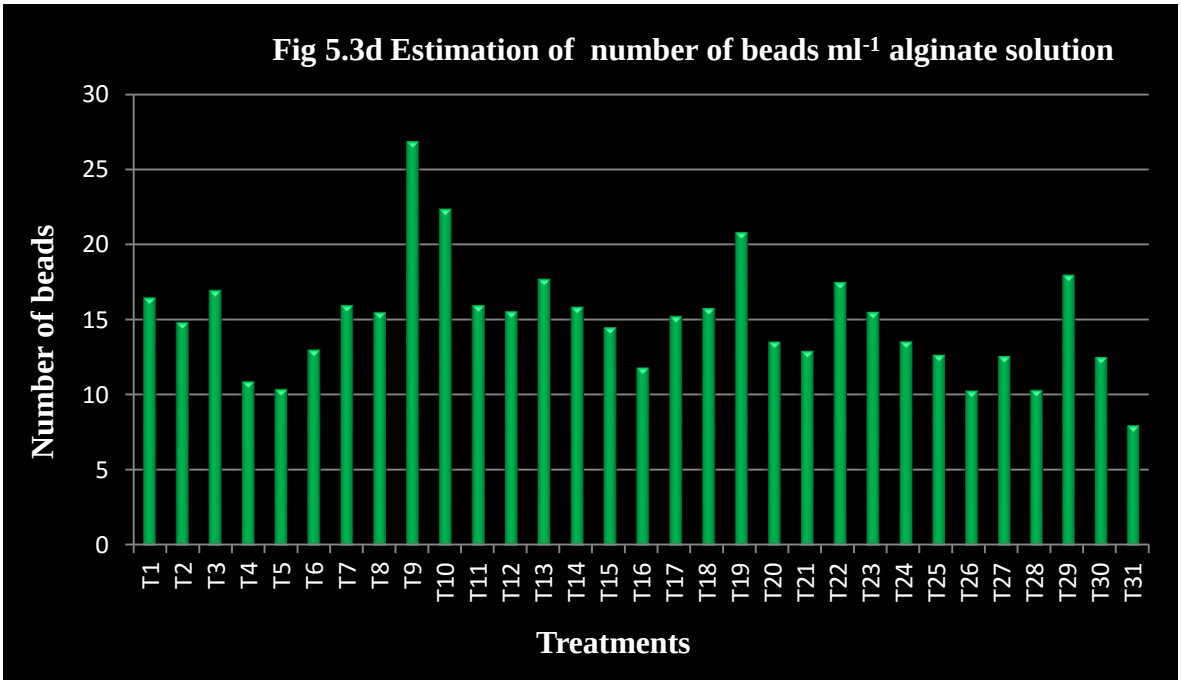


Fig 5.3d Estimation of number of beads ml-1 alginate solution
Number of beads
30
25
20
15
10
5
0
T1 T2 T3 T4 T5 T6 T7 T8 T9 T10 T11 T12 T13 T14 T15 T16 T17 T18 T19 T20 T21 T22 T23 T24 T25 T26 T27 T28 T29 T30 T31
Treatments

5.2.4 Evaluation of shelf life of *T. viride* encapsulated alginate beads

According to John *et al.* (2011), encapsulation 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. The shelf life of *T. viride* encapsulated alginate beads was evaluated at monthly intervals upto six months, employing the technique of serial dilution and plating. After six months of evaluation, it was observed that all the treatments with the additives trehalose (15mM), PVP (1%), CMC (0.5%), and tween 80 (0.5%) were superior in comparison to others. Over six months of evaluation, the population of *T. viride* ranged from 6.6 x 10^6 - 34.6 x 10^6 cfu g^{-1} bead in different treatments (T_{19} - T_{24}) with above mentioned additives, whereas, the cfu in control ranged from 2.0 x 10^6 - 13.6 x 10^6 cfu g^{-1} bead. Among these six treatments, T_{21} (1.5% sodium alginate and 3.5% $CaCl_2$) showed a consistent performance in terms of colony count. Hence, this treatment was selected for the encapsulation of *Trichoderma*.

According to Krasaekoopt *et al.* (2004), the amalgamation of additional components into the polymer matrix or coating of the capsules with them enhanced cell viability, capsule stability and improved storage ability of encapsulated formulation. Szczech and Maciorowski (2016) opined that the kind of entrapped bioagent had a significant effect on the product durability, where they observed better survival of conidia of *Trichoderma* in storage. However, they observed that the type of microbeads influenced the viability of *Trichoderma* in beads, additive compounds like peat powder, chitosan and skim milk were not found to influence the same.

Similar line of work was recorded by Lewis and Papavizas (1987), where they reported that the efficiency of *Trichoderma* captured pellets attenuated after a storage period of six weeks at 5^0C or 25^0C against *Rhizoctonia solani*. Similarly, Thilagavathi *et al*. (2015) enumerated the population of encapsulated *T. asperellum* strain TTH1, at monthly intervals upto 12 months employing serial dilution and plating technique. Results showed that the population was maintained upto 12 months without much

Fig. 5.4a Population of *T. viride* (x 10^6cfu g^{-1} bead) at monthly intervals

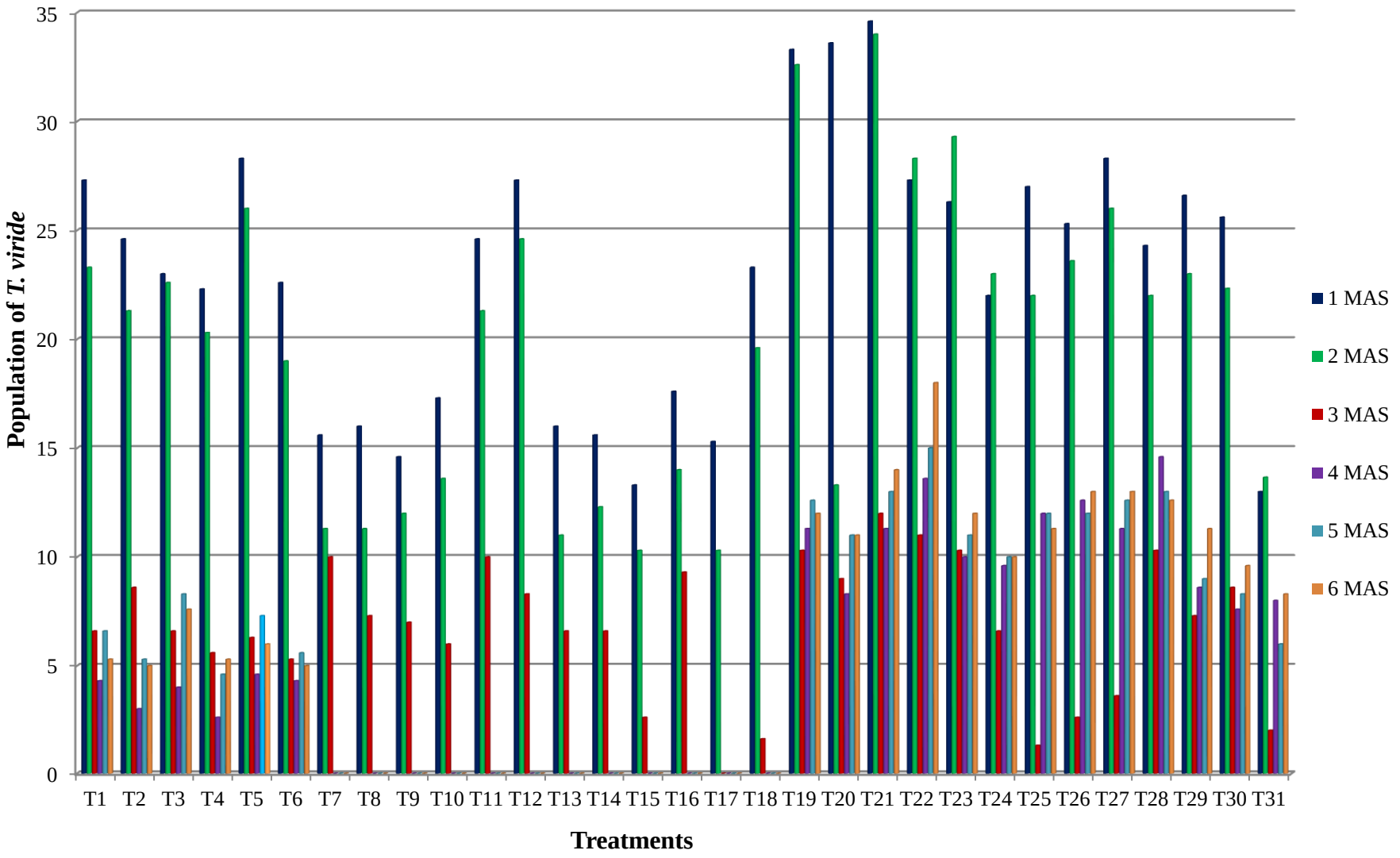


Fig. 5.4b Population of *T. viride* (x10^8 cfu g^{-1} bead) at monthly intervals

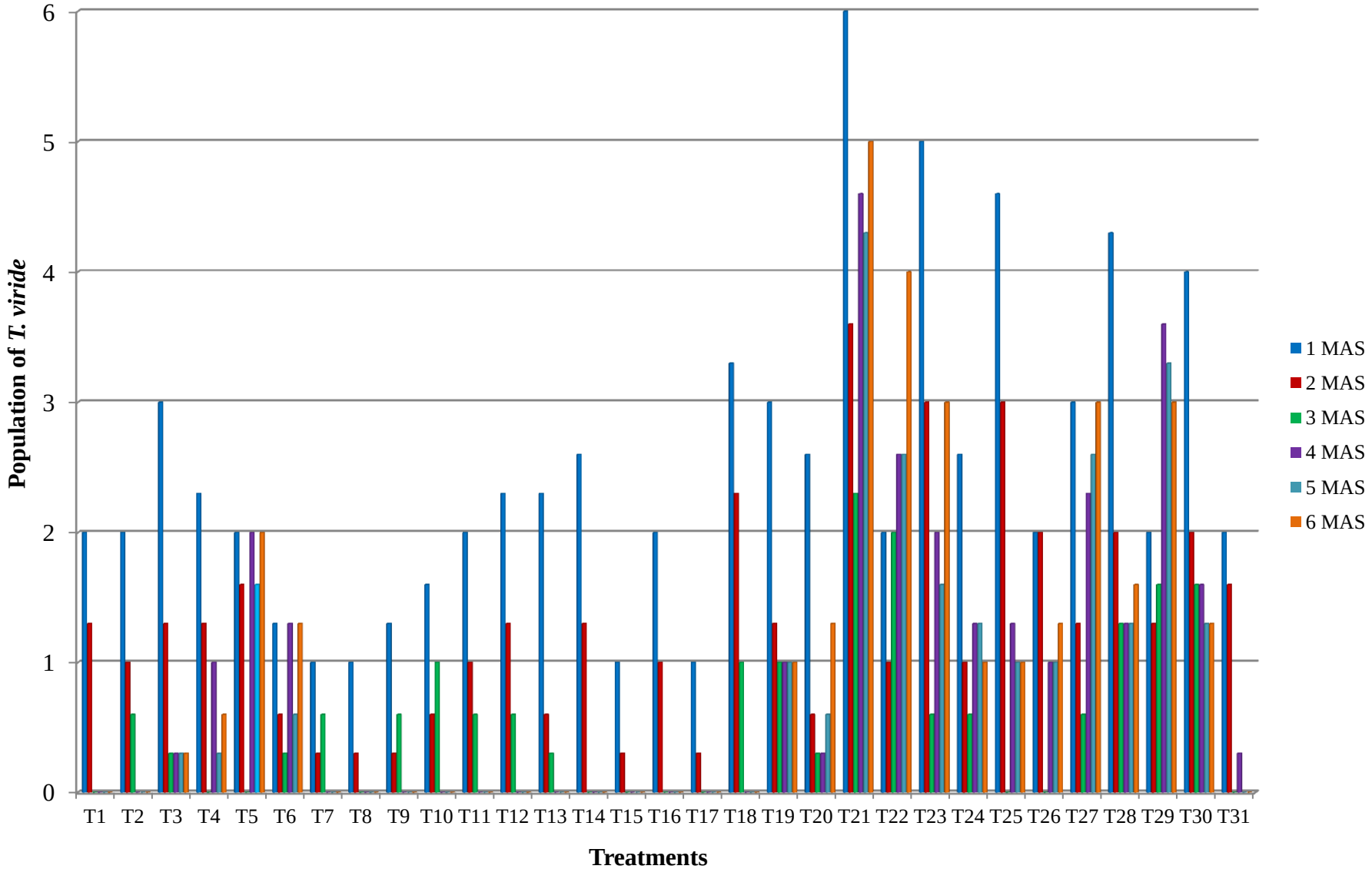
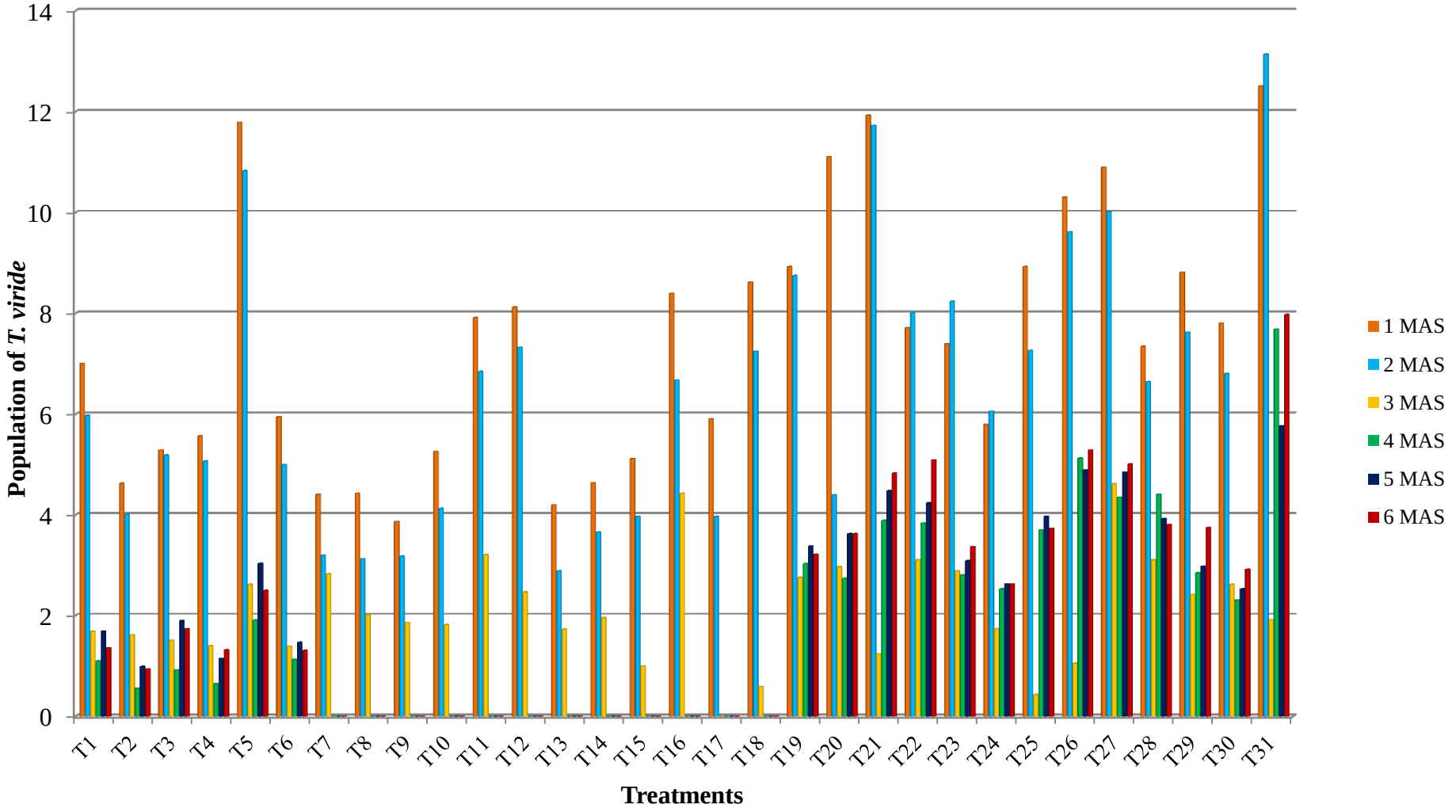


Fig. 5.4c Population of *T. viride* (x10^5 cfu per bead) at monthly intervals



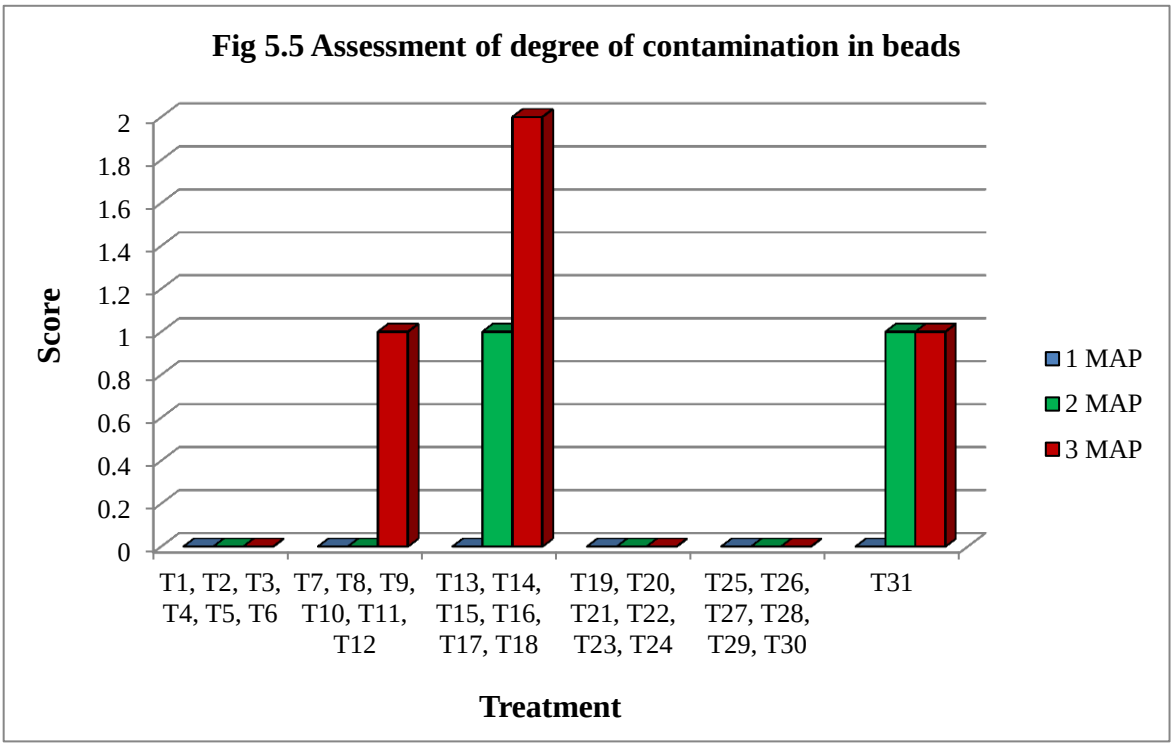
decline. In a similar work conducted by Adzmi *et al*. (2012), the population of *T. harzianum* UPM40 conidia in the beads was recorded as 2.69 x 10^4 to 1.56 x 10^3 cfu g^{-1} beads during six months of storage at 5 ± 2^0C.

5.2.5 Assessment of degree of contamination in microbeads

In the present investigation, the degree of contamination of *T. viride* entrapped microbeads was estimated at 1, 3 and 6 months after preparation. For the estimation, a scoring index with 0 - 4 score range was used, which was supported by Szczech and Maciorowski (2016). One month after preparation, no sign of contamination was detected in any of the treatments including control. After three months, control beads along with $T_{13,}$ $T_{14,}$ $T_{15,}$ $T_{16,}$ $T_{17,}$ T_{18} consists of mannitol, PEG, liquid paraffin and tween 80 showed slight discoloration without any typical smell, which was assigned with score 1. The results of the present study were supported by Szczech and Maciorowski (2016), where they documented that the first symptoms of spoilage in wet microcapsules was generally observed three months after preparation.

Further, the beads with mannitol in combination with PVP and CMC ($T_{1,}$ $T_{2,}$ $T_{3,}$ $T_{4,}$ T_5 and T_6) and the beads amended with trehalose ($T_{19,}$ $T_{20,}$ $T_{21,}$ $T_{22,}$ $T_{23,}$ $T_{24,}$ $T_{25,}$ $T_{26,}$ $T_{27,}$ $T_{28,}$ T_{29} and T_{30}) remained free of contamination even after six months of preparation. In contrast, the beads supplemented with mannitol in combination with PEG and paraffin oil showed slight discolouration without any typical smell at third month of storage. Further, these beads showed distinct discoloration with unpleasant smell. In addition to that, control beads devoid of any additives turned light brown from ivory colour and no mold growth was detected on them. A peculiar combination of different adjuvants in other treatments might have contributed to the contaminants on the bead surface. The significant effect of the composition of microcapsules on the degree of contamination and shelf life of wet product was identified by Szczech and Maciorowski (2016). Among the three different additives they used, peat reduced the contamination and significantly enhanced bead quality, while skim milk deteriorated the quality of the microcapsules during storage.

Fig 5.5 Assessment of degree of contamination in beads



5.2.6 Other parameters for standardization of microbeads

Some of the additional parameters like per cent potentially infective beads, moisture content of microbeads, swelling behaviour of microbeads at different pH, per cent shrinking, sphericity factor, time of gelation and population of *T. viride* in soil at different pH was also estimated, where many of these characteristics were found to influence each other.

5.2.6.1 Per cent potentially infective beads

The concept of per cent potentially infective bead (%PIB) was devised by Declerck *et al.* (1996). The beads with atleast one germinated spore crossing calcium alginate coating are counted as potentially infective. The germination assay of microbeads was carried out by embedding five beads on potato dextrose agar plates. The observations evidenced that no germination was noticed in any of the treatments after 24 h incubation. However, after 48 h, 84.22 ± 8 per cent germination was recorded in T_1, T_2, T_4 and T_5, while in T_3 and T_6, 79.03 ± 9.8 per cent germination in each was observed. After 72 h of incubation, cent per cent germination was obtained from all the six treatments other than control. Analysis of results indicated that the beads from all the treatments are potentially infective and thus fit for field application.

These findings are in congruence with the observations of De Jaeger *et al.* (2011), where they recorded 72 ± 4.5 per cent germination of the entrapped conidia of *T. harzianum* on the second day of incubation. Further, 86 ± 3.5 and 96.19 per cent germination was recorded on third and sixth day respectively. After sixth day, per cent germination remained constant.

5.2.6.2 Moisture content of beads

Moisture per cent of beads for treatments T_1 - T_7 including control was estimated using an instant moisture analyser. Immediately after preparation of beads, the moisture per cent ranged from 90.2 ± 0.86 (T_4) to 94.0 ± 1.63 (T_1) per cent. Thereafter, it decreased gradually upto 72 h. It was observed that 24 h after bead preparation, the highest moisture per cent was noticed with control (84.5 ± 1.14) and the minimum was

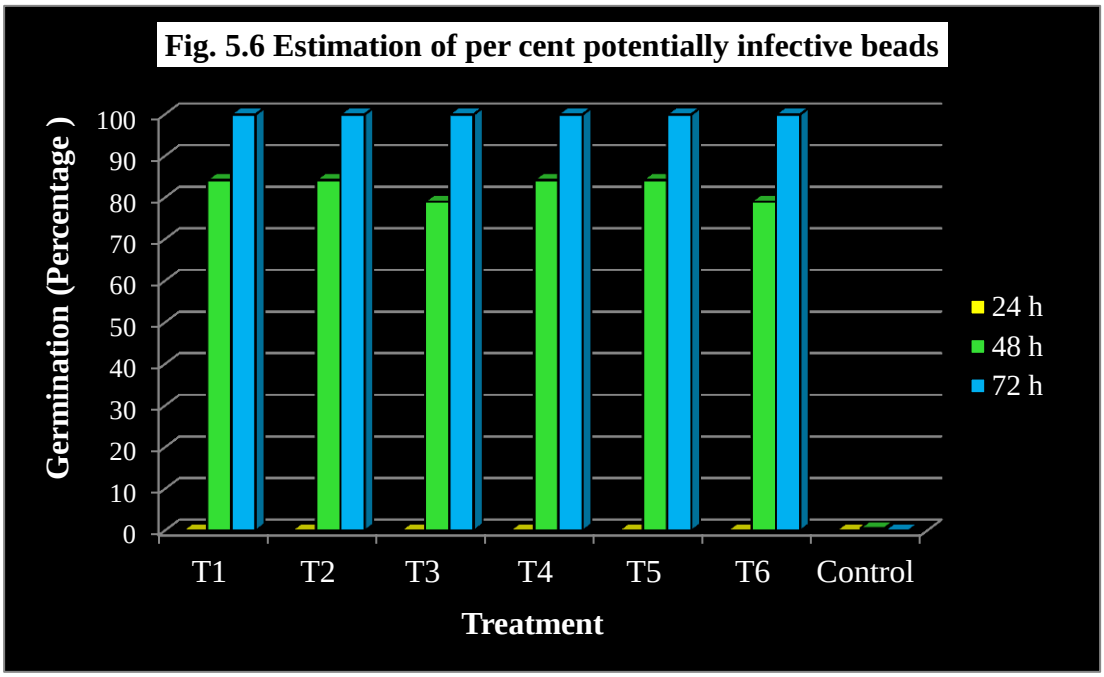


Fig. 5.6 Estimation of per cent potentially infective beads
Germination (Percentage)
100
90
80
70
60
50
40
30
20
10
0
T1
T2
T3
T4
T5
T6
Control
Treatment
24 h
48 h
72 h

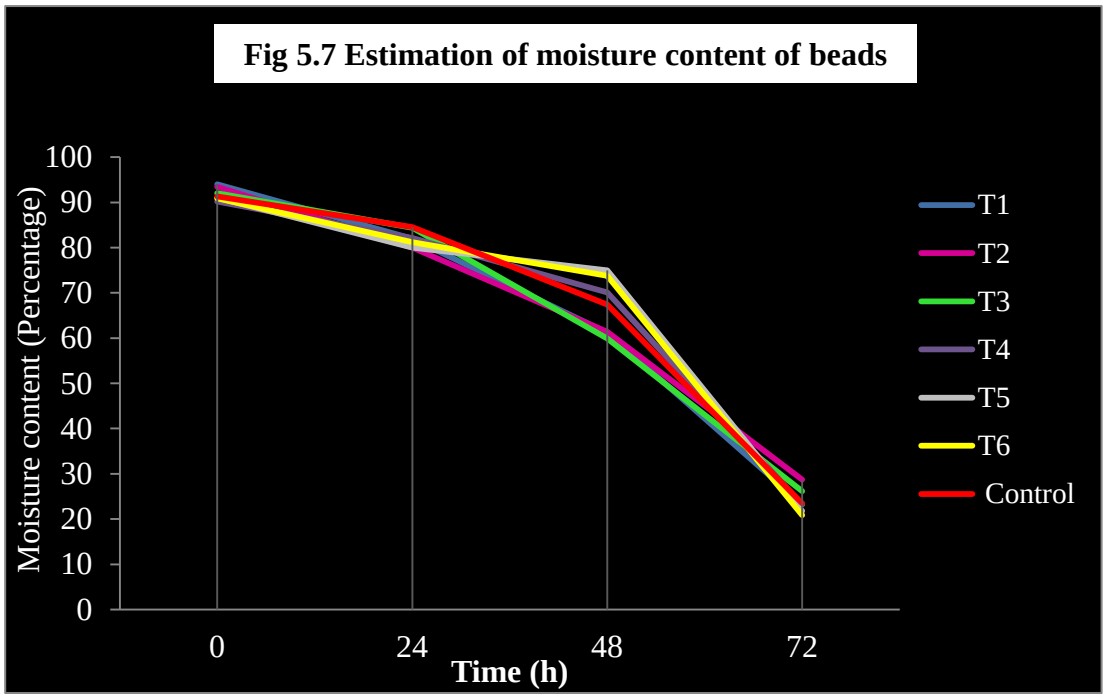


Fig 5.7 Estimation of moisture content of beads
Moisture content (Percentage)
100
90
80
70
60
50
40
30
20
10
0
0
24
48
72
Time (h)
T1
T2
T3
T4
T5
T6
Control

recorded in T_5 (80.0 ± 3.74) and T_2 (80.0 ± 2.44). After 48 h of drying, a moisture per cent ranging from 59.9 ± 1.95 (T_3) to 75.0 ± 1.63 (T_5) was observed. Further, it reduced drastically to 20.9 ± 0.63 (T_6) to 28.7 ± 1.28 (T_2) after 72 h of open drying, where the beads became hard, shriveled and turned dark brown. The above findings were also closely supported by Tous *et al.* (2014) as they recorded 90 - 93 per cent water content in drug loaded sodium alginate beads after 24 h open drying followed by 24 h oven drying at 45°C. Similarly, Adzmi *et al.* (2012) estimated the moisture content of microcapsules containing *T. harzianum* UPM40 as 5.39 per cent after 24 h of drying at 30^0C.

5.2.6.3 Swelling behaviour of microbeads at different pH

Swelling and erosion are two important phenomena that lead to degradation and dissolution of beads in the soil. According to Bajpai and Kirar (2016) degradation of alginate beads were achieved by exchange of cross-linking Ca^{2+} ions present within the so called 'egg-box' cavity by counter ions in the swelling medium. The basic factor contributing to this was revealed by Lin and Ayres (1992), where they observed that the alginate matrix was highly permeable in nature. Moreover, Sherina *et al*. (2012) attributed the phenomenon of swelling of dry microbeads to the hydration of the hydrophilic groups of alginate. George and Nikolaos (2006) described that the hydration resulted in the increased repulsion between the negatively charged carboxylate group (-COO-) in the alginate. Along with that, the degree of cross-linking was reduced due to the loss of Ca^{2+} ions from calcium alginate matrix. This subsequently led to a loose matrix which takes up more water and finally disintegrates.

In the present investigation, swelling behaviour of adjuvant amalgamated sodium alginate bead formulation of *T. viride* was analysed at pH 1 and 7.4. Maximum swelling was exhibited by T_4, T_5 and T_6 with lower concentration (0.75%) of sodium alginate. Control beads which contain 2.5 per cent sodium alginate were highly resistant to swelling. Further, maximum swelling ratio was recorded in T_5 (0.70) with 70.47 per cent swelling and minimum in control (0.03) with 3.95 per cent swelling. In the present study, maximum swelling was observed in beads that immersed in solution with pH 7.4 compared to that kept at pH 1. Similar findings were documented by Turkoglu *et al*.

Fig. 5.8a Estimation of swelling of beads at pH 7.4 in phosphate buffer

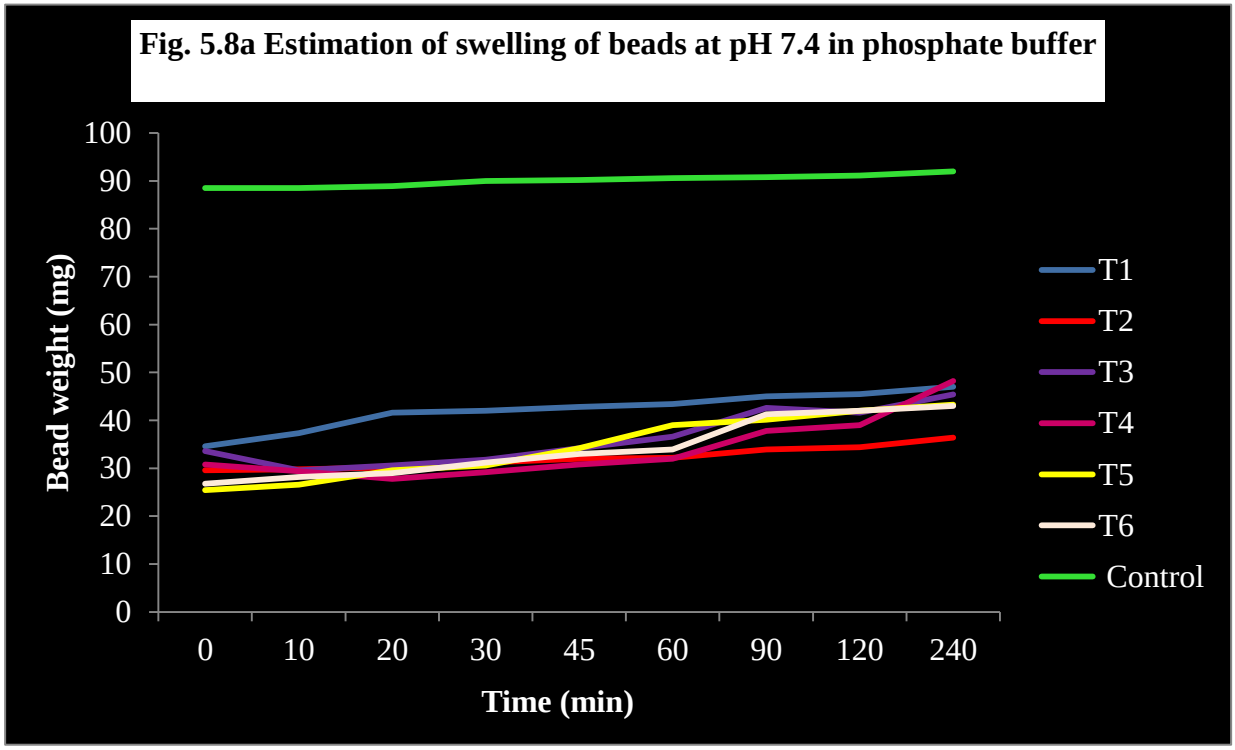
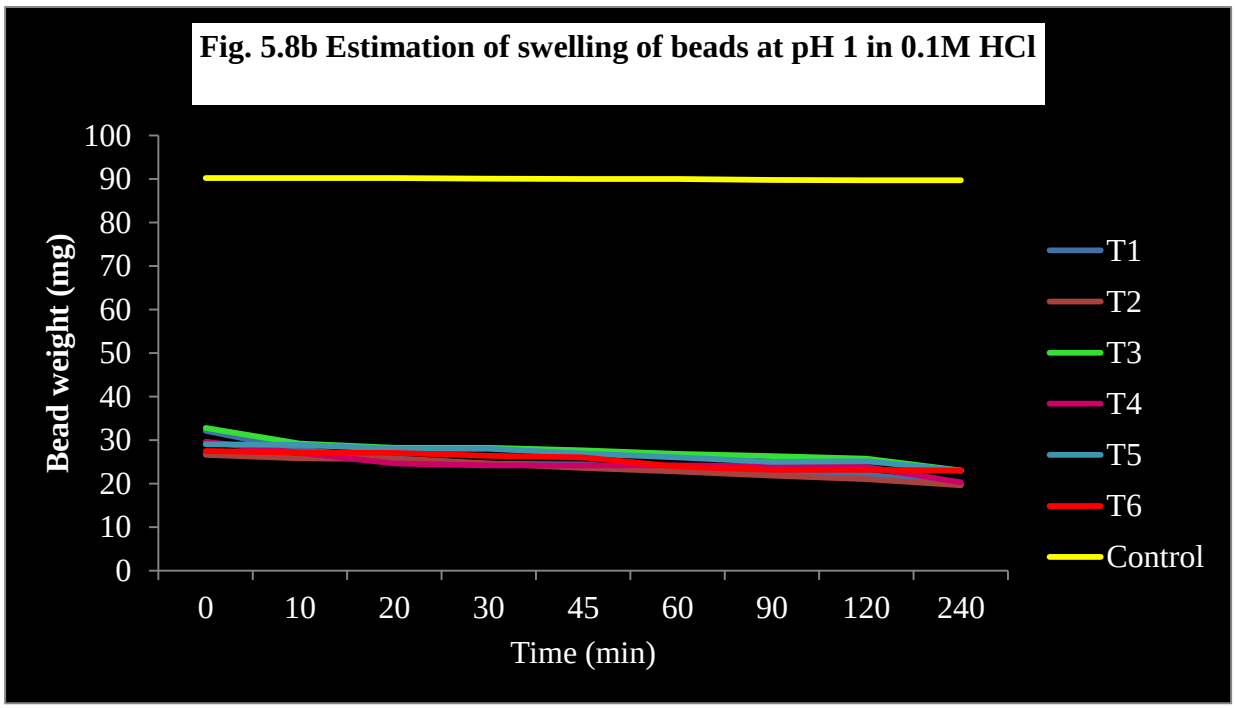


Fig. 5.8b Estimation of swelling of beads at pH 1 in 0.1M HCl



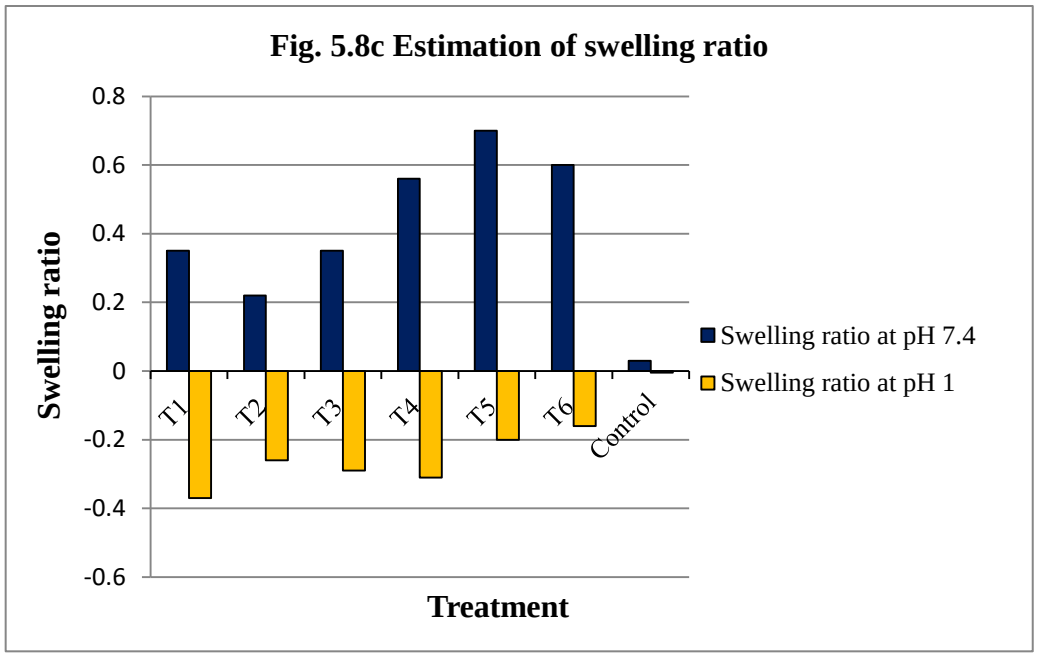


Fig. 5.8c Estimation of swelling ratio
Swelling ratio
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
T1
T2
T3
T4
T5
T6
Control
Treatment
Swelling ratio at pH 7.4
Swelling ratio at pH 1

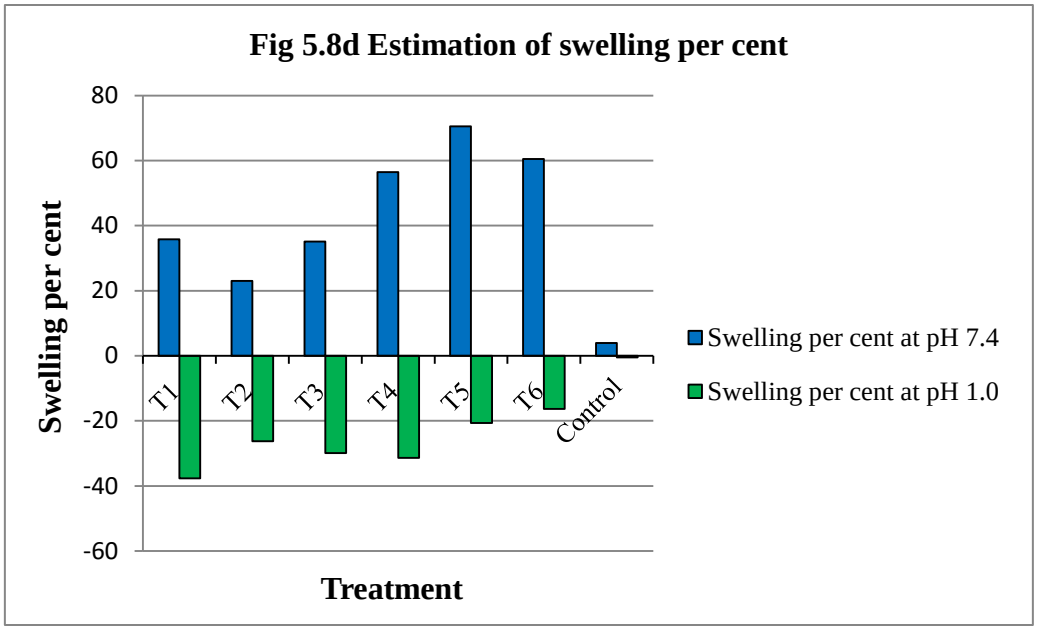


Fig 5.8d Estimation of swelling per cent
Swelling per cent
80
60
40
20
0
-20
-40
-60
T1
T2
T3
T4
T5
T6
Control
Treatment
Swelling per cent at pH 7.4
Swelling per cent at pH 1.0

(1997), where he reported that the alginate beads exhibited highest rate of swelling at pH 7.4, while lowest rate was recorded at pH 1. He explained that the swelling of beads at higher pH was due to the interchange of Ca^{2+} with anti-gelling ions like K^{+} in the buffer. Furthermore, Sherina *et al*. (2012) also recorded a lowest swelling ratio at pH 1.2 and the highest at a pH of 6.8.

In the present experiment, minimum swelling was recorded for beads kept at pH 1. The result was supported by Patel *et al.* (2016), where they described that the low swelling index of calcium alginate polymer beads in acidic pH is due to the proton - Ca^{2+} exchange. This exchange results in the formation of almost insoluble alginic acid. They added that the influx of water through the pores in the bead surface caused a marginal swelling even in the acidic environment, which was observed in the present experiment as well. In the present investigation, the rate of swelling was less even at pH 7.4, probably due to the presence of additive substances in the bead. These findings are comparable with the studies conducted by Patel *et al*. (2016), where they observed less swelling in beads with additional pH-sensitive enteric coating polymer than uncoated beads. Similarly, Sherina *et al*. (2012) observed significant decline in the swelling properties of alginate beads as the coating polymer ratio increased.

In the present study, a gradual and continuous weight gain of the beads were observed. An increased bead weight confirmed that the beads were swollen due to the influx of water. The results of the present study was supported by Sherina *et al*. (2012) where they stated that the swelling of dry microbeads occur due to the hydration of the hydrophilic groups in alginate, which in turn caused the weight gain of microbeads. Hence, the beads swell and gradually increase the weight upon absorption of water. Similar observation was recorded by Crcarevska *et al.* (2008), where they reported that the volume of ionically cross-linked, dried beads started to increase a few minutes after plunging in water or buffers. In the present experiment, the beads were stable and did not degrade even after 240 min of incubation. It may be the additives which impart stability to the microbeads. The above conclusion was supported by Bajpai and Kirar (2016) where they reported that the various polymer amended sodium alginate beads remained stable for 18-48 h at pH 7.4, while the plain alginate beads attained maximum swelling in

30 min and completely disintegrated within a couple of hours. However, Al-Kassas (2007) demonstrated that the maximum swelling of beads was achieved in 1.5 h in phosphate buffer (pH 7.4) after which the erosion and degradation of microbeads were observed. Likewise, Tous *et al.* (2014) noticed the retention of the intact form of microbeads without any erosion up to 4 h in acidic pH and only upto 1.5 h at pH 7.4.

In the present investigation, it was observed that the beads incubated in solution with pH 1 showed gradual reduction in weight. The results were supported by Ouwerx *et al*. (1998), where they stated that at low pH values (< 4), carboxylate (-COO-) groups of alginate are protonized and hence, the electrostatic repulsion gets reduced. This favours bead shrinkage, expulsion of water out of the bead and subsequent weight loss. The present findings are also in line with the report of Tonnesen and Karlsen (2002) where they observed that the swelling of calcium alginate beads under acidic conditions is insignificant. Similar findings were also documented by Sherina *et al.* (2012), where they reported that alginate beads shrunk and encapsulated drugs were not released in acidic pH, whereas they readily swell and release in an alkaline environment. However, Patel *et al.* (2016) observed that the inflow of water through the pores present on the bead surface caused a marginal swelling even in the acidic condition. Moreover, Smrdel *et al*. (2008) reported that open drying left only less number of pores on bead surface, which was observed in the present study as well.

In general, swelling of microbeads was influenced by several factors. Vincekovic *et al*. (2016) observed that the swelling behaviour and release pattern are substantially influenced by the size of microbeads and entrapped bioagent. Moreover, Patel *et al.* (2016) reported that the swelling-dissolution-erosion process in alginate hydrogels depends on the osmotic pressure gradient between the gel and the environment. Tous *et al.* (2014) reported that the process of swelling was strongly dependent on pH of the medium. Tonnesen and Karlsen (2002) also made a similar observation. Moreover, the method of drying influence the swelling properties of microbeads (Gal and Nussinovitch, 2007; George and Abraham, 2007). Crcarevska *et al.* (2008) reported that the swelling, resulted from the matrix rehydration strongly depending on the degree of cross-linking. Therefore it is concluded that, a cumulative effect of all these factors contributed to the

present result.

5.2.6.4 Shrinking percentage

The shrinking of microbeads was estimated as the ratio of bead diameter before drying to the bead diameter after drying and expressed in percentage. In the present study, it was observed that the microbeads subjected to open drying for 48 h undergo shrinking in various degree. This happens probably due to the evaporation of water from the beads. This observation was supported by John *et al.* (2011), where they stated that the alginate encapsulation provides a protective hydrophilic matrix that facilitates smooth water evaporation. Similar observation was made by dos Santos *et al*. (2015) also. The results are in conformity with Smrdel *et al*. (2008) where they reported that the air-dried beads substantially shrank and became smaller during drying.

In the present experiment, maximum shrinking percentage was observed in T_4 (47.05%) followed by T_5 (46.98%), where the concentration of sodium alginate was minimum (0.75%). Minimum shrinking percentage was recorded in control beads (5.57%) and T_3 (8.98%), where the concentration of sodium alginate was 2.5 and 1.5 per cent respectively. Among the beads with same concentration of sodium alginate, maximum shrinking was recorded for the beads with minimum concentration of cross linking Ca^{2+} and *vice versa*. The results of the present study are in line with the findings of Huang and Lin (2017), where they observed that the beads which gelled in high concentrations of cross linkers rarely shrunk.

From the present studies, it was concluded that the degree of shrinking is inversely proportional to the concentration of both sodium alginate and Ca^{2+} in the bead. The above observations is in line with the observations of El-Zatahry *et al.* (2006), where they reported that the adequate divalent cation concentration in crosslinker solution lead to a compact matrix with little structural rearrangements of beads to cause shrinkage. Also calcium alginate beads were not sufficiently bound due to less affinity between sodium alginate and $CaCl_2$ (Loh *et al.*, 2012; Chuang *et al*., 2017). Likewise, Tous *et al*. (2014) observed less shrinkage of alginate beads with 5 per cent sodium alginate compared to that with lower concentrations.

In general, the shrinking behaviour of microbeads were influenced by several factors like affinity between polymer and cross linker (Morch *et al.*, 2006) and incubation temperature (Huang and Lin, 2017). From the present study, it is concluded that the shrinkage may lead to the desiccation of beads which impair the storage and shelf life.

5.2.6.5 Sphericity factor

Sphericity factor assumes significance while defining the shape of a microbead. An exact spherical shape is essential in determining the bead quality. According to Tal *et al.* (1997), any defects like distorted shapes or uneven size of microbeads may deteriorate the viability and stability of encapsulated cell during storage. Chan *et al*. (2011) reported that the value of the sphericity factor ranges between zero and one, the former is assigned to an exactly spherical bead and the latter to an elongated one. In the present study, the maximum value of sphericity factor was recorded in T_6 (0.117) followed by T_4 (0.106), which represents non-spherical beads. On the other hand, control (0.034) followed by T_3 (0.044) were the beads with minimum recorded value of sphericity and thus, almost spherical in shape.

From these observations, it is concluded that the beads with high concentration of sodium alginate assumes an almost spherical shape with least value for sphericity factor. As the concentration of sodium alginate decreases, beads shrink disproportionately and sphericity factor approaches unity. Moreover, it was observed that most of the beads was spherical in shape at the time of formation. However, deviation of sphericity from zero is probably due to the evaporation of water from the beads during drying. The results are in line with the findings of Adzmi *et al*. (2012), where they prepared plain microbeads with 3 per cent sodium alginate and adjuvant amended beads by adding 1 per cent montmorillonite. They reported that the sphericity factor of amended beads and plain beads of *T. harzianum* UPM40 as 0.0650 ± 0.06 and 0.1230 ± 0.03 respectively. They concluded that variation in the sphericity factor was noticed due to the loss of moisture content during the process of drying. Moreover, they inferred that additive substances have some influence on the sphericity of microbeads.

Further, Gal and Nussinovitch (2007) revealed the influence of the method of drying on bead shape.

5.2.6.6 Estimation of time of gelation or curing

The principle of gelation or cross-linking of sodium alginate with $CaCl_2$ is based on the tight junctions between the guluronic acid residues in the sodium alginate solution (Rajinikanth *et al*., 2003). Yotsuyanagi *et al*. (1991) remarked the gelation of alginate as an instantaneous process with rapid expulsion of water from the gel matrix. Owing to this phenomenon, the progress of gelation or cross linking is noticeable with the gradual reduction of bead weight until the process of cross linking gets completed. At the point of completion, beads achieve a stable weight. This point is significant in determining the time period required for complete incubation of beads in $CaCl_2$ bath. For estimation of the same, 10 individual beads were weighed at specific intervals of time after formation. The weight was recorded till the bead weight became stable or no more reduction in bead weight was noticed.

From the observations, it was clear that the weight of individual beads from each treatment reduced gradually until 60 min of formation. Hence, the time for complete gelation was estimated to be 60 min, which demands the complete immersion of a bead in $CaCl_2$ solution for 60 min from the time of formation. This is in accordance with the study conducted by Velings and Mestdagh (1995), where they observed a gradual reduction in bead weight, which was attributed to the phenomenon of ‘syneresis’, where the carboxylate groups of guluronate monomers in the polymer complex the cations. Similar line of work was recorded by Tateshita *et al.* (1993), where the rate of gelling was assessed from weight loss of beads in $CaCl_2$ solution during bead formation. However, depending on the concentration of sodium alginate, $CaCl_2$ and availability of a stirrer or a shaker, time required for curing also varies. Aslani and Kennedy (1996) reported that the process of cross linking proceeded at a lower rate, where the concentrations of sodium alginate is more. Huang and Lin (2017) stated that calcium alginate beads (2% w/v sodium alginate, 10% w/v $CaCl_2$) required minimum time

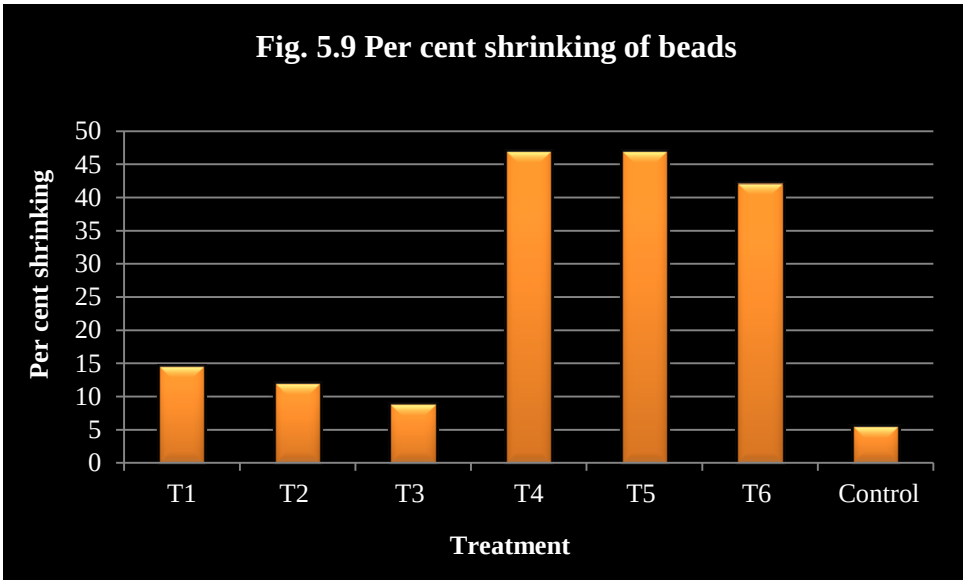


Fig. 5.9 Per cent shrinking of beads
Per cent shrinking
50
45
40
35
30
25
20
15
10
5
0
T1
T2
T3
T4
T5
T6
Control
Treatment

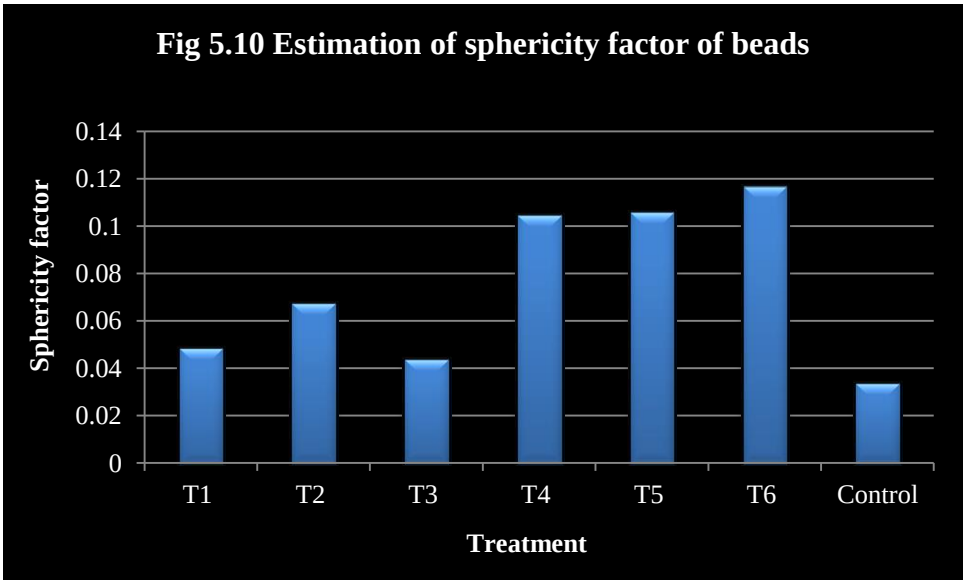


Fig 5.10 Estimation of sphericity factor of beads
Sphericity factor
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
T1
T2
T3
T4
T5
T6
Control
Treatment

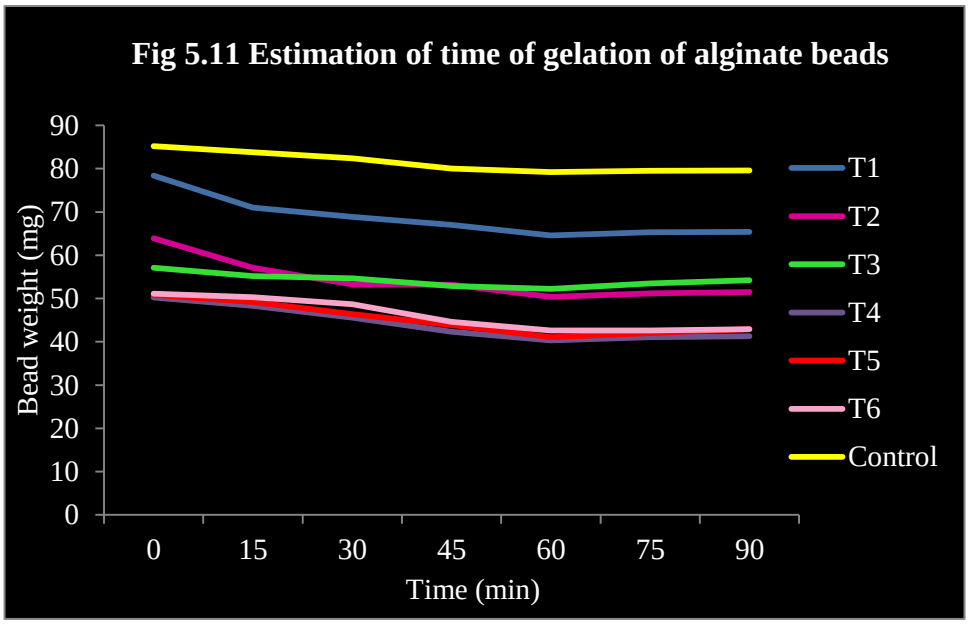


Fig 5.11 Estimation of time of gelation of alginate beads
Bead weight (mg)
90
80
70
60
50
40
30
20
10
0
0
15
30
45
60
75
90
Time (min)
T1
T2
T3
T4
T5
T6
Control

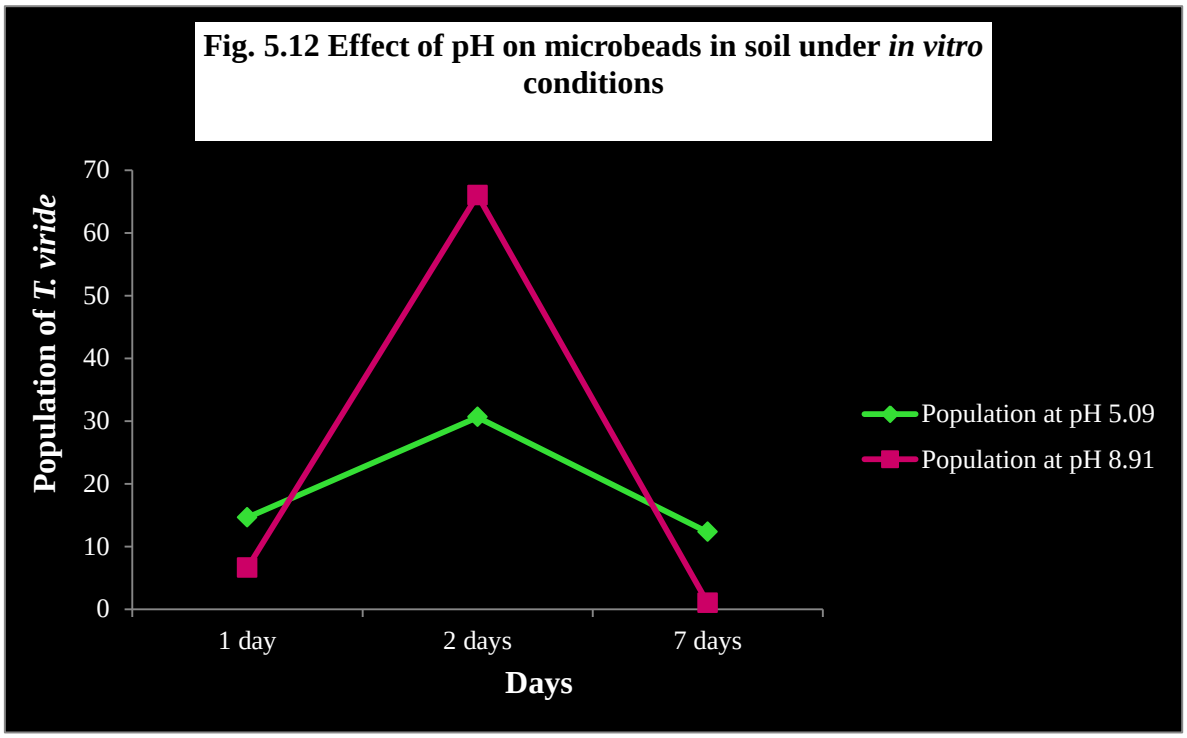
duration of 120 min after ionic gelation process to acquire stability. Furthermore, Andresen and Smidsorod (1977) showed that the modulus of elasticity of alginate gel was time dependent and it attained a steady state after approximately 6 h. Similar line of work was recorded by Declerck *et al.* (1996), where they incubated *Glomus versiforme* entrapped sodium alginate (2% w/v) beads in 0.1 M $CaCl_2$ solution under agitation for 30 min. The findings of Thilagavathi *et al*. (2015) are also in line with the present investigation, where they hardened *T. asperellum* encapsulated sodium alginate (2% w/v) beads for 60 - 180 min in 0.1 M $CaCl_2$ solution to harvest regular beads. Similarly, Bashan *et al.* (2002) cured *A. brasilense* entrapped sodium alginate (2% w/v) beads for 30 min in 0.1 M $CaCl_2$ solution. The results of the present investigation are in conformity with Szczech and Maciorowski (2016), where they reported that the sodium alginate (2% w/v) microbeads of fungal and bacterial bioagents were retained in 0.1 M $CaCl_2$ solution for 60 min at room temperature. Further, they provided a constant agitation on a magnetic stirrer to complete the process of hardening.

Likewise, Das and Senapati (2007), immersed the drug loaded alginate beads in $CaCl_2$ for 15 min to complete the gelation of the beads. The present findings are also in line with the report of Zommere and Nikolajeva (2017), where they maintained sodium alginate (3% w/v) beads in 0.2 M $CaCl_2$ bath for 60 min to complete gelation at room temperature. The findings of Mandal *et al.* (2010) are also in line with the present investigation, where they allotted 60 min for complete curing of drug amended microbeads. Similarly, Sherina *et al.* (2012) incubated the drug loaded beads on a stirrer for 60 min and further rigidization of the beads were achieved by adding one ml of 25 per cent glutraldehyde solution. Likewise, Mattam and Sailaja (2016) incubated the drug incorporated microbeads in $CaCl_2$ solution for 30 min under continuous agitation at 100 rpm.

5.2.6.7 Effect of pH on microbeads in soil under in vitro condition

Erosion and degradation of microbeads primarily depend upon their swelling behaviour, which in turn is defined by the pH of the surrounding medium. Deasy (1984) stated that the hydrogel forming property of sodium alginate facilitates pH dependent

Fig. 5.12 Effect of pH on microbeads in soil under *in vitro* conditions



controlled release of the encapsulated drug from formulations. Similarly, Tous *et al.* (2014) reported that the mechanism of release depends on swelling and erosion of microbeads.

In the present investigation, at pH 5.09, about 14.6 x 10^6 cfu g^{-1} soil was released at 24 h of incubation under *in vitro* conditions. After 48 h, it was increased to 30.6 x 10^6 cfu g^{-1} soil. After one week incubation period, the colony count was reduced to 12.3 x 10^6 cfu g^{-1} soil. At pH 8.91, the population of *T. viride* was estimated as 6.6 x10^6, 66.0 x10^6 and 1.0 x10^6 cfu g^{-1} soil at 1, 2 and 7 days after incubation respectively. Observations revealed that the release of bioagent in acidic environment is gradual and sustained due to reluctant swelling. But in alkaline pH, beads readily swell and suddenly release the bioagent. Turkoglu *et al.* (1997) explained that the rapid swelling in phosphate buffer created porous structure and brought more liquid inside the beads. Along with that, the ion exchange with phosphate buffer and formation of the solute alginate, lead to bead dissolution. A similar line of work was recorded by Polk *et al*. (1994), where they reported that the release of protein from a polymer derived slow release protein delivery system was significantly influenced by the pH of extra capsular environment. They noticed only 15 per cent release at a pH 3.0 while, 73 per cent release was recorded at pH 8.0 over 24 h time period. Similarly, Tous *et al* (2014) identified the influence of pH of the surrounding medium on the swelling and erosion behaviour of microbeads, where they recorded complete drug release in phosphate buffer (pH 7.4) whereas, the release profile was poor in 0.1 M HCl (pH 1) within 2 h of immersion.

5.3 *In vivo* EVALUATION OF BIOCONTROL EFFICACY AND PLANT GROWTH PROMOTION OF ENCAPSULATED *Trichoderma viride*

According to Papavizaz (1985), laboratory grown conidia of *Trichoderma* spp. are more vulnerable to soil fungistasis and depending on the species, germination varies from 1 - 22 per cent. In contrast, chlamydospores from potato dextrose broth or a liquid fermentation system readily germinated in soil upto 39 - 99 per cent. For the first time, Caldwell (1958) observed that the chlamydospores survive better in soil than conidia. Interestingly, Lewis and Papavizas (1987) demonstrated the potential of various

Trichoderma species to form chlamydospores readily and abundantly in natural soil and organic matter after being introduced as conidia.

Hence, to evaluate and compare the aggressiveness of encapsulated *Trichoderma* against talc and liquid formulations, the present investigation was shifted from *in vitro* to *in vivo* conditions with three different pot culture experiments. Bush cowpea var. Bhagyalakshmi served as the test crop while plant pathogenic fungi *viz*., *Pythium aphanidermatum*, *Rhizoctonia solani* and *Fusarium oxysporum* were employed as test pathogens. In all the three experiments, treatments exhibited higher yield and biometric features such as plant height, number of leaves and earliness in flowering compared to control. Sodium alginate beads applied @ 5 g plant^{-1} performed superior to talc based and liquid based formulations. Similar results on improved yield and biometric aspects were recorded by Anis *et al.* (2012) where they reported that the sunflower seeds coated with *T. viride* using 2 per cent sodium alginate showed significantly higher vigour index, plant length and biomass compared to non treated control. According to several researchers, growth promotion is achieved through auxin phytohormones like indole-3-acetic acid (IAA) (Nieto-Jacobo *et al.*, 2017), indole-3-acetaldehyde, indole-3-ethanol (Contreras-Cornejo *et al.*, 2009) and volatile organic compounds (Lee *et al*., 2016) as well as secondary metabolites (Vinale *et al*., 2012). Eventhough, considerable quantum of disease incidence (70% for *P. aphanidermatum* and 80% for *R. solani*) was recorded in plants treated with sodium alginate beads @ 5 g plant^{-1}, further events of disease incidence was not observed. This observation suggests that the build-up of pathogen inoculum was restricted by the bioagent applied in the form of formulations. Hence, it is concluded that the sodium alginate bead formulation which is under investigation is better than or as efficient as already available talc and liquid based formulations.

There are several reports on similar *in vivo* antagonistic studies. Lewis and Papavizas (1987) recorded that alginate pellets with eight different isolates of *Trichoderma* reduced 34-78 per cent survival of *R. solani* in infested beet seeds, prevented damping-off of cotton and controlled damping-off of sugar beet under greenhouse conditions. However, Szczech and Maciorowski (2016) observed that *T. virens* TRS106 entrapped microcapsules neither protect tomato plants from *Fusarium*

Fig. 5.13 Effect of treatments on plant height (Experiment 1)

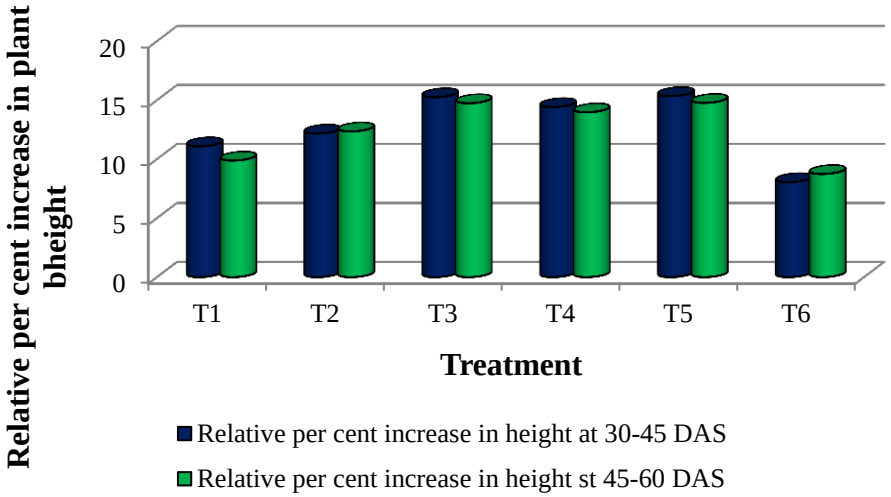
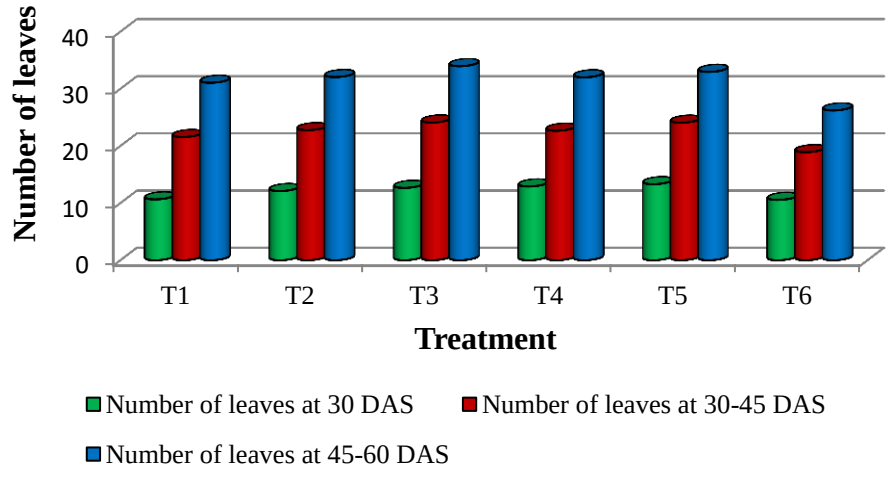


Fig. 5.14 Effect of treatments on number of leaves (Experiment 1)



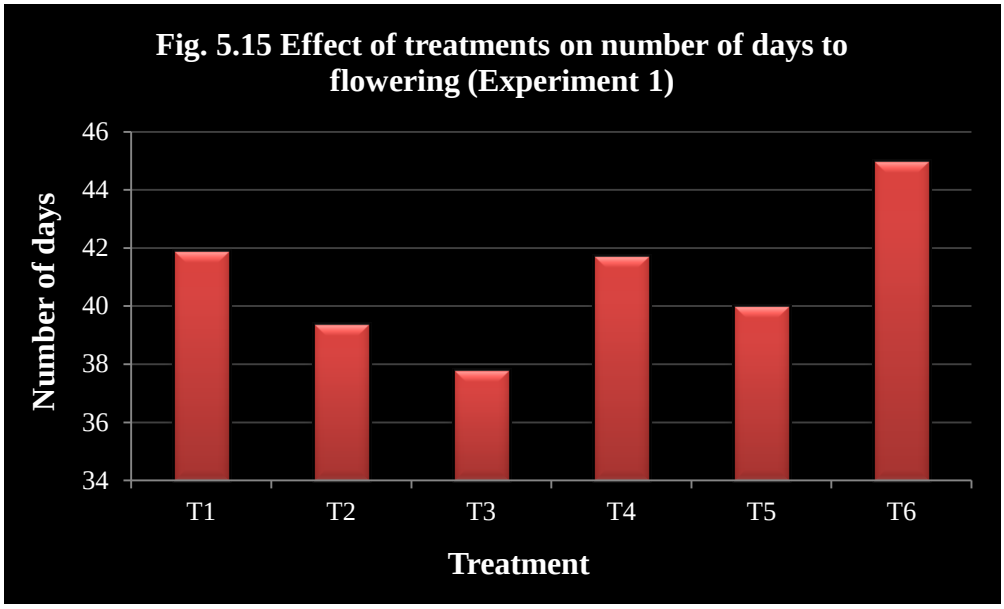


Fig. 5.15 Effect of treatments on number of days to flowering (Experiment 1)

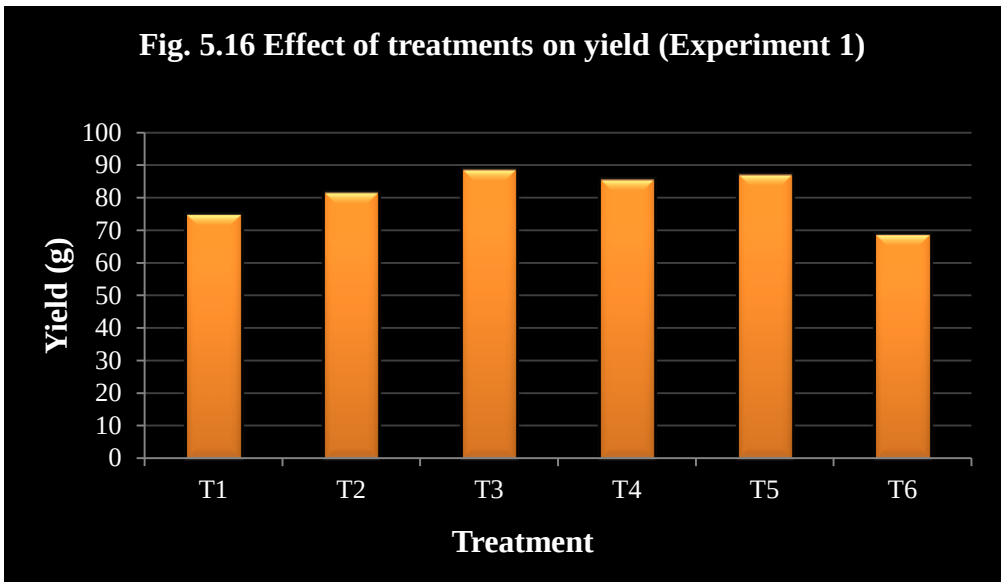


Fig. 5.16 Effect of treatments on yield (Experiment 1)

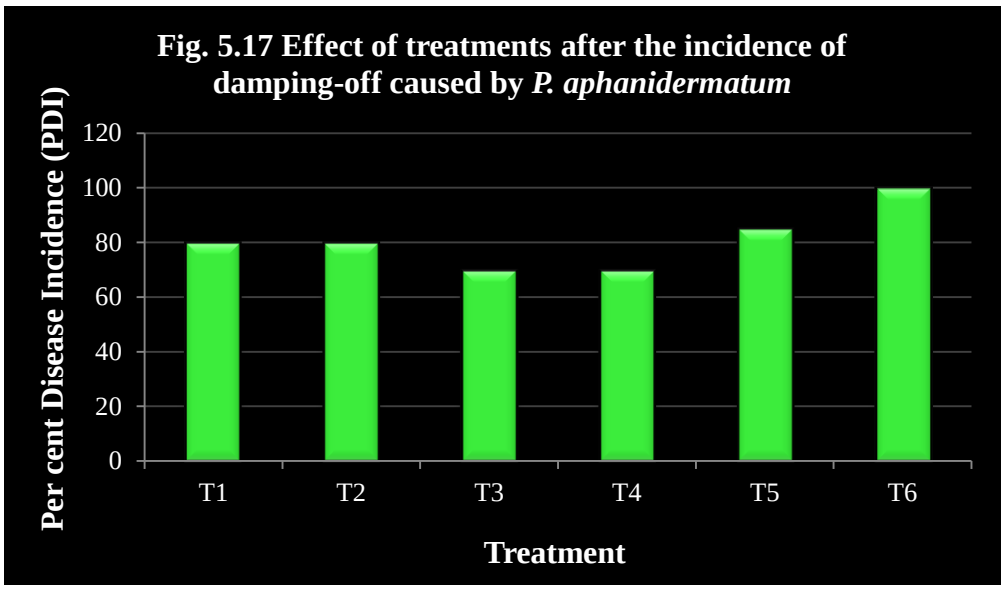


Fig. 5.17 Effect of treatments after the incidence of damping-off caused by *P. aphanidermatum*

Fig 5.18 Effect of treatments on plant height (Experiment 2)

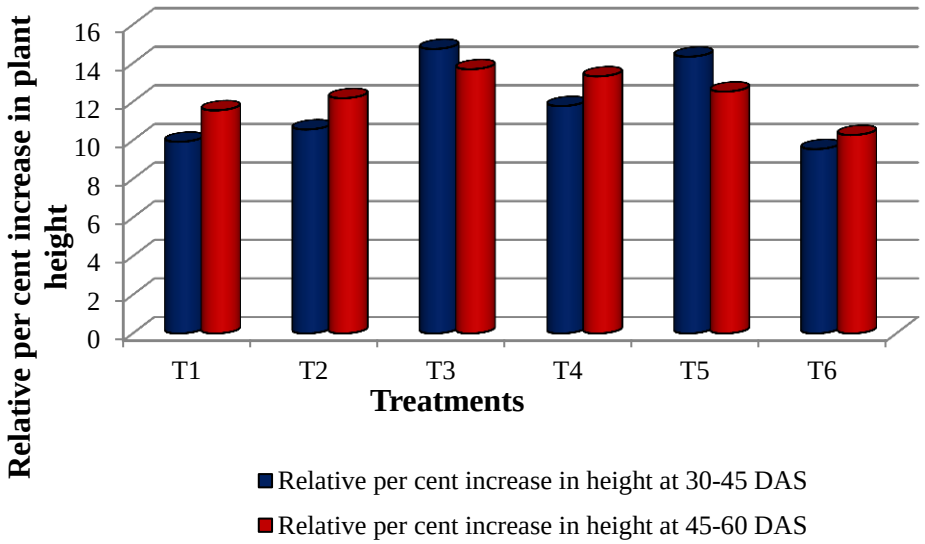
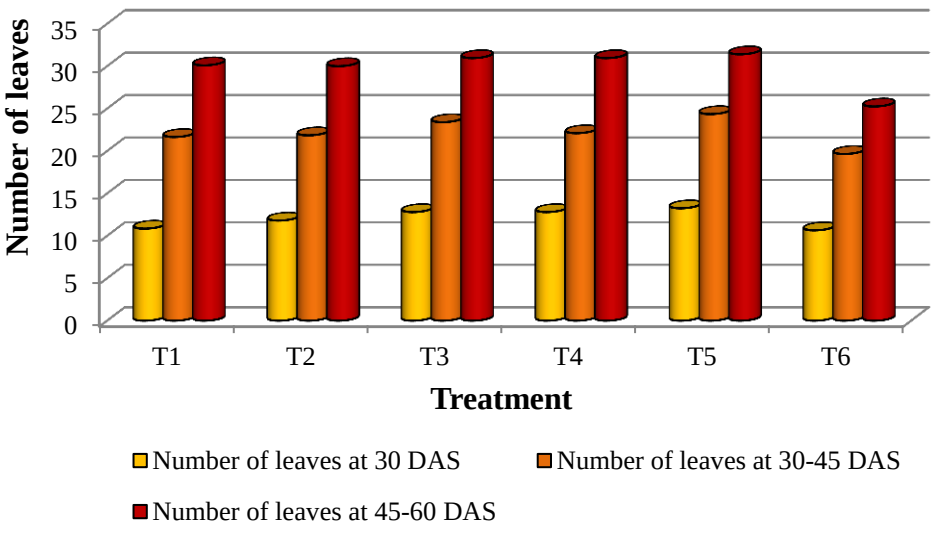


Fig. 5.19 Effect of treatments on number of leaves (Experiment 2)



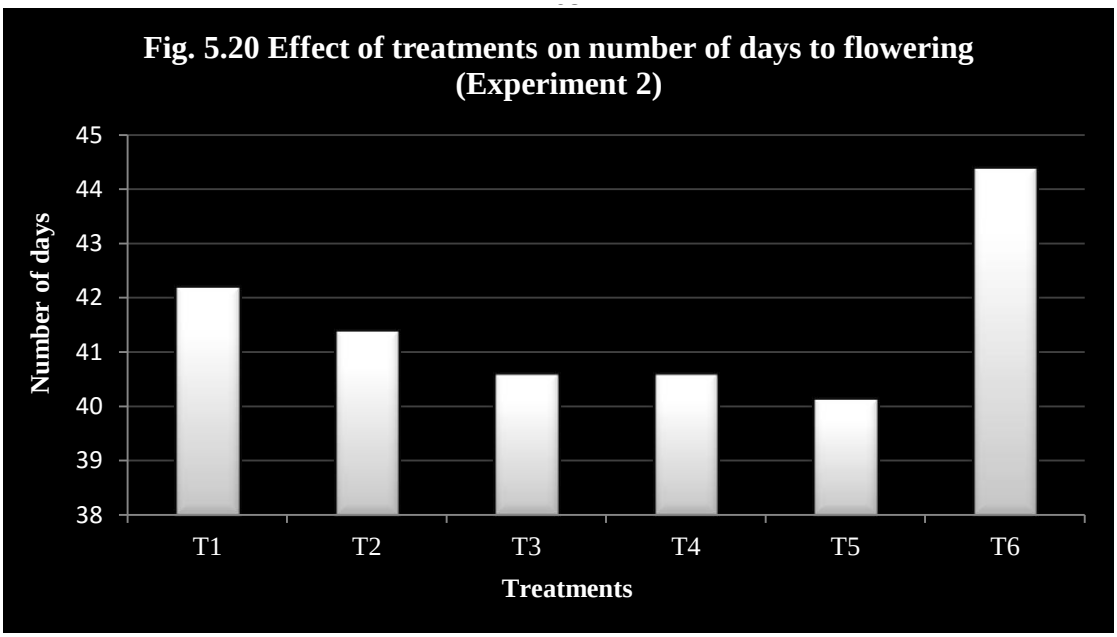


Fig. 5.20 Effect of treatments on number of days to flowering (Experiment 2)

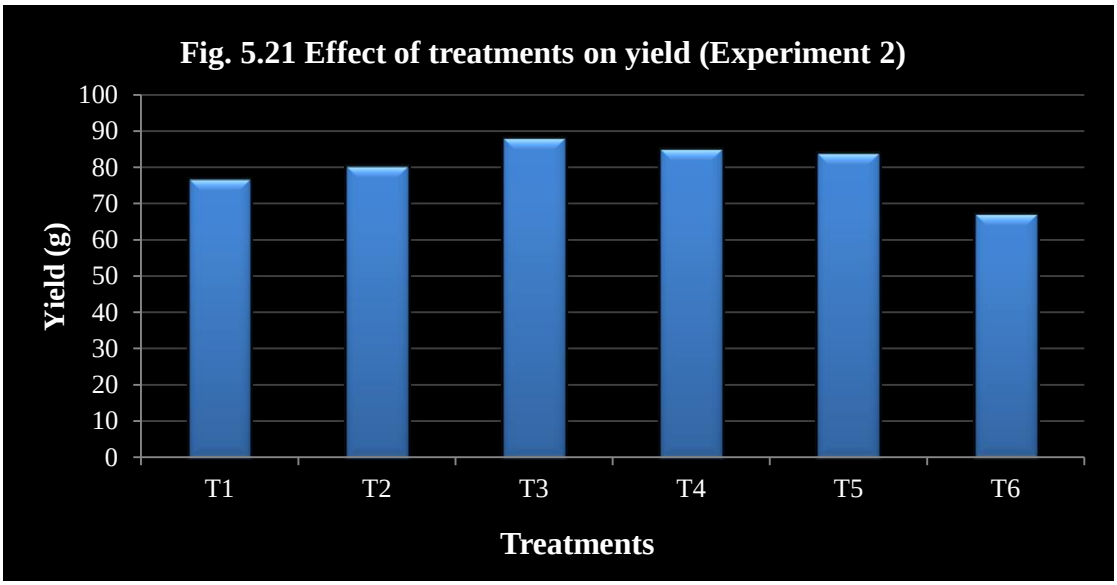


Fig. 5.21 Effect of treatments on yield (Experiment 2)

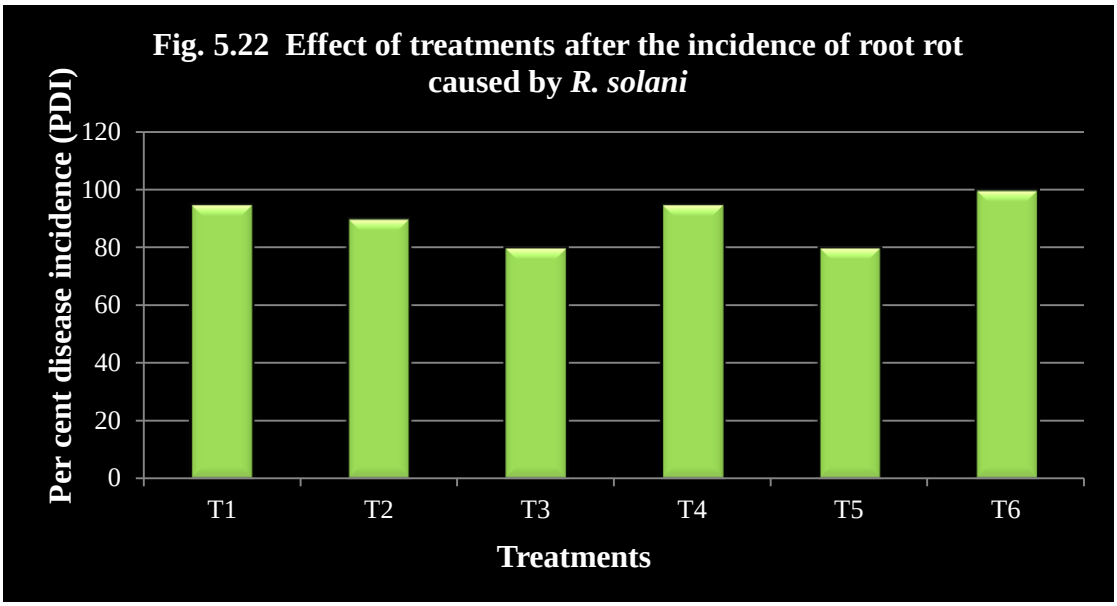


Fig. 5.22 Effect of treatments after the incidence of root rot caused by *R. solani*

Fig. 5.23 Effect of treatments on plant height (Experiment 3)

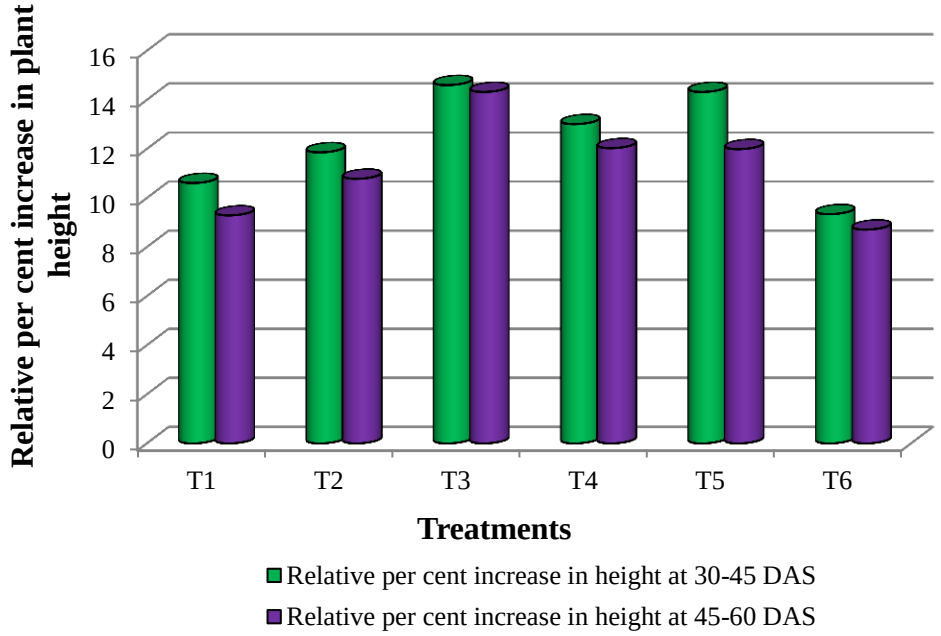
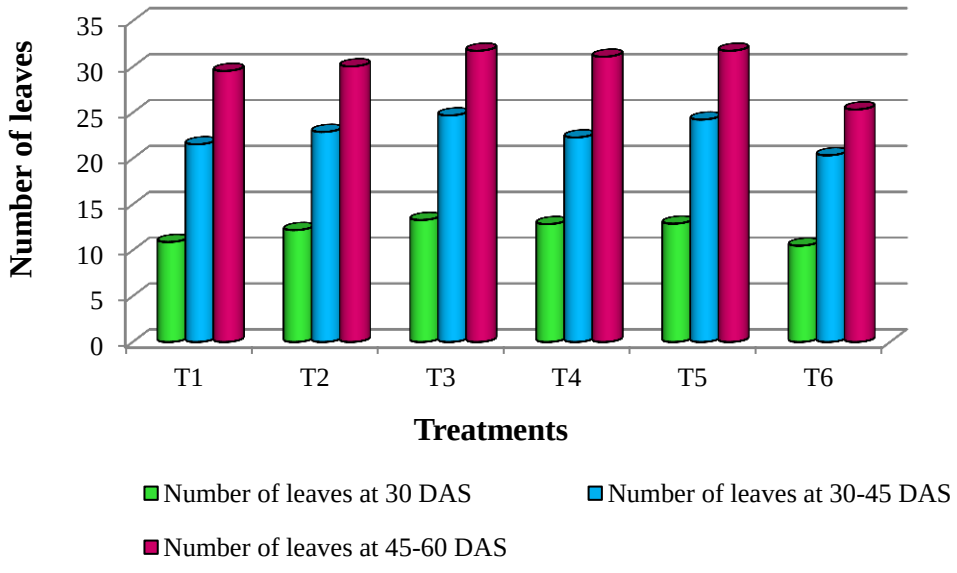


Fig. 5.24 Effect of treatments on number of leaves (Experiment 3)



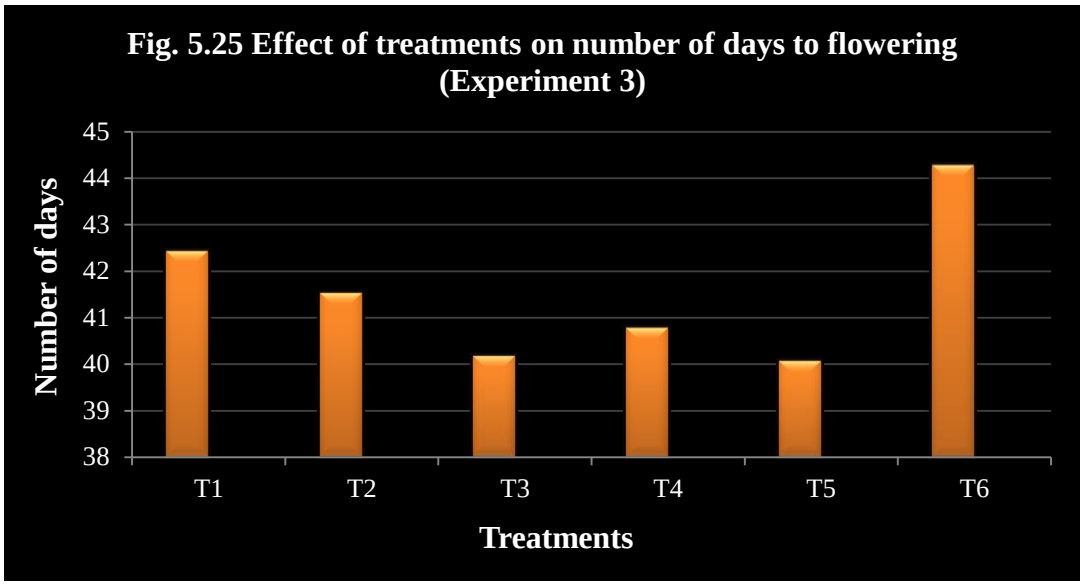


Fig. 5.25 Effect of treatments on number of days to flowering (Experiment 3)
Number of days
45
44
43
42
41
40
39
38
T1
T2
T3
T4
T5
T6
Treatments

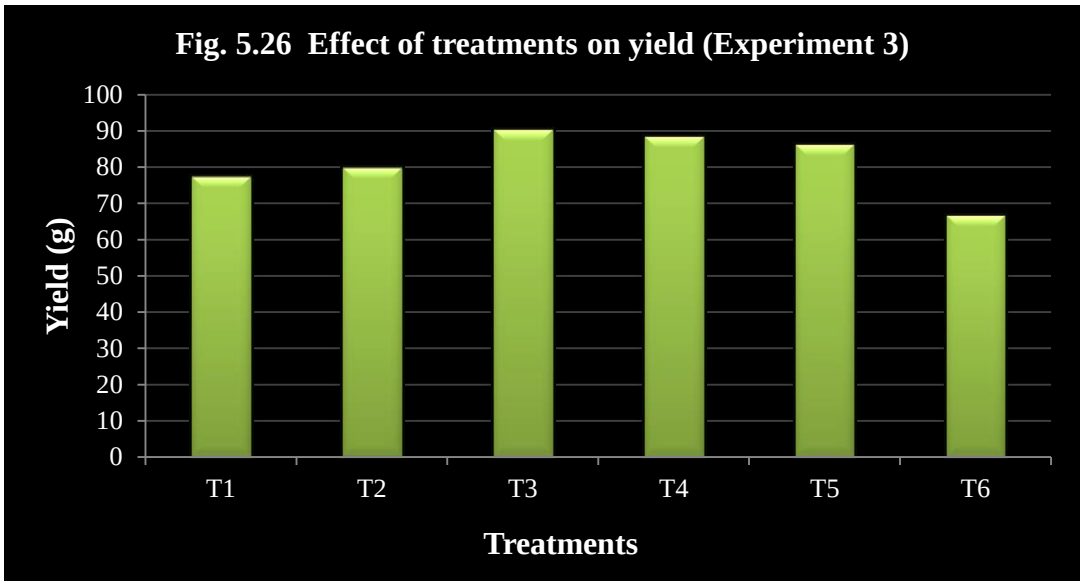


Fig. 5.26 Effect of treatments on yield (Experiment 3)
Yield (g)
100
90
80
70
60
50
40
30
20
10
0
T1
T2
T3
T4
T5
T6
Treatments

wilt nor reduce pathogen density in the soil. Similarly, Lewis *et al*. (1998) reported that sodium alginate prills of *T. hamatum* TRI - 4 with additives like bran, soy fibre, castor pomace and chitin controlled the damping off cotton caused by *R. solani*.

5.3.4 Estimation of population of *T. viride* in different treatments

The population of *T. viride* present in the potting mixture was enumerated prior to the application of treatment and at monthly intervals after the application. Owing to the process of fumigation, population was nil before the application of treatments in all the six treatments. The results of the present study were supported by Warcup (1951), where he observed the significant impact of formalin on the fungal flora including *Trichoderma* sp. in nursery soil. Similar findings were also reported by Mollison (1953), where he stated that the treatment with formalin devastated the population of *Trichoderma* sp. in nursery soil. Enumeration was carried out at 10^6 dilution, where 3.0 x 10^6 cfu g^{-1} soil was recorded in T_3 while population was nil in control at one month after application. The population in T_3 was increased to 3.6 x 10^6 and 4.0 x 10^6 cfu g^{-1} soil in subsequent months. The present findings are also in line with the report of Lewis and Papavizas (1987), where they observed the proliferation of the propagules of 11 isolates of *Trichoderma* spp. and *Gliocladium* sp. into 10^6 - 10^{11} cfu g^{-1} alginate pellet formulation applied in the soil. Similar reports were also putforth by Szczech and Maciorowski (2016), where the wet microbeads of *T. virens* TRS106 were incubated in soil and after 4 days, the population was estimated to 5.5 x 10^5 cfu·g-^{1} bead.

5.4 ASSESSMENT OF BIODEGRADATION OF *Trichoderma viride* ENCAPSULATED ALGINATE BEADS IN SOIL

The biodegradation of *T. viride* encapsulated microbeads was estimated, where the beads were bundled in meshed nylon bags and buried in garden soil. On third day, no beads were observed without any erosion while maximum number of completely degraded beads was observed in T_4 and minimum in T_2 and control. On sixth day, maximum number of completely degraded beads was recorded in in T_4 and T_5, while minimum was in control. On ninth and twelfth day of observation, again T_5 recorded the maximum number of completely degraded beads while control recorded the minimum.

Fig. 5.27 Estimation of the population of *T. viride* in different treatments

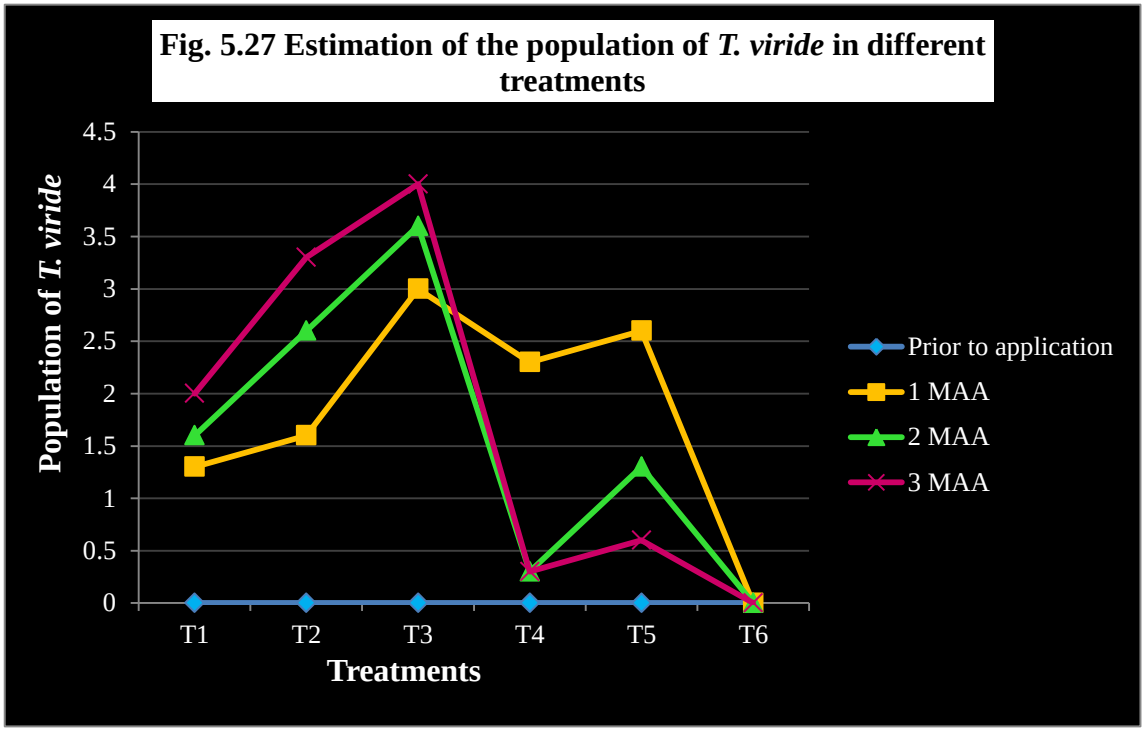


Fig 5.28 Estimation of biodegradation of microbeads

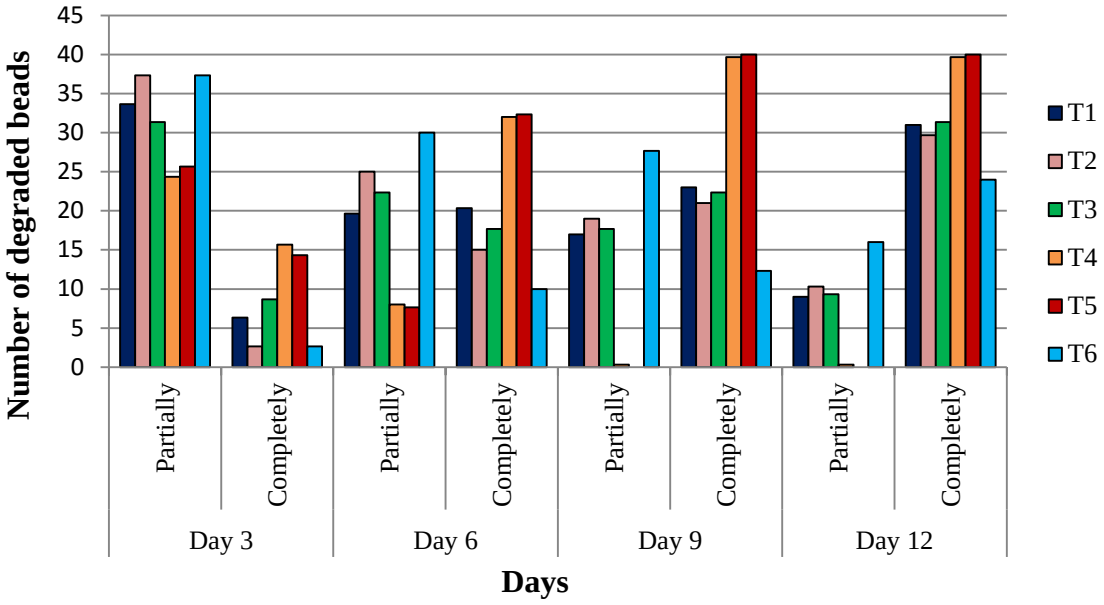
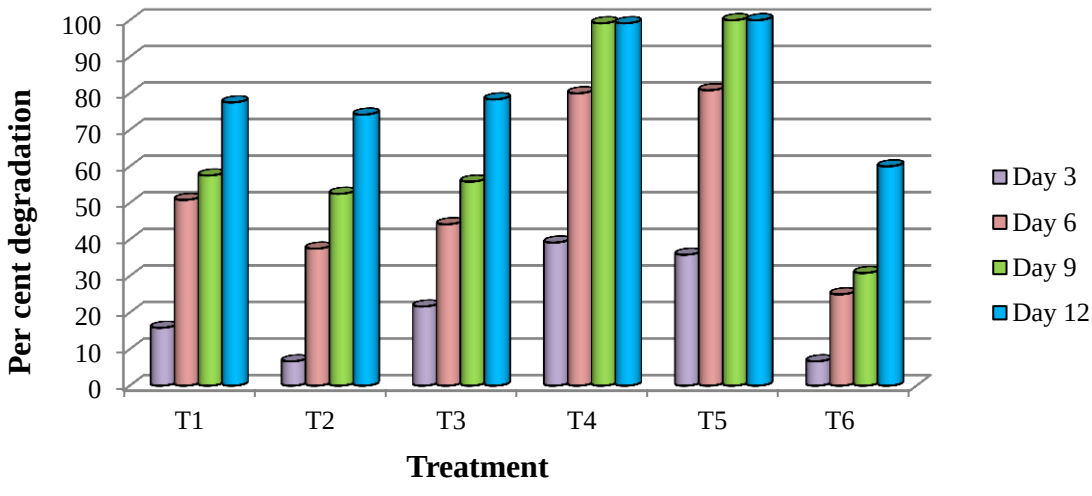


Fig 5.29 Per cent degradation of microbeads



From the observations, it was concluded that the rate of biodegradation diminished as the concentration of sodium alginate increased. The above conclusions are in agreement with Kakita and Kamishima (2008), where they reported that wet tensile strengths of gel fibres derived from high viscosity sodium alginate were higher than those from low viscosity sodium alginate. Further, thermo gravimetric analysis (TGA) conducted by Bajpai and Tankhiwale (2006) revealed that the strength of alginate beads depend on the concentration of sodium alginate and $CaCl_2$, the nature of the ionic salt used and the presence of other polymers.

5.5 BENEFIT - COST RATIO ANALYSIS

The recurring cost involved in the production of 1 Kg bead is INR 200.00 where a benefit cost ratio of 2.09:1 was calculated over talc based formulation.

Thus, by analysing the results of various experiments, it is concluded that sodium alginate bead formulation treatment $T_{21,}$ composed of Trehalose (15 mM), PVP (1%), CMC (0.5%), tween 80 (0.5%) , sodium alginate (1.5%) and $CaCl_2$ (3.5%) was selected as the best treatment among the other treatments with respect to colony count. The population of *T. viride* ranged from 11.3 x 10^6 - 34.6 10^6 cfu g^{-1} bead. The beads from this treatment are spherical in shape with smooth texture and ivory colour. The mean weight of these beads was estimated as 28.30 ± 1.5 mg with an average diameter of 1.76 ± 0.06 mm. The bead yield was estimated to 40.95 per cent with 12.94 beads formed from each ml of the sodium alginate solution. It is pertinent to note that these beads remained free of contamination even after 6 months of storage.

While analysing per cent potentially infective beads, though the germination was nil after 24 h of incubation, 79.03 ± 9.8 per cent germination was recorded at 48 h and cent per cent at 72 h. Thus the beads were proven to be potentially infective and hence, fit for field application. Immediately after preparation, the moisture content was estimated to be 92.0 ± 1.63 per cent. However, after 24, 48 and 72 h of incubation, a moisture content of 84.4 ± 0.43, 59.9 ± 1.95 and 26.2 ± 0.62 per cent respectively was recorded. It was also

noticed that the beads remained intact even after 240 min of incubation in phosphate buffer (pH 7.4) and gradually shrank in HCl (pH 1.0). A swelling ratio of 0.35 with 35.11 per cent swelling in phosphate buffer (pH 7.4) and a swelling ratio of -0.29 with -29.87 per cent swelling in HCl (pH 1.0) was also recorded. The beads showed a shrinking per cent of 8.98 which is minimum among the different treatments. Sphericity factor was recorded as 0.044 which is also least among different treatments, indicating the spherical shape of the bead. Also, the bead weight increased gradually till 60 min after formation, indicating the time of gelation as 60 min. Moreover, the bioagent release pattern varied for different pH, where the release was sudden at pH 5.09.

Further, the field performance of the sodium alginate bead based formulation was superior or was on par with talc and liquid based formulations and population of *T. viride* was also readily recovered from treated pots even after three months of application. Hence, the alginate bead based formulation of *T. viride* may thus represent a reliable alternative to talc and liquid based formulations in agro environments as the most important advantage of this formulation is the non-toxic nature and the slow release of the microorganism into the soil with a prolonged shelf life. Further, these microbeads can be produced easily unlike other formulations with optimum population of the bioagent for practical applications. Moreover, the *in vivo* results clearly confirmed the capacity of *T. viride* entrapped in alginate beads to inhibit the growth of soil borne pathogens. However, broad scale field application with multilocational trials should be necessitated to prove the efficacy of this novel formulation.

In conclusion, a simple protocol has been formulated in this study to produce microbeads useful for the application of *Trichoderma* sp. to agricultural plants. Moreover, the entire process is feasible in an industrial scale but demands pilot study.

SUMMARY

6. SUMMARY

Trichoderma, a genus of fungi well known for its biocontrol activity against a number of plant pathogens can promote plant growth and induce tolerance against abiotic stresses. However, to tackle the constraints associated with field application, a number of formulations based on diverse range of carrier materials have been developed in the recent past. Still, most of these formulations are suffering from severe drawbacks like bulkiness, contamination, desiccation *etc*. Hence, the present investigation on "Microencapsulation of *Trichoderma viride* for management of major soil borne fungal pathogens" was undertaken with an objective to standardize and prepare the alginate based bead formulation of *Trichoderma viride.*

1. Seven additive substances belonging to four different classes *viz*., sugar, wetting agent, adhesive and surfactant, in eight possible combinations were evaluated to improve the shelf life of alginate based bead formulation of *T. viride.* The study employed serial dilution and plating technique which was conducted at monthly intervals for seven months.
    - Combination consisting of trehalose (15 mM), PVP (1%), CMC (0.5%) and tween 80 (0.5%) (T_5) were found to outperform the other combinations with respect to colony count.
    - At 10^6 dilution, the colony count in T_5 ranged from 23.0 x 10^6 to 45.0 x 10^6 cfu ml^{-1}, while control recorded only 0.3 x 10^6 - 13 x 10^6 cfu ml^{-1}.
    - At 10^8 dilution, T_5 documented 6.0 x 10^8 - 21 x 10^8 cfu ml^{-1} whereas control recoded a maximum population of 1.3 x 10^8 cfu ml^{-1}.

2. Experimental parameters like concentration of sodium alginate and calcium chloride, volume of *T. viride* spore suspension required per 100 ml of sodium alginate solution and optimum vertical distance between the orifice of the funnel to surface of $CaCl_2$ bath were standardized.
    - At 0.75 and 1.5 per cent sodium alginate solution without additives, no beads were formed, while 2.5, 3.0 and 3.5 per cent sodium alginate solution yielded beads.

- However, at 2.5, 3.0 and 3.5 per cent sodium alginate solution with additives, no beads were formed, while 0.75 and 1.5 per cent sodium alginate solution yielded beads.
- Calcium chloride, at all the three selected concentrations *viz*., 2.5, 3.0 and 3.5 per cent yielded good quality spherical beads.
- Hence, 0.75 and 1.5 per cent sodium alginate and 2.5, 3.0 and 3.5 per cent $CaCl_2$ solution was selected to prepare the microbeads in 31 combinations.
- A volume of 5 ml spore suspension of *T. viride* in 95 ml sodium alginate solution yielded 2.3 x 10^8 cfu g^{-1} bead which was standardized for bead preparation.
- Based on the shape of the beads, a vertical range of 8 - 12 cm was selected for bead preparation.

3. The microbeads were prepared for 31 treatments including control by employing ionotropic gelation and cross linking technique and dried at room temperature for 48 h.

4. The beads were characterized for properties like weight, diameter, per cent yield and number of beads formed per ml sodium alginate solution.

    - Average bead weight ranged from 22.84 ± 4.1 to 96.20 ± 1.8 mg, with highest weight in control (T_{31}) and the least in T_4 (mannitol, PVP, CMC, tween 80, 0.75% sodium alginate and 2.5% $CaCl_2$).
    - Bead yield ranged from 24.51 to 69.09 per cent with the maximum yield in T_{19} (trehalose, PVP, CMC, tween 80, 1.5% sodium alginate, 2.5% $CaCl_2$) and the least in T_4 (mannitol, PVP, CMC, tween 80, 0.75% sodium alginate and 2.5% $CaCl_2$).
    - Maximum number of beads formed per ml of sodium alginate solution was recorded in T_9 (mannitol, PVP, liquid paraffin, tween 80, 1.5% sodium alginate and 3.5% $CaCl_2$) (26.91) and the minimum in control (T_{31}) (8.00).
    - Bead diameter ranged from 1.31 ± 0.01 to 2.42 ± 0.10 mm with the maximum in control (T_{31}) and minimum in T_{22} (trehalose, PVP, CMC, tween 80, 0.75% sodium alginate and 2.5% $CaCl_2$). Diameter was directly proportional to concentration of sodium alginate and $CaCl_2$.

5. Shelf life of *T. viride* encapsulated alginate beads were carried out employing serial dilution and plating technique at monthly intervals upto six months.
    - Treatment T_{21} (trehalose, PVP, CMC, tween 80, 1.5% sodium alginate and 3.5 % $CaCl_2$) outperformed the other treatments with respect to colony count and it was selected as the best treatment. The population ranged from 11.3×10^6 - 34.6×10^6 cfu g^{-1} bead.

6. Beads were inspected for the degree of contamination using 0 - 4 scoring index.
    - Treatment T_1 to T_6 and T_{19} to T_{30} remained free of contamination even after six months, while control beads showed discolouration.

7. Some additional parameters like per cent potentially infective beads, moisture content in microbeads, swelling behaviour of microbeads at different pH, per cent shrinking, sphericity factor, time of gelation and population of *T. viride* in soil at different pH was estimated for the seven selected treatments.
    - All the selected treatments were potentially infective with cent per cent germination at 72 h after incubation and hence, fit for field application.
    - Immediately after preparation, moisture per cent ranged from 90.2 ± 0.86 (T_4) (mannitol, PVP, CMC, tween 80, 0.75% sodium alginate and 2.5% $CaCl_2$) to 94.0 ± 1.63 (T_1) (mannitol, PVP, CMC, tween 80, 1.5% sodium alginate and 2.5% $CaCl_2$) per cent, whereas it was reduced to 20.9 ± 0.63 (T_6) (mannitol, PVP, CMC, tween 80, 0.75% sodium alginate and 3.5% $CaCl_2$) to 28.7 ± 1.28 (T_2) (mannitol, PVP, CMC, tween 80, 1.5% sodium alginate and 3.0% $CaCl_2$) after 72 h of open drying.
    - Microbeads were found to swell in phosphate buffer with pH 7.4 and shrank in 0.1M HCl with pH 1.0.
    - Maximum per cent shrinking was observed in T_5 (mannitol, PVP, CMC, tween 80, 0.75% sodium alginate and 3.0% $CaCl_2$) (47.05%) and the minimum in control (T_{31}) (5.57 %). Degree of shrinking is inversely proportional to the concentration of sodium alginate in the bead.

- Maximum value of sphericity factor was noticed in T_6 (mannitol, PVP, CMC, tween 80, 0.75% sodium alginate and 3.5% $CaCl_2$) (0.117) and the minimum in control (T_{31}) (0.034). It was proved that the magnitude of sphericity factor is inversely proportional to the concentration of sodium alginate.
- Time of gelation was estimated as 60 min.
- Release of entrapped bioagent depends on the pH of the external medium. Release in acidic environment is gradual and sustained and in alkaline pH, beads readily swell and suddenly release the bioagent.

8. Biocontrol efficacy and plant growth promoting activity of sodium alginate bead based formulation of *T. viride* was studied using bush cowpea as the test crop and *Pythium aphanidermatum*, *Rhizoctonia solani* and *Fusarium oxysporum* as the test pathogen. Biometric characters like plant height and number of leaves were recorded along with the assessment of number of days required for first flowering and crop yield and per cent disease incidence.
    - Sodium alginate based bead formulation of *T. viride* performed superior to talc and liquid formulations.
    - Though considerable quantum of disease incidence was recorded in all the three experiments, recurrence of the disease incidence was not observed.
    - The recurring cost involved in the production of 1 Kg bead is INR 200.00, where a benefit cost ratio of 2.09:1 was calculated over talc based formulation.

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APPENDICES

APPENDIX-I

Composition of media used for preservation of *T. viride*

Potato Dextrose Agar

Potato	:	200.0 g
Dextrose	:	20.0 g
Agar	:	20.0 g
Distilled water	:	1000 ml

APPENDIX-II

Composition of media used for growing *T. viride* in shelf life evaluation study

Potato Dextrose Broth

Potato	:	200.0 g
Dextrose	:	20.0 g
Distilled water	:	1000 mL

APPENDIX-III

Composition of medium used for recovery of *T. viride* from soil

***Trichoderma* Selective Medium**

Agar	:	15.0 g
Glucose	:	3.0 g
K_2HPO4	:	0.9 g
$MgSO_4.7H2O$	:	0.2 g
NH_4NO_3	:	1.0 g
KCl	:	0.5 g
Rose Bengal	:	0.033 g
Metalaxyl	:	0.3 g
Pentachloronitrobenzene	:	0.2 g
Chloramphenicol	:	0.25 g
Distilled water	:	1000 mL

MICROENCAPSULATION OF *Trichoderma viride* FOR MANAGEMENT OF MAJOR SOIL BORNE FUNGAL PATHOGENS

Abstract of a Thesis

Submitted in partial fulfillment of
the requirement for the degree of

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by

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Microencapsulation of *Trichoderma viride* for management of major soil borne fungal pathogens

Abstract

Trichoderma spp, one of the widely exploited biocontrol agents in the arena of crop disease management, are known for promoting growth and inducing abiotic stress tolerance in plants. However, direct application in the field limits their efficiency due to several adverse factors. Hence, they should be adequately formulated to escalate the efficacy in field application. Eventhough, a number of such formulations have been developed in the recent past, many of them are bulky in nature, having reduced shelf life with a high risk of contamination and desiccation. Thus, a study was conducted to develop a novel formulation of *T. viride*, with sodium alginate, a biodegradable polymer as the encapsulating carrier material.

Evaluation of the effect of additive substances *viz*., mannitol (2%), trehalose (15 mM), polyvinyl pyrrolidone (1%), polyethylene glycol (0.25%), carboxymethyl cellulose (0.5%), liquid paraffin (1%) and tween 80 (0.5%) in eight combination on the shelf life of *T. viride* was carried out. After seven months of evaluation, a combination of trehalose, PVP, CMC and tween 80 outperformed the other treatments. Beads were prepared using 0.75, 1.5, 2.5, 3.0 and 3.5 per cent sodium alginate and 2.5, 3.0 and 3.5 per cent $CaCl_2$ solution with and without additives. Without additives, no beads were formed at 0.75 and 1.5 per cent sodium alginate and spherical beads were formed at 2.5, 3.0 and 3.5 per cent. When additives were amalgamated, 0.75 and 1.5 per cent sodium alginate yielded spherical beads, while beads were not formed at 2.5, 3.0 and 3.5 per cent. Hence, the concentration of sodium alginate was standardized as 0.75 and 1.5 per cent. The impact of height of air column between the orifice of the separating funnel and the level of $CaCl_2$ bath on the bead shape was evaluated at different heights where 2 and 4 cm yielded tailed beads while, rest of the heights yielded spherical beads. Hence, based on the shape of the bead, 8 - 12 cm was selected as the optimum height of air column.

Previously standardized parameters along with 2.5, 3.0 and 3.5 per cent $CaCl_2$ solution was adopted to prepare beads by employing ionotropic gelation and cross linking technique in 31 treatments. The beads were dried for 48 h at room temperature and stored in air tight containers.

Properties like bead weight, diameter, yield and number of beads formed per ml sodium alginate solution was estimated. A maximum bead weight of 96.20 ± 1.8 mg and bead diameter of 2.42 ± 0.13 mm was recorded in control (T_{31}-sodium alginate 2.5% and $CaCl_2$ 2.5%). Maximum per cent yield was documented in T_{19} (trehalose, PVP, CMC, tween 80, sodium alginate 1.5%, $CaCl_2$ 2.5%) (69.09%) and a maximum of 26.91 beads were formed from each ml of sodium alginate in T_9 while, it was 8.0 in control. Shelf life evaluation was carried out for six months, where T_{21} (trehalose, PVP, CMC, tween 80, sodium alginate 1.5%, $CaCl_2$ 3.5%) outperformed the other treatments even after six months hence, T_{21} was selected as the best treatment. Degree of contamination was estimated at 1, 3 and 6 months after preparation, where T_1 - T_6 and T_{19} - T_{30} remained free of contamination even after six months of preparation.

Per cent of potentially infective beads were estimated where cent per cent germination was recorded at 72 h after incubation. Moisture content of the beads were assessed where the beads retained its intact shape at 48 h and therefore was selected as the optimum period of drying. Swelling behaviour of microbeads were estimated at pH 7.4 in phosphate buffer and at pH 1.0 in 0.1M HCl. A gradual increase in weight of beads at pH 7.4 confirmed the swelling behaviour while, reduction in weight at pH 1.0 revealed its shrinking nature. Time of gelation was standardized as 60 min as the bead weight declined until this time and attained stability after this time. The effect of pH on the selected bead (T_{21}) was evaluated at pH 5.09 and 8.91 in soil under *in vitro* conditions where the release in former was sustained while a sudden release was observed in latter. Observations on biometric parameters, yield and the per cent disease incidence from pot culture experiments revealed that sodium alginate bead based formulation @ 5.0 g $plant^{-1}$ performed superior to talc based and liquid formulations. The study on degradation revealed that the beads were biodegradable in nature.

Thus, the present investigation succeeded in formulating *T. viride* using sodium alginate as the encapsulating carrier material, which would help to compensate the drawbacks associated with presently available formulations. However, the study should be complimented with multilocational trials to confirm its efficacy under field conditions.