ALGINATE BASED ENCAPSULATION OF *Pseudomonas fluorescens* FOR MANAGEMENT OF SOIL BORNE PATHOGENS

By

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(2018-11-146)

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THESIS

Submitted in partial fulfillment of the requirement for the degree of

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DEPARTMENT OF PLANT PATHOLOGY
COLLEGE OF HORTICULTURE
VELLANIKKARA, THRISSUR – 680656
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2020

DECLARATION

I, hereby declare that the thesis entitled **"Alginate based encapsulation of *Pseudomonas fluorescens* for management of soil borne 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.

Vellanikkara,
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(2018-11-146)

CERTIFICATE

Certified that this thesis entitled **"Alginate based encapsulation of *Pseudomonas fluorescens* for management of soil borne pathogens"** is a record of research work done independently by **Ms. SIVADHARSHANAPRIYA, R. (2018-11-146)** 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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INTRODUCTION

1. INTRODUCTION

Agriculture is heavily dependent on the use of chemical fertilizers and pesticides for plant nutrition and disease control which is a practice that had significant negative impacts on human health and environment. Globally, increasing knowledge of ill effects of synthetic pesticides has increased a demand for safer alternatives (Singh, 2006; Keswani *et al.*, 2014; Mishra *et al.*, 2015). In this context, biocontrol approaches help to develop an eco-friendly control strategy for plant disease management (Bharathi *et al.*, 2004; Heydari and Gharedaghli, 2007).

Inoculation of plants with microorganisms to control plant diseases has been practiced for several decades. *Pseudomonas fluorescens* is one such biocontrol agent which is used to combat many phytopathogens. They are fast growing, culturable could be manipulated easily in the laboratory, promotes plant growth, produce antimicrobial compounds, siderophores and compete with disease causing microbes (Walsh *et al.*, 2001; Haas and Defago, 2005; Morales *et al*., 2010; Özyilmaz and Benlioglu, 2013).

Microorganisms applied in the field encounters several problems due to unpredictable conditions in the environment. They have to compete with indigenous microorganisms, variable temperature and pH, drought, adsorption by soil particles, or washing-off by rain (Bashan, 1986b; Young *et al.*, 2006; Guo *et al.*, 2012). Therefore, for commercial application, microbial inoculum should be supported by appropriate formulation preventing rapid decline of introduced biocontrol agents and extending their shelf-life.

The formulations are the industrial “secret art” of converting a promising laboratory-proven microorganism, developed by professional experts into a commercial field products which can be effectively used by the farmers under uncontrolled field

conditions. Hence, the formulations must be developed to ensure that biocontrol agents could be present in enough concentrations to give effective and consistent control of the target disease. The application of *Pseudomonas fluorescens* to soil was always considered a challenge for scientists due to the dynamic chemical, physical and biological state of soil environment which makes difficult to hold bacteria as long as possible under the best conditions of viability and operation. Therefore, developing a good formulation is the key to the commercial success of biocontrol agents.

A number of formulations based on several carriers were developed for application in agriculture against various soil borne pathogens *viz*., *Fusarium, Pythium, Sclerotium, Ralstonia, Rhizoctonia* etc. Among the different carriers, talc and peat based formulation of *P. fluorescens* are widely accepted by the farmers in commercial scale. But the major drawbacks faced by farmers while using these formulations are poor shelf life of carrier-based biocontrol agents and biofertilizers (3-4 months), unavailability of good carrier materials, temperature sensitive, more chances of contamination, bulkiness to transport, poor cell protection and low moisture retention capacity (Verma *et al.*, 2011).

Liquid formulation of *P. fluorescens* are also widely used to control plant pathogens, as liquid formulations can be packed and stored for longer period of time but survival rates of bacterial inoculants on liquid formulation decreases, because of unpredictable environmental conditions. Biocontrol agents in liquid formulations are subject to abiotic stress, thermal shock or hypoxia, resulting in a drastic reduction of viable cells (He *et al*., 2015; Berger *et al*., 2018; Bernabeu *et al.*, 2018) and the distribution of number of bacteria is quite heterogeneous during application of liquid formulations. Liquid formulations are prone to contamination during storage, transport and application into the soil (Bashan *et al*., 2002). The constraints of powder and liquid formulations necessitated studies with an objective of extending shelf life, quality and efficiency of inoculants. Therefore, developing an alternate formulation is necessary for preparation of microbial inoculants.

Several experimental formulations based on polymers have been evaluated for decades. These polymers were demonstrated as potential bacterial carriers (Jung *et al.*, 1982; Bashan, 1998), offering considerable practical advantages over talc and peat-based formulations (Amiet-Charpentier *et al.,* 1999) which include prolonged shelf life, maintain sufficient cell density, ease of manufacturing and improved performance at the destination field (Bashan 1998; John *et al.,* 2011). Encapsulation is a process by which active ingredients are packaged within a polymer matrix for the purpose of shielding biocontrol agents from the surrounding environment (Anal and Singh, 2007). Alginate is the most common polymeric material obtained from macroalgae is used for encapsulation of microorganisms for commercial use (Cassidy *et al*., 1997). Sodium alginate has a unique gelation property with calcium ions that leads to the formation of egg box- like gel structure.

The preparation of alginate beads is quite easy, straight forward and beads are formed instantaneously at room temperature with sufficient mechanical strength. The resulting granular preparation are lighter in weight than liquid and also beads are more uniform and less bulkier than other commercially available formulations.

Advantages of alginate based encapsulation are non toxic nature, biodegradability, release of the entrapped microorganisms gradually into the soil when polymers are degraded, protects biocontrol agents from biotic and abiotic stress, possible to store under dry condition at ambient temperature for prolonged periods, simple to use for the farmers, storage requires little space and less prone to contamination after production (Kim *et al.,* 1996; Bashan *et al.,* 2002; Zohar Perez *et al*., 2002).

Stability of the formulation is enhanced by the addition of nutrient amendments during encapsulation which makes the active ingredient retain its population for the considerable period of time, thus improving the shelf life of the formulation. Several

experimental alginate-based formulations were evaluated *viz*., encapsulation of the ectomycorrhizal fungi (Tacon *et al*., 1985), Plant growth promoting bacteria (Trevors, 1992), VA-mycorrhizal fungi (Ganry *et al.*, 1982), bacteria (Aino, 1997) and fungi (DeLucca *et al.*, 1990) used as biocontrol agents against soil-borne pathogens. Although, commercial alginate beads are not yet available for bacterial plant inoculation against phytopathogens, considering the status of research in this area, this investigation was conducted with the following objectives.

- Standardization of media with additives for optimum growth of *P. fluorescens*
- Preparation of alginate bead based formulation of *P. fluorescens*
- Evaluation of shelf life of *P. fluorescens* encapsulated alginate beads
- *In vitro* inhibition of alginate formulation against major soil borne pathogens

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

There is an increasing public concern about the continuous use of agrochemicals, which harm the environment and human health. Such concerns drive the search for more eco-friendly methods of plant disease control that will contribute to sustainable goal in agriculture. It was at this time that the idea emerged, regarding the use of microorganisms to control plant diseases. Rovira and Bowen (1966) reported that the application of plant growth promoting rhizobacteria into agricultural soils have been proven to increase the crop yield tremendously. Inoculation of plants with bio-control agents has been practiced for several decades to control plant diseases and also to enhance crop yield.

Among the different biocontrol agents, *Pseudomonas* spp. is one such biocontrol agent that colonize the roots of crop plants, promotes plant growth, produce antimicrobial compounds, siderophores and compete with microbes causing diseases, an alternative to the utility of chemical fungicides (Walsh *et al*., 2001; Haas and Defago, 2005; Morales *et al*., 2010; Özyilmaz and Benlioglu, 2013).

The key components to the commercial success of a biological control agent were dependent on the development of formulation. Formulation can stabilize the biocontrol agent during production, distribution and storage, protect the microorganisms from adverse environmental conditions, helps in the handling and application of the product and enhance the activity of the organisms (Jones and Burges, 1998). Specifically, bacterial inoculants were formulated as fluid suspensions, powder formulations and granules for seed, soil and spray application. Although powder and liquid based formulations are widely used, few factors contributing to the failure of inoculated products *viz*., shorter shelf life, high contamination, reduced quality, death of organisms upon inoculation and unpredictable field performances.

These limitations necessitated studies with an objective of developing suitable alternate formulation with greater shelf life, quality and efficiency of inoculants. The research work on formulations has therefore focused on alginate as the carrier material and has harnessed enormous data to develop an efficient formulation which can increase the shelf life. Hence, an attempt was made to review some of the recently published work on "Alginate based encapsulation of *Pseudomonas fluorescens* for the management of soil borne pathogens" relevant to the study.

2.1 *Pseudomonas fluorescens*- Dual role as PGPR and biocontrol agent

Pseudomonas possesses many characteristics which makes them suitable as both biocontrol and growth promoting agents (Banasco *et al*., 1998). These include the ability to (i) grow rapidly *in vitro* (ii) colonize and multiply rapidly in the rhizosphere and spermosphere environments and in the interior of the plant; (iii) utilize seed and root exudates; (iv) produce a broad spectrum of bioactive metabolites such as antibiotics, HCN, growth promoting hormones, and catalase, siderophores, chitinolytic enzymes and can also solublize phosphorous (Seong and Shin, 1996) (v) compete aggressively with other microorganisms and (vi) adapt to environmental stresses (Sivasakthi *et al.*, 2014).

Pseudomonas spp. has been reported to have biocontrol activity against wide range of plant pathogens. They are fast growing, culturable and manipulate easily in the laboratory for experimentation and are common rhizosphere organisms and must be adapted to life in the rhizosphere to a large extent (Marilley and Aragno, 1999). *Pseudomonas* spp. has been used as biocontrol agents against various plant pathogens (Khan and Khan, 2002; Guo *et al.*, 2004). It is a rod shaped, gram negative bacterium which is ubiquitous as it is found in all agricultural soils and has many characteristics that make them suitable as PGPR. Several soil edaphic factors such as soil temperature, soil pH, moisture and clay content affect bacterial growth and survival and also their interaction

with pathogens. *P. fluorescens* found in rice and sugarcane rhizosphere, exhibits strong antifungal activity against *Rhizoctonia bataticola* and *Fusarium oxysporum* (Kumar *et al.*, 2004). Gaur *et al*. (2004) found that 50 – 60 per cent of *Pseudomonas* spp. isolated from the rhizosphere and endorhizosphere of wheat cultivated in Indo-gangetic plains are antagonistic towards *Helminthosporium sativum*. Siddiqui (2005) reported thar *P. fluorescens* CHA0 suppresses black root rot of tobacco and also in the biocontrol of *Meloidogyne javanica*, the root-knot nematode under in situ conditions. Paul *et al*. (2006) reported *P. fluorescens* MSP-393 as an efficient biocontrol agent in rice grown in coastal saline soils. Paul and Sarma (2006) observed that *P. fluorescens* antagonizes all the reproductive phases of the *Phytophthora capsici*, the causal organism of foot rot disease in all seasons of plant growth.

Reddy *et al*. (2007) obtained isolates of *P. fluorescens* from rice rhizosphere and showed antifungal activity against *Dreschelaria oryzae*, *Magnaporthe grisea*, *Sarocladium oryzae* and *Rhizoctonia solani* that are known to attack rice plants. Indigenous Pseudomonas strains from rhizosphere of rice grown in coastal agri - ecosystem was found to be effective against *Xanthomonas oryzae* and *Rhizoctonia solani* under both natural and saline soil conditions (Reddy *et al*., 2008). Maleki *et al*. (2010) observed that *Pseudomonas fluorescens* isolated from cucumber rhizosphere act as potential biological control agents against *Phytophthora drechsleri*, causal agent of cucumber root rot under greenhouse conditions and significantly promoted plant growth under *in vitro* condition.

According to Gaskins *et al.* (1985), *Pseudomonas fluorescens* typically enhance plant growth by directly influencing plants through generating plant growth promoting substances by both direct and indirect means, but not all of the particular mechanisms were well characterized (Glick *et al*., 1995). *Pseudomonas fluorescens* having phosphate solubilizing ability have been found to enhance growth rate of peanut under pot culture conditions (Pal *et al*., 2000). Johri (2001) reported that field trials of a *Pseudomonas* strain lead to a significant increase in legume yield. *Pseudomonas* spp. are used as biofertilizers

and also increase crop yield by direct and indirect mechanisms (Walsh *et al.,* 2001). *Pseudomonas* strains are capable of solubilizing phosphorous in soil and increasing its availability to plants (Sundara *et al.,* 2002). The inoculation of soil with *Pseudomonas* spp. increases yield and productivity in two cultivars of rice (Mirza *et al.,* 2006), tomato (Minorsky, 2008) and asparagus (Liddycoat *et al.,* 2009). Gulati *et al*. (2008) stated that some species of the genus *Pseudomonas* such as *P. corrugta, P. aeruginosa, P. fluorescens, P. rhizosphareae, P. lutea* and *P. stutzeri* are known to be good phosphate solubilizers.

Siddiqui (2004) reported that *P. fluorescens* was good at increasing growth of tomato plants and reducing nematode and galling multiplication. Plant growth promoting rhizobacteria *P. fluorescens* B16 isolated from graminaceous roots has been shown to colonize the roots of different plants and increase in height, flower number, fruit weight and fruit number of tomato plants (Minorsky, 2008). The sawdust-based formulations of *Pseudomonas fluorescens* and *Rhizobium leguminosarum* were found to be better carrier-based inoculant in improving nodule number and seedling biomass of *Trifolium repense* (Arora *et al.,* 2008).

Karnwal (2009) stated that *P. fluorescens* are effective in IAA production than *Pseudomonas aeruginosa*. IAA production in fluorescent *Pseudomonas* isolates increased with increase in tryptophan concentration from 1-5 mg ml^{-1}. Auxin's effect on plant seedlings is dependent on concentration and low concentration may improve growth while high concentration may be inhibitory (Ahmad *et al.,* 2008). Sarma *et al.* (2011) used vermiculite formulations of two strains of fluorescent Pseudomonads R62 and R81. The combined inoculation effect of these two formulations resulted in marked improvement in dry root weight, dry shoot weight, and fruit yield of tomato plants.

Thus, it is evident from various studies that *P. fluorescens* are capable of producing antifungal and antibacterial substances and function as plant growth promoting substances. Hence, it can be used as potential biofertilizer and also as biocontrol agent in

agriculture field. However, microorganisms introduced in the field encompasses several problems due to unpredictable conditions in the environment. They have to compete with indigenous microorganisms, high temperature, drought, soil particle adsorption, variable pH, or wash-off by rain (Bashan, 1986b; Young *et al*., 2006; Guo *et al*., 2012). Therefore, for commercial application, microbial inoculum should be supported by appropriate formulation which has an extended shelf life of introduced biocontrol agents.

2.2 Formulation -a key step towards commercialization

The success of potential biocontrol activity depends on the development of appropriate formulations. Formulation of inoculant carrier is an industrial art of converting a promising laboratory proven bacterium to a commercial field product (Bashan, 1998). An effective biocontrol agent must survive formulation and storage and should be an aggressive and competitive colonizer after inoculation (Beatty and Jensen, 2002; Selim *et al*., 2005).

Usually, the formulation consists of establishing viable bacteria in a suitable carrier, together with additives that help to stabilize and protect microbial cells during storage and transportation (Hynes and Boyetchko, 2006). Xavier *et al*. (2004) reported that the formulation should also be easy to handle and apply so that it is administered in the most effective manner and form, which protects bacteria from harmful environmental factors and maintains or enhances activity of the organism within the field. Numerous formulations were developed based on microorganisms, with applications for various crops around the world (Saleem and Khan, 2017). A good bioformulation should be effective, readily biodegradable, non-polluting, with high water retention capacity and with adequate shelf life (Malusa *et al*., 2012; Sahu *et al*., 2018). The formulation process must increases the product's efficiency and shelf life.

Several attempts have been made by researchers on various formulations of *Pseudomonas* spp. and according to Smith (1992), various formulations are available such as powder, liquid and granular formulations on legume inoculant.

2.2.1 Carrier based inoculant formulation

This formulation has been typically prepared as carrier-based inoculants that contain effective microorganisms. The incorporation of microorganisms in carrier material provides an environment for microbial inoculants which are conducive for their growth. Various types of materials like talc, peat, lignite, compost, vermiculite are used as carriers (Vidhyasekaran and Muthamilan, 1995).

2.2.1.1 Talc as carrier material

Talc is a natural material composed of various minerals along with chloride and carbonate obtained as raw material from soapstone industries. It is used as a carrier for microbial inoculants as it is readily available and also maintains sufficient population of inoculants during storage (Nakeeran *et al.,* 2005).

Kloepper and Schroth, (1981) reported that the population of fluorescent pseudomonads did not decrease in talc mixture containing 20 per cent xanthum gum after two months of storage at 4^{o}C and that *P. fluorescens* were first developed as a talc-based formulation for the treatment of potato seed tubers. Likewise, Senthil *et al.* (2003) stated that the talc-based formulation has been reported for the management of several crop diseases in India. Rajappan and Ramaraj (1999) observed that in field studies, soil application of the talc-based formulation of *P. fluorescens* effectively controlled cauliflower wilt caused by *F. moniliformae*.

Vimala *et al.* (2009) studied the effect of carrier materials against *Macrophomina phaseolina*. Among the carriers, talc formulation of *P. fluorescens* maintained a population of 16.25×10^7 cfu g^{-1} of product and the efficacy of *P. fluorescens* in inhibiting the growth of *Macrophomina phaseolina* was 47.77 per cent in talc even after 90 days of storage. Chitra *et al*. (2006) reported that the application of talc formulation of *P. fluorescens* through seed treatment, seed treatment plus foliar spray and foliar spray alone significantly reduced the incidence of groundnut leaf blight under both greenhouse and field conditions. Similarly, Chandar *et al*. (2013) recorded higher colony forming units (cfu) in talc based formulation of *P. fluorescens* compared to lignite and vermiculite-based formulations. Gade *et al.* (2014) observed that talc formulations promoted the maximum number of viable *P. fluorescens* cells as compared to lignite formulation.

2.2.1.2 Peat as carrier material

Peat is the commonly used and most preferred carrier material. Callan *et al.* (1990) reported that soil application of peat-based formulations of *P. fluorescens* effectively controlled seedling rot and blight disease of cotton. Moreover, Rabindran and Vidhyasekaran (1996) reported that application of the peat-based formulation of *P. fluorescens* ALR2 in the field trials effectively controlled the disease, increased yield and efficacy was comparable to that of commercially available carbendazim fungicide. Vidhyasekaran *et al.* (1997) tested talc, vermiculite, peat, lignite and kaolinite as carrier materials for survival of *P. fluorescens* strains Pf1 and Pf2 and found that both strains could survive (10^6 cfu g^{-1}) in talc and peat-based formulations.

Survival of *P. fluorescens* was analysed by Sivakumar and Narayanaswamy (1998) and they noticed that among the different carrier-based formulations, peat and talc-based formulations could maintain the highest level of population even after 40 days of storage. Sivakumar *et al*. (2000) also observed that foliar spraying and soil application of peat based formulation of *P. fluorescens* provided protection against *R. solani* f. sp. *sasaki*

in rice. Gasoni *et al.* (1998) examined the effectiveness of different carrier combinations in maintaining the *B. cereus* and *P. fluorescens* population. They observed that *P. fluorescens* in peat formulation could effectively control the disease compared to vermiculite and clay formulations but the size of population of *B. cereus* in peat-based formulation increased to 20 folds after 150 days of storage whereas, population size of *P. fluorescens* in peat formulation declined after 30 days of storage.

According to Parker and Vincent (1981), peat being an organic carrier, difficulties are found in complete sterilization by steam or gamma radiation and it was noted that high temperature and high radiation dosages are undesirable, as it produces toxic by-products. They also noticed that the unavailability of good quality peat is a major restriction for the inoculant production.

2.2.1.3 Lignite as carrier material

Kandaswamy and Prasad (1971) reported that lignite could be an alternative to peat for inoculant formulation and according to Khungar (1998), lignite is one of the preferred and commonly used carrier in biofertilizers manufactured across India. Kalaivani (1998) reported that lignite formulation enabled higher survival of *P. fluorescens* and *Bradyrhizobium japonicum* than peat based formulations. However, Chandar *et al.* (2013) noticed that commercially, lignite is preferred less compared to talc and peat-based formulation because survival of biocontrol agent is less compared to peat and talc based formulations and according to them the poor survival of inoculants may be due to the formation of hard clumps which upon drying significantly reduced the population in lignite inoculants. They also observed that talc based formulations possess higher colony forming units of *P. fluorescens*, *Trichoderma viride*, *Trichoderma harzianum* than lignite or vermiculite based formulations.

Karunya and Reetha (2014) revealed that saline tolerant PGPR strains *viz*., *Azospirillum brasilense* PA-17, *Bacillus subtilis* PB-15 and *Pseudomonas fluorescens* PP-15 are maintained at a population of 1×10^8 cells g^{-1} in both carriers and liquid formulation. Among the carriers, lignite possesses higher population compared to pressmud and vermiculite. According to Gade *et al*. (2014), maximum number of viable cells of *P. fluorescens* was obtained from talc formulation than lignite based formulations.

2.2.1.4 Vermiculite and vermicompost as carrier material

Graham-weiss *et al.* (1987) found that vermiculite formulation of *Bacillus megaterium* and several strains of *Pseudomonas* supported higher population when supplemented with nutrients and moisture. Sarma *et al.* (2009) observed that vermiculite formulations of fluorescent pseudomonads strains is effective for positive crop response of *Vigna mungo*. Gandhi and Saravanakumar (2009) noticed that shelf life of bioinoculants such as *Azospirillum lipoferum*, *Bacillus megaterium* and *P. fluorescens* in vermicompost formulation was higher compared to lignite formulations. Chakravarty and Kalita (2011) observed that survival of *P. fluorescens* in vermicompost based formulation proved to be the best in management of bacterial wilt of brinjal followed by farmyard manure, decomposed mustard oil cake, rice bran, wheat bran, rice straw and banana leaf respectively.

However, Verma *et al.* (2011) stated that powder based formulations are not very satisfactory due to certain limitations related with poor shelf life (3-4 months) of carrier-based biofertilizers, temperature sensitive, more chances of contamination, bulkiness to transport and therefore, high transport cost, less scope for export, proper packing problems, poor cell protection and low moisture retention capacity. Hence, the above studies substantiate the fact that powder based formulation has several disadvantages among the various formulations developed with respect to field application and shelf life.

2.3 Liquid based formulations

A perusal of the literature revealed that reports on liquid formulations are meagre and scanty. However, attempts have been made to include some of the available literature on the same. Liquid formulations contain inoculants combined with liquid carriers which may be water, oil or some solvents in the form of suspension, emulsion or concentrates which can increase stability, adhesion and dispersion capacity (Lee *et al.,* 2016). The advantages of liquid formulations are ease in application and processing and lower cost compared to solid-based formulations (Kumaresan and Reetha, 2011).

Karunya and Reetha (2014) found that population of (1×10^8 cells g^{-1}) is maintained in liquid and carrier based formulation of *Azospirillum brasilense* PA-17, *Bacillus subtilis* PB-15 and *Pseudomonas fluorescens* PP-15. Various workers reported that liquid inoculants can be packed and stored for longer periods especially when biocontrol agents are subjected to abiotic stress which is caused by depletion of nutrients, thermal shock or hypoxia, resulting in a drastic reduction in viable cells (He *et al*., 2015; Berger *et al*., 2018; Bernabeu *et al.*, 2018). However, Bashan *et al*. (2002) reported that survival rates of bacterial inoculants in liquid formulations decrease because of unpredictable environmental conditions and according to them, distribution of number of bacteria is quite heterogeneous during application of liquid formulations. These formulations are also prone to contamination during storage, transport and also during application into the soil.

2.4 Granular based formulations

The constraints of powder and liquid-based formulations could be overcome by formulating granular microbial inoculants, which is simple and fast, which has longer shelf-life and easy to apply by farmers and is also free from pollutants and has supported large

microbial populations. The granular formulations are prepared using different substrates and carriers.

According to Bezdicek *et al.* (1978), granular inoculants are effective means for delivery of microbial inoculants. Rice *et al.* (2000) reported that increasing interest in granular form of inoculants may be due to the fact that granular inoculants are simple to use and easy to apply and less dense than powder formulations. In granular formulations, several experiments based on polymers have been evaluated. These polymers were demonstrated as potential bacterial carriers (Jung *et al.*, 1982; Bashan, 1998), offering considerable practical benefits over peat-based formulations (Amiet-Charpentier *et al.*, 1999).

Mugnier and Jung (1985) observed that natural and synthetic polymers are able to limit heat transfer and promote high water activity (aw) thereby, these polymers provide a protective microenvironment for bacterial cells to survive under various storage conditions. The immobilization of microbial cells into a polymer matrix is more advantageous than direct inoculation in the soil (Cassidy *et al.*, 1997). Moreover, polymer formulations offer a long shelf-life even at room temperature, as they provide protection against environmental stresses and good batch quality due to standardized production. Nevertheless, preservation at cool temperature (4°C) helps encapsulated cells to remain viable for prolonged periods (Bashan, 1998). Park and Chang (2000) opined that encapsulation of cells in polymeric matrix is a well established technology in a broad range of applications. This polymeric matrix allows the cells to remain viable for longer duration with its catalytic ability.

Many workers reported that the main aim of encapsulation is to protect biocontrol agents from harsh environment, reduce microbial competition and release them gradually to the soil, thus facilitate colonization of plant roots (Vassilev *et al*., 2001; Bashan *et al*.,

2002). Anal and Singh (2007) reported that microencapsulation is an advanced technology, an alternate viable option to overcome the drawbacks of other formulations resulting in extended shelf-life and controlled microbial release from formulations improving their application efficacy. It is a method whereby tiny packages of an active ingredient are packed into a second substance in order to protect the active ingredient from the environment.

Many workers have conducted several studies on microencapsulation and they noticed effective applications in many fields including pharmaceuticals, agricultural, therapeutics and in nutrition (Aghbashlo *et al.*, 2012; Rubilar *et al*., 2012; Shin *et al.*, 2012).

2.4.1 Alginate as the carrier material

For successful field application of any biocontrol agent or biofertilizer, a suitable carrier material is needed. A carrier material according to Bashan (1998) is that which functions as the delivery vehicle of live microorganisms from the factory to the field. It provides a protective niche for microbial inoculants in soil, either physically, through the provision of a protective surface or nutritionally, through the provision of a specific substrate (Arora *et al*., 2008). The carrier should be free from toxic substances, biodegradable and non-polluting products, with limited environmental risks. Also several workers earlier had reported that a good carrier material should ensure adequate shelf life, stick well and should be able to survive on seeds, enable rapid and controlled release of microorganisms into the soil near the host's roots at the right time (Bashan, 1986c, 1991; Fages, 1990, 1992; Smith, 1992; Trevors *et al.,* 1993).

Alginate is one of the biopolymers derived from macroalgae, most often used for microbial encapsulation (Yabur *et al.*, 2007). Commercially, it is extracted from marine weeds such as *Macrocystis pyrifera, Ascophyllum nodosum, Laminaria* etc. Its production is massive and cheap. This compound is non-toxic, quality-consistent, nearly sterile and

biocompatible with microorganisms. Alginate can be dissolved in water or in liquid microbial culture and it forms thermally stable hydrogel globules called "beads" in contact with solution of di-or tri-cations (Chan *et al.,* 2011).

Witter (1996) conducted several studies on alginate as an encapsulating material, as it forms microbeads instantly in the presence of polyvalent cations by binding the cation to units of guluronic acid. The use of encapsulated cells for environmental applications has several advantages over free cell formulations which offer protection from biotic stress (Smit *et al*., 1996) and abiotic stress (Cassidy *et al.,* 1997). According to Murua *et al.* (2008), alginate is a linear polysaccharide occurring in marine brown algae as a structural component of the cell wall, and is known to be the most common biomaterial in bacterial encapsulation. The alginate polymer consists of two monomeric units: β-(1,4) related residues of D-mannuronic acid (M) and α-(1,4)-linked residues of L-guluronic acid (G).The basic alginate structure consists of linear unbranched polymer units consisting of monomers arranged in blocks of M and G residues interspersed with regions containing alternating M-G sequences within the structure (Donati and Paoletti, 2009; Draget, 2009). However, the attractive property of alginate is the calcium induced gelation between calcium ions and guluronate residues in alginate.

Earlier, Draget *et al*. (2000) reported that gelation occurs when a zone of union is formed between the blocks of α-L-guluronic acid (G) of an alginate molecule which is physically connected by calcium ions to another block of α-L-guluronic acid (G) of another alginate molecule and according to him the system forecast is called the egg box model. Alginate is used as the encapsulating material as it forms microbeads instantly in the presence of polyvalent cations by binding the cation to units of guluronic acid with sufficient mechanical strength. Park and Chang (2000) revealed that gel-like matrix helps the cell to remain viable for longer periods of time with its catalytic ability.

Sriamornsak *et al.* (2007) also noticed that sodium alginate, a hydrophilic biopolymer has been found to be highly promising drug delivery agent because of its high biological safety. According to them, usually alginates are sold as powders so, they must be dissolved in water before use. Nussinovitch (1997) earlier had observed that alginates form lumps when they are added to water as they possess high affinity for water. Moreover, wetting and hydration of alginate is achieved by heating the alginate solution to atleast 70^{o}C before it is used. ISP (2007) also reported that heating the alginate solution to around 80^{o}C causes the alginate structure to open and allows the water to enter inside and hydrate the alginate structure.

Alginates are capable of producing gels with divalent cations. One such divalent cation is calcium. Typically, aqueous solution of sodium alginate is dripped into the calcium solution (Draget *et al.,* 2006). Through ionotropic gelation, alginate binds with Ca^{+2} ions under mild conditions which has made it possible to encapsulate biocontrol agents (Polk *et al.,* 1994; Kikuchi *et al.,* 1996; Bowersock *et al.,* 1999). Simpson *et al.* (2003) reported that use of alginate as an immobilizing agent has ability to form heat stable strong gels, under room temperature and it does not undergo excessive swelling or shrinking, during crosslinking, thus maintains its form for long period. Alginate beads are mechanically stable and biodegradable which is a preferable trait of the formulation. The confinement of microbial cells in alginate spheres offers protection against both mechanical and environmental stresses, retaining growth and metabolic activities for longer periods of time (Anal and Singh, 2007).

Preparation of bacteria-containing beads is fairly simple and involves a multistep process (Bashan, 1986a; Digat, 1991). In some cases, the biomass of the entrapped strain is low or not sufficient, and hence secondary multiplication of the entrapped bacteria is required in the already developed beads (Bashan, 1986a). Several workers reported the mechanism behind bead formation where the droplet grows at the dripping tip and once it reaches its maximum volume, it detaches from the tip as a single droplet by the action of

gravitational force (Dulieu *et al*., 1999; Del Gaudio *et al*., 2005; Chan *et al*., 2009). According to Andersen *et al*. (2014), the beads typically hold high water content, have adjustable mechanical and chemical properties depending on the type of crosslinking agent used and alginate gel particles are desirable as a natural product for biological applications, because they are biocompatible, non-toxic, biodegradable and relatively cheap. Likewise, alginate bead encapsulation protects the inoculants from stress factors and slowly releases them as a viable source of inoculum for a longer period of time to promote their rhizosphere establishment. Alginate beads are also capable of entrapping sufficient number of bacteria (Zohar-Perez *et al.*, 2002). Vassilev *et al.* (2001) and Bashan *et al.* (2002) reported that the main objective of encapsulation is to shield microorganisms from harsh soil factors, to reduce microbial competition and to gradually release them to plant to fight against diseases. Fenice *et al.* (2000); Zohar-Perez *et al.* (2002); Bashan *et al*. (2002) and Young *et al*. (2006) found that alginate beads are capable of encapsulating sufficient number of viable bacterial cells and the cell density may reach upto 10^{11} cfu g^{-1} of the bead.

Clayton *et al.* (2004) studied the effect of soil inoculation using granular and liquid inoculants. They noticed that seed inoculation using peat powder along with an uninoculated control, on nitrogen fixation and pea nodulation and effects on nodule number accumulation were higher in granular formulation compared to powder and liquid formulations. Ivanova *et al*. (2005) noticed that encapsulation of nitrogen fixing *Azospirillum* in alginate showed a better yield than application of liquid and powdered formulations in field conditions. The dried micro beads of *A. brasilense* used for seed inoculation resulted in increased growth of tomato plants Yabur *et al*. (2007). Immobilization of unicellular micro algae *Chlorella* sp. with *Azospirillum* sp. in alginate beads, helps in close interaction between two microorganisms and get rid from bacterial contaminants (De- Bashan *et al.*, 2008). Devi *et al*. (2012) reported that the survival of *Bacillus megaterium* and *Bradyrhizobium* sp. was higher in alginate beads compared with liquid lignite inoculant formulations. Schoebitz *et al*. (2013) reported that inoculation of wheat plants with microcapsules of sodium alginate and sodium alginate combined with

potato starch and with strains of *Pseudomonas fluorescens* and *Serratia* sp. significantly promoted plant height, foliar content of phosphorous and dry biomass. They also found that this encapsulation improved population, protection and stabilization of the bacterial cells around the plant rhizosphere.

According to Thilagavathi *et al.* (2015), *Trichoderma* and *Pseudomonas* formulation of alginate beads could control root rot of sugarbeet by gradual and continuous release of the biocontrol agent into the soil and rhizosphere. Similarly, Abo-Kora *et al.* (2016) reported that tomato plants when inoculated with microcapsules and bacterial suspension of *Pseudomonas fluorescens*, *Azotobacter chroorcoccum*, *Bacillus polymyxa* and *Azospirillum brasilense*, it was found that both types of inoculation had beneficial effects on plant growth with microcapsules of *P. fluorescens* and *Azospirillum brasilense* which stood out in plant height and root length respectively.

2.4.2 Effect of different adjuvants in enhancing the shelf life of bioagent

Shelf life is the first and foremost problem of formulations. To enhance the survival of biocontrol agent in formulations, inoculant media should be supplemented with adjuvants. Use of additives improves the population density and thereby enhances its shelf life. Nutrient amendments enhance the efficacy of inoculation process by stimulating the growth of introduced microbial population (Acea *et al.*, 1988; Devliegher *et al.*, 1995). Incorporation of additional nutrient components into the polymer matrix or coating of the capsules increased the cell viability, stability and storage ability of encapsulated microorganisms inside the capsules (Krasaek-oopt *et al.*, 2004). Deaker *et al.* (2004) reported that polymers with good water solubility, non-toxicity and a complex chemical composition are good additives.

Additives are selected based on their ability to protect bacterial cells in storage and on seeds under extreme temperature, desiccation and toxic conditions (Bashan, 1986a).

In formulations, some widely used additives include polyvinyl pyrrolidone (PVP), methyl cellulose, polyvinyl alcohol, polyethylene glycol, gum arabica, trehalose, glycerol, Fe-EDTA, tapioca flour, etc. (Singleton *et al.,* 2002). It should be chemically stable, non toxic, non reactive, economical and should meet efficiency in regards with the intended use. Brahmaprakash and Sahu (2012) suggested that formulation containing additives promotes prolonged survival of desired microorganism during storage conditions and also after application to soil or seed. The main advantage of encapsulation along with nutrients provide better survival conditions for the bacteria to withstand stresses and to increase cell vigour (O'Callaghan, 2016).

Knudsen *et al*. (1991) observed that alginate pellets treated with PEG possessed higher population of bioagent than from untreated beads which improved the biocontrol efficacy. Supplementation of alginate beads with poly vinyl pyrrolidone (Doria-Serrano *et al*. 2001) and carboxy methyl cellulose (Joo *et al*., 2001) for hardening of alginate beads showed that both additives enhanced the stability of beads. The sticky property of PVP with high water binding ability enhanced seed adhesion (Singleton *et al.,* 2002, Deaker *et al.,* 2004). Navi (2004) suggested that additives such as PVP, trehalose, glucose and mannitol increased the shelf life of bacteria in liquid formulations. Formulation containing PVP as an osmoprotectant promoted shelf life than those without PVP amendment (Girisha *et al.,* 2006). Tittabutr *et al.* (2007) reported that liquid inoculant formulations of *Bradyrhizobium* supplemented with PVP was found effective compared to gum arabic, polyvinyl alcohol (PVA), polyethylene glycol (PEG) and cassava starch, as PVP slowed down the drying process of the inoculant due to their adhesive nature under field conditions. Alamraj *et al*. (2013) reported that liquid formulation supplemented with PVP (2%), CMC (0.1%) and polysorbate 20 (0.025%) maintained prolonged survival of bacterial inoculants even after 480 days of inoculation when stored at 30^{o}C. Likewise, Trivedi *et al*. (2016) observed that addition of PVP (2.5%) + glycerol (2%) showed maximum survival rate of inoculants in formulations upto 720 days of storage. Biradar and Santhosh (2018) observed that supplementation of PVP as one of the additive combination

(King's B broth + 2% PVP + 0.3% xanthan gum + 0.5% Tween-20 + 0.2% potassium sorbate) in King's B broth enhanced the population rate of *P. fluorescens* even after six months followed by PEG and CMC against *Fusarium* wilt of tomato under greenhouse conditions.

Leslie *et al.* (1995) found that formulations containing trehalose after encapsulation increased cell survival and viability and this confirmed the protective effect of trehalose on proteins and cell membrane components growing inside the beads. Surendra Gopal and Baby (2016) reported that there will be enhanced shelf life in *Azospirillum* sp. and PSB upto nine months at room temperature, when the basal media was amended with chemical additives like trehalose (15 mM) and PVP (2.5%) respectively. Zago *et al.* (2019) noticed that cell viability is maintained when trehalose (0.1M) was added during encapsulation process.

Li *et al*. (2009) reported that glycerol, an economical polyol, possesses the ability to protect and defend bacterial cells from abiotic stress and it assisted in osmotic pressure balance and transmembrane traffic regulation. In addition, glycerol was a source of carbon for bacteria, which has high water-binding ability and can protect cells from desiccation. Manikandan *et al.* (2010) also reported that addition of glycerol to nutrient broth enhanced the survival of *P. fluorescens* Pf1 which enhanced the viability of antagonists for six months against *Fusarium* wilt of tomato.

According to Elegba and Rennie (1984), formulations prepared without adhesives reported lower viable counts compared to formulations with adhesives like carboxy methyl cellulose and gum arabic as these adhesives used were effective in maintaining the shelf-life of biocontrol agents. Vidhyasekaran and Muthamilan (1995) observed that addition of CMC as adhesive recovered a population of more than 10^7 cfu g^{-1} in the preparation of different *P. fluorescens* based formulations and upto 240 days of storage. Several other workers also reported that granular and dry powder formulations, carboxymethyl cellulose

was used as an adhesive/protective substance along with carrier material (Martínez-Álvarez *et al.*, 2016; Prasad and Babu, 2017; Basheer *et al.*, 2019). Anis *et al.* (2012) reported that sunflower seeds coated with sodium alginate along with calcium carbonate and carboxy methyl cellulose (CMC) showed significant increase in plant biometric characters compared to seed coated with sodium alginate along with calcium carbonate and gum arabic.

2.4.3 Effect of skim milk on alginate beads

Bashan (1986c) reported a novel combination of sodium alginate beads containing a wide reservoir of *Azospirillum* or *Pseudomonas* cells along with skim milk which could release bioagents at a slow and constant rate. Fages (1990) also noticed the highest survival of bacterial cells and controlled dehydration in alginate beads amended with skim milk. Later, encapsulation of *Pseudomonas fluorescens* in alginate beads supplemented with skim milk and bentonite clay showed good survival rate in both dried and wet beads in wheat plants (Trevors *et al*., 1993). Bashan and Gonzalez (1999) reported that there will be reduction in survival of bacteria in alginate beads supplemented with skim milk.

Similarly, Vassilev *et al*. (2001) found that supplementation of skim milk and clay amendments to alginate beads entrapped microbial inoculants showed better performance in soil. Bashan *et al.* (2002) observed that alginate beads produced with and without skim milk was capable of releasing the bioagent at the rate of 10^5 cfu g^{-1} and 10^7 cfu g^{-1} respectively. Trivedi *et al.* (2005) reported that among carrier-based preparations of *Bacillus subtilis* and *Pseudomonas corrugata* in five different formulations *viz*., alginate beads, alginate beads supplemented with skim milk, alginate-coated seeds, charcoal-based and broth-based preparations, maximum number of inoculated bacteria were recovered

from alginate beads and alginate beads supplemented with skim milk formulations even after 180 days of storage.

Saxena (2011) studied on immobilization using sodium alginate and alginate + skim milk as carriers on two bacterial strains *viz. Pseudomonas fluorescens* BAM- 4 and *Burkholderia cepacian* BAM -12 to check the phosphate solubilization in wheat plants under *in vitro* conditions and found that alginate + skim milk was the best carrier as skim milk favoured profuse multiplication of cells compared to control. Additional supplementation of skim milk and glycerol during encapsulation of *P. fluorescens, A. brasilense* and *Aspergillus* (filamentous fungi) strains in alginate formulations enhanced the viability of the strains (Singh *et al.*, 2011). Archana and Brahmaprakash (2014) observed that survival rate of *Azotobacter chroococcum*, *Acinetobacter sp* and *P. fluorescens* was higher in alginate formulations followed by alginate + skim milk, alginate + charcoal and alginate + lignite formulations. However, Szczech and Maciorowski (2016) observed that addition of skim milk had no effect on the yield of microcapsules and its diameter. In addition, they noticed that the application of skim milk made it more difficult to produce micro-capsules, primarily because of the less efficient separation from the oil phase. They also experimented on production of microcapsules amended with peat, chitosan and skim milk containing *Burkholderia cepacia*, *Bacillus* spp. and *Trichoderma virens* and found that skim milk reduced the quality of the final product.

2.4.4 Effect of different parameters on bead formation and quality

Several authors studied the effect of different factors responsible for bead formation *viz.*, sodium alginate and calcium chloride concentration, gelation and curing time, harvesting, storage, concentration of entrapped cells and cell leakage for the production of effective bead formulations.

2.4.4.1 *Concentration of sodium alginate and calcium chloride on bead formation*

Yotsuyanagi *et al.* (1987) observed that increase in initial concentration of alginate solution resulted in increased bead weight due to increase in density of the beads. This led to the production of fully cured state of gel structure with almost completely spherical shape and they also found that drug release from the beads is directly proportional to sodium alginate concentration. Ouwerx *et al*. (1998) studied the mechanical property of alginate beads and they noticed that it determines the nature of the polymer, cation, cation concentration and ionic strength and the gelling nature of sodium alginate solution depends on the ion binding properties. Calcium is the most used ion in the preparation of alginate beads particularly in encapsulating cells which are served as a biocatalyst or to release entrapped biomolecules such as pesticides, drugs, preservatives, etc (Martinesen *et al*., 1992).

Bashan *et al.* (2002) reported that alginate beads were produced by low pressure spraying of alginate solution mixed with liquid medium containing bacteria through the small nozzle. The droplets when it came in contact with calcium chloride solution, immediately hardened into beads of required size. Similarly, El-Kamel *et al.* (2003) stated that drug loading inside the alginate beads was directly proportional to polymer concentration. Rajinikanth *et al.* (2003) observed that increase in sodium alginate concentration lead to the formation of large sized beads and also found that use of calcium chloride concentration (2.5% w/v) was sufficient to reduce the porosity of alginate matrix and thus increased the entrapment efficiency of the drug. Krasaek-oopt *et al.* (2004) reported that addition of calcium chloride during encapsulation resulted in the production of micro-hydrogel globules which were then harvested by filtration. Several reports showed that average diameter of beads formed in dripping mode decreased, as the concentration of alginate solution increased (Thu *et al.,* 1996; Del Gaudio *et al.,* 2005; Huang *et al.*, 2011). Rui Rodrigues and Lagoa (2006) noticed that lowest concentration of calcium chloride limits the gelation leading to formation of large wet beads with higher

percentage of water content. So, increase in concentration of calcium chloride resulted in proper gelation and formation of rigid beads.

Maximum survival rate and excellent encapsulation efficiency of beads were obtained at 4 per cent sodium alginate in 75 mM calcium chloride solution respectively and this lead to the production of isodiametric and compact beads (Mondal *et al.*, 2002). Mandal *et al.* (2010) observed that alginate beads were prepared using calcium chloride (3% w/v) and higher alginate concentration showed more rigid gel network due to more crosslinking, resulting in greater sustained release characteristics. Simpson *et al*. (2004) found that during the process of bead formation, calcium cations in calcium chloride solution interacted and formed ionic crosslinks with L-guluronic acid of sodium alginate solution. Therefore, increase in concentration of calcium chloride increased the crosslinking with sodium alginate in three-dimensional network and thus increases bead stability. Bajpai and Kirar (2016) observed that polymerization mixture of sodium alginate solution was dropped into calcium chloride solution where Ca^{2+} ions instantaneously entered into the bead matrix and cross-link alginate chains via an ‘egg-box’ formation. Kaur *et al.* (2018) revealed that increase in sodium alginate (2%) and calcium chloride concentration (3%) increased the particle size and swelling rate of alginate beads. Iqbal *et al.* (2019) found that the beads produced using sodium alginate (3%) and 100 mM calcium chloride were firm, clear, compact and isodiametric in nature compared to other combinations.

2.4.4.2 *Gelation and curing of beads*

The gelation rate is a critical factor in maintaining gel uniformity and strength when using divalent cations and slower gelation produced more uniform beads with greater mechanical integrity (Kuo and Ma, 2001). According to Rezende *et al.* (2009) gelation indicates the formation of three dimensional structures by crosslinking between divalent ions and G blocks of the polymer chain. The time at which the weight of the alginate bead

becomes stable was considered as the time period required for complete curing of the bead (Tous *et al.*, 2014).

Trevors *et al*. (1993) reported that prepared alginate beads when allowed to cure for only one hour attained complete gelation. Cassidy *et al*. (1995) reported that encapsulation of *Pseudomonas aeruginosa* UG2Lr cells in alginate beads when allowed to cure for two hours at 10^{o}C attained complete gelation with sterile distilled water and were thereafter dried aseptically for 24 h in a laminar flow hood. Such beads were then aseptically transferred to a sterile glass bottle, sealed with a screw-cap and stored at 4°C in the dark. Bashan *et al.* (2002) reported that microbeads were formed instantly upon contact of the droplets with calcium chloride solution and were allowed to cure in the solution for additional 30 min to obtain regular spherical beads. Sarmah *et al*. (2010) prepared alginate beads by testing different concentrations of sodium alginate and calcium chloride and reported that curing of beads for 30 min in calcium chloride solution produced firm, clear, round and uniform optimal beads. But, Sivakumar *et al.* (2014) noticed that the instantaneously formed alginate beads were hardened for 3-6 h at room temperature to attain spherical beads.

Various concentrations of sodium alginate (0.4-1.0% w/v) and calcium chloride (0.2-4.0%) were used for the study by Patel *et al.* (2016) and Segale *et al*. (2016). They reported that the beads were produced instantaneously when drug loaded sodium alginate solution came in contact with calcium chloride solution and the beads were allowed to cure for 15 more minutes to increase its rigidity. Likewise, He *et al*. (2016) observed that an additional two hours were required for the beads in calcium chloride solution to obtain constant weight. Chuang *et al.* (2017) and Zacchetti *et al*. (2018) observed that alginate droplets were cross-linked with $BaCl_2$ or $CaCl_2$ solutions to form alginate particles in five minutes.

2.4.4.3 *Harvest and storage of beads*

Scanty literature is available on the harvest and storage of beads. However, attempts were made to include some studies pertaining to the same. Cassidy *et al.* (1995) washed the beads encapsulated with *Pseudomonas aeruginosa* UG2Lr cells collected from calcium chloride solution in sterile distilled water and were dried for 24 h in a laminar flow hood aseptically. These beads were aseptically transferred to a sterile glass bottle, sealed and were stored at 4°C. Russo *et al.* (2001) reported that moist beads of *Pseudomonas fluorescens* F113 LacZY were air dried for about 20 h in a laminar flow hood at 25ºC to obtain regular bold beads and were stored in air tight container for future use.

Bashan *et al.* (2002) reported that microbeads were collected from the suspension by filtration using Whatman no. 4 filter paper and were rinsed in 500 ml saline solution for three times. Sivakumar *et al.* (2014) harvested the beads by sieving and were washed with sterile water for several times and were stored at 4°C in saline (0.85% w/v) for future use. He *et al*. (2016) washed the wet microcapsules with sterile water two or three times and were dried in oven at 40^{o}C until a constant weight was obtained.

2.4.4.4 *Cell viability of alginate beads*

For effective application of beads, it is necessary to enumerate the number of viable microbial cells in alginate spheres to carry out the desired function effectively. It has been found that the viability of the encapsulated cells is affected not only by the physicochemical properties of alginate beads but also by the processing parameters used for microencapsulation (Groboillot *et al.,* 1993; O'Riordan *et al.,* 2001; Weinbreck *et al*., 2010; Burgain *et al.,* 2011). Measurement of cell viability inside alginate beads are usually conducted by disrupting the beads using physical or chemical methods, followed by culturing and counting of the released cells on agar surface (Doria-Serrano *et al.,* 2001; Desmond *et al.,* 2002; Lian *et al.,* 2003; Young *et al*., 2006; Özer *et al*., 2008). Lievense *et*

al. (1994) and Pedroso *et al.* (2012) reported that storage temperature is found to be indirectly proportional to viability of encapsulated cells.

Bacterial entrapment inside the beads were counted by dissolving 10 bead samples of *Azospirillum brasiliense* and *Pseudomonas fluorescens* in 10 ml potassium phosphate buffer (0.25 M, pH 6.8) in a test-tube for 16±24 h at 30.2 °C (Bashan 1986a; Levanony *et al*., 1987). Similarly, Trevors *et al.* (1993) determined initial cell density of beads by dissolving 10 alginate beads in 0.1M potassium phosphate buffer, pH 7.0 for 45 min at 20°C and they found that initial population of the bioagent was higher in wet beads compared to the dry alginate beads. Russo *et al*. (2001) determined the number of viable encapsulated cells by dissolving the microbeads in sterile sodium citrate solution (1%) completely at pH 6.5 for 30 min at room temperature and were enumerated by serial dilution plating technique.

2.4.4.5 *Cell leakage determination*

Reducing cell leakage while bead production is very important in the cultivation of immobilized cells. Cell leakage determination was obtained by measuring the cell density in different $CaCl_2$ solution after recovering the beads from $CaCl_2$ solution. (Callone *et al.*, 2008). Shibasaki-Kitakawa *et al*. (2000) reported that decreased cell leakage from the beads is observed with an increase in $CaCl_2$ concentration. Zhang *et al.* (2013) observed that in case of sodium alginate beads, an increase in $CaCl_2$ concentration from 0.03 M to 0.1 M minimized the cell leakage and increased the chitosanase yield.

2.5 Characteristics of bead

2.5.1 Bead size

Size is a measure of an object's physical magnitude 'how big or small it is'. Beads are formed in the nanometer, micrometer or millimeter size ranges and application and

performance of alginate beads depends on their size and shape. Several workers reported that spherical alginate particles were produced using a standard droplet forming method to reduce the free energy interfacial between particles and gelation solution (Hu *et al*., 2012; Kregiel *et al.,* 2013).

According to Gentile *et al.* (1995), size of beads designed for encapsulation of microbial cells ranged from few millimeters to centimeters. Poncelet *et al.* (1999) observed that bead diameter was approximately twice the times larger than the dripping tip diameter. Optimum frequency of production of beads of equal size from a given nozzle diameter depends on the physicochemical properties and the flow rate of the extruded fluid (Serp *et al.* 2000). The dimension of the microbeads was evaluated using a scanning electron microscope (Russo *et al.,* 2001). Increase in bead diameter is associated with increase in the flow rate from a dripping tip (Klokk and Melvik, 2002; Strand *et al*., 2002; Herrero *et al.,* 2006). Zacchetti *et al.* (2018) used stereo microscope to visualize microcapsules and encapsulated mycelium and observed that diameter of microcapsules was found between 4-7 μm.

Trevors *et al.* (1993) and Weir *et al*. (1996) reported that alginate beads of *P. fluorescens* R2f and *P. aeruginosa* were of 2-3 mm in diameter. Klokk and Melvik (2002) reported that the size of the bead decreased as the concentration of calcium ion increased. Similarly, Ivanova *et al*. (2005) found that with increase in the size of alginate spheres form 1-5 mm, it enhanced the survival of bacteria by 36 per cent. Das and Senapati (2007) also observed that increase in alginate concentration resulted in high degree of crosslinking which caused the development of larger microbeads and greater drug entrapment inside the beads. Soni *et al.* (2010) and Kaur *et al.* (2018) noticed that increase in sodium alginate concentration (1% - 3%) increased the bead size based on the fact that sodium alginate binds more calcium chloride by cross linking. They also observed that increase in calcium chloride concentration resulted in reduced bead size, which was explained by the fact that

calcium (divalent ions) penetrated into the inner space of the beads resulting in expulsion of water molecules which lead to the formation of compact beads.

2.5.2 Bead shape

The shape of alginate bead is tailored by the balance between polymer viscosity and interfacial tension (Yoo and Mitragotri, 2010). Formation of spherical alginate beads is inter-influenced by the physical properties and flow rate of alginate solution as well as dripping tip diameter (Heinzen *et al*., 2004). Lee *et al*. (2013) also observed that the impact force led by alginate droplet upon hitting the surface of the gelation bath had a positive effect on the shape of the calcium alginate beads.

Yotsuyanagi *et al*. (1987) noticed that with increase in initial concentration of alginate, the weight of beads increased because of increasing bead density. According to King *et al.* (1989), alginate solution should be sterilized for preparing calcium alginate beads. They also stated that alginate should be subjected to boiling water sterilization at 100 °C for 15 min. Al- Hajry *et al*. (1999) observed that if the surface tension of alginate solution is much lower compared to that of water, perfect sphere of the droplet cannot be formed which lead to the formation of tear-drop shaped beads. They also noticed that nonspherical beads reduced the strength of the bead as compared to spherical beads. Breakage and cracking was observed on nonspherical and tear shape beads, resulting in uneven release of the encapsulant material (Woo *et al.,* 2007).

Fundueanu *et al*. (1999) and Prusse *et al.* (2008) reported that the collecting distance *i.e*, distance between dripping tip and surface of gelation solution determines the shape of the bead. They observed that shorter collecting distance tends to form tail shaped beads whereas longer collecting distance form spherical bold bead. Chan *et al*. (2009) reported that the shape transition of the alginate droplet with increasing falling distance has

been distinguished into three phases as tear shape, egg shape and spherical shape. They also observed increase in collecting distance led to the formation of spherical beads.

2.5.3 Role of pH in determining swelling and shrinkage of beads

The effect of pH has a significant role on alginate bead formation. The pH value is another parameter in gelation process of alginate and is directly related to the interfacial tension (Yoo and Mitragotri, 2010; Beaman *et al.,* 2012).

Yotsuyanagi *et al*. (1987) also reported that the magnitude of swelling was determined by the ratio of the diameter of a swelling particle to the corresponding diameter of the fully-cured bead before drying. They observed no swelling of bead at pH 1.6 KC1-HCl buffer and distilled water. At the same time, at pH 7.0 phosphate buffer, the dried alginate bead swelled to its original size in 1 h, then it gradually disintegrated and dispersed over several hours. King (1993) reported that alginate beads were successfully formed at a low pH of 2.8-4.0. Alginate beads were sensitive to acidic environment which could cause capsule disruption or dissolution (Shah and Ravula, 2000; Qi *et al*., 2006). Sriamornsak and Kennedy (2010) observed that swelling of beads occured due to water intake, when beads come in contact with aqueous solution, resulting in hydration of alginate molecules. The penetration of water caused the bead's shell to slowly disentangle, which made water migrate into the centre of beads which resulted in swelling of alginate beads. Vreeker *et al*. (2008) noticed that swelling of beads were due to ions exchange between polymer chain (Ca^{2+}) and phosphate buffer (Na+) respectively. The presence of salt such as K, Na etc disrupted the dimer–dimer associations resulting in swelling of beads.

Several reports showed that about 50 per cent of water was squeezed out from the alginate bead when it was fully cured, irrespective of the initial alginate concentration (Voo *et al.,* 2011) This phenomenon was known as syneresis as noticed by Chrastil (1991) and Velings and Mestdagh (1995) and according to them an alginate sphere usually shrinks

during gelation process. Sathali and Varun (2012) reported that presence of hydrophilic groups on the polymer chain increased the swelling rate. As these groups interacted with water, it contributed to a greater degree of swelling. According to Mukhopadhyay *et al*. (2013) alginate, being a pH responsive polymer will shrink in a low pH medium but swells or dissolves in a high pH medium. Bajpai and Kirar (2016) observed that maximum per cent of swelling in alginate beads was observed after 30 min and they began to deteriorate after 3 h where they completely dissolved in the swelling medium. Patil *et al*. (2016) observed that with the increase in calcium chloride concentration, the swelling index declined because the strong alginate bead formed at high calcium chloride concentration resulted in slow penetration of dissolution medium into the alginate matrix.

2.5.4 Shelf life of alginate beads

Commercialization of developed formulation depends upon the shelf life of the biocontrol agents. An effective formulation should maintain viable count of antagonists with a minimum shelf life of 8-12 months for industrialization (Nakkeran *et al.,* 2005). Hence, research should be concentrated to enhance the shelf life of the formulation. Several workers reported that microencapsulation is a viable option to support maximum shelf life with low level of contamination, thus making biocontrol a commercial venture and therefore, inoculants should be developed as formulation with prolonged storage stability. The requirements with respect to their shelf life vary from 2-3 months at room temperature according to Malusa *et al*. (2012) to 1-1.5 years (Catroux *et al.,* 2001) to a minimum of 1-2 years (Bashan *et al.,* 2014; He *et al.,* 2015; Martinez-Alvarez *et al.,* 2016; Oliveira *et al.,* 2017).

According to Bashan and Gonzalez (1999), significant increase in plant height was observed, when wheat and tomato plants were inoculated with *Azospirillum brasilense* as alginate microbeads in wet and dry formulations. They also reported that bacteria could survive in alginate beads, even after 14 years. Russo *et al.* (2001) observed that

encapsulation of *P. fluorescens F113* LacZY in alginate formulation retained it's viability upto one year. Young *et al*. (2006) observed that alginate matrix protects encapsulated cells from mechanical stress and limits their mortality and enhanced prolonged storage conditions. Trivedi and Pandey (2008) reported that immobilization of *B. subtilis* and *Pseudomonas corrugata* in sodium alginate beads, exhibited higher survival rate upto 10^8 cfu g^{-1} even after three years. Furthermore, both biocontrol agents maintained the ability to promote plant growth. Power *et al.* (2011) also reported 100 per cent recovery of viable *P. fluorescens F113* in alginate beads even after 250 days of storage under ambient temperature.

Sangeetha and Stella (2012) tested that among the survival of different isolates of *Azospirillum lipoferum* VAZS-18, *Azotobacter chroococcum* VAZB-6, *Bacillus megaterium* VBA-2, *Pseudomonas fluorescens* VPS-19 by using various carrier materials, *P. fluorescens* maintained higher population (63.89 x10^8 cfu g^{-1}) in alginate beads followed by pressmud, vermiculite and lignite based carriers after six months of storage. A perusal of the literature revealed that the viability of the microorganism was highly influenced by the shelf life of the product that ultimately depends on the culture medium, bacterial species, physiological state, concentration of water in the inoculum and storage temperature (Schoebitz *et al*., 2013; Sivasakthivelan and Saranraj, 2013). Archana *et al.* (2013) reported maximum survival of *Pseudomonas* in alginate formulation even after 240 days of storage and also observed that there was no harmful effect of alginate polymer on plant growth. According to Sivakumar *et al*. (2014), high viability and continuous release of cells from the bead was observed for one week and encapsulated bacteria maintained its population for five months with minimal cell loss at storage. Alginate gel matrix had the capability for extending the shelf life of microbial strain under biotic and abiotic stressful conditions. Schoebitz and Belchi (2016) reported that during encapsulation process, nutritional additives are also used to maintain the viable populations of bioagent for the longer period under both anaerobic and aerobic conditions.

2.6 *In vitro* inhibition of different formulations of *P. fluorescens* against plant pathogens

Many workers have conducted several studies on *in vitro* inhibition studies of different formulations of *P. fluorescens* against wide range of plant pathogens and they tested under greenhouse and field trials. Attempts have been made to include some of the available literature on the same.

Vidhyasekaran *et al.* (1997) observed that talc formulations of *P. fluorescens* Pf1 and Pf2 effectively controlled *Fusarium udum* (pigeon pea wilt) under both *in vitro* and *in vivo* conditions. Rajappan and Ramaraj (1999) reported that application of talc based formulation of *P. fluorescens* effectively controlled wilt disease of cauliflower caused by *F. moniliformae* under field conditions. Thiribhuvanamala *et al.* (1999) observed reduced mycelial growth and sclerotial production of *Sclerotium rolfsii* the causal agent of stem rot in tomato with powder formulation of *P. fluorescens* under *in vitro* conditions. Soaking the rice seeds in water containing mixture of two strains of *P. fluorescens* PF1 and PF2 in talc formulation for 24h significantly reduced rice sheath blight under field conditions (Nandakumar *et al*., 2001). According to Commare *et al*. (2002) combined application of talc formulation of *P. fluorescens* through seed, root, soil and foliar spray significantly supressed the sheath blight (62.1%) and leaf folder (47.7–56.1%) incidence of rice under greenhouse and field conditions. Singh and Sinha (2005) reported that talc amended CMC based formulation of *P. fluorescens* strain 1 and 5 exhibited maximum reduction of disease incidence (32.2%) and severity (56.6%) of sheath blight caused by *Rhizoctonia solani* of rice under glass house conditions.

The antagonistic activity of *P. fluorescens* B5 formulated in bentonite, diatomaceous earth and peat with 60% moisture significantly reduced the *in vitro* growth of *P. ultimum* var. *ultimum* in dual culture method (Wiyono *et al*., 2008). Ardakani *et al*. (2010) reported that cotton seeds treated with bentonite based formulation of *P. fluorescens*

strain Q18 reduced damping-off caused by *R. solani* by 81.75 per cent. Sallam *et al*. (2013) observed wood flour formulation of *P. fluorescens* supressed the disease index of *Fusarium solani* compared to talc formulation under *in vitro* and field conditions. Al-Waily *et al.* (2018) observed higher inhibition percentage of *R.solani* and *F.solani* with powder formulation of *P. fluorescens* upto six months of storage.

Several authors reported that bacterial cell suspension of *P. fluorescens* showed inhibitory property against the growth of a wide range of plant pathogens *viz*. *S. rolfsii* (Ganesan and Gnanamanickam, 1987), *R. solani* (Sakthivel and Gnanamanickam, 1987), *F. oxysporum* f. sp. *cubense* (Sivamani and Gnanamanickam, 1988) under *in vitro* conditions by dual culture method. Application of liquid suspension of *Pseudomonas* strain BC-8 inhibited the incidence of *R. solanacearum* under *in vitro* conditions (Gallardo *et al.*, 1989). Aneja (2005) observed that application of bacterial cell suspension of *P. fluorescens* through seed, root and soil treatment significantly reduced bacterial wilt of brinjal under *in vivo* conditions. According to Manikandan *et al.* (2010) the liquid formulation of *P. fluorescens* Pf1 inhibited the growth *F. oxysporum* f. sp. *lycopersici* with least mycelial growth of 54 mm in dual plate method. They also observed that seed treatment, seedling dip and soil drenching of liquid formulation of Pf1 significantly reduced the percentage of disease occurrence of *Fusarium* wilt of tomato under glasshouse and field conditions.

Zegeye *et al.* (2011) tested the biocontrol potential of cell suspensions of strains of *Trichoderma viride*-ES1 and *Pseudomonas fluorescens*-Bak150 against *Phytophthora infestans* under *in vitro* and greenhouse conditions. They observed that *P. fluorescens* inhibited the radial growth of the pathogen by 88 per cent. Gargi and Kalita (2012) reported that antagonistic strain of *P. fluorescens* was applied as liquid suspensions during pot culture experiment. They observed that combination of root +soil and seed + root+ soil treatments of the antagonists effectively reduced the per cent wilt incidence in brinjal.

Russo *et al.* (2001) observed that alginate beads encapsulating *P. fluorescens* F113 LacZY inhibited the growth of both *Pythium ultimum* and *Rhizoctonia solani* under *in vitro* conditions. Russo *et al*. (2005) also observed that *P. fluorescens* 134 encapsulated in alginate beads exhibited antifungal activity against *R. solani* under both *in vitro* and *in planta* conditions. They further confirmed that alginate-immobilized cells could maintain the biocontrol ability near 90% in infected soil compared to free cells and chemical treatment. Dhanya and Adeline (2014) noticed that alginate beads of *P. fluorescens* showed higher percentage inhibition of *Rhizoctonia solani* (39.2%) compared with the control plate. Soesanto *et al*. (2019) reported that granular formulation of *P. fluorescens* P60 bacteria inhibited the growth of *R. solanacearum* under *in vitro* conditions.

MATERIALS & METHODS

3. MATERIALS AND METHODS

The present investigation on "Alginate based encapsulation of *P. fluorescens* for management of soil borne pathogens" was conducted in the Department of Plant Pathology, College of Horticulture, Vellanikkara during 2018-2020. The details of the methodologies followed during the course of the research work are given below:

3.1 PRESERVATION OF STANDARDIZED CULTURE OF *Pseudomonas fluorescens*

Pseudomonas fluorescens, the reference culture of KAU was used as the biocontrol agent for preparation of alginate beads. The culture was sourced from the Department of Agricultural Microbiology, College of Horticulture, Vellanikkara and were subcultured into King's B (KB) slants (Annexure-I). The culture after 72 h of incubation was stored under refrigerated condition at 4º C for further studies.

3.2 STANDARDIZATION OF MEDIA WITH ADDITIVES

To promote the shelf life of the bead based formulation, King's B broth was amended with different adjuvants so as to enhance the survival rate of the biocontrol agent. The broth was supplemented each with a sugar source, wetting agent, adhesive and surfactant and thus eight different combinations were obtained. The following adjuvants were amended in the media:

Sugar : Mannitol (3%) and trehalose (15mM)
Wetting agent : PVP (2%) and PEG (0.25%)
Adhesive : CMC (1%) and liquid paraffin (1%)
Surfactant : Tween-80 (0.5%)

The treatments with different combinations of additives supplemented in King's B broth are given below:

T_1- Mannitol (3%) + PVP (2%) + CMC (1%) + Tween-80 (0.5%)

T_2- Mannitol (3%) + PVP (2%) + Liquid paraffin (1%) + Tween-80 (0.5%)

T_3- Mannitol (3%) + PEG (0.25%) + CMC (1%) + Tween-80 (0.5%)

T_4- Mannitol (3%) + PEG (0.25%) + Liquid paraffin (1%) + Tween-80 (0.5%)

T_5- Trehalose(15mM) + PVP (2%) + CMC (1%) + Tween-80 (0.5%)

T_6- Trehalose(15mM) + PVP (2%) + Liquid paraffin (1%) + Tween-80 (0.5%)

T_7- Trehalose(15mM) + PEG (0.25%) + CMC (1%) + Tween-80 (0.5%)

T_8- Trehalose(15mM) + PEG (0.25%) + Liquid paraffin (1%) +Tween-80 (0.5%)

T_9- Control

These additive combinations amended in King's B broth (100 ml) were sterilized at 121ºC at 15 psi for 20 min in an autoclave. Thereafter, the broth was inoculated with loopful of *P. fluorescens* and were incubated at room temperature for 48 h for further shelf life studies.

3.2.1 Effect of additives on shelf life of *P. fluorescens*

To evaluate the best additive combination which enhances the shelf life of *P.fluorescens,* the experiment was carried out at monthly intervals upto nine months using serial dilution and plating technique. One ml of the bacterial suspension was pipetted out into a 9 ml sterile water which gives 10^{-1} dilution. The process was continued till a final required concentration of 10^{-6} and 10^{-8} was achieved. One ml of the aliquot each from 10^{-6} and 10^{-8} dilution was poured in the sterile Petri plates separately and 10-15 ml of melted and cooled King's B agar media was poured in the plate and thereafter, rotated it in both clockwise and anti-clockwise direction to achieve uniform distribution of bacterial cells. After solidification of the media, the plates were incubated in an inverted position at room temperature for 48 h. A control without additives was also maintained for comparison. Observations were recorded after 48 h of incubation and results were expressed in cfu ml^{-1}

$$\text{Colony forming units (cfu/ ml)} = \frac{\text{No. of colonies x dilution factor}}{\text{Volume of the sample}}$$

After nine months of storage, the best two additive combinations were selected based on the maximum colony count.

3.3 PREPARATION OF ALGINATE BEAD BASED FORMULATION OF *P. fluorescens*

The two best additive combinations obtained from 3.2.1 was selected for the preparation of alginate based formulations of *P. fluorescens.* It was prepared by entrapping the bioagent in the polymer matrix as per the standard protocol of Bashan *et al*. (2002) with slight modifications. Beads with alginate alone and alginate with skim milk (0.75%) were used for the study.

3.3.1 Preparation of bacterial cell suspension

A loopful of the reference culture of *P. fluorescens* was inoculated in 250 ml conical flask containing 100 ml of sterilized King's B broth supplemented with selected additives and were kept for incubation for 48 h at room temperature in an orbital shaker at 180 rpm (Bashan *et al*., 2002).

3.3.2 Concentration of sodium alginate and calcium chloride

The experiment was conducted to determine the optimum concentration of sodium alginate and calcium chloride required for the preparation of alginate beads. Five different concentrations of sodium alginate (1%, 1.5%, 2%, 2.5%, 3%) and four different concentrations of calcium chloride (2%, 2.5%, 3%, 3.5%) were used to prepare alginate beads so as to determine the best concentration of sodium alginate and calcium chloride required for developing good quality formulation. Calcium chloride solution was sterilized at 121^{o}C temperature at 15 psi for 20 min. Freshly prepared sodium alginate and $CaCl_2$ solutions were used for the preparation of beads. A control treatment of beads without additives and an absolute control without the biocontrol agent was also maintained.

3.3.3 Preparation of microbeads

Ten ml of bacterial cell suspension was added to the standardised sodium alginate solution as in 3.2 aseptically and mixed thoroughly at 100 rpm for 1 h under ambient temperature conditions. This viscous solution was poured into a sterile separating funnel fitted on to a stand and then the solution was added drop wise from the separating funnel fitted into a pre-cooled sterile calcium chloride solution taken in the conical flask kept below the funnel which was standardised as in 3.2.1 under mild agitation. The beads formed upon contact of the droplets with the $CaCl_2$ solution were allowed to cure for one hour in $CaCl_2$ solution at room temperature. Thereafter, beads collected by a sieve and were rinsed 3 to 4 times with sterile distilled water and allowed to air dry in sterile plastic trays for 48 h and then stored in air tight sealed containers for further use (Bashan *et al*., 2002).

3.3.4 Preparation of alginate beads amended with skim milk

Sodium alginate was amended with skim milk (0.75 %) in 250 ml conical flask and mixed thoroughly at 100 rpm for 30 min to obtain a homogeneous solution. Ten ml of the bacterial cell suspension was added to the above solution and mixed thoroughly at 100 rpm for 1 h under ambient temperature conditions. This viscous solution taken in a separating funnel fitted on a stand was added drop wise into pre-cooled sterile $CaCl_2$ solution taken in a beaker kept below the funnel. Beads were allowed to stabilize for 1h in $CaCl_2$ solution, washed aseptically 3 to 4 times with sterile distilled water and allowed to air dry for 48 h and stored in air tight sealed containers for further use (Bashan *et al*., 2002).

Table 3.1 Different treatment combinations of sodium alginate and calcium chloride for preparation of beads

Treatment	Alginate combinations			Treatment	Alginate + Skim milk combinations		
	Treatments	Sodium alginate (%)	Calcium chloride (%)		Treatments (Alginate + Skim milk)	Sodium alginate (%)	Calcium chloride (%)
T_1A_1	T_1 - mannitol + PVP + CMC + tween-80	2.5	2.5	T_1S_1	T_1 - mannitol + PVP + CMC + tween-80 +0.75% skim milk	2.5	2.5
T_1A_2		2.5	3.0	T_1S_2		2.5	3.0
T_1A_3		2.5	3.5	T_1S_3		2.5	3.5
T_1A_4		3.0	2.5	T_1S_4		3.0	2.5
T_1A_5		3.0	3.0	T_1S_5		3.0	3.0
T_1A_6		3.0	3.5	T_1S_6		3.0	3.5
T_3A_7	T_3- mannitol + PEG + CMC + tween -80	2.5	2.5	T_3S_7	T_3- mannitol + PEG + CMC + tween -80 + 0.75% skim milk	2.5	2.5
T_3A_8		2.5	3.0	T_3S_8		2.5	3.0
T_3A_9		2.5	3.5	T_3S_9		2.5	3.5
T_3A_{10}		3.0	2.5	T_3S_{10}		3.0	2.5
T_3A_{11}		3.0	3.0	T_3S_{11}		3.0	3.0
T_3A_{12}		3.0	3.5	T_3S_{12}		3.0	3.5
C_1A_{13}	King's B broth	3.0	3.0	C_1S_{13}	King's B broth + 0.75% skim milk	3.0	3.0
C_1A_{14}		3.0	3.5	C_1S_{14}		3.0	3.5

A total of 28 different treatment combinations were used to estimate the optimum concentration of sodium alginate and $CaCl_2$ needed for preparing the microencapsulated beads (Table 3.1). Those treatment combinations with alginate alone were abbreviated as 'TA' where T_1A_1 to T_1A_6 were the beads prepared from treatment combination (T_1) obtained in 3.2.1 with different concentration of sodium alginate and calcium chloride solution while from T_3A_7 to T_3A_{12} were the beads prepared from treatment combination (T_3) obtained in 3.2.1 with different concentration of sodium alginate and calcium chloride solution. Whereas, Treatment combinations of alginate amended skim milk was designated a 'TS' where from T_1S_1 to T_1S_6 were the beads prepared from combination of treatment (T_1) along with skim milk and different concentration of sodium alginate and calcium chloride solution while treatments T_3S_7 to T_3S_{12} were the beads prepared from treatment (T_3) amended with skim milk and different concentration of sodium alginate and calcium chloride solution. Control treatments for alginate beads alone were abbreviated by 'CA' (C_1A_{13} and C_1A_{14}) whereas alginate amended with skim milk was designated as 'CS' (C_1S_{13} and C_1S_{14}) which were the beads produced from King's B broth (without additives) combined with sodium alginate and calcium chloride solution.

3.3.4. Secondary multiplication of alginate beads

The prepared alginate beads were added to fresh King's B broth and were kept for incubation on a rotary shaker at 100 rpm at room temperature. Bacteria were allowed multiplied in this mixture for an additional 24 h and the beads were collected with a sieve. The beads were then washed several times with saline water (0.85% w/v) and stored in air tight containers.

Beads were thus produced in three different batches *viz*., beads produced from alginate alone, alginate amended with skim milk and beads after secondary multiplication.

3.4 STANDARDIZATION OF DIFFERENT PARAMETERS FOR BEAD PREPARATION

3.4.1 Gelation and curing of beads

The time at which the weight of alginate beads attains stability is considered as time of gelation or time for bead curing (Tous *et al.,* 2014). Five randomly selected beads from 28 different treatments were subjected to six different time periods *viz*., 30, 45, 60, 75 and 90 min in the experiment, as gelation rate is a critical factor which maintains gel uniformity and strength. At each time period, the beads were taken out from the $CaCl_2$ solution and their weight was recorded using a weighing balance, after removal of water present on the surface of the bead using a blotting paper.

3.4.2 Determination of cell leakage

In the process of bead preparation, the viscous alginate solution taken in a separating funnel fitted on a stand was added drop wise into a pre-cooled sterile $CaCl_2$ solution taken in a beaker kept below the funnel whereby alginate beads were formed instantly. Leakage of bacterial cells from the polymer matrix of sodium alginate and calcium chloride occurs during gelation period. The leakage of the cells was determined by measuring the cell density. For this, one ml of the solution was pipetted from $CaCl_2$ solution after recovering the beads and was transferred to 9 ml sterile water which gives a dilution of 10^{-1}. Likewise, the process was continued until it reached dilution of 10^{-6} and 10^{-8} where one ml of the solution from each of these dilutions were pipetted into sterile Petri plates. Melted and cooled King's B agar media of 10-15 ml was added to these plates and rotated in both clockwise and anti-clockwise direction for uniform distribution of bacterial cells (Callone *et al.,* 2008). The plates were allowed to solidify and were incubated at room temperature for 24-48 h. The numbers of colonies produced were enumerated after 48 h and the results were expressed in cfu ml^{-1}.

3.4.3 Standardization of optimum height of air column

The shape of alginate beads depends on the distance between dripping tip of the separating funnel and the surface of gelation solution and it is decided by the height of the air column *i.e,* optimum height of air column is determined between the orifice of separating funnel and the level of the calcium chloride solution maintained in the beaker kept below the funnel. The height was adjusted from 1- 25cm and was standardized from the analysis of shape of beads.

3.5 CHARACTERIZATION OF MICROENCAPSULATED BEADS

3.5.1 Bead size and shape

The size and shape of beads depends on the concentration of sodium alginate and calcium chloride solution. Bead diameter and shape were estimated by using a stereomicroscope available at College of Forestry, Kerala Agricultural University. Diameter and shape of five randomly selected beads from 28 different treatments were estimated.

3.5.1.1 Sphericity factor

Shape of bead was estimated by sphericity factor. Using a stereomicroscope, mean diameter of five randomly selected beads of different treatments in three directions were taken (Chan *et al.,* 2009). From the data obtained, sphericity factor was calculated by the formula:

$$\text{Spherecity factor (SF)} = \frac{d_{max} - d_{min}}{d_{max} + d_{min}}$$

Where, d_{max} is the largest diameter of the beads

d_{min} is the smallest diameter of the beads

3.5.2 Bead weight and number of beads per gram of formulation

Weight of 10 randomly selected beads was estimated from each treatment. Mean bead weight was calculated and expressed in mg. Number of beads per gram of the formulation for each treatment combination was estimated using a weighing balance for selecting the best combination.

3.5.3 Bead yield

The prepared alginate beads were filtered, collected, dried and weighed to determine bead yield. The yield of production of alginate beads of various batches were estimated from the final weight of beads after drying with respect to the initial total weight of components used for bead preparation (Sherina *et al*., 2012; Sabry, 2018). The per cent production yield of beads was calculated by

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

where, Practical yield - total weight of beads produced

Theoretical yield - sum of different components used for bead preparation

3.5.4 Swelling ratio of alginate beads

Swelling ratio was estimated by measuring the percentage uptake of water by the beads. About five beads from 16 treatments were accurately weighed and placed in 10 ml of 0.2M phosphate buffer (pH 7.0) and sterile water separately. The beads were removed after 1 h, dried with tissue paper and weighed. The swelling ratio was estimated as the ratio of the increase in bead weight after swelling to the dry weight of

the beads (Sherina *et al.*, 2012; Santhi *et al.*, 2013; Sabry, 2018). Swelling percentage was determined by the following equation:

$$\text{Swelling ratio} = \frac{\text{Swollen weight - initial weight}}{\text{Initial weight}} \times 100$$

3.5.5 Water content in beads

The per cent water content of beads or water holding capacity was estimated. The beads from 16 treatments were weighed immediately after preparation and also after being subjected to drying (Tous *et al.*, 2014; Abdellatif *et al.*, 2016). The mean water loss from the beads was calculated using the equation:

$$\text{Water content (\%)} = \frac{\text{Weight before drying-Weight after drying}}{\text{Weight before drying}} \text{ X } 100$$

3.5.6 Shrinkage percentage of beads

It is the percentage of water expelled from the beads during gelation process. The diameter of five alginate beads for three dimensions from each treatment was estimated using a stereomicroscope immediately after preparation and also after drying of beads. The mean diameter of five beads were calculated (Tous *et al.*, 2014). Shrinkage percentage was estimated using the formula:

$$\text{Shrinkage \%} = \frac{\text{Diameter of beads before drying}}{\text{Diameter of beads after drying}} \text{ X } 100$$

3.6 EVALUATION OF SHELF LIFE OF *P. fluorescens* ENCAPSULATED BEADS UNDER *IN VITRO* CONDITIONS

3.6.1 Rate of release of bioagent from beads

The *in vitro* release of bioagent *P. fluorescens* from prepared beads was estimated in 0.2 M phosphate buffer (pH 7.0) and sterile distilled water. One gram of prepared beads were weighed and added separately each to 10 ml of phosphate buffer and 10 ml sterile water and was kept in an orbital shaker at 100 rpm at room temperature. One ml of the sample from each of these was pipetted at 30 min, 1h, 2h and after 24 h interval and at each time interval, one ml of the sample was pipetted from the buffer solution and sterile distilled water. At the same time, one ml of fresh buffer solution and sterile water was added to the respective lots to maintain sink condition (Sherina *et al.*, 2012). The samples were then subjected to serial dilution and plating technique. For this one ml of the sample pipetted from the respective solutions was transferred to nine ml sterile water which gives a dilution of 10^{-1}. Likewise, the process continued until it reached a dilution of 10^{-8} and 10^{-16} where 1 ml of the solution from each of these dilutions was pipetted into sterile Petri plates. Melted and cooled King's B agar media of 10-15 ml was added to these plates and rotated it in both clockwise and anti-clockwise directions for uniform distribution of bacterial cells. Three replications were maintained for each treatment. The plates were allowed to solidify and incubated at room temperature for 24-48 h. The number of colonies produced were enumerated after 48 h and the results were expressed in cfu ml^{-1}.

3.6.2 Population density of *P. fluorescens* per bead

Population density of the bioagent was estimated by dissolving one bead in one ml of 0.2M phosphate buffer of pH 7.0 and was incubated overnight at 120 rpm. Population was enumerated by using serial dilution and plating technique in King's B media. Dilutions were prepared until it reached a final required concentration of 10^{-8} and 10^{-12}. One ml of the aliquot from each of these dilutions were plated on the respective medium in three replications. After solidification of the media, the plates were incubated at room temperature for 48 h and the results were expressed in cfu ml^{-1}.

3.6.3 Survival of bacteria entrapped in beads at monthly intervals

This experiment is done by using serial dilution and plating technique. 1 g of alginate beads were accurately weighed and dissolved in 0.2 M phosphate buffer pH 7.0 and incubate at 120 rpm until beads get dissolved. Population of microorganisms from the beads was estimated by serial dilution and plating technique by pipetting 1 ml from the bacterial buffer solution and serial dilute up to 10^{-34} dilution. One ml of the aliquot from the dilutions was plated on the respective medium. Observations on colony forming units (cfu) were recorded as per 3.6.1 in three replications for each treatment upto four months and results were expressed as cfu g^{-1} of beads.

3.7 *In vitro* INHIBITION OF FORMULATION AGAINST MAJOR SOIL BORNE PLANT PATHOGENS

The two best promising treatment combinations selected from 3.6.3 were tested for *in vitro* inhibition against major soil borne plant pathogens *viz.*, *Fusarium oxysporum*, *Phytophthora nicotiana*, *Pythium aphanidermatum*, *Sclerotium rolfsii* and *Rhizoctonia solani* by dual culture technique and that with *Ralstonia solanacearum* were tested by filter paper disc method. For this, one gram of alginate bead from the best two treatments were dissolved in 0.2 M of phosphate buffer and were incubated for 48 h to facilitate release of bacteria from the beads in buffer solution.

3.7.1 Dual culture technique

One gram of bead was weighed and dissolved completely in phosphate buffer for 48 h. From this, one ml of the bacterial buffer solution was pipetted and poured into Petri plates to prepare a lawn of the bacterium. Melted and cooled potato dextrose agar (PDA) media (Annexure-I) of 10-15 ml was added to these plates and rotated it in both clockwise and anti-clockwise direction for uniform distribution of bacterial cells. The plates were allowed to solidify and a nine mm fungal mycelial disc from an actively growing culture of the respective pathogen was placed in the centre of the Petri plate and incubated at room temperature. Three replications were maintained and fungal disc

of the pathogen alone served as control. Observations on inhibition zone and mycelial growth of test pathogen were recorded and per cent inhibition of pathogen growth was calculated by using the formula:

$$\text{Per cent inhibition (PI)} = \frac{C-T}{C} \times 100$$

Where, C- mycelial growth of pathogen in control
T- mycelial growth of pathogen in dual culture plate

3.7.2 Filter paper disc method

One ml of the bacterial suspension was pipetted and poured to Petri plates and 10-15 ml of melted and cooled King's B (KB) agar media was poured in the same plate and thereafter rotated it in both clockwise and anti-clockwise direction to achieve uniform distribution of bacterial cells. Then, filter paper discs of one cm diameter, was dipped in alginate bead based bacterial suspension and were placed in the centre of the KB plate and were incubated at room temperature. Filter paper immersed in sterile water served as control. Three replications were maintained for each treatment. Per cent inhibition was calculated as per 3.7.1.

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

3.8 STATISTICAL ANALYSIS

Data were subjected to analysis of variance (ANOVA) and the data collected in various experiments were analysed using the statistical package Web Agri Stat Package (WASP 2.0). Mean, critical difference and level of significance were obtained for various data sets. Multiple comparisons between the treatment means where the F test was significant was done with Duncan's Multiple range Test (DMRT). The data wherever needed was subjected to appropriate transformation as suggested by Gomez and Gomez (1984).

RESULTS

4. RESULTS

The results of the study on "Alginate based encapsulation of *P. fluorescens* for management of soil borne plant pathogens" conducted during the period 2018-2020 at Department of Plant Pathology, College of Horticulture, Vellanikkara are presented in this chapter.

4.1 STANDARDIZATION OF MEDIA WITH ADDITIVES AND ITS EFFECT ON SHELF LIFE OF *P. fluorescens*

Survival of *P. fluorescens*, the reference culture of KAU, in King's B broth amended with selected additives in nine different treatments including control was monitored upto nine months of storage. Observations on the enumeration of viable count carried out by serial dilution plating technique were recorded at 48 h and 15 days after inoculation (DAI) and also at monthly intervals and are presented in Tables 4.1a and 4.1b and is depicted in plate 4.1a, b, c, d, e and f. It was evident from the data that there was a significant different among various treatments where the population of *P. fluorescens* increased in the initial 60 days of storage in all the additive amended combinations when compared to control (Plate 4.1). After one month of incubation period, it was observed that population of *P. fluorescens* was highest in control T_9 (8.0 x 10^{12} cfu ml^{-1}) closely followed by T_1 (7.0 x 10^{12} cfu ml^{-1}). Population of *P. fluorescens* in T_3 (2.33 x 10^{12} cfu ml^{-1}) and T_5 (2.66 x 10^{12} cfu ml^{-1}) were statistically on par with each other. Minimum population of *P. fluorescens* was recorded in T_4, T_6, T_8 and no population was noticed in treatment T_7. After two months of storage, it was noticed that except in T_2, there was a rise in the population with the maximum count in T_3 (17.0 x 10^8 cfu ml^{-1}) and T_1 (15.0 x 10^8 cfu ml^{-1}).

However, after three months of storage, the population P. *fluorescens* started declining drastically and after nine months, highest population of *P. fluorescens* was observed in **T_1 (1.33 x 10^8 cfu ml^{-1})** and **T_3 (1.66 x 10^8 cfu ml^{-1})** which were statistically on par with each other followed by treatment T_2 (0.33 x 10^8 cfu ml^{-1}). However, treatments T_4, T_5, T_6, T_7, T_8 and including control T_9 at nine months of storage showed

Table 4.1a Population of *P. fluorescens* in King's B broth upto two months of storage period

Treatments	Population of *P.fluorescens* (X 10^{12} cfu ml^{-1})			
	48 h	**15 DAI**	**1MAI**	**2MAI**
T_1	10.0 ± 1 (1.040)^{b}	11.33±0.57 (1.091)^{a}	7.0 ± 2 (0.894)^{a}	12.0 ± 1 (1.113)^{a}
T_2	5.0 ± 1 (0.774)^{c}	5.66±1.52 (0.816)^{b}	3.33 ± 2.30 (0.600)^{b}	1.66 ± 0.57 (0.418)^{c}
T_3	1.0 ± 0 (0.301)^{d}	3.66±1.52 (0.651)^{bc}	2.33 ± 0.57 (0.519)^{bc}	13.33 ± 1.52 (1.155)^{a}
T_4	0 ± 0 (0.000)^{e}	3.33±0.57 (0.634)^{bc}	1.0 ± 0 (0.301)^{cd}	5.66 ± 1.52 (0.816)^{b}
T_5	4.0 ± 2 (0.674)^{c}	4.0 ±1 (0.693)^{bc}	2.66 ± 2.08 (0.519)^{bc}	10.0 ± 1 (0.816)^{b}
T_6	0 ± 0 (0.000)^{e}	2.33± 1.15 (0.502)^{cd}	0.66 ± 0.57 (0.201)^{de}	1.33 ± 0.57 (0.360)^{c}
T_7	0 ± 0 (0.000)^{e}	1.33±1.52 (0.301)^{d}	0 ± 0 (0.000)^{e}	8.0 ± 1 (0.952)^{ab}
T_8	0 ± 0 (0.000)^{e}	2.33±0.57 (0.519)^{cd}	0.33 ± 0.57 (0.100)^{de}	1.33 ± 1.52 (0.301)^{c}
T_9	16.33 ± 1.52 (1.238)^{a}	14.0 ± 2.64 (1.172)^{a}	8.0 ± 2 (0.947)^{a}	8.33 ± 2.08 (0.962)^{ab}
CD (0.05)	0.118	0.241	0.250	0.221

DAI: Days after inoculation, MAI: Month after inoculation

T_1- Mannitol +PVP +CMC +Tween-80; T_2- Mannitol +PVP +Liquid paraffin +Tween-80; T_3- Mannitol +PEG +CMC +Tween-80; T T_4- Mannitol +PEG +Liquid paraffin +Tween-80; T_5- Trehalose +PVP +CMC +Tween-80; T_6- Trehalose +PVP +Liquid paraffin + Tween-80; T_7- Trehalose +PEG +CMC +Tween-80; T_8- Trehalose + PEG +Liquid paraffin +Tween-80; T_9- Control

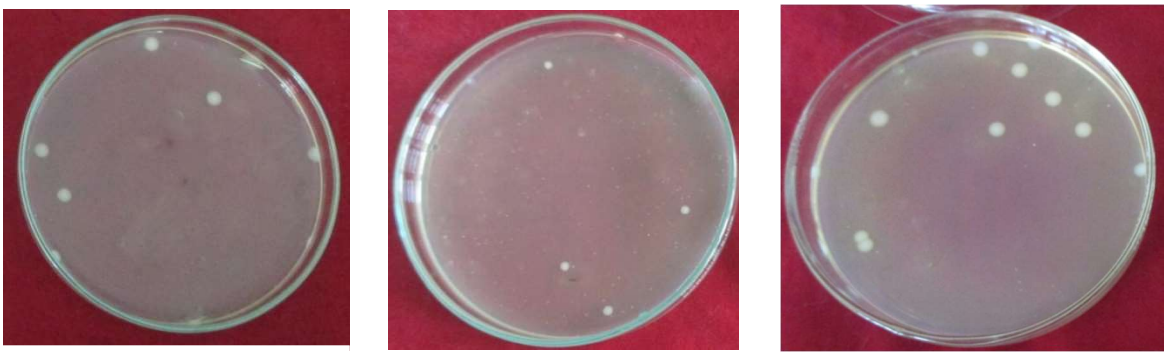
*Values are arranged in Mean ± SD. Log transformations are given in parentheses. In each column figures followed by same letter do not differ significantly according to DMRT.

T_1 T_3 T_9

Plate 4.1a Population of *P. fluorescens* (x 10^{12} cfu ml^{-1}) in different treatments at 48 h

T_1 T_3 T_9

Plate 4.1b Population of *P. fluorescens* (x 10^{12} cfu ml^{-1}) in different treatments at 15 DAI



T_1 T_3 T_9

Plate 4.1c Population of *P. fluorescens* (x 10^{12} cfu ml^{-1}) in different treatments at 1 MAI

Contd:

Table 4.1b. Population of *P. fluorescens* in King's B broth at monthly intervals

Treatments	Population of *P.fluorescens* (X 10^8 cfu ml^{-1})						
	3 MAI	**4MAI**	**5MAI**	**6MAI**	**7 MAI**	**8 MAI**	**9MAI**
T_1	15.0 ± 2.64 (1.200)^{a}	5.0 ± 1.73 (0.764)^{ab}	4.0 ± 2.64 (0.661)^{a}	1.0 ± 1.0 (0.593)^{a}	2.0 ± 1.0 (0.460)^{a}	1.33 ± 0.57 (0.360)^{a}	1.33± 0.57 (0.360)^{a}
T_2	1.33 ± 0.57 (0.360)^{c}	1.0±0 (0.301)^{de}	0.33 ± 0.57 (0.100)^{bc}	1.66 ± 1.15 (0.401)^{abc}	0.66 ± 1.15 (0.159)^{bc}	0.33 ± 0.57 (0.100)^{bc}	0.33± 0.57 (0.100)^{b}
T_3	17.0 ± 2.64 (1.252)^{a}	9.0 ± 3.0 (0.986)^{a}	3.0 ± 1.73 (0.577)^{a}	2.0 ± 0 (0.477)^{ab}	2.0 ± 1.0 (0.460)^{a}	2.0 ± 0 (0.477)^{a}	1.66± 1.15 (0.401)^{a}
T_4	4.66 ± 2.08 (0.735)^{b}	0 (0.000)^{f}	0 (0.000)^{c}	1.0 ± 0 (0.301)^{bcd}	0.33 ± 0.57 (0.100)^{bc}	0 (0.000)^{c}	0 (0.000)^{b}
T_5	15.0 ±2.64 (1.200)^{a}	4.0 ± 1.0 (0.693)^{b}	3.0±1.0 (0.593)^{a}	1.0 ± 1.0 (0.259)^{cd}	0 (0.000)^{c}	0 (0.000)^{c}	0 (0.000)^{b}
T_6	2.0 ±0 (0.318)^{c}	0.33 ± 0.57 (0.100)^{ef}	0.33±0.57 (0.100)^{bc}	0.33 ± 0.57 (0.100)^{de}	0 (0.000)^{c}	0 (0.000)^{c}	0 (0.000)^{b}
T_7	4.33 ± 1.52 (0.715)^{b}	2.0 ± 1.0 (0.460)^{cd}	0 (0.000)^{c}	0 (0.000)^{e}	0 (0.000)^{c}	0 (0.000)^{c}	0 (0.000)^{b}
T_8	3.66 ± 1.15 (0.661)^{b}	2.66 ± 0.57 (0.560)^{bc}	1.0±1.0 (0.259)^{bc}	0 (0.000)^{e}	0 (0.000)^{c}	0 (0.000)^{c}	0 (0.000)^{b}
T_9	5.66 ± 2.08 (0.810)^{b}	1.5 ± 0.70 (0.259)^{de}	1.0 ±0 (0.301)^{b}	1.0 ± 0 (0.301)^{bcd}	1.0 ± 0 (0.301)^{ab}	0.66 ± 0.57 (0.201)^{b}	0 (0.000)^{b}
CD (0.05)	0.234	0.230	0.262	0.207	0.223	0.152	0.152

T_1- Mannitol +PVP +CMC +Tween-80; T_2- Mannitol +PVP +Liquid paraffin +Tween-80; T_3- Mannitol +PEG +CMC +Tween-80; T_4- Mannitol +PEG +Liquid paraffin +Tween-80; T_5- Trehalose +PVP +CMC +Tween-80; T_6- Trehalose +PVP +Liquid paraffin Tween-80; T_7- Trehalose +PEG +CMC +Tween-80; T_8- Trehalose + PEG +Liquid paraffin +Tween-80; T_9- Control

*Values are arranged in Mean ± SD

Log transformations are given in parentheses. In each column figures followed by the same letter do not differ significantly according to DMRT.

no population of *P. fluorescens*. Hence, the best two treatments T_1 (Mannitol +PVP +CMC +tween-80) and T_3 (Mannitol +PEG +CMC +tween-80) were selected for the preparation of alginate beads.

4.2 STANDARDIZATION OF DIFFERENT PARAMETERS FOR BEAD PREPARATION

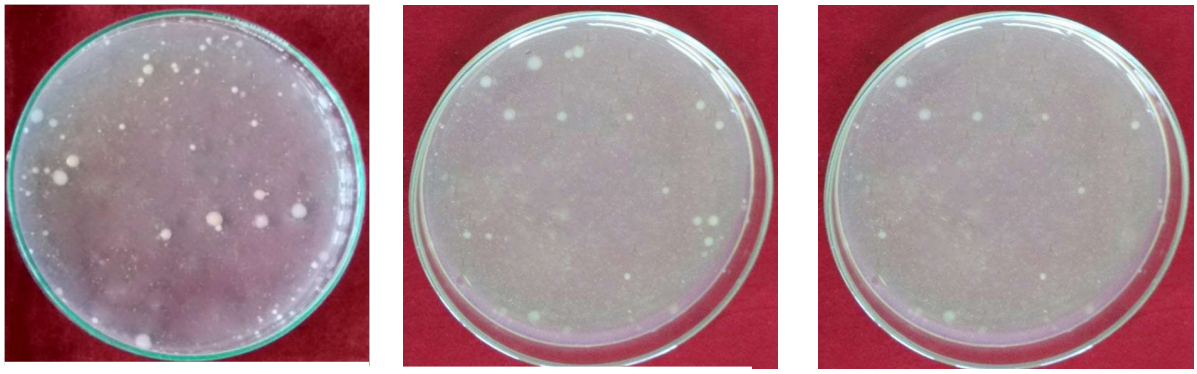
The alginate beads were prepared in three different batches *viz*., beads from alginate alone, beads from alginate amended with skim milk and beads after secondary multiplication.

4.2.1 Concentration of sodium alginate and calcium chloride

As per the protocol of Bashan *et al.* (2002), the optimum concentration of sodium alginate and calcium chloride for bead preparation was standardised. Five different concentrations of sodium alginate (1.0%, 1.5%, 2.0%, 2.5% and 3.0%) and four different concentrations of calcium chloride (2.0%, 2.5%, 3.0% and 3.5%) were used for the study and the data is illustrated in Table 4.2.

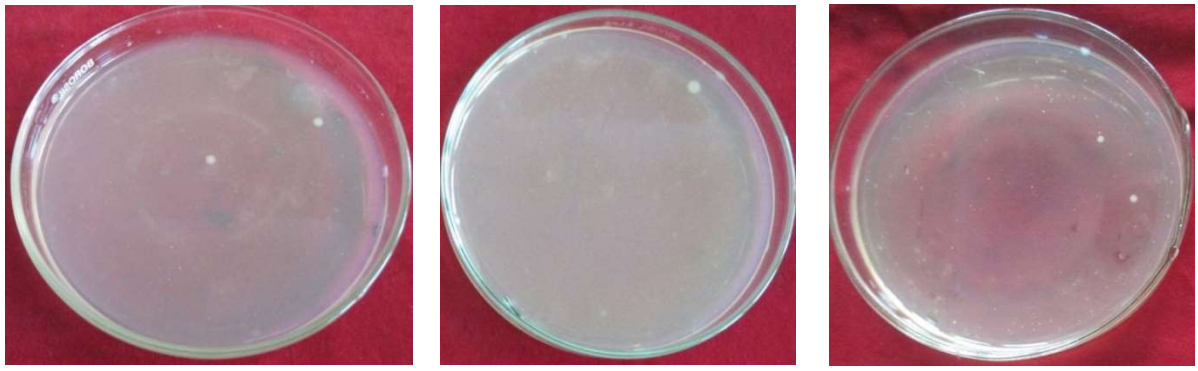
The data clearly indicates that lower concentration of sodium alginate (1.0% and 1.5%) with different concentrations of calcium chloride solution (2.0%, 2.5%, 3.0% and 3.5%) resulted in improper and irregular beads in both alginate and alginate amended skim milk preparation. It was noticed that beads were formed at higher concentrations of sodium alginate (2.0%, 2.5% and 3.0%) and calcium chloride (2.0%, 2.5%, 3.0% and 3.5%), whereas treatments containing low concentration of sodium alginate (2.0%) and calcium chloride solution (2.0%) resulted in irregular soft bead, which was not a desirable character and hence, it was not used for further studies. Large and rigid beads were formed when the concentration of sodium alginate was standardized to 2.5 and 3.0 per cent combined with calcium chloride solution of 2.5, 3.0 and 3.5 per cent (Plate 4.2).

Therefore, beads formed at 2.5 and 3.0 per cent concentration of sodium alginate with each of 2.5, 3.0 and 3.5 per cent concentration of calcium chloride solution



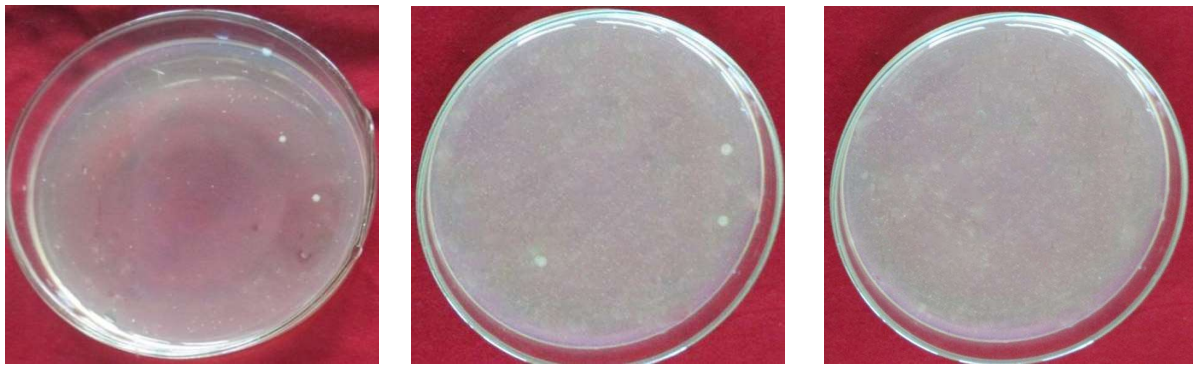
T_1 **T_3** **T_9**

Plate 4.1d Population of *P. fluorescens* (x 10^8 cfu ml^{-1}) in different treatments at 3 MAI



T_1 **T_3** **T_9**

Plate 4.1e Population of *P. fluorescens* (x 10^8 cfu ml^{-1}) in different treatments at 6 MAI



T_1 **T_3** **T_9**

Plate 4.1f Population of *P. fluorescens* (x 10^8 cfu ml^{-1}) in different treatments at 9 MAI

T_1- Mannitol (3%) +PVP (2%) +CMC (1%) +Tween-80 (0.5%)
T_3- Mannitol (3%) +PEG (0.25%) +CMC (1%) +Tween-80 (0.5%)
T_9- Control

Table 4.2 Optimum concentration of sodium alginate and calcium chloride on bead formation

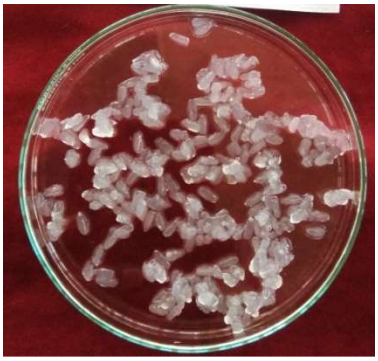
Concentration of sodium alginate (%)	Concentration of calcium chloride (%)	Characteristics of beads formed (control)	Characteristics of beads formed (with additives)
1.0	**2.0**	_	Irregular shape
1.0	**2.5**	_	Irregular shape
1.0	**3.0**	_	Irregular shape
1.0	**3.5**	_	Irregular shape
1.5	**2.0**	_	Irregular shape
1.5	**2.5**	_	Irregular shape
1.5	**3.0**	_	Irregular shape
1.5	**3.5**	_	Irregular soft beads
2.0	**2.0**	Irregular shape	Small to medium sized beads
2.0	**2.5**	Irregular shape	Small to medium sized beads
2.0	**3.0**	Irregular shape	Medium sized beads
2.0	**3.5**	Irregular shape	Medium sized to strong beads
2.5	**2.0**	Irregular shape	Small soft beads
2.5	**2.5**	Irregular shape	Medium sized to uniform shape
2.5	**3.0**	Irregular shape	Uniform shape
2.5	**3.5**	Irregular shape	Medium sized to bold beads
3.0	**2.0**	Soft beads	Medium sized to bold uniform beads
3.0	**2.5**	Soft beads	Medium sized to bold uniform beads
3.0	**3.0**	Soft beads	Uniform shape and bold beads
3.0	**3.5**	Soft beads	Uniform shape and bold beads

in both alginate and alginate amended skim milk beads were selected for further studies. Whereas, in control treatment beads were formed only at 3.0 per cent sodium alginate with each of 3.0 and 3.5 per cent $CaCl_2$ solution respectively. Thus, a total of 28 different treatment combinations in two different formulations (alginate beads and alginate amended skim milk beads) of two best treatments T_1 (Mannitol + PVP + CMC + tween-80) and T_3 (Mannitol + PEG + CMC + tween-80) obtained in 4.1 were used for the preparation of beads in order to estimate the best concentration of sodium alginate and $CaCl_2$ needed for commercial formulation of bead preparation as depicted in Table 4.3.

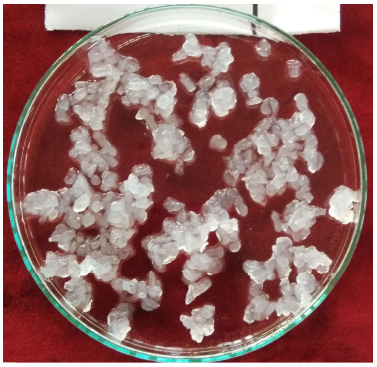
4.2.2 Gelation and curing of beads

Five randomly selected beads from 28 different treatments were subjected to five different time periods *viz*., 30, 45, 60, 75 and 90 min for studying gelation and curing time of beads. At each time period, weight of five beads from each treatment was recorded and average bead weight was calculated (Table 4.4 and Table 4.5). From the Table 4.4, it was observed that a higher bead weight was obtained in treatment combination T_1A_6, T_3A_{12} and T_3A_{11} which are statistically on par with each other at 30, 45, 60 and 75 min of gelation time. While, at 90 min of gelation time, maximum bead weight was observed in treatment combination T_1A_6 and T_3A_9 followed by T_3A_{11} and T_3A_{12}. whereas, minimum bead weight was observed in control beads C_1A_{13} and C_1A_{14} at all different time periods. It was also noticed that the average weight of beads gradually varied with increased time period. After 60 min of gelation period, average bead weight was gradually maintained till 75 min in beads prepared with sodium alginate (2.5 and 3.0%) and calcium chloride (3.0% and 3.5%) respectively. However, in control treatment average bead weight could not be maintained even after 60 minutes and also in beads prepared with 2.5 per cent calcium chloride solution.

In alginate amended skim milk beads, higher bead weight was observed in treatments T_1S_6 and T_3S_{12} which are on par with each other followed by T_1S_5, T_3S_{11}, T_3S_{10} and T_1S_4 at 30 and 45 min of gelation time (Table 4.5). Whereas, treatments T_1S_6 and T_3S_{12} on par were also observed with higher average bead weight followed by T_3S_{11}



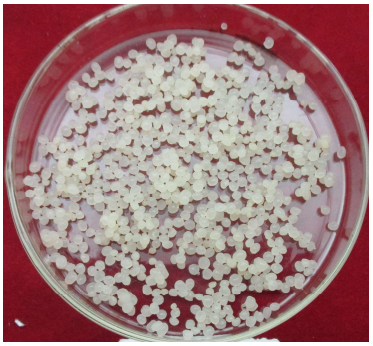
Sodium alginate (1.0%) + $CaCl_2$ (3.0%)



Sodium alginate (1.0%) + $CaCl_2$ (3.5%)

Sodium alginate (1.5%) + $CaCl_2$ (3.0%)

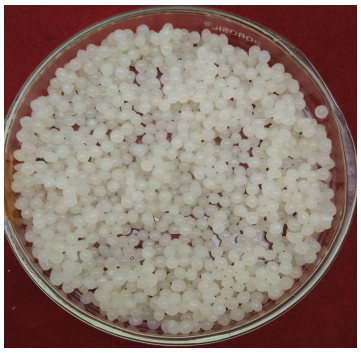
Sodium alginate (1.5%) + $CaCl_2$ (3.5%)



Sodium alginate (2.0%) + $CaCl_2$ (3.0%)

Sodium alginate (2.0%) + $CaCl_2$ (3.5%)

Sodium alginate (3.0%) + $CaCl_2$ (3.0%)



Sodium alginate (3.0%) + $CaCl_2$ (3.5%)

Plate 4.2 Different concentrations of sodium alginate and calcium chloride on bead formation

Table 4.3 Different treatment combinations of sodium alginate and calcium chloride for preparation of beads

Treatments	Alginate combination alone			Treatments	Alginate + Skim milk combination		
	Treatments	Sodium alginate (%)	Calcium chloride (%)		Treatments	Sodium alginate (%)	Calcium chloride (%)
T_1A_1	T_1 - Mannitol + PVP + CMC + Tween-80	2.5	2.5	T_1S_1	T_1 - Mannitol + PVP + CMC + Tween-80 +0.75% Skim milk	2.5	2.5
T_1A_2		2.5	3.0	T_1S_2		2.5	3.0
T_1A_3		2.5	3.5	T_1S_3		2.5	3.5
T_1A_4		3.0	2.5	T_1S_4		3.0	2.5
T_1A_5		3.0	3.0	T_1S_5		3.0	3.0
T_1A_6		3.0	3.5	T_1S_6		3.0	3.5
T_3A_7	T_3- Mannitol + PEG + CMC + Tween -80	2.5	2.5	T_3S_7	T_3- Mannitol + PEG + CMC + Tween -80 + 0.75% Skim milk	2.5	2.5
T_3A_8		2.5	3.0	T_3S_8		2.5	3.0
T_3A_9		2.5	3.5	T_3S_9		2.5	3.5
T_3A_{10}		3.0	2.5	T_3S_{10}		3.0	2.5
T_3A_{11}		3.0	3.0	T_3S_{11}		3.0	3.0
T_3A_{12}		3.0	3.5	T_3S_{12}		3.0	3.5
C_1A_{13}	King's B broth	3.0	3.0	C_1S_{13}	King's B broth + 0.75% Skim milk	3.0	3.0
C_1A_{14}		3.0	3.5	C_1S_{14}		3.0	3.5

*PEG : Polyethylene glycol
PVP : polyvinyl pyrrolidone
CMC: Carboxyl methyl cellulose

Table 4.4 Mean weight of beads at different gelation period for alginate bead formulation

Treatments (Alginate alone)	*Weight of beads (mg)				
	Gelation time (min)				
	30	45	60	75	90
T_1A_1 (T_1 +2.5% NA+ 2.5% $CaCl_2$)	6.2 ± 0.83 (2.485)^{f}	7.8 ± 1.30 (2.785)^{g}	9.2 ± 0.83 (3.031)^{e}	11.2 ± 0.83 (3.345)^{de}	13.6 ± 2.8 (3.671)^{de}
T_1A_2 (T_1 + 2.5% NA +3.0% $CaCl_2$)	12 ± 1.0 (3.462)^{c}	13.4 ± 2.19 (3.650)^{cd}	14.6 ± 1.14 (3.819)^{c}	15.6 ± 1.14 (3.948)^{c}	15.6 ± 3.64 (3.926)^{bcd}
T_1A_3 (T_1 + 2.5% NA +3.5% $CaCl_2$)	15 ± 1.58 (3.869)^{b}	16.6 ± 1.51 (4.071)^{ab}	18.8 ± 0.83 (4.335)^{a}	21.2 ± 1.92 (4.601)^{a}	20.8 ± 9.47 (4.451)^{ab}
T_1A_4 (T_1 + 3.0% NA +2.5% $CaCl_2$)	7.4 ± 1.14 (2.714)^{e}	8.2 ± 0.83 (2.861)^{fg}	9.4 ± 0.54 (3.065)^{e}	10.8 ± 1.92 (3.277)^{de}	14.6 ± 3.91 (3.792)^{cd}
T_1A_5 (T_1 + 3.0% NA +3.0% $CaCl_2$)	13.4 ± 0.89 (3.659)^{bc}	14.6 ± 0.54 (3.820)^{bc}	17.0 ± 1.0 (4.122)^{b}	18.2 ± 0.44 (4.266)^{b}	18.4 ± 0.54 (4.289)^{abc}
T_1A_6 (T_1 + 3.0% NA +3.5% $CaCl_2$)	17.2 ± 1.48 (4.144)^{a}	18.6 ± 0.54 (4.312)^{a}	20.0 ± 0.70 (4.472)^{a}	20.2 ± 0.44 (4.494)^{ab}	20.4 ± 0.54 (4.516)^{a}
T_3A_7 (T_3 + 2.5% NA +2.5% $CaCl_2$)	5.8 ± 0.83 (2.403)^{f}	5.6 ± 1.14 (2.356)^{h}	8.4 ± 1.14 (2.893)^{e}	9.4 ± 1.67 (3.056)^{ef}	13.0 ± 2.73 (3.590)^{de}
T_3A_8 (T_3 + 2.5% NA +3.0% $CaCl_2$)	9.2 ± 0.83 (3.031)^{d}	10.6 ± 1.14 (3.252)^{e}	11.8 ± 0.83 (3.433)^{d}	12.2 ± 1.92 (3.484)^{d}	14.6 ± 4.33 (3.785)^{cd}
T_3A_9 (T_3 + 2.5% NA +3.5% $CaCl_2$)	10 ± 0.70 (3.161)^{d}	12.6 ± 2.30 (3.537)^{d}	15.2 ± 1.30 (3.896)^{c}	15.2 ± 3.11 (3.883)^{c}	21.2 ± 4.14 (4.587)^{a}
T_3A_{10} (T_3 + 3.0% NA +2.5% $CaCl_2$)	7.8 ± 1.30 (2.785)^{e}	9.4 ± 1.14 (3.061)^{ef}	11.0 ± 1.0 (3.314)^{d}	12.0 ± 1.58 (3.458)^{d}	14.4 ± 3.64 (3.769)^{cde}
T_3A_{11} (T_3 + 3.0% NA +3.0% $CaCl_2$)	17.8 ± 0.83 (4.218)^{a}	17.4 ± 0.89 (4.170)^{a}	19.0 ± 0.70 (4.358)^{a}	19.4 ± 0.54 (4.404)^{ab}	19.2 ± 0.44 (4.382)^{ab}
T_3A_{12} (T_3 + 3.0% NA +3.5% $CaCl_2$)	17.4 ± 1.14 (4.170)^{a}	18.6 ± 0.54 (4.312)^{a}	19.4 ± 0.54 (4.404)^{a}	20.4 ± 0.54 (4.516)^{ab}	20.2 ± 0.44 (4.494)^{ab}
C_1A_{13} (KB + 3.0% NA +3.0% $CaCl_2$)	3.6 ± 0.89 (1.886)^{g}	3.4 ± 1.14 (1.823)^{i}	4.8 ± 0.83 (2.184)^{g}	8.0 ± 2.0 (2.809)^{f}	11.4 ± 2.40 (3.360^{de}
C_1A_{14} (KB + 3.0% NA +3.5% $CaCl_2$)	3.8 ± 0.83 (1.940)^{g}	5.8 ± 0.83 (2.403)^{h}	5.8 ± 0.83 (2.403)^{f}	5.4 ± 1.81 (2.295)^{g}	10.2 ± 1.30 (3.189)^{e}
CD (0.05)	0.221	0.262	0.172	0.310	0.558

*NA- Sodium alginate, $CaCl_2$- Calcium chloride, T_1- Mannitol + PVP + CMC + Tween-80, T_3- Mannitol + PEG + CMC + Tween-80, C_1- Control, KB- King's B broth. Transformed values are given in parentheses. In each column figures followed by same letter do not differ significantly according to DMRT.

Table 4.5 Mean weight of beads at different gelation period for alginate bead + skim milk formulation

Treatments (Alginate + Skim milk)	*Weight of beads (mg)				
	Gelation time (min)				
	30	45	60	75	90
T_1S_1 (T_1 +2.5% NA+ 2.5% $CaCl_2$+ 0.75% SM)	17.2 ± 0.83 (4.146)^{f}	19.4 ± 1.14 (4.403)^{g}	20.4 ± 0.54 (4.516)^{i}	22.0 ± 1.58 (4.688)^{f}	25.0 ± 3.16 (4.992)^{de}
T_1S_2 (T_1 + 2.5% NA +3.0% $CaCl_2$+ 0.75% SM)	23.2 ± 0.83 (4.816)^{e}	21.6 ± 4.15 (4.629)^{f}	25.4 ± 0.54 (5.040)^{h}	26.2 ± 1.30 (5.117)^{e}	28.0 ± 1.58 (5.290)^{cd}
T_1S_3 (T_1 + 2.5% NA +3.5% $CaCl_2$+ 0.75% SM)	28.4 ± 1.14 (5.328)^{c}	29.6 ± 0.54 (5.440)^{d}	30.6 ± 0.54 (5.532)^{e}	32.8 ± 1.92 (5.725)^{c}	32.0 ± 1.58 (5.655)^{b}
T_1S_4 (T_1 + 3.0% NA +2.5% $CaCl_2$+ 0.75% SM)	40.6 ± 0.54 (6.372)^{b}	43.4 ± 0.54 (6.588)^{c}	44.4 ± 0.54 (6.663)^{d}	43.6 ± 2.40 (6.601)^{b}	44.8 ± 1.92 (6.692)^{a}
T_1S_5 (T_1 + 3.0% NA +3.0% $CaCl_2$+ 0.75% SM)	42.0 ± 0.70 (6.481)^{b}	44.4 ± 0.54 (6.663)^{bc}	45.6 ± 0.54 (6.753)^{c}	46.6 ± 0.54 (6.826)^{a}	46.6 ± 0.54 (6.826)^{a}
T_1S_6 (T_1 + 3.0% NA +3.5% $CaCl_2$+ 0.75% SM)	45.6 ± 0.54 (6.753)^{a}	47.4 ± 0.54 (6.885)^{a}	48.6 ± 054 (6.971)^{a}	48.0 ± 0.70 (6.928)^{a}	47.6 ± 0.54 (6.899)^{a}
T_3S_7 (T_3 + 2.5% NA +2.5% $CaCl_2$+ 0.75% SM)	17.2 ± 0.83 (4.146)^{f}	18.2 ± 0.44 (4.266)^{g}	19.6 ± 0.54 (4.427)^{j}	22.2 ± 2.38 (4.706)^{f}	23.0 ± 5.24 (4.769)^{e}
T_3S_8 (T_3 + 2.5% NA +3.0% $CaCl_2$+ 0.75% SM)	23 ± 1 (4.795)^{e}	24.2 ± 0.83 (4.919)^{e}	26.2 ± 0.44 (5.118)^{g}	25.4 ± 1.51 (5.038)^{e}	28.0 ± 1.58 (5.290)^{cd}
T_3S_9 (T_3 + 2.5% NA +3.5% $CaCl_2$+ 0.75% SM)	26.4 ± 0.54 (5.138)^{d}	27.6 ± 1.14 (5.253)^{d}	28.4 ± 0.54 (5.329)^{f}	29.0 ± 1.22 (5.384)^{d}	28.8 ± 1.92 (5.364)^{bc}
T_3S_{10} (T_3 + 3.0% NA +2.5% $CaCl_2$+ 0.75% SM)	41 ± 0.70 (6.403)^{b}	42.6 ± 0.54 (6.527)^{c}	44.4 ± 0.54 (6.663)^{d}	43.0 ± 1.58 (6.557)^{b}	44.8 ± 3.11 (6.690)^{a}
T_3S_{11} (T_3 + 3.0% NA +3.0% $CaCl_2$+ 0.75% SM)	41.8 ± 0.44 (6.465)^{b}	44.6 ± 1.67 (6.677)^{bc}	46.6 ± 0.54 (6.826)^{b}	47.4 ± 0.54 (6.885)^{a}	47.4 ± 0.54 (6.885)^{a}
T_3S_{12} (T_3 + 3.0% NA +3.5% $CaCl_2$+ 0.75% SM)	45.6 ± 0.54 (6.753)^{a}	46.4 ± 1.14 (6.811)^{ab}	47.8 ± 0.44 (6.914)^{a}	47.6 ± 0.54 (6.899)^{a}	47.6 ± 0.54 (6.899)^{a}
C_1S_{13} (KB + 3.0% NA +3.0% $CaCl_2$+ 0.75% SM)	4.6 ± 0.54 (2.142)^{h}	6.4 ± 0.89 (2.525)^{i}	9.4 ± 0.54 (3.065)^{l}	12.0 ± 2.23 (3.452)^{g}	13.2 ± 3.27 (3.611)^{f}
C_1S_{14} (KB + 3.0% NA +3.5% $CaCl_2$+ 0.75% SM)	6.8 ± 0.83 (2.604)^{g}	9.2 ± 0.83 (3.031)^{h}	10.6 ± 0.54 (3.255)^{k}	12.4 ± 2.07 (3.512)^{g}	14.8 ± 3.63 (3.823)^{f}
CD (0.05)	0.111	0.198	0.069	0.220	0.345

*NA- Sodium alginate, $CaCl_2$- Calcium chloride, T_1- Mannitol + PVP + CMC + Tween-80, T_3- Mannitol + PEG + CMC + Tween-80, C_1- Control, KB- King's B broth, SM- Skim milk. Transformed values are given in parentheses. In each column figures followed by same letter do not differ significantly according to DMRT.

and T_1S_5 at 60 min of gelation time. At 75 min, the treatments T_1S_6 and T_3S_{12} as well as T_3S_{11} and T_1S_5 were statistically on par with each other as these could also maintain higher average bead weight. Minimum bead weight was observed in control beads C_1S_{13} followed by C_1S_{14} and T_1S_1 at all different time periods of gelation. It was noticed that rigid and uniform bead weight was obtained at 60 minutes of gelation in most of the treatments. Where maximum bead weight was obtained in treatment combination containing sodium alginate (3.0%) amended each with calcium chloride (3.0% and 3.5%) in T_1S_6 , T_3S_{12}, T_3S_{11} and T_1S_5 compared to other treatment combinations. However, the least bead weight was obtained in control beads and beads produced from 2.5% sodium alginate solution. Hence, for preparation of beads, the gelation time in $CaCl_2$ solution was standardised at 60 min.

4.2.3 Determination of cell leakage

Cell leakage was determined from the population of *P. fluorescens* in the remaining $CaCl_2$ solution which was enumerated by serial dilution and plating technique after recovering the alginate beads from the $CaCl_2$ solution. The data is presented in Table 4.6.

From the data, it was observed that, higher cell leakage in $CaCl_2$ solution was observed in control C_1A_{13} (9.0×10^6 cfu ml^{-1}) and C_1A_{14} (7.0×10^6 cfu ml^{-1}) in alginate beads, and C_1S_{13} (11.0×10^6 cfu ml^{-1}) and C_1S_{14} (9.0×10^6 cfu ml^{-1}) in alginate skim milk beads. These treatments were followed by beads prepared from lower concentration of sodium alginate (2.5%) and calcium chloride solution (2.5%). In treatments where alginate alone was used for preparation of beads, minimal leakage was found in treatments **T_1A_3 (2×10^6 cfu ml^{-1})** followed by **T_3A_{12} (2.33×10^6 cfu ml^{-1})**, T_1A_6 (3.0×10^6 cfu ml^{-1}) and T_1A_5 (3.33×10^6 cfu ml^{-1}). However, in treatment combination with skim milk, it was noticed that the cell leakage was lower in **T_1S_6 (1.0×10^6 cfu ml^{-1})** followed by treatments **T_1S_3 (2.33×10^6 cfu ml^{-1})**, T_1S_5, T_3S_{11} and T_3S_{12} where the population was found to be 2.0×10^6 cfu ml^{-1} where the treatments were statistically on par with each other.

Table 4.6 Population of *P. fluorescens* in $CaCl_2$ solution in various treatment combinations as a measure of cell leakage

Treatments (Alginate alone)	Population of *P. fluorescens* (x 10^6 cfu ml^{-1})	Treatments (Alginate + Skim milk)	Population of *P. fluorescens* (x 10^6 cfu ml^{-1})
T_1A_1 (T_1 +2.5% NA+ 2.5% $CaCl_2$)	5.0 ± 1 (0.693)^{bcd}	T_1S_1 (T_1 +2.5% NA+ 2.5% $CaCl_2$+ 0.75% SM)	6.33 ± 0.57 (0.800)^{bc}
T_1A_2 (T_1 + 2.5% NA +3.0% $CaCl_2$)	4.0 ± 2 (0.560)^{cde}	T_1S_2 (T_1 + 2.5% NA +3.0% $CaCl_2$+ 0.75% SM)	5.0 ± 1 (0.693)^{cd}
T_1A_3 (T_1 + 2.5% NA +3.5% $CaCl_2$)	2.0 ± 1 (0.259)^{f}	T_1S_3 (T_1 + 2.5% NA +3.5% $CaCl_2$+ 0.75% SM)	2.33 ± 0.57 (0.360)^{e}
T_1A_4 (T_1 + 3.0% NA +2.5% $CaCl_2$)	4.0 ± 1 (0.593)^{cde}	T_1S_4 (T_1 + 3.0% NA +2.5% $CaCl_2$+ 0.75% SM)	4.0 ± 1 (0.593)^{d}
T_1A_5 (T_1 + 3.0% NA +3.0% $CaCl_2$)	3.33 ± 0.57 (0.519)^{de}	T_1S_5 (T_1 + 3.0% NA +3.0% $CaCl_2$+ 0.75% SM)	2.0 ± 1 (0.259)^{e}
T_1A_6 (T_1 + 3.0% NA +3.5% $CaCl_2$)	3.0 ± 0 (0.477)^{def}	T_1S_6 (T_1 + 3.0% NA +3.5% $CaCl_2$+ 0.75% SM)	1.0 ± 0 (0.000)^{f}
T_3A_7 (T_3 + 2.5% NA +2.5% $CaCl_2$)	6.0 ± 1 (0.774)^{abc}	T_3S_7 (T_3 + 2.5% NA +2.5% $CaCl_2$+ 0.75% SM)	9.0 ± 1 (0.952)^{ab}
T_3A_8 (T_3 + 2.5% NA +3.0% $CaCl_2$)	5.0 ± 1 (0.693)^{bcd}	T_3S_8 (T_3 + 2.5% NA +3.0% $CaCl_2$+ 0.75% SM)	6.0 ± 1 (0.774)^{bcd}
T_3A_9 (T_3 + 2.5% NA +3.5% $CaCl_2$)	5.0 ± 1 (0.693)^{bcd}	T_3S_9 (T_3 + 2.5% NA +3.5% $CaCl_2$+ 0.75% SM)	6.33 ± 1.52 (0.793)^{bc}
T_3A_{10} (T_3 + 3.0% NA +2.5% $CaCl_2$)	4.66 ± 3.05 (0.602)^{cd}	T_3S_{10} (T_3 + 3.0% NA +2.5% $CaCl_2$+ 0.75% SM)	4.0 ± 1 (0.593)^{d}
T_3A_{11} (T_3 + 3.0% NA +3.0% $CaCl_2$)	4.0 ± 1 (0.593)^{cde}	T_3S_{11} (T_3 + 3.0% NA +3.0% $CaCl_2$+ 0.75% SM)	2.0 ± 0 (0.301)^{e}
T_3A_{12} (T_3 + 3.0% NA +3.5% $CaCl_2$)	2.33 ± 0.57 (0.360)^{ef}	T_3S_{12} (T_3 + 3.0% NA +3.5% $CaCl_2$+ 0.75% SM)	2.0 ± 1 (0.259)^{e}
C_1A_{13} (KB + 3.0% NA +3.0% $CaCl_2$)	9.0 ± 1 (0.952)^{a}	C_1S_{13} (KB + 3.0% NA +3.0% $CaCl_2$+ 0.75% SM)	11.0 ± 2 (1.037)^{a}
C_1A_{14} (KB + 3.0% NA +3.5% $CaCl_2$)	7.0 ± 1 (0.842)^{ab}	C_1S_{14} (KB + 3.0% NA +3.5% $CaCl_2$+ 0.75% SM)	9.0 ± 1 (0.952)^{ab}
CD (0.05)	0.237	CD (0.05)	0.194

*NA- Sodium alginate, $CaCl_2$- Calcium chloride, T_1- Mannitol + PVP + CMC + Tween-80, T_3- Mannitol + PEG + CMC + Tween-80, C_1- Control, KB- King's B broth, SM- Skim milk. Values are arranged in Mean ± SD. Log transformations are given in parentheses. In each column figures followed by same letter do not differ significantly according to DMRT.

From the data, it was observed that higher the concentration of sodium alginate and calcium chloride, lower will be the cell leakage or cell wastage, as high cross linking during bead formation leads to higher bacterial entrapment in beads and vice versa.

4.2.4 Standardization of optimum height of air column

The shape of bead is decided by the height of air column which is the distance between dripping tip and surface of $CaCl_2$ solution. Different falling distance of 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23 and 25 cm were studied to analyse the optimum falling distance for the spherical bead production of spherical beads (Table 4.7). It was observed that the minimum collecting distance of 1, 3, 5 and 7 cm between tip of the separating funnel and surface of $CaCl_2$ solution led to formation of tailed beads whereas, spherical shaped beads were formed from 9 cm onwards and shape was maintained upto 25 cm (Plate 4.3). It was also noticed that when length of the collecting distance was highest, it resulted in the splitting of alginate droplet. This led to the formation of very minute beads while the beads travelled in the air column, in addition to the formation of spherical alginate beads. Moreover, increased height of the collecting distance led to an inconvenience in handling the separating funnel during the preparation of beads. Thus, optimum height of air column maintained during bead preparation was standardised at 9 -15 cm.

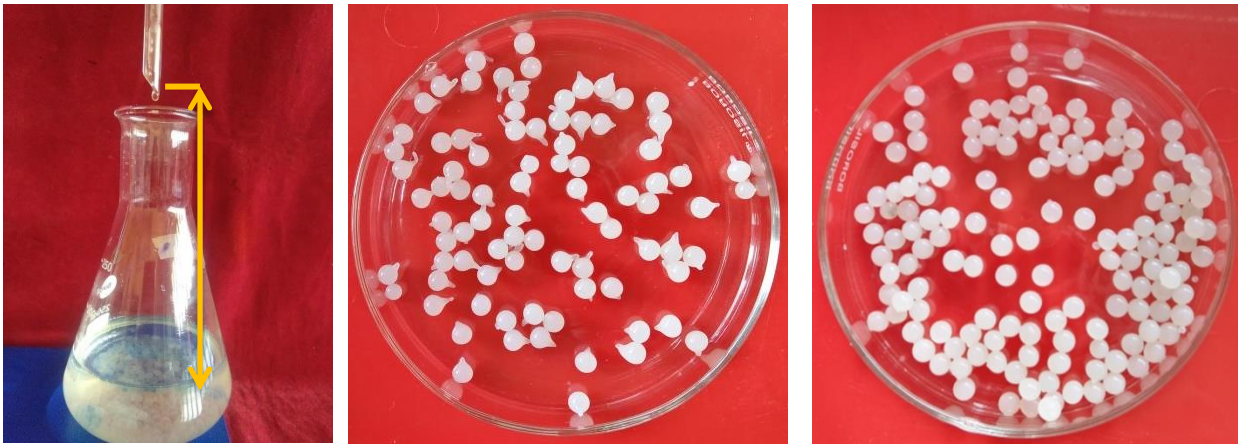
4.3 CHARACTERIZATION OF MICROENCAPSULATED BEADS

4.3.1 Bead size and shape

Diameter and shape of alginate beads were determined using a stereomicroscope. The results furnished in the Table 4.8 and 4.9 revealed that in general, mean diameter of the beads increased with increase in sodium alginate (3%) and calcium chloride concentration (3.5% and 3.0%) in both alginate and alginate amended skim milk formulations compared to other treatments. It was noticed that beads produced from alginate alone recorded maximum diameter in T_1A_6 (1.86 mm)

Table 4.7 Effect of length of air column on bead shape

Length of air column (cm)	Shape of bead
1	Tailed beads
3	Tailed beads
5	Tailed beads
7	Tailed beads
9	Spherical beads
11	Spherical beads
13	Spherical beads
15	Spherical beads
17	Spherical and minute beads
19	Spherical and minute beads
21	Spherical and minute beads
23	Spherical and minute beads
25	Spherical and minute beads



a) Length of air column b) Tailed beads c) Spherical beads

Plate 4.3 Effect of length of air column on bead shape

and T_3A_{12} (1.84 mm) which were statistically on par with each other, followed by T_1A_5 (1.73 mm) and T_3A_{11} (1.72 mm). While, maximum diameter was observed in treatments is T_3S_{12} (2.02 mm) followed by T_1S_6 (1.94 mm), T_1S_5 (1.86 mm) and T_3S_{11} (1.83 mm) in alginate + skim milk beads. It was also observed that treatment combinations containing alginate and skim milk produced beads with larger diameter compared to alginate alone. The lowest diameter was observed in control C_1A_{13} (1.11 mm) and C_1A_{14} (1.13 mm) in alginate beads, and in C_1S_{13} (1.14 mm) and C_1S_{14} (1.16 mm) in alginate skim milk beads.

As the concentration of sodium alginate solution increased, the beads became more and more rigid when compared to the beads obtained at its lower concentration. From the observation, it is therefore noted that, increased concentration of sodium alginate (3%) and calcium chloride (3.5% and 3.0%) produced uniform spherical beads compared to the lower concentration of sodium alginate and calcium chloride solution.

4.3.1.1 *Sphericity factor (SF)*

Sphericity factor is determined using a stereomicroscope which depicts the shape of a bead. For this, mean diameter of five randomly selected beads of different treatments in three directions were taken and sphericity factor was estimated and the results are presented in Table 4.9. As per the literature, beads are considered spherical if SF varies from zero for a perfect sphere to less than 0.05.

The data clearly indicates that although all the beads formed from different treatments were generally spherical (SF < 0.05), perfect spherical beads were formed from high concentration of alginate (3 %) and calcium chloride (3% and 3.5%) solution after gelation. Sphericity factor of 0.01 was noticed in alginate treatments like T_1A_6 (0.010), T_3A_{11} (0.011), T_3A_{12} (0.013) and T_1A_5 (0.017) and in alginate amended skim milk treatments like T_1S_5 (0.008), T_3S_{11} (0.013), T_1S_6 (0.015) and T_3S_{12} (0.015). From the above results, it was observed that perfect sphere near to zero (*i.e* 0.008) was obtained in alginate beads amended with skim milk which was produced from equal concentrations of sodium alginate (3%) and calcium chloride (3%) (Plate 4.4 and 4.5). It was also observed that minor deformations occurred in other treatment droplets when

Table 4.8 Mean diameter of microencapsulated beads

Treatments (Alginate alone)	Mean diameter (mm)	Treatments (Alginate + Skim milk)	Mean diameter (mm)
T_1A_1 (T_1 +2.5% NA+ 2.5% $CaCl_2$)	1.16±0.03^{g}	T_1S_1 (T_1 +2.5% NA+ 2.5% $CaCl_2$+ 0.75% SM)	1.18±0.04^{hi}
T_1A_2 (T_1 + 2.5% NA +3.0% $CaCl_2$)	1.31±0.03^{ef}	T_1S_2 (T_1 + 2.5% NA +3.0% $CaCl_2$+ 0.75% SM)	1.31±0.03^{g}
T_1A_3 (T_1 + 2.5% NA +3.5% $CaCl_2$)	1.39±0.04^{d}	T_1S_3 (T_1 + 2.5% NA +3.5% $CaCl_2$+ 0.75% SM)	1.36±0.05^{ef}
T_1A_4 (T_1 + 3.0% NA +2.5% $CaCl_2$)	1.59±0.04^{c}	T_1S_4 (T_1 + 3.0% NA +2.5% $CaCl_2$+ 0.75% SM)	1.62±0.05^{d}
T_1A_5 (T_1 + 3.0% NA +3.0% $CaCl_2$)	1.73±0.02^{b}	T_1S_5 (T_1 + 3.0% NA +3.0% $CaCl_2$+ 0.75% SM)	1.86±0.01^{c}
T_1A_6 (T_1 + 3.0% NA +3.5% $CaCl_2$)	1.86±0.01^{a}	T_1S_6 (T_1 + 3.0% NA +3.5% $CaCl_2$+ 0.75% SM)	1.94±0.02^{b}
T_3A_7 (T_3 + 2.5% NA +2.5% $CaCl_2$)	1.27±0.02^{f}	T_3S_7 (T_3 + 2.5% NA +2.5% $CaCl_2$+ 0.75% SM)	1.21±0.04^{h}
T_3A_8 (T_3 + 2.5% NA +3.0% $CaCl_2$)	1.33±0.02^{e}	T_3S_8 (T_3 + 2.5% NA +3.0% $CaCl_2$+ 0.75% SM)	1.32±0.01f^{g}
T_3A_9 (T_3 + 2.5% NA +3.5% $CaCl_2$)	1.39±0.03^{d}	T_3S_9 (T_3 + 2.5% NA +3.5% $CaCl_2$+ 0.75% SM)	1.41±0.05^{e}
T_3A_{10} (T_3 + 3.0% NA +2.5% $CaCl_2$)	1.60±0.03^{c}	T_3S_{10} (T_3 + 3.0% NA +2.5% $CaCl_2$+ 0.75% SM)	1.61±0.01^{d}
T_3A_{11} (T_3 + 3.0% NA +3.0% $CaCl_2$)	1.72±0.01^{b}	T_3S_{11} (T_3 + 3.0% NA +3.0% $CaCl_2$+ 0.75% SM)	1.83±0.02^{c}
T_3A_{12} (T_3 + 3.0% NA +3.5% $CaCl_2$)	1.84±0.01^{a}	T_3S_{12} (T_3 + 3.0% NA +3.5% $CaCl_2$+ 0.75% SM)	2.02±0.05^{a}
C_1A_{13} (KB + 3.0% NA +3.0% $CaCl_2$)	1.11±0.02^{h}	C_1S_{13} (KB + 3.0% NA +3.0% $CaCl_2$+ 0.75% SM)	1.14±0.03^{i}
C_1A_{14} (KB + 3.0% NA +3.5% $CaCl_2$)	1.13±0.02^{gh}	C_1S_{14} (KB + 3.0% NA +3.5% $CaCl_2$+ 0.75% SM)	1.16±0.03^{i}
CD (0.05)	0.038	CD (0.05)	0.049

*NA- Sodium alginate, $CaCl_2$- Calcium chloride, T_1- Mannitol + PVP + CMC + Tween-80, T_3- Mannitol + PEG + CMC + Tween-80, C_1- Control, KB- King's B broth, SM- Skim milk.

* Mean of 5 replications

Values are presented in mean ± SD. In each column figures followed by same letter do not differ significantly according to DMRT

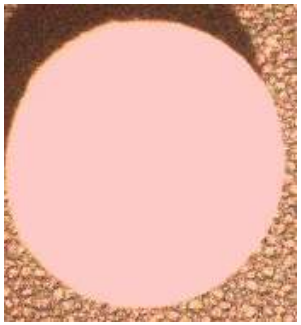
Table 4.9 Sphericity factor of microencapsulated beads

Treatments (Alginate alone)	Sphericity factor	Treatments (Alginate + Skim milk)	Sphericity factor
T_1A_1 (T_1 +2.5% NA+ 2.5% $CaCl_2$)	0.03	T_1S_1 (T_1 +2.5% NA+ 2.5% $CaCl_2$+ 0.75% SM)	0.046
T_1A_2 (T_1 + 2.5% NA +3.0% $CaCl_2$)	0.034	T_1S_2 (T_1 + 2.5% NA +3.0% $CaCl_2$+ 0.75% SM)	0.037
T_1A_3 (T_1 + 2.5% NA +3.5% $CaCl_2$)	0.04	T_1S_3 (T_1 + 2.5% NA +3.5% $CaCl_2$+ 0.75% SM)	0.044
T_1A_4 (T_1 + 3.0% NA +2.5% $CaCl_2$)	0.028	T_1S_4 (T_1 + 3.0% NA +2.5% $CaCl_2$+ 0.75% SM)	0.037
T_1A_5 (T_1 + 3.0% NA +3.0% $CaCl_2$)	0.017	T_1S_5 (T_1 + 3.0% NA +3.0% $CaCl_2$+ 0.75% SM)	0.008
T_1A_6 (T_1 + 3.0% NA +3.5% $CaCl_2$)	0.010	T_1S_6 (T_1 + 3.0% NA +3.5% $CaCl_2$+ 0.75% SM)	0.015
T_3A_7 (T_3 + 2.5% NA +2.5% $CaCl_2$)	0.023	T_3S_7 (T_3 + 2.5% NA +2.5% $CaCl_2$+ 0.75% SM)	0.046
T_3A_8 (T_3 + 2.5% NA +3.0% $CaCl_2$)	0.022	T_3S_8 (T_3 + 2.5% NA +3.0% $CaCl_2$+ 0.75% SM)	0.015
T_3A_9 (T_3 + 2.5% NA +3.5% $CaCl_2$)	0.028	T_3S_9 (T_3 + 2.5% NA +3.5% $CaCl_2$+ 0.75% SM)	0.042
T_3A_{10} (T_3 + 3.0% NA +2.5% $CaCl_2$)	0.021	T_3S_{10} (T_3 + 3.0% NA +2.5% $CaCl_2$+ 0.75% SM)	0.012
T_3A_{11} (T_3 + 3.0% NA +3.0% $CaCl_2$)	0.011	T_3S_{11} (T_3 + 3.0% NA +3.0% $CaCl_2$+ 0.75% SM)	0.013
T_3A_{12} (T_3 + 3.0% NA +3.5% $CaCl_2$)	0.013	T_3S_{12} (T_3 + 3.0% NA +3.5% $CaCl_2$+ 0.75% SM)	0.015
C_1A_{13} (KB + 3.0% NA +3.0% $CaCl_2$)	0.031	C_1S_{13} (KB + 3.0% NA +3.0% $CaCl_2$+ 0.75% SM)	0.035
C_1A_{14} (KB + 3.0% NA +3.5% $CaCl_2$)	0.030	C_1S_{14} (KB + 3.0% NA +3.5% $CaCl_2$+ 0.75% SM)	0.043

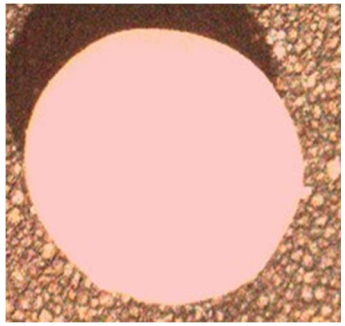
*NA- Sodium alginate, $CaCl_2$- Calcium chloride, T_1- Mannitol + PVP + CMC + Tween-80, T_3- Mannitol + PEG + CMC + Tween-80, C_1- Control, KB- King's B broth, SM- Skim milk.

* Mean of 5 replications

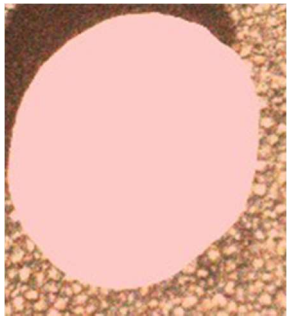
T_1A_1



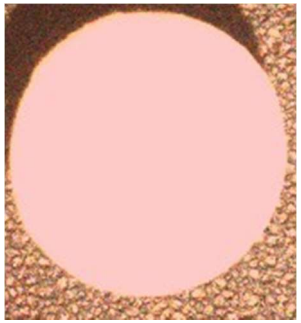
T_1A_2



T_1A_3

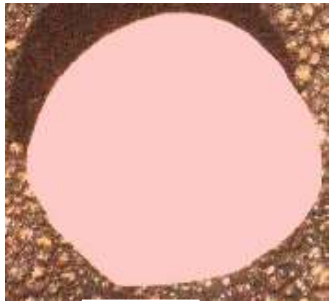


T_1A_4

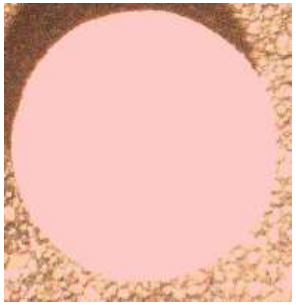


T_1A_5

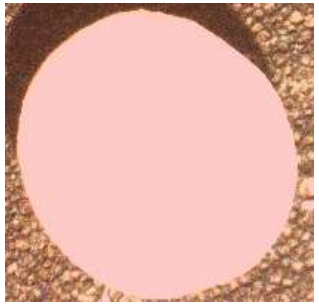
T_1A_6



T_3A_7

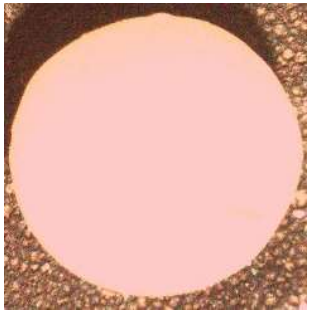


T_3A_8



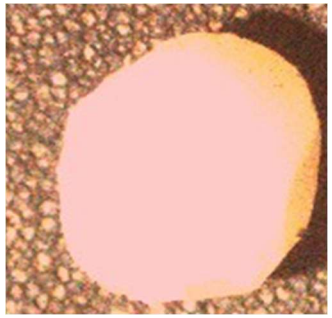
T_3A_9

T_3A_{10}

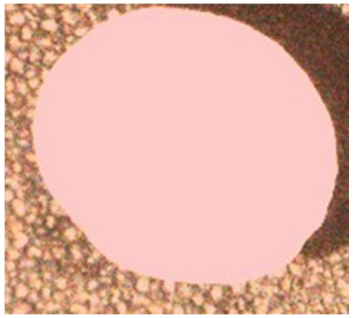


T_3A_{11}

T_3A_{12}



C_1A_{13}



C_1A_{14}

Plate 4.4 Shape of alginate beads at 100X magnification under stereomicroscope

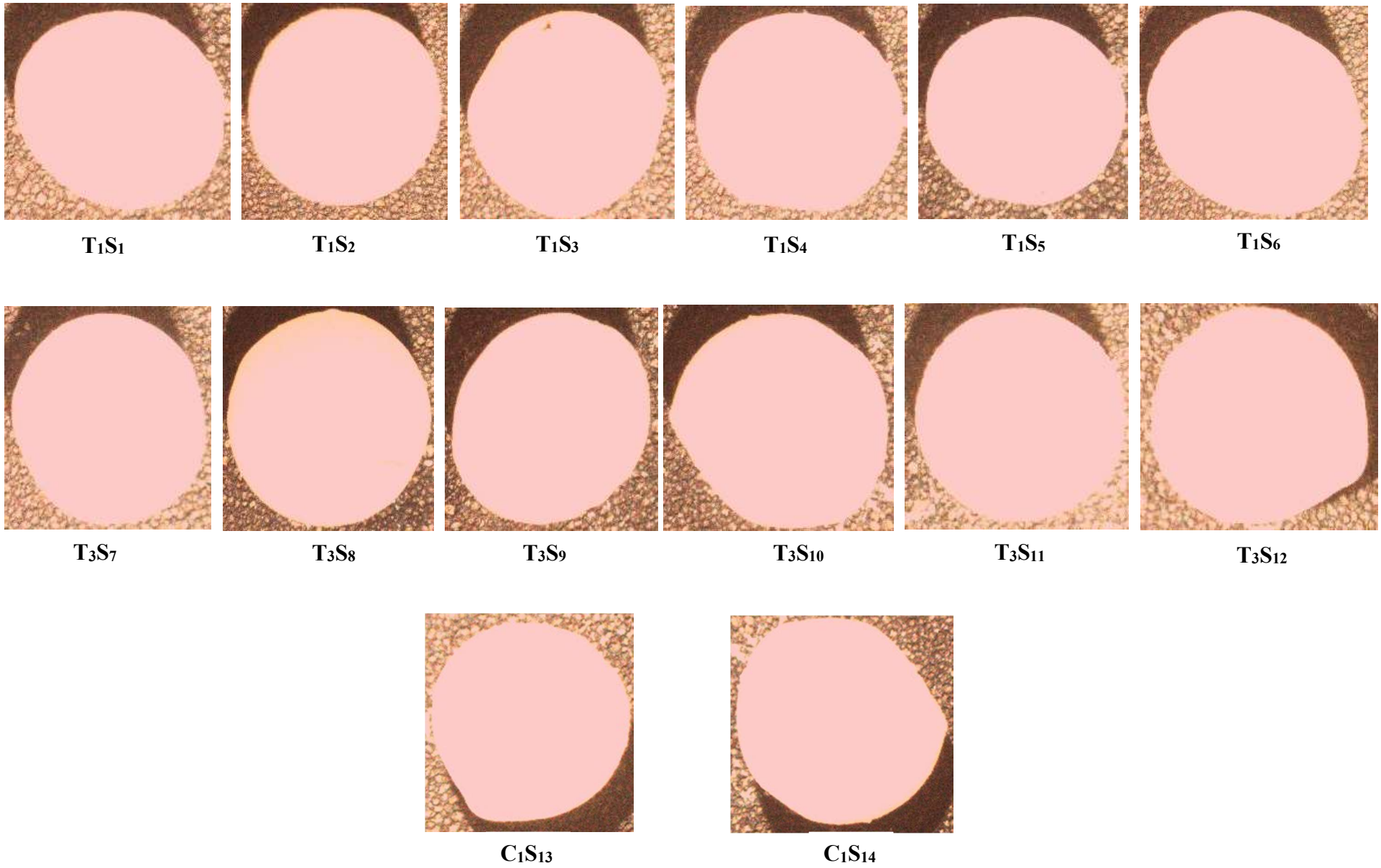


Plate 4.5 Shape of alginate + skim milk beads at 100X magnification under stereomicroscope

impacted on the gelation bath surface. As a result, beads produced from such treatments were not perfectly spherical in shape after gelation.

4.3.2 Bead weight and number of beads per gram of formulation

For estimation of bead weight, average weight of 10 randomly selected beads (mg) were determined and the results are presented in Table 4.10. Results revealed that in general, the bead weight was noticed more in beads prepared from alginate + skim milk combinations compared to beads of alginate alone. The maximum bead weight was noticed in alginate amended skim milk treatments T_1S_6 (48.7 mg) followed by T_3S_{12} (47.7 mg), T_3S_{11} (47 mg) and T_1S_5 (46 mg). whereas in alginate beads treatment T_1A_6 (20.3 mg) produced the maximum bead weight followed by T_3A_{12} (19.6 mg), T_3A_{11} (18.5 mg) and T_1A_5 (16.6 mg). The minimum weight was observed in control beads in both the treatment formulations with the least in C_1A_{13} (4.3 mg) and C_1S_{13} (9.5 mg). It can be inferred that higher bead weight was obtained when the beads are produced from the combination of 3 per cent sodium alginate with 3 and 3.5 per cent calcium chloride solution respectively in both the formulations of alginate alone and alginate-skim milk combinations. From the results, it was observed that bead weight is directly proportional to the concentration of sodium alginate and calcium chloride used for the preparation.

Number of beads per gram varied among different treatment combinations and the results are tabulated in Table 4.11. Observations recorded revealed that increased concentration of sodium alginate and calcium chloride resulted in reduced bead number per gram of beads compared to other treatment combinations. In general, it was noticed that maximum number of beads were obtained in case of beads prepared from 2.5 per cent each of sodium alginate alone and calcium chloride solution. However, an exception was noticed where beads prepared in control treatment with alginate alone produced 232 (C_1A_{13}) and 168 (C_1A_{14}) beads per gram. The highest number of beads per gram in additive amended alginate (alone) beads treatments was noticed in T_1A_1

Table 4.10 Weight of the microencapsulated beads as influenced by the concentration of calcium chloride and sodium alginate

Treatments (Alginate alone)	Mean weight (mg)	Treatments (Alginate + Skim milk)	Mean weight (mg)
T_1A_1 (T_1 +2.5% NA+ 2.5% $CaCl_2$)	8.9± 0.73^{g}	T_1S_1 (T_1 +2.5% NA+ 2.5% $CaCl_2$+ 0.75% SM)	20.05 ± 0.43^{i}
T_1A_2 (T_1 + 2.5% NA +3.0% $CaCl_2$)	14.2 ± 0.78^{e}	T_1S_2 (T_1 + 2.5% NA +3.0% $CaCl_2$+ 0.75% SM)	25.9 ± 1.19^{h}
T_1A_3 (T_1 + 2.5% NA +3.5% $CaCl_2$)	19.0 ± 0.66^{bc}	T_1S_3 (T_1 + 2.5% NA +3.5% $CaCl_2$+ 0.75% SM)	30.0 ± 0.57^{e}
T_1A_4 (T_1 + 3.0% NA +2.5% $CaCl_2$)	9.6 ± 0.51^{g}	T_1S_4 (T_1 + 3.0% NA +2.5% $CaCl_2$+ 0.75% SM)	44.6 ± 2.01^{d}
T_1A_5 (T_1 + 3.0% NA +3.0% $CaCl_2$)	16.6 ± 3.40^{d}	T_1S_5 (T_1 + 3.0% NA +3.0% $CaCl_2$+ 0.75% SM)	46.0 ± 0.88^{c}
T_1A_6 (T_1 + 3.0% NA +3.5% $CaCl_2$)	20.3 ± 0.48^{a}	T_1S_6 (T_1 + 3.0% NA +3.5% $CaCl_2$+ 0.75% SM)	48.7 ± 0.58^{a}
T_3A_7 (T_3 + 2.5% NA +2.5% $CaCl_2$)	8.9 ±0.87^{g}	T_3S_7 (T_3 + 2.5% NA +2.5% $CaCl_2$+ 0.75% SM)	19.0 ± 0.57^{j}
T_3A_8 (T_3 + 2.5% NA +3.0% $CaCl_2$)	11.5 ± 0.52^{f}	T_3S_8 (T_3 + 2.5% NA +3.0% $CaCl_2$+ 0.75% SM)	27.1 ± 0.99^{g}
T_3A_9 (T_3 + 2.5% NA +3.5% $CaCl_2$)	15.8 ± 0.78^{d}	T_3S_9 (T_3 + 2.5% NA +3.5% $CaCl_2$+ 0.75% SM)	29.0 ± 1.15^{f}
T_3A_{10} (T_3 + 3.0% NA +2.5% $CaCl_2$)	10.9 ± 0.73^{f}	T_3S_{10} (T_3 + 3.0% NA +2.5% $CaCl_2$+ 0.75% SM)	44.8 ± 0.78^{d}
T_3A_{11} (T_3 + 3.0% NA +3.0% $CaCl_2$)	18.5 ± 0.52^{c}	T_3S_{11} (T_3 + 3.0% NA +3.0% $CaCl_2$+ 0.75% SM)	47.0 ± 0.81^{b}
T_3A_{12} (T_3 + 3.0% NA +3.5% $CaCl_2$)	19.6 ± 0.51^{ab}	T_3S_{12} (T_3 + 3.0% NA +3.5% $CaCl_2$+ 0.75% SM)	47.7 ± 0.82^{b}
C_1A_{13} (KB + 3.0% NA +3.0% $CaCl_2$)	4.3 ± 0.67^{i}	C_1S_{13} (KB + 3.0% NA +3.0% $CaCl_2$+ 0.75% SM)	9.5 ± 0.97^{l}
C_1A_{14} (KB + 3.0% NA +3.5% $CaCl_2$)	5.9 ± 0.87^{h}	C_1S_{14} (KB + 3.0% NA +3.5% $CaCl_2$+ 0.75% SM)	10.5 ± 0.52^{k}
CD (0.05)	0.985	CD (0.05)	0.844

*NA- Sodium alginate, $CaCl_2$- Calcium chloride, T_1- Mannitol + PVP + CMC + Tween-80, T_3- Mannitol + PEG + CMC + Tween-80, C_1- Control, KB- King's B broth, SM- Skim milk.

Values are presented in Mean ± SD of 10 replications. In each column figures followed by same letter do not differ significantly according to DMRT.

Table 4.11 Number of beads per gram in different treatment combinations

Treatments (Alginate alone)	Number/gram of bead	Treatments (Alginate + Skim milk)	Number/gram of bead
T_1A_1 (T_1 +2.5% NA+ 2.5% $CaCl_2$)	112^{c}	T_1S_1 (T_1 +2.5% NA+ 2.5% $CaCl_2$+ 0.75% SM)	48^{d}
T_1A_2 (T_1 + 2.5% NA +3.0% $CaCl_2$)	71^{g}	T_1S_2 (T_1 + 2.5% NA +3.0% $CaCl_2$+ 0.75% SM)	39^{e}
T_1A_3 (T_1 + 2.5% NA +3.5% $CaCl_2$)	52^{k}	T_1S_3 (T_1 + 2.5% NA +3.5% $CaCl_2$+ 0.75% SM)	34^{g}
T_1A_4 (T_1 + 3.0% NA +2.5% $CaCl_2$)	104^{d}	T_1S_4 (T_1 + 3.0% NA +2.5% $CaCl_2$+ 0.75% SM)	23^{h}
T_1A_5 (T_1 + 3.0% NA +3.0% $CaCl_2$)	60^{i}	T_1S_5 (T_1 + 3.0% NA +3.0% $CaCl_2$+ 0.75% SM)	22^{hi}
T_1A_6 (T_1 + 3.0% NA +3.5% $CaCl_2$)	49^{l}	T_1S_6 (T_1 + 3.0% NA +3.5% $CaCl_2$+ 0.75% SM)	20^{i}
T_3A_7 (T_3 + 2.5% NA +2.5% $CaCl_2$)	112^{c}	T_3S_7 (T_3 + 2.5% NA +2.5% $CaCl_2$+ 0.75% SM)	53^{c}
T_3A_8 (T_3 + 2.5% NA +3.0% $CaCl_2$)	86^{f}	T_3S_8 (T_3 + 2.5% NA +3.0% $CaCl_2$+ 0.75% SM)	37^{ef}
T_3A_9 (T_3 + 2.5% NA +3.5% $CaCl_2$)	63^{h}	T_3S_9 (T_3 + 2.5% NA +3.5% $CaCl_2$+ 0.75% SM)	34^{fg}
T_3A_{10} (T_3 + 3.0% NA +2.5% $CaCl_2$)	93^{e}	T_3S_{10} (T_3 + 3.0% NA +2.5% $CaCl_2$+ 0.75% SM)	23^{h}
T_3A_{11} (T_3 + 3.0% NA +3.0% $CaCl_2$)	55^{j}	T_3S_{11} (T_3 + 3.0% NA +3.0% $CaCl_2$+ 0.75% SM)	20^{i}
T_3A_{12} (T_3 + 3.0% NA +3.5% $CaCl_2$)	50^{kl}	T_3S_{12} (T_3 + 3.0% NA +3.5% $CaCl_2$+ 0.75% SM)	21^{hi}
C_1A_{13} (KB + 3.0% NA +3.0% $CaCl_2$)	232^{a}	C_1S_{13} (KB + 3.0% NA +3.0% $CaCl_2$+ 0.75% SM)	106^{a}
C_1A_{14} (KB + 3.0% NA +3.5% $CaCl_2$)	168^{b}	C_1S_{14} (KB + 3.0% NA +3.5% $CaCl_2$+ 0.75% SM)	95^{b}
CD (0.05)	2.048	CD (0.05)	2.862

*NA- Sodium alginate, $CaCl_2$- Calcium chloride, T_1- Mannitol + PVP + CMC + Tween-80, T_3- Mannitol + PEG + CMC + Tween-80, C_1- Control, KB- King's B broth, SM- Skim milk.

Values are presented in Mean of 3 replications. In each column figures followed by same letter do not differ significantly according to DMRT.

and T_3A_7 with 112 beads followed by T_1A_4 (104 beads) and T_3A_{10} (93 beads). The least number of beads of 49 was noticed with T_1A_6.

In treatment combination of alginate and skim milk, the number of beads per gram was comparatively less with the maximum number noticed in T_1S_1 (48) followed by T_1S_2 (39) and T_3S_8 (37) and the least number of 20 in T_1S_6 and T_3S_{11} respectively. Thus, it can be concluded that when the bead weight was reduced, control produced highest number of beads compared to treatments in both the additive amended formulations.

4.3.4 Bead yield

Bead yield was estimated from the final weight of beads after drying with respect to the initial total weight of components used for bead preparation. The results are depicted in the Table 4.12. It was noticed that in general, bead yield of more than 60 per cent was obtained in both alginate and alginate + skim milk bead formulations prepared from 3.0 per cent sodium alginate and 3.0 per cent and 3.5 per cent calcium chloride respectively. Higher yield of 74.62 per cent was obtained in treatment T_3A_{12} followed by 73.20 per cent (T_1A_6), 68.49 per cent (T_1A_5) and 68.38 per cent (T_3A_{11}) in alginate bead formulations. While, in alginate + skim milk bead formulation higher bead yield of 74.13 per cent was noticed with T_1S_6 followed by 72.24 per cent (T_3S_{12}), 69.90 per cent (T_1S_5) and 69.01 per cent (T_3S_{11}). Lower yield of production of beads was obtained in control treatment in both the additive amended formulations.

From the above estimated parameters, it was observed that lower concentration of sodium alginate (2.5 %) leads to the formation of irregular spherical beads of uneven size and with fluctuating bead weight compared to 3.0 per cent sodium alginate concentration. Hence, sodium alginate concentration of (2.5%) was eliminated and beads prepared from concentration of 3.0 per cent sodium alginate and calcium chloride (2.5%, 3.0%, 3.5%) were used for further studies in both alginate and skim milk amended alginate formulations.

Table 4.12 Yield of production of beads in various treatments

Treatments (Alginate alone)	Yield (%)	Treatments (Alginate + skim milk)	Yield (%)
T_1A_1 (T_1 +2.5% NA+ 2.5% $CaCl_2$)	38.35	T_1S_1 (T_1 +2.5% NA+ 2.5% $CaCl_2$+ 0.75% SM)	41.17
T_1A_2 (T_1 + 2.5% NA +3.0% $CaCl_2$)	42.05	T_1S_2 (T_1 + 2.5% NA +3.0% $CaCl_2$+ 0.75% SM)	46.34
T_1A_3 (T_1 + 2.5% NA +3.5% $CaCl_2$)	47.05	T_1S_3 (T_1 + 2.5% NA +3.5% $CaCl_2$+ 0.75% SM)	52.73
T_1A_4 (T_1 + 3.0% NA +2.5% $CaCl_2$)	62.87	T_1S_4 (T_1 + 3.0% NA +2.5% $CaCl_2$+ 0.75% SM)	64.24
T_1A_5 (T_1 + 3.0% NA +3.0% $CaCl_2$)	68.49	T_1S_5 (T_1 + 3.0% NA +3.0% $CaCl_2$+ 0.75% SM)	69.90
T_1A_6 (T_1 + 3.0% NA +3.5% $CaCl_2$)	73.20	T_1S_6 (T_1 + 3.0% NA +3.5% $CaCl_2$+ 0.75% SM)	74.13
T_3A_7 (T_3 + 2.5% NA +2.5% $CaCl_2$)	37.34	T_3S_7 (T_3 + 2.5% NA +2.5% $CaCl_2$+ 0.75% SM)	41.63
T_3A_8 (T_3 + 2.5% NA +3.0% $CaCl_2$)	43.17	T_3S_8 (T_3 + 2.5% NA +3.0% $CaCl_2$+ 0.75% SM)	45.30
T_3A_9 (T_3 + 2.5% NA +3.5% $CaCl_2$)	47.72	T_3S_9 (T_3 + 2.5% NA +3.5% $CaCl_2$+ 0.75% SM)	52.49
T_3A_{10} (T_3 + 3.0% NA +2.5% $CaCl_2$)	62.92	T_3S_{10} (T_3 + 3.0% NA +2.5% $CaCl_2$+ 0.75% SM)	62.40
T_3A_{11} (T_3 + 3.0% NA +3.0% $CaCl_2$)	68.38	T_3S_{11} (T_3 + 3.0% NA +3.0% $CaCl_2$+ 0.75% SM)	69.01
T_3A_{12} (T_3 + 3.0% NA +3.5% $CaCl_2$)	74.62	T_3S_{12} (T_3 + 3.0% NA +3.5% $CaCl_2$+ 0.75% SM)	72.24
C_1A_{13} (KB + 3.0% NA +3.0% $CaCl_2$)	31.17	C_1S_{13} (KB + 3.0% NA +3.0% $CaCl_2$+ 0.75% SM)	35.87
C_1A_{14} (KB + 3.0% NA +3.5% $CaCl_2$)	34.51	C_1S_{14} (KB + 3.0% NA +3.5% $CaCl_2$+ 0.75% SM)	38.91

*NA- Sodium alginate, $CaCl_2$- Calcium chloride, T_1- Mannitol + PVP + CMC + Tween-80, T_3- Mannitol + PEG + CMC + Tween-80, C_1- Control, KB- King's B broth, SM- Skim milk

4.3.5 Swelling ratio of alginate beads

Swelling percentage of beads in phosphate buffer and sterile water medium was estimated for different treatment combinations and were studied by measuring the percentage uptake of water by the beads. A dynamic weight change of beads was noticed in phosphate buffer medium compared to sterile water (Table 4.13 and Table 4.14). From the data, it was observed that swelling ratio of beads was higher in phosphate buffer compared to sterile water in alginate and skim milk amended alginate beads. However, control beads showed higher water uptake in phosphate buffer as well as in sterile water medium in both alginate and alginate skim milk formulations. Moreover, a reduced swelling ratio was observed in general, in treatment combinations prepared from higher concentrations of cross linking agent (3% and 3.5%).

Decreased swelling ratio of beads in alginate bead formulations in phosphate buffer solution was observed in treatments *viz.*, T_3A_6 (17.36%), T_3A_5 (21.91%), T_1A_3 (22.42%) which are statistically on par with each other followed by the treatment T_1A_2 (27.68%). While, in alginate supplemented with skim milk beads, reduced swelling percentage was observed in treatments T_3S_6 (8.39%), T_1S_3 (8.64%), T_3S_5 (11.63%) and T_1S_2 (14.01%). Neverthless, a decreased swelling ratio in sterile water in alginate bead formulation was noticed in the treatment T_3A_6 (9.20%) which is statistically on par with T_1A_3 (12.26%) followed by the treatment T_1A_2 (19.36%) and T_3A_4 (21.27%). While, treatments T_3S_6 (5.04%), T_1S_3 (5.33%), T_3S_5 (8.17%) and T_1S_2 (8.76%) showed a reduced swelling ratio in sterile water in alginate supplemented with skim milk formulations.

4.3.6 Water content in beads

The mean water loss from beads was estimated to determine the percentage of water withheld by the beads. From the Table 4.15 shown, it was noticed that the percentage water content was more in alginate (alone) treatments compared to alginate + skim milk combinations. The results revealed that percentage water content was

Table 4.13 Swelling ratio of microencapsulated beads in 0.2 M phosphate buffer medium

Treatments (Alginate alone)	Swelling ratio (%)	Treatments (Alginate + Skim milk)	Swelling ratio (%)
T_1A_1 (T_1 + 3.0% NA + 2.5% $CaCl_2$)	40.66 ± 10.04^{c}	T_1S_1 (T_1 + 3.0% NA + 2.5% $CaCl_2$+ 0.75% SM)	20.25 ± 2.69^{c}
T_1A_2 (T_1 + 3.0% NA + 3.0% $CaCl_2$)	27.68 ± 2.74^{de}	T_1S_2 (T_1 + 3.0% NA + 3.0% $CaCl_2$+ 0.75% SM)	14.01 ± 2.38^{d}
T_1A_3 (T_1 + 3.0% NA + 3.5% $CaCl_2$)	22.42 ± 2.39^{e}	T_1S_3 (T_1 + 3.0% NA + 3.5% $CaCl_2$+ 0.75% SM)	8.64 ± 1.74^{d}
T_3A_4 (T_3 + 3.0% NA + 2.5% $CaCl_2$)	36.36 ± 6.64^{cd}	T_3S_4 (T_3 + 3.0% NA + 2.5% $CaCl_2$+ 0.75% SM)	19.82 ± 1.98^{c}
T_3A_5 (T_3 + 3.0% NA + 3.0% $CaCl_2$)	21.91 ± 2.62^{e}	T_3S_5 (T_3 + 3.0% NA + 3.0% $CaCl_2$+ 0.75% SM)	11.63 ± 1.16^{d}
T_3A_6 (T_3 + 3.0% NA + 3.5% $CaCl_2$)	17.36 ± 2.92^{e}	T_3S_6 (T_3 + 3.0% NA + 3.5% $CaCl_2$+ 0.75% SM)	8.39 ± 1.42^{d}
C_1A_7 (KB + 3.0% NA + 3.0% $CaCl_2$)	77.00 ± 17.88^{a}	C_1S_7 (KB + 3.0% NA + 3.0% $CaCl_2$+ 0.75% SM)	55.33 ± 8.62^{a}
C_1A_8 (KB + 3.0% NA + 3.5% $CaCl_2$)	64.75 ± 12.52^{b}	C_1S_8 (KB + 3.0% NA + 3.5% $CaCl_2$+ 0.75% SM)	26.36± 7.44^{b}
CD (0.05)	11.620	CD (0.05)	5.634

*NA- sodium alginate, $CaCl_2$- calcium chloride, T_1- mannitol + PVP + CMC + tween-80, T_3- mannitol + PEG + CMC + tween-80, C_1- control, KB- King's B broth, SM- skim milk.

Values are arranged in Mean ± SD of five replications. In each column figures followed by same letter do not differ significantly according to DMRT with 5% significance level.

Table 4.14 Swelling ratio of microencapsulated beads in sterile water

Treatments (Alginate alone)	Swelling ratio (%)	Treatments (Alginate + Skim milk)	Swelling ratio (%)
T_1A_1 (T_1 + 3.0% NA + 2.5% $CaCl_2$)	27.55 ± 5.05^{c}	T_1S_1 (T_1 + 3.0% NA + 2.5% $CaCl_2$+ 0.75% SM)	16.67 ± 2.09^{c}
T_1A_2 (T_1 + 3.0% NA + 3.0% $CaCl_2$)	19.36 ± 3.27^{cd}	T_1S_2 (T_1 + 3.0% NA + 3.0% $CaCl_2$+ 0.75% SM)	8.76 ± 1.53^{d}
T_1A_3 (T_1 + 3.0% NA + 3.5% $CaCl_2$)	12.26 ± 2.87^{d}	T_1S_3 (T_1 + 3.0% NA + 3.5% $CaCl_2$+ 0.75% SM)	5.33 ± 1.14^{d}
T_3A_4 (T_3 + 3.0% NA + 2.5% $CaCl_2$)	21.27 ± 8.12^{cd}	T_3S_4 (T_3 + 3.0% NA + 2.5% $CaCl_2$+ 0.75% SM)	14.43 ± 2.69^{c}
T_3A_5 (T_3 + 3.0% NA + 3.0% $CaCl_2$)	19.61 ± 11.8^{cd}	T_3S_5 (T_3 + 3.0% NA + 3.0% $CaCl_2$+ 0.75% SM)	8.17 ± 2.77^{d}
T_3A_6 (T_3 + 3.0% NA + 3.5% $CaCl_2$)	9.20 ± 4.40^{d}	T_3S_6 (T_3 + 3.0% NA + 3.5% $CaCl_2$+ 0.75% SM)	5.04 ± 1.16^{d}
C_1A_7 (KB + 3.0% NA + 3.0% $CaCl_2$)	63.00 ± 13.9^{a}	C_1S_7 (KB + 3.0% NA + 3.0% $CaCl_2$+ 0.75% SM)	36.22 ± 5.85^{a}
C_1A_8 (KB + 3.0% NA + 3.5% $CaCl_2$)	47.71 ± 16.4^{b}	C_1S_8 (KB + 3.0% NA + 3.5% $CaCl_2$+ 0.75% SM)	22.54 ± 4.37^{b}
CD (0.05)	12.375	CD (0.05)	4.019

*NA- sodium alginate, $CaCl_2$- calcium chloride, T_1- mannitol + PVP + CMC + tween-80, T_3- mannitol + PEG + CMC + tween-80, C_1- control, KB- King's B broth, SM- skim milk.

Values are arranged in Mean ± SD of five replications. In each column figures followed by same letter do not differ significantly according to DMRT with 5% significance level.

highest in alginate (alone) treatment T_1A_3 (65.29%) followed by T_3A_6 (61.96%), T_1A_2 (54.50%) and T_3A_5 (48.20%). In alginate + skim milk combinations, the treatments T_3S_6 (44.18 %) and T_1S_3 (39.21%) showed higher water content followed by treatments T_1S_2 (21.27%) and T_3S_5 (21.50%) which are statistically on par with each other. Minimum water content was observed in control treatments in both alginate (C_1A_7 and C_1A_8) and alginate + skim milk beads (C_1S_7 and C_1S_8). It was also noticed that higher concentration of sodium alginate (3%) and calcium chloride (3.0 and 3.5%) during bead preparation enabled the beads to hold more water content which in turn enabled survival of bacteria for longer period inside the beads compared to other treatments.

4.3.7 Shrinkage percentage of beads

The percentage of shrinkage of alginate liquid drops after gelation was calculated and is presented in Table 4.16. The results showed that shrinkage percentage was influenced by the concentration of cross linking agent. In this case, control beads in both formulations showed higher shrinkage compared to other treatments. It was also observed that degree of shrinkage was higher for the beads produced from low alginate concentration (2.5%). The lowest shrinkage percentage was recorded in treatment T_1A_3 (114.41%) which is statistically on par with T_3A_6 (115.78%), T_3A_5 (120.54%) and T_1A_2 (120.42%) in alginate bead formulation. Whereas, in alginate + skim milk beads, the treatment T_1S_3 (114.74%) recorded lowest shrinkage percentage which is statistically on par with T_3S_6 (115.23%), T_3S_5 (118.01%) and T_1S_2 (117.65%). From the result, we can also infer that reduced shrinkage percentage was observed in beads produced from equal concentrations of sodium alginate (3%) and calcium chloride (3%) in both alginate and alginate + skim milk beads.

Table 4.15 Percentage of water content in microencapsulated beads

Treatments (Alginate alone)	Water content (%)	Treatments (Alginate + Skim milk)	Water content (%)
T_1A_1 (T_1 + 3.0% NA + 2.5% $CaCl_2$)	45.35 ± 1.07^{e}	T_1S_1 (T_1 + 3.0% NA + 2.5% $CaCl_2$+ 0.75% SM)	20.87 ± 0.95^{cd}
T_1A_2 (T_1 + 3.0% NA + 3.0% $CaCl_2$)	54.50 ± 1.73^{c}	T_1S_2 (T_1 + 3.0% NA + 3.0% $CaCl_2$+ 0.75% SM)	21.27 ± 0.69^{c}
T_1A_3 (T_1 + 3.0% NA + 3.5% $CaCl_2$)	65.29 ± 1.22^{a}	T_1S_3 (T_1 + 3.0% NA + 3.5% $CaCl_2$+ 0.75% SM)	39.21 ± 0.33^{b}
T_3A_4 (T_3 + 3.0% NA + 2.5% $CaCl_2$)	46.55 ± 0.99^{de}	T_3S_4 (T_3 + 3.0% NA + 2.5% $CaCl_2$+ 0.75% SM)	19.90 ± 0.12^{d}
T_3A_5 (T_3 + 3.0% NA + 3.0% $CaCl_2$)	48.20 ± 1.10^{d}	T_3S_5 (T_3 + 3.0% NA + 3.0% $CaCl_2$+ 0.75% SM)	21.50 ± 0.48^{c}
T_3A_6 (T_3 + 3.0% NA + 3.5% $CaCl_2$)	61.96 ± 0.88^{b}	T_3S_6 (T_3 + 3.0% NA + 3.5% $CaCl_2$+ 0.75% SM)	44.18 ± 0.46^{a}
C_1A_7 (KB + 3.0% NA + 3.0% $CaCl_2$)	37.78 ± 0.98^{f}	C_1S_7 (KB + 3.0% NA + 3.0% $CaCl_2$+ 0.75% SM)	18.41 ± 0.66^{e}
C_1A_8 (KB + 3.0% NA + 3.5% $CaCl_2$)	38.24 ± 0.51^{f}	C_1S_8 (KB + 3.0% NA + 3.5% $CaCl_2$+ 0.75% SM)	14.01 ± 0.82^{f}
CD (0.05)	1.925	CD (0.05)	1.081

*NA- sodium alginate, $CaCl_2$- calcium chloride, T_1- mannitol + PVP + CMC + tween-80, T_3- mannitol + PEG + CMC + tween-80, C_1- control, KB- King's B broth, SM- skim milk.

Values are arranged in Mean ± SD of three replications. In each column figures followed by same letter do not differ significantly according to DMRT with 5% significance level.

Table 4.16 Shrinkage percentage of microencapsulated beads

Treatments (Alginate alone)	Shrinkage (%)	Treatments (Alginate + Skim milk)	Shrinkage (%)
T_1A_1 (T_1 + 3.0% NA + 2.5% $CaCl_2$)	127.56 ± 3.49^{c}	T_1S_1 (T_1 + 3.0% NA + 2.5% $CaCl_2$+ 0.75% SM)	125.94 ± 2.21^{c}
T_1A_2 (T_1 + 3.0% NA + 3.0% $CaCl_2$)	120.42 ± 1.43^{d}	T_1S_2 (T_1 + 3.0% NA + 3.0% $CaCl_2$+ 0.75% SM)	117.65 ± 0.85^{d}
T_1A_3 (T_1 + 3.0% NA + 3.5% $CaCl_2$)	114.41 ± 1.32^{e}	T_1S_3 (T_1 + 3.0% NA + 3.5% $CaCl_2$+ 0.75% SM)	114.74 ± 0.68^{e}
T_3A_4 (T_3 + 3.0% NA + 2.5% $CaCl_2$)	126.02 ± 2.47^{c}	T_3S_4 (T_3 + 3.0% NA + 2.5% $CaCl_2$+ 0.75% SM)	127.10 ± 0.79^{c}
T_3A_5 (T_3 + 3.0% NA + 3.0% $CaCl_2$)	120.54 ± 1.58^{d}	T_3S_5 (T_3 + 3.0% NA + 3.0% $CaCl_2$+ 0.75% SM)	118.01 ± 0.46^{d}
T_3A_6 (T_3 + 3.0% NA + 3.5% $CaCl_2$)	115.78 ± 0.48^{de}	T_3S_6 (T_3 + 3.0% NA + 3.5% $CaCl_2$+ 0.75% SM)	115.23 ± 1.50^{de}
C_1A_7 (KB + 3.0% NA + 3.0% $CaCl_2$)	168.20 ± 6.84^{a}	C_1S_7 (KB + 3.0% NA + 3.0% $CaCl_2$+ 0.75% SM)	182.12 ± 4.61^{a}
C_1A_8 (KB + 3.0% NA + 3.5% $CaCl_2$)	163.31 ± 6.43^{b}	C_1S_8 (KB + 3.0% NA + 3.5% $CaCl_2$+ 0.75% SM)	172.23 ± 2.61^{b}
CD (0.05)	4.844	CD (0.05)	2.783

*NA- sodium alginate, $CaCl_2$- calcium chloride, T_1- mannitol + PVP + CMC + tween-80, T_3- mannitol + PEG + CMC + tween-80, C_1- control, KB- King's B broth, SM- skim milk.

Values are arranged in Mean ± SD of five replications. In each column figures followed by same letter do not differ significantly according to DMRT with 5% significance level.

4.4 EVALUATION OF SHELF LIFE OF *P. fluorescens* ENCAPSULATED BEADS UNDER *IN VITRO* CONDITIONS

4.4.1 Rate of release of bioagent from beads

The rate of release of bacteria from alginate and alginate + skim milk beads were studied under *in vitro* conditions in two media *viz*., 0.2 M phosphate buffer (pH 7.0) and sterile water. Rate of release of bacteria was analysed by dilution plate count technique at 30 min, 1 h, 2 h and 24 h and the results are presented in Table 4.17 and 4.18. It was noticed that in general, higher bacterial release from beads was observed in phosphate buffer compared to sterile water. Moreover, two fold increase in bacterial release in both phosphate buffer and sterile water was noticed at 24 h compared to initial time (30 min) in both the additive amended formulation.

It was found that the rate of release of bacteria in alginate and alginate + skim milk beads were significantly higher with increasing period of time. However, the release profile of beads prepared from higher concentrations of sodium alginate (3%) and $CaCl_2$ (3% and 3.5%) depicted a slower rate and lower extent of bacterial release compared to other treatments. From the Table 4.17, in alginate bead treatments, after 30 min of dissolution studies, higher bacterial release form the beads was observed in treatment C_1A_7 (8.66 x 10^8 cfu g^{-1}) followed by T_1A_1 (7.66 x 10^8 cfu g^{-1}), C_1A_8 and T_3A_4 (6.66 x 10^8 cfu g^{-1}) which were statistically on par with each other in phosphate buffer medium while, lower bacterial release was observed in treatments T_1A_3 (3.0 x 10^8 cfu g^{-1}) and T_3A_6 (3.33 x 10^8 cfu g^{-1}). Whereas, in sterile water, higher release of bioagents from the beads was observed in treatments C_1A_7 (6 x 10^8 cfu g^{-1}) followed by T_1A_1 (5.0 x 10^8 cfu g^{-1}) and lower bacterial release in T_3A_6 (1.66 x 10^8 cfu g^{-1}) and T_1A_3 (2.0 x 10^8 cfu g^{-1}) after 30 min of dissolution.

After 24 h of dissolution studies, increased bacterial release from alginate beads was observed in T_1A_1 (18.33 x 10^{16} cfu g^{-1}) and C_1A_8 (16.66 x 10^{16} cfu g^{-1}) which were statistically on par with each other. Whereas, lower bacterial release was observed in T_1A_3 (2.0 x 10^{16} cfu g^{-1}) and T_1A_6 (2.66 x 10^{16} cfu g^{-1}) in phosphate buffer medium

Table 4.17 Release of bioagent from alginate beads in phosphate buffer and sterile water at different time intervals under *in vitro*

Treatments	Population in phosphate buffer				Population in sterile water			
	30 min	1 h	2 h	24 h	30 min	1 h	2 h	24 h
	X 10^8 cfu g^{-1}	X 10^8 cfu g^{-1}	X 10^8 cfu g^{-1}	X 10^{16}cfu g^{-1}	X 10^8 cfu g^{-1}	X 10^8 cfu g^{-1}	X 10^8 cfu g^{-1}	X 10^{16} cfu g^{-1}
T_1A_1 (T_1 + 3.0% NA + 2.5% $CaCl_2$)	7.66 ± 0.57 (0.884)^{a}	16.33 ± 2.51 (1.210)^{a}	22.0 ± 1 (1.342)^{b}	18.33 ± 5.50 (1.248)^{a}	5.0 ± 1 (0.693)^{ab}	9.0 ± 1 (0.952)^{a}	8.33 ± 1.15 (0.968)^{a}	4.33 ± 0.57 (0.725)^{a}
T_1A_2 (T_1 + 3.0% NA + 3.0% $CaCl_2$)	6.66 ± 1.52 (0.816)^{ab}	13.33 ± 1.52 (1.123)^{b}	18.33 ± 1.15 (1.263)^{cd}	3.33 ± 1.15 (0.502)^{d}	3.66 ± 0.57 (0.560)^{bc}	6.66 ± 0.57 (0.823)^{b}	6.66 ± 2.08 (0.874)^{a}	2.66 ± 0.57 (0.560)^{bc}
T_1A_3 (T_1 + 3.0% NA + 3.5% $CaCl_2$)	3.0 ± 1 (0.460)^{d}	9.33 ± 0.57 (0.969)^{cd}	16.66 ± 2.08 (1.220)^{d}	2.0 ± 1 (0.259)^{e}	2.0 ± 0 (0.301)^{de}	4.66 ± 0.57 (0.667)^{c}	2.0 ± 1 (0.460)^{bc}	1.0 ± 0 (0.301)^{ef}
T_3A_4 (T_3 + 3.0% NA + 2.5% $CaCl_2$)	6.66±1.15 (0.820)^{a}	14.0 ± 1 (1.145)^{ab}	17.33 ± 1.52 (1.238)^{d}	9.0 ± 1 (0.952)^{bc}	4.0 ± 1 (0.593)^{b}	7.33 ± 0.57 (0.864)^{b}	7.33 ± 1.52 (0.916)^{a}	3.0 ± 1 (0.593)^{abc}
T_3A_5 (T_3 + 3.0% NA + 3.0% $CaCl_2$)	4.66 ± 0.57 (0.667)^{bc}	11.0 ± 1 (1.040)^{c}	13.0 ± 1 (1.113)^{e}	6.33 ± 0.57 (0.800)^{c}	2.66 ± 0.57 (0.418)^{cd}	5.33 ± 0.57 (0.725)^{c}	3.0 ± 1 (0.593)^{b}	2.0 ± 0 (0.477)^{cd}
T_3A_6 (T_3 + 3.0% NA + 3.5% $CaCl_2$)	3.33 ± 0.57 (0.519)^{cd}	8.33 ± 1.15 (0.918)^{d}	11.0 ± 1 (1.040)^{f}	2.66 ± 0.57 (0.418)^{de}	1.66 ± 0.57 (0.201)^{e}	3.33 ± 0.57 (0.519)^{d}	1.0 ± 1 (0.259)^{c}	0.66 ± 0.57 (0.201)^{f}
C_1A_7 (KB + 3.0% NA + 3.0% $CaCl_2$)	8.66 ± 1.52 (0.933)^{a}	16.66 ± 1.15 (1.221)^{a}	25.33 ± 1.52 (1.403)^{a}	13.0 ± 2 (1.110)^{ab}	6.0 ± 1 (0.774)^{a}	11.0 ± 1 (1.040)^{a}	10.33 ± 1.52 (1.052)^{a}	3.66 ± 0.57 (0.667)^{ab}
C_1A_8 (KB + 3.0% NA + 3.5% $CaCl_2$)	6.66 ± 1.15 (0.820)^{a}	13.33 ± 1.15 (1.124)^{b}	20.33 ± 1.15 (1.308)^{bc}	16.66 ± 1.52 (1.221)^{a}	3.66 ± 0.57 (0.560)^{bc}	7.0 ± 1 (0.842)^{b}	7.66 ± 0.57 (0.937)^{a}	1.33 ± 0.57 (0.360)^{de}
CD(0.05)	0.150	0.078	0.059	0.221	0.169	0.088	0.212	0.154

*NA- sodium alginate, $CaCl_2$- calcium chloride, T_1- mannitol + PVP + CMC + tween-80, T_3- mannitol + PEG + CMC + tween-80, C_1- control, KB- King's B broth, SM- skim milk.

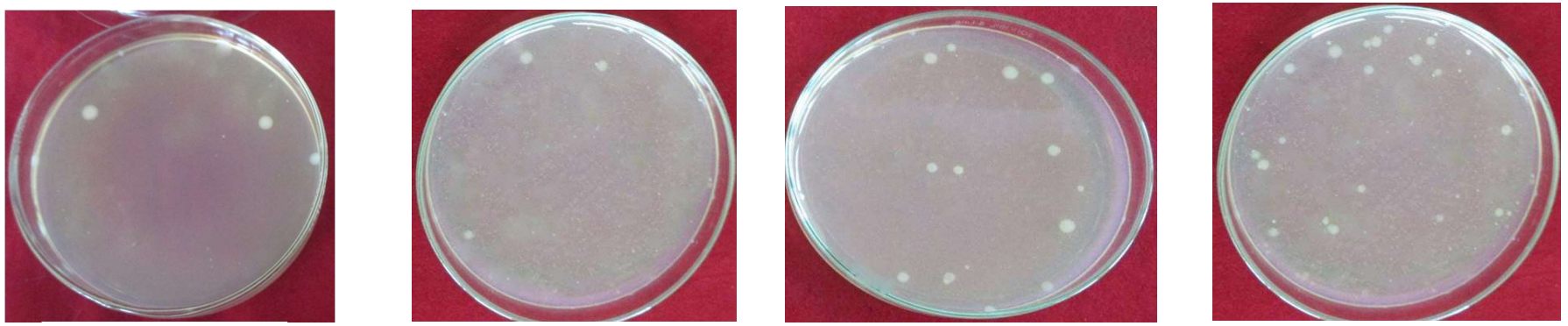
Values are arranged in Mean ± SD. Log transformations are given in parentheses. In each column figures followed by same letter do not differ significantly according to DMRT.

Table 4.18 Release of bioagent from alginate + skim milk beads in phosphate buffer and sterile water at different time intervals under *in vitro*

Treatments	Population in phosphate buffer				Population in sterile water			
	30 min	1 h	2 h	24 h	30 min	1 h	2 h	24 h
	X 10^{8} cfu g^{-1}	X 10^{8} cfu g^{-1}	X 10^{16} cfu g^{-1}	X 10^{32} cfu g^{-1}	X 10^{8} cfu g^{-1}	X 10^{8} cfu g^{-1}	X 10^{10} cfu g^{-1}	X 10^{24} cfu g^{-1}
T_1S_1 (T_1 + 3.0% NA + 2.5% $CaCl_2$+ 0.75% SM)	16.66 ± 0.57 (1.222)^{c}	24.66 ± 1.52 (1.392)^{ab}	16.33 ± 1.52 (1.212)^{bc}	11.66 ± 0.57 (1.067)^{e}	13.33 ± 0.57 (1.125)^{bc}	17.66 ± 1.52 (1.246)^{ab}	11.33 ± 1.52 (1.052)^{ab}	28.33 ± 0.57 (1.452)^{a}
T_1S_2 (T_1 + 3.0% NA + 3.0% $CaCl_2$+ 0.75% SM)	14.0 ± 1 (1.145)^{de}	22.33 ± 1.52 (1.348)^{b}	12.33 ± 1.15 (1.090)^{cd}	31.66 ± 0.57 (1.501)^{c}	10.66 ± 0.57 (1.028)^{d}	14.66 ± 1.52 (1.165)^{cd}	8.66 ± 1.52 (0.933)^{b}	21.66 ± 1.15 (1.335)^{cd}
T_1S_3 (T_1 + 3.0% NA + 3.5% $CaCl_2$+ 0.75% SM)	11.66 ± 0.57 (1.067)^{f}	18.0 ± 1 (1.255)^{c}	6.66 ± 2.08 (0.810)^{e}	41.66 ± 1.15 (1.620)^{a}	5.66 ± 0.57 (0.752)^{e}	13.33 ± 1.52 (1.123)^{d}	5.0 ± 2 (0.674)^{cd}	13.33 ± 1.52 (1.123)^{f}
T_3S_4 (T_3 + 3.0% NA + 2.5% $CaCl_2$+ 0.75% SM)	17.33 ± 0.57 (1.239)^{c}	22.33 ± 1.52 (1.348)^{b}	13.66 ± 1.52 (1.134)^{bc}	12.33 ± 0.57 (1.091)^{e}	14.0 ± 1 (1.145)^{b}	17.0 ± 1 (1.230)^{b}	10.66 ± 1.52 (1.025)^{ab}	25.66 ± 0.57 (1.409)^{b}
T_3S_5 (T_3 + 3.0% NA + 3.0% $CaCl_2$+ 0.75% SM)	14.66 ± 0.57 (1.166)^{d}	18.0 ± 1 (1.255)^{c}	8.33 ± 0.57 (0.920)^{de}	35.0 ± 1 (1.544)^{b}	11.33 ± 0.57 (1.054)^{cd}	14.66 ± 1.15 (1.165)^{cd}	6.0 ± 1.73 (0.767)^{c}	20.0 ± 1 (1.301)^{d}
T_3S_6 (T_3 + 3.0% NA + 3.5% $CaCl_2$+ 0.75% SM)	13.33 ± 0.57 (1.125)^{e}	11.0 ± 1 (1.040)^{d}	2.0 ± 1 (0.259)^{f}	44.66 ± 2.51 (1.650)^{a}	5.0 ± 1 (0.693)^{e}	10.33 ± 0.57 (1.014)^{e}	3.33 ± 0.57 (0.519)^{d}	16.33 ± 0.57 (1.213)^{e}
C_1S_7 (KB + 3.0% NA + 3.0% $CaCl_2$+ 0.75% SM)	28.0 ± 1 (1.447)^{a}	26.66 ± 0.57 (1.426)^{a}	25.33 ± 1.52 (1.403)^{a}	15.33 ± 0.57 (1.185)^{d}	19.66 ± 1.52 (1.293)^{a}	20.33 ± 1.52 (1.307)^{a}	13.66 ± 2.08 (1.132)^{a}	23.66 ± 1.52 (1.374)^{bc}
C_1S_8 (KB + 3.0% NA + 3.5% $CaCl_2$+ 0.75% SM)	24.66 ± 0.57 (1.392)^{b}	22.66 ± 1.52 (1.355)^{b}	20.33 ± 1.15 (1.308)^{ab}	12.0 ± 1 (1.078)^{e}	18.0 ± 1 (1.255)^{a}	16.0 ± 1 (1.204)^{bc}	12.33 ± 1.15 (1.090)^{ab}	20.33 ± 0.57 (1.308)^{d}
CD(0.05)	0.033	0.048	0.177	0.036	0.072	0.062	0.165	0.042

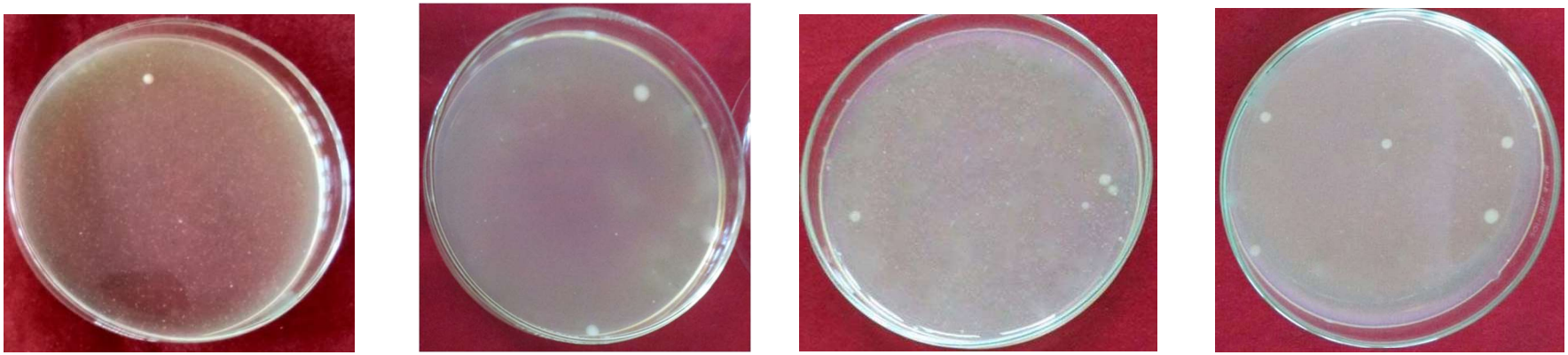
*NA- sodium alginate, $CaCl_2$- calcium chloride, T_1- mannitol + PVP + CMC + tween-80, T_3- mannitol + PEG + CMC + tween-80, C_1- control, KB- King's B broth, SM- skim milk.

Values are arranged in Mean ± SD. Log transformations are given in parentheses. In each column figures followed by same letter do not differ significantly according to DMRT.



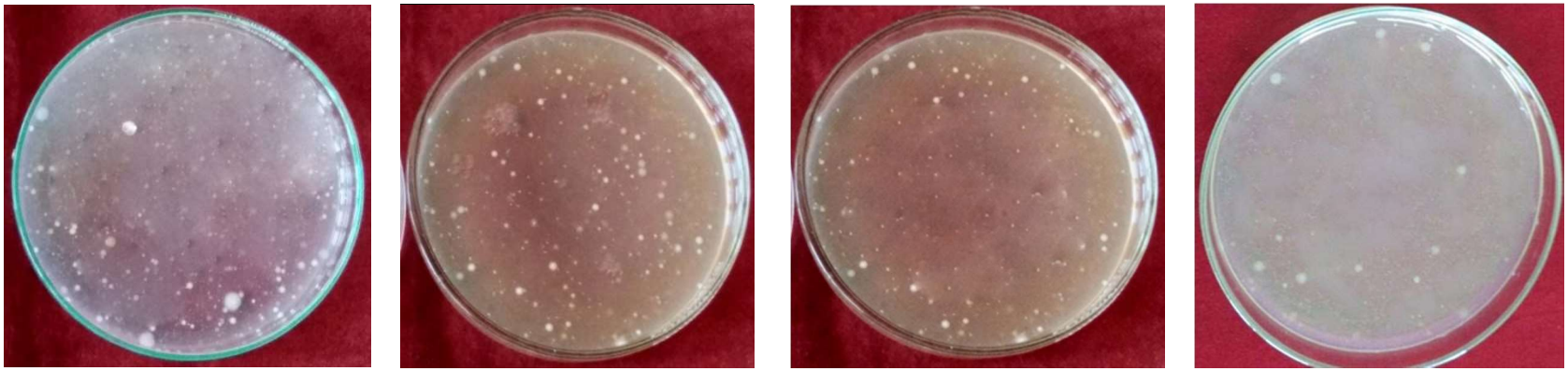
T_1A_3 T_3A_6 C_1A_7 C_1A_8

Plate 4.6a Release of *P. fluorescens* from alginate beads in 0.2 M phosphate buffer medium after 24 h of dissolution under *in vitro*



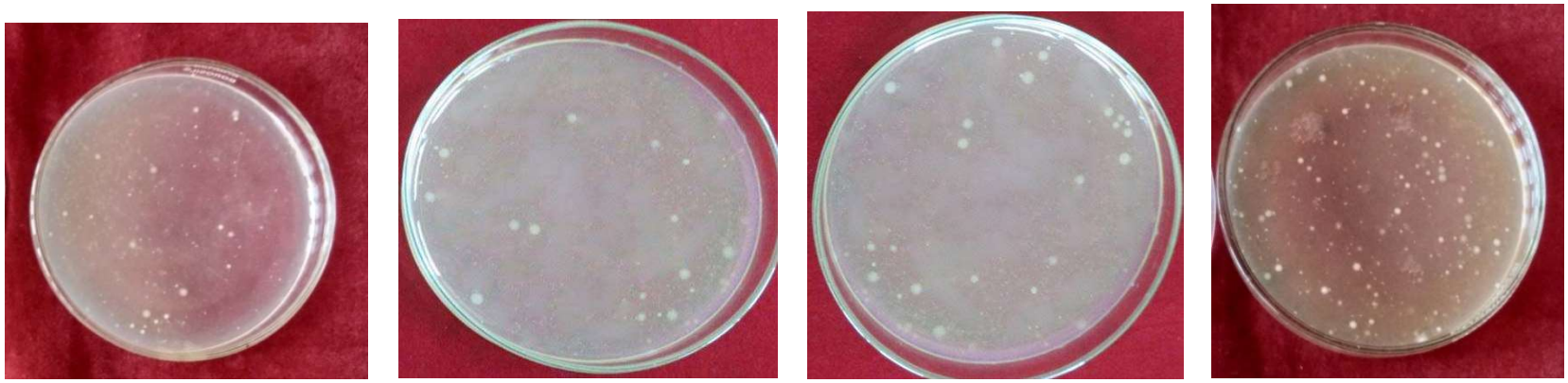
T_1A_3 T_3A_6 C_1A_7 C_1A_8

Plate 4.6b Release of *P. fluorescens* from alginate beads in sterile water after 24 h of dissolution under *in vitro*



T_1S_3 T_3S_6 C_1S_7 C_1S_8

Plate 4.7a Release of *P. fluorescens* from alginate + skim milk beads in 0.2 M phosphate buffer medium after 24 h of dissolution under *in vitro*



T_1S_3 T_3S_6 C_1S_7 C_1S_8

Plate 4.7b Release of *P. fluorescens* from alginate + skim milk beads in sterile water after 24 h of dissolution under *in vitro*

which were statistically on par with each other after 24 h followed by treatments T_1A_2 (3.33 x 10^{16} cfu g^{-1}) and T_3A_5 (6.33 x 10^{16} cfu g^{-1}). However, rate of release of bioagent from sterile water was lower compared to phosphate buffer medium. However, treatments T_1A_1 (4.33 x 10^{16} cfu g^{-1}) followed by C_1A_7 (3.66 x 10^{16} cfu g^{-1}) showed a higher release while, T_3A_6 (0.66 x 10^{16} cfu g^{-1}) and T_1A_3 (1.0 x 10^{16} cfu g^{-1}) was noticed with a lower release of bacteria in sterile water at the end of 24 h of dissolution studies (Plate 4.6a and Plate 4.6b).

Though, a slow and constant release of bacteria was observed in alginate beads, however, in alginate + skim milk formulations the beads completely dissolved in 0.2 M of phosphate buffer medium compared to sterile water at the end of 24 h. As a result, rate of bacterial release from alginate + skim milk beads was found higher in buffer medium than sterile water. Results are presented in Table 4.18. In alginate + skim milk beads, after 30 min of dissolution studies, bacterial release from the beads was higher in control beads C_1S_7 (28 x 10^8 cfu g^{-1}) followed by C_1S_8 (24.66 x 10^8 cfu g^{-1}) and also from beads produced from sodium alginate (2.5% and 3.0%) and calcium chloride (2.5%) solution as noticed in treatments T_3S_4 (17.33 x 10^8 cfu g^{-1}) and T_1S_1 (16.66 x 10^8 cfu g^{-1}) which were statistically on par with each other. However, lower bacterial release was noticed in skim milk beads produced from sodium alginate (3%) and calcium chloride (3.5% and 3.0%) in treatments T_1S_3 (11.66 x 10^8 cfu g^{-1}) followed by T_3S_6 (13.33 x 10^8 cfu g^{-1}), T_1S_2 (14.0 x 10^8 cfu g^{-1}) and T_3S_5 (14.66 x 10^8 cfu g^{-1}) in phosphate buffer medium whereas, in sterile water after 30 min of dissolution studies, higher bacterial release was observed from beads in control treatments C_1S_7 (19.66 x 10^8 cfu g^{-1}) and C_1S_8 (18 x 10^8 cfu g^{-1}) which are statistically on par with each other. A reduction in bacterial release was noticed in beads produced from sodium alginate (3%) and calcium chloride (3.5% and 3.0%) in treatments T_3S_6 (5.0 x 10^8 cfu g^{-1}) and T_1S_3 (5.66 x 10^8 cfu g^{-1}) followed by T_1S_2 (10.66 x 10^8 cfu g^{-1}) and T_3S_5 (11.33 x 10^8 cfu g^{-1}).

Likewise, bacterial release was found higher in buffer medium compared to sterile water after 24 h. At the end of 24 h of dissolution studies, higher bacterial entrapment was observed in beads produced from sodium alginate (3%) and calcium

chloride (3.5% and 3.0%) in treatments T_1S_3 (41.66 x 10^{32} cfu g^{-1}) and T_3S_6 (44.66 x 10^{32} cfu g^{-1}) in phosphate buffer medium which were statistically on par with each other followed by T_3S_5 (35.0 x 10^{32} cfu/g) and T_1S_2 (31.66 x 10^{32} cfu/g) while reduced bacterial entrapment was observed in beads produced from 2.5 per cent calcium chloride solution and also in control beads (Plate 4.7a). After 24 h of dissolution studies in sterile water, alginate + skim milk beads not dissolved completely in sterile water compared to the phosphate buffer. As the result, higher bacterial release was observed in treatment beads prepared from 2.5 per cent calcium chloride solution followed by control beads in treatments T_1S_1 (28.33 x 10^{24} cfu g^{-1}) followed by T_3S_4 (25.66 x 10^{24} cfu g^{-1}) and C_1S_7 (23.66 x 10^{24} cfu g^{-1}).Whereas, beads prepared from 3.0 per cent sodium alginate and calcium chloride (3.5% and 3.0 %) showed lower release of bacteria in sterile water at the end of 24 hours of dissolution studies in treatments T_1S_3 (13.33 x 10^{24} cfu g^{-1}) followed by T_3S_6 (16.33 x 10^{24} cfu g^{-1}), T_3S_5 (20.0 x 10^{24} cfu g^{-1}) and T_1S_2 (21.66 x 10^{24} cfu g^{-1}) (Plate 4.7b).

4.4.2 Population density of *P. fluorescens* per bead

To estimate population density, a single bead was immersed in 1 ml of 0.2 M phosphate buffer (pH 7.0) and was incubated for 24 - 48 h at 100 rpm for better dissolution of beads. It was observed that the formulation of alginate amended with skim milk dissolved better in buffer solution at 24 h, whereas the formulation of beads with alginate alone took nearly 48 h to dissolve completely in phosphate buffer medium. From the Table 4.19 and Plate 4.10**,** results revealed that higher bacterial entrapment of alginate and alginate + skim milk beads were observed in beads prepared from sodium alginate (3%) and calcium chloride (3.5% and 3.0%) solution. Treatments T_1A_3 (17.66 x 10^8 cfu/bead) and T_3A_6 (17.0 x 10^8 cfu/ bead) which were statistically on par with each other were noticed with higher bacterial release followed by treatments T_1A_2 (12.0 x 10^8 cfu/bead) and T_3A_5 (12.0 x 10^8 cfu/ bead) at one month after production of alginate beads (1MAP) (Plate 4.8). While, in alginate + skim milk bead formulation, higher bacterial release was observed in treatments T_1S_6 (23.33 x 10^8 cfu/bead) and T_1S_3 (23.0 x 10^8 cfu/ bead) followed by treatments T_3S_5 (18.33 x 10^8 cfu/bead) and T_1S_2 (18 x 10^8 cfu/ bead) at one month after production (MAP) of beads. Lower bacterial entrapment

Table 4.19 Population of *P.fluorescens* in single bead in microencapsulated formulation

Treatments (alginate beads)	Population of bioagent /bead		Treatments (alginate + skim milk beads)	Population of bioagent /bead	
	0 MAP	1 MAP		0 MAP	1 MAP
	X 10^{12} cfu $bead^{-1}$	X 10^{8} cfu $bead^{-1}$		X 10^{12} cfu $bead^{-1}$	X 10^{8} cfu $bead^{-1}$
T_1A_1 (T_1 + 3.0% NA + 2.5% $CaCl_2$)	6.0 ± 1 (0.774)^{d}	11.33 ± 2.08 (1.049)^{b}	T_1S_1 (T_1 + 3.0% NA + 2.5% $CaCl_2$+ 0.75% SM)	11.66 ± 0.57 (1.067)^{c}	15.33 ± 0.57 (1.185)^{c}
T_1A_2 (T_1 + 3.0% NA + 3.0% $CaCl_2$)	8.33 ± 0.57 (0.920)^{b}	12.0 ± 1 (1.078)^{b}	T_1S_2 (T_1 + 3.0% NA + 3.0% $CaCl_2$+ 0.75% SM)	14.66 ± 0.57 (1.166)^{b}	18.0 ± 1 (1.255)^{b}
T_1A_3 (T_1 + 3.0% NA + 3.5% $CaCl_2$)	12.0 ± 1 (1.078)^{a}	17.66 ± 1.52 (1.246)^{a}	T_1S_3 (T_1 + 3.0% NA + 3.5% $CaCl_2$+ 0.75% SM)	18.66 ± 1.52 (1.270)^{a}	23.0 ± 1 (1.361)^{a}
T_3A_4 (T_3 + 3.0% NA + 2.5% $CaCl_2$)	7.33 ± 1.52 (0.859)^{bc}	11.66 ± 1.52 (1.064)^{b}	T_3S_4 (T_3 + 3.0% NA + 2.5% $CaCl_2$+ 0.75% SM)	12.66 ± 0.57 (1.102)^{c}	12.66 ± 0.57 (1.102)^{d}
T_3A_5 (T_3 + 3.0% NA + 3.0% $CaCl_2$)	8.66 ± 0.57 (0.937)^{b}	12.0 ± 1 (1.078)^{b}	T_3S_5 (T_3 + 3.0% NA + 3.0% $CaCl_2$+ 0.75% SM)	15.33 ± 0.57 (1.185)^{b}	18.33 ± 1.15 (1.263)^{b}
T_3A_6 (T_3 + 3.0% NA + 3.5% $CaCl_2$)	12.33 ± 0.57 (1.091)^{a}	17.0 ± 2 (1.228)^{a}	T_3S_6 (T_3 + 3.0% NA + 3.5% $CaCl_2$+ 0.75% SM)	18.33 ± 0.57 (1.263)^{a}	23.33 ± 0.57 (1.368)^{a}
C_1A_7 (KB + 3.0% NA + 3.0% $CaCl_2$)	6.33 ± 0.57 (0.800)^{cd}	4.33 ± 0.57 (0.634)^{c}	C_1S_7 (KB + 3.0% NA + 3.0% $CaCl_2$+ 0.75% SM)	9.66 ± 1.15 (0.983)^{d}	7.66 ± 0.57 (0.884)^{f}
C_1A_8 (KB + 3.0% NA + 3.5% $CaCl_2$)	7.33 ± 0.57 (0.864)^{bc}	6.66 ± 0.57 (0.823)^{d}	C_1S_8 (KB + 3.0% NA + 3.5% $CaCl_2$+ 0.75% SM)	12.0 ± 1 (1.078)^{c}	10.66 ± 0.57 (1.028)^{e}
CD(0.05)	0.085	0.090	CD(0.05)	0.050	0.040

*NA- sodium alginate, $CaCl_2$- calcium chloride, T_1- mannitol + PVP + CMC + tween-80, T_3- mannitol + PEG + CMC + tween-80, C_1- control, KB-King's B broth, SM- skim milk. MAP – Month after preparation. Values are arranged in Mean ± SD. Log transformations are given in parentheses. In each column figures followed by same letter do not differ significantly according to DMRT

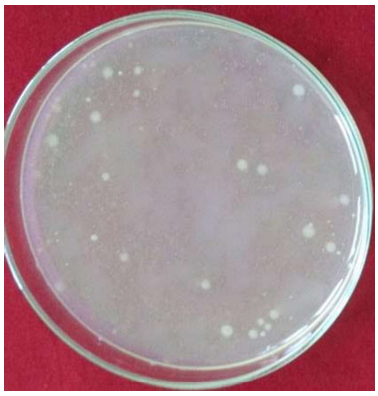
was observed in beads prepared from 2.5 per cent calcium chloride and control beads in both alginate and alginate + skim milk formulations.

4.4.3 Survival of bacteria entrapped in beads at monthly intervals

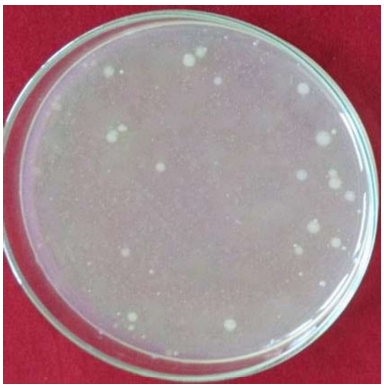
The survival of bacteria entrapped in alginate beads and alginate + skim milk formulations was studied at monthly intervals upto four months by using serial dilution and plating technique. The results are presented in Table 4.20.

In general, the initial population of bioagent were significantly higher in alginate beads when compared to the population of bacteria after fourth month of bead production (MAP). The initial population of *P. fluorescens* was higher in treatment T_3A_{12} (56.33 x 10^{32} cfu g^{-1}) followed by treatments T_1A_6 (56.0 x 10^{32} cfu g^{-1}) and T_3A_{11} (55.0 x 10^{32} cfu g^{-1}) which were statistically on par with each other followed by T_1A_5 (53.0 x 10^{32} cfu g^{-1}). Gradually, the population declined after fourth month of bead production where, higher bacterial population was noticed in treatment T_3A_{12} (10.33 x 10^{20} cfu g^{-1}) followed by treatments T_3A_{11} (8.33 x 10^{20} cfu g^{-1}), T_1A_6 (6.33 x 10^{20} cfu g^{-1}) and T_1A_5 (5.0 x 10^{20} cfu g^{-1}) respectively. The least population after 4 MAP was noticed with T_1A_1 (0.66 x 10^{20} cfu g^{-1}) closely followed by T_1A_4 (1.33 x 10^{20} cfu g^{-1}), T_1A_2 (2.0 x 10^{20} cfu g^{-1}) and also with the control treatment C_1A_{13} (2.0 x 10^{20} cfu g^{-1}) which were on par with each other (Plate 4.9a and Plate 4.9b).

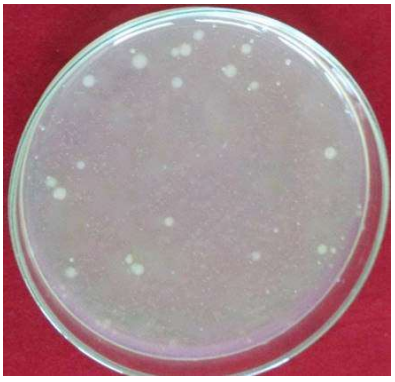
Likewise, the initial population of bioagent from alginate amended skim milk beads were estimated and it was noticed that the population was significantly higher in treatments T_1S_6 (55.33 x 10^{32} cfu g^{-1}), T_3S_{12} (55.0 x 10^{32} cfu g^{-1}) and T_3S_{11} (54.0 x 10^{32} cfu g^{-1}) which were statistically on par with each other (Table 4.21). It was also observed that the population was higher at one month of bead preparation. Higher bacterial population was observed in beads produced from sodium alginate (3.0%) and calcium chloride (3.0% and 3.5%) in treatments T_1S_6 (6.0 x 10^{36} cfu g^{-1}), T_3S_{12} (6.0 x 10^{36} cfu g^{-1}) and T_1S_5 (5.0 x 10^{32} cfu g^{-1}) which were statistically on par with each other followed by T_3S_{11} (4.33 x 10^{36} cfu g^{-1}) at one month of beads preparation. It was noticed



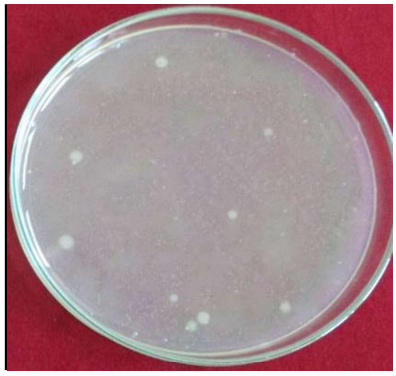
T_1A_3



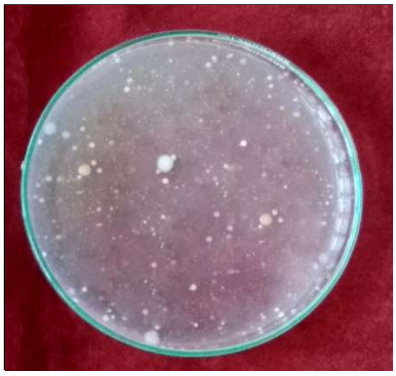
T_3A_6



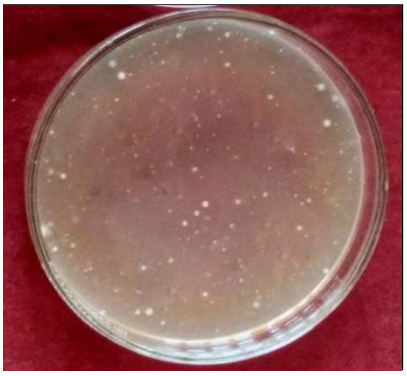
T_1A_2



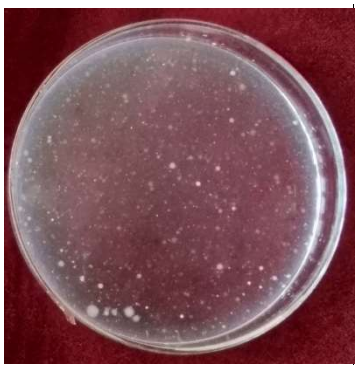
T_3A_5



T_1S_3



T_3S_6



T_3S_5

T_1S_2

Plate 4.8 Population of *P. fluorescens* (x 10^8 cfu per bead) at one month of bead preparation

that the beads prepared from alginate + skim milk were subjected rapidly to dehydration and hence got deteriorated as it lost its entire spherical structure after one month of preparation. Hence, the population of bacteria entrapped in such beads was counted only for two months and it was noticed that the population in the 2nd month was higher in T_1S_6 (9.33×10^{32} cfu g^{-1}) followed by T_1S_5 (8.66×10^{32} cfu g^{-1}) and T_3S_{12} (8.0×10^{32} cfu g^{-1}), whereas the least count was noticed with the control treatment C_1S_{13} (2.33×10^{32} cfu g^{-1}) followed by T_3S_7 (2.66×10^{32} cfu g^{-1}) and T_3S_{10} (3.66×10^{32} cfu g^{-1}) (Plate 4.10 a and Plate 4.10b).

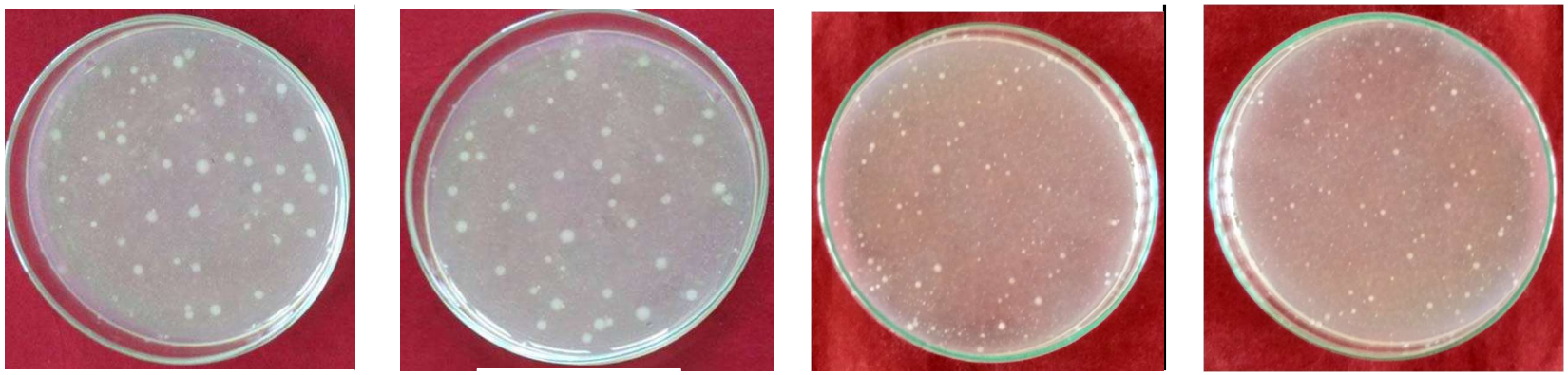
From the results on the characterization of beads, it was observed that alginate amended skim milk beads holds higher bead weight, bead diameter and yield of production. However, their shelf life was poor compared to those prepared with alginate alone amended with additives. Moreover, the alginate amended skim milk beads dehydrated after one month of preparation and thereby lost its water content to a greater extent by the end of one month of bead preparation. Moreover, the bead lost its entire structure at the end of second month and easily broke which made them difficult to handle. Addition of skim milk also didn't improve the population of the bioagent inside the bead and as a result of dehydration, the population in the treatment and control beads were statistically on par with each other after one month of bead production.

Alginate and alginate amended skim milk beads were subjected to secondary multiplication as per the procedure of Bashan *et al*. (2002). In this experiment, beads were incubated in the basal broth used initially for bead preparation for additional 24 h at 100 rpm. It was noticed that the alginate amended skim milk beads when dissolved in the basal broth for secondary multiplication, degraded and hence were not used for further studies. However, from the observations recorded, it was noticed that there was two fold increase in bacterial population entrapped inside alginate beads compared to beads without secondary multiplication. Results are presented in Table 4.22. Results revealed that population of the bioagent after secondary multiplication in alginate beads was higher in T_3A_{12} (50.33×10^{38} cfu g^{-1}) followed by treatments T_1A_6 (48.33×10^{38} cfu g^{-1}), T_3A_{11} (46.0×10^{38} cfu g^{-1}) and T_1A_5 (45.33×10^{38} cfu g^{-1}) but it was observed that the beads started dehydrating within one week after preparation.

Table 4.20 Survival rate of *P. fluorescens* in alginate beads at monthly intervals

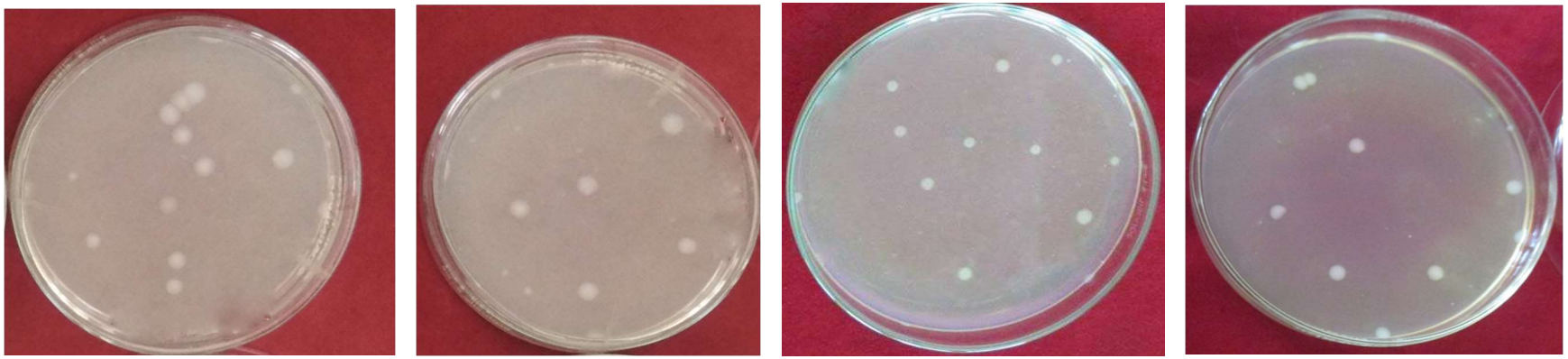
Treatments	Population of bioagent (cfu g^{-1})				
	0 MAP	1 MAP	2 MAP	3 MAP	4 MAP
	X 10^{32} cfu g^{-1}	X 10^{36} cfu g^{-1}	X 10^{32} cfu g^{-1}	X 10^{24} cfu g^{-1}	X 10^{20} cfu g^{-1}
T_1A_1 (T_1 +2.5% NA+ 2.5% $CaCl_2$)	29.0 ± 1 (1.462)^{h}	2.66 ± 0.57 (0.418)^{ef}	4.0 ± 1 (0.593)^{ef}	0.66 ± 0.57 (0.201)^{f}	0.66 ± 0.57 (0.201)^{h}
T_1A_2 (T_1 + 2.5% NA +3.0% $CaCl_2$)	32.0 ± 1 (1.505)^{g}	5.33 ± 0.57 (0.725)^{cd}	5.66 ± 1.52 (0.742)^{de}	2.0 ± 1 (0.460)^{e}	2.0 ± 1 (0.460)^{fg}
T_1A_3 (T_1 + 2.5% NA +3.5% $CaCl_2$)	42.66 ± 2.51 (1.630)^{d}	6.0 ± 1 (0.774)^{c}	9.66 ± 0.57 (0.985)^{bc}	3.66 ± 0.57 (0.667)^{bcd}	3.33 ± 0.57 (0.634)^{de}
T_1A_4 (T_1 + 3.0% NA +2.5% $CaCl_2$)	35.33 ± 1.52 (1.548)^{f}	5.66 ± 0.57 (0.752)^{c}	10.0 ± 1 (0.999)^{bc}	2.0 ± 1 (0.460)^{e}	1.33 ± 0.57 (0.360)^{g}
T_1A_5 (T_1 + 3.0% NA +3.0% $CaCl_2$)	53.0 ± 2 (1.724)^{b}	9.66 ± 1.52 (0.981)^{ab}	12.33 ± 0.57 (1.091)^{ab}	5.33 ± 0.57 (0.800)^{ab}	5.0 ± 1 (0.774)^{cd}
T_1A_6 (T_1 + 3.0% NA +3.5% $CaCl_2$)	56.0 ± 1 (1.748)^{ab}	12.0 ± 1 (1.078)^{a}	15.33 ± 0.57 (1.185)^{a}	7.33 ± 1.52 (0.916)^{a}	6.33 ± 0.57 (0.864)^{bc}
T_3A_7 (T_3 + 2.5% NA +2.5% $CaCl_2$)	30.0 ± 1 (1.477)^{h}	2.66 ± 0.57 (0.418)^{ef}	3.0 ± 1 (0.460)^{f}	2.0 ± 1 (0.460)^{e}	2.33 ± 0.57 (0.519)^{ef}
T_3A_8 (T_3 + 2.5% NA +3.0% $CaCl_2$)	37.0 ± 2 (1.568)^{ef}	3.66 ± 1.52 (0.534)^{e}	5.0 ± 1 (0.693)^{e}	2.66 ± 1.15 (0.551)^{de}	3.66 ± 0.57 (0.667)^{de}
T_3A_9 (T_3 + 2.5% NA +3.5% $CaCl_2$)	48.66 ± 0.57 (1.687)^{c}	5.66 ± 1.52 (0.742)^{cd}	8.0 ± 1 (0.901)^{cd}	4.33 ± 1.52 (0.715)^{bcd}	3.33 ± 0.57 (0.634)^{de}
T_3A_{10} (T_3 + 3.0% NA +2.5% $CaCl_2$)	38.33 ± 0.57 (1.584)^{e}	7.66 ± 1.15 (0.881)^{bc}	10.0 ± 1 (0.999)^{bc}	5.0 ± 1 (0.774)^{abc}	2.33 ± 0.57 (0.519)^{ef}
T_3A_{11} (T_3 + 3.0% NA +3.0% $CaCl_2$)	55.0 ± 1 (1.740)^{ab}	10.0 ± 1.73 (0.995)^{ab}	13.0 ± 1 (1.113)^{ab}	7.33 ± 0.57 (0.920)^{a}	8.33 ± 0.57 (0.969)^{ab}
T_3A_{12} (T_3 + 3.0% NA +3.5% $CaCl_2$)	56.33 ± 0.57 (1.751)^{a}	13.33 ± 1.15 (1.124)^{a}	17.0 ± 1 (1.230)^{a}	8.0 ± 1 (0.952)^{a}	10.33 ± 0.57 (0.054)^{a}
C_1A_{13} (KB + 3.0% NA +3.0% $CaCl_2$)	32.33 ± 1.15 (1.509)^{g}	2.0 ± 1 (0.259)^{f}	3.0 ± 1 (0.460)^{f}	1.66 ± 0.57 (0.418)^{e}	2.0 ± 1 (0.460)^{fg}
C_1A_{14} (KB + 3.0% NA +3.5% $CaCl_2$)	35.33 ± 1.15 (1.548)^{f}	3.66 ± 0.57 (0.560)^{de}	5.0 ± 2.64 (0.661)^{e}	3.0 ± 1 (0.593)^{cde}	3.33 ± 0.57 (0.634)^{de}
CD(0.05)	0.025	0.185	0.167	0.185	0.154

NA- sodium alginate, $CaCl_2$- calcium chloride, T_1- mannitol + PVP + CMC + tween-80, T_3- mannitol + PEG + CMC + tween-80, C_1- control, KB- King's B broth, MAP- Month after production. Values are arranged in Mean ± SD. Log transformations are given in parentheses. In each column figures followed by same letter do not differ significantly according to DMRT.



T_3A_{12} T_3A_{11} T_1A_6 T_1A_5

Plate 4.9a Survival of entrapped bacteria in beads immediately after preparation (0 MAP) (x 10^{32} cfu g^{-1})



T_3A_{12} T_3A_{11} T_1A_6 T_1A_5

Plate 4.9b Survival of entrapped bacteria in alginate beads after 4 MAP (x 10^{20} cfu g^{-1})

Table 4.21 Survival rate of *P. fluorescens* in alginate amended skim milk beads at monthly intervals

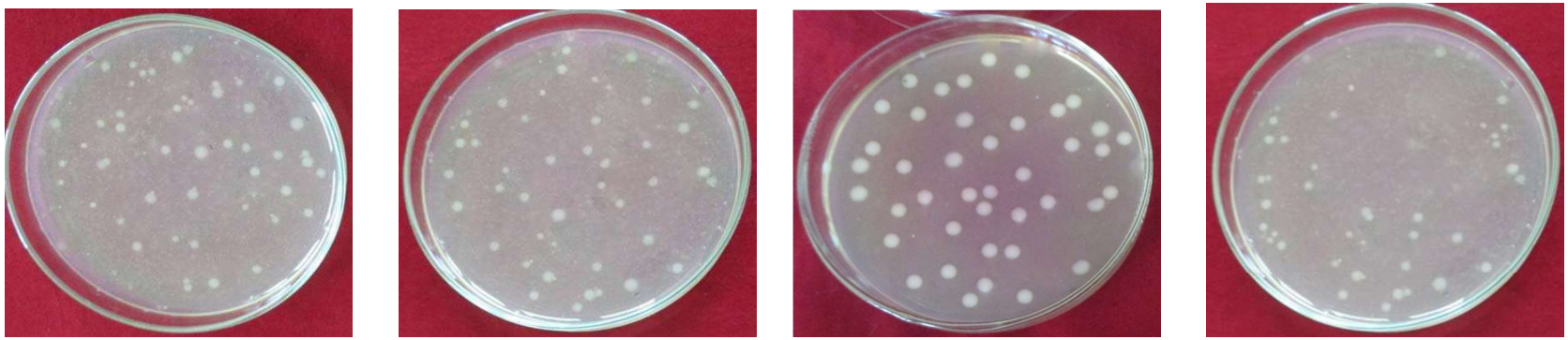
Treatments	Population of bioagent (cfu g^{-1})		
	0 MAP	1 MAP	2 MAP
	X 10^{32} cfu g^{-1}	X 10^{36} cfu g^{-1}	X 10^{32} cfu g^{-1}
T_1S_1 (T_1 +2.5% NA+ 2.5% $CaCl_2$ + 0.75% SM)	28.0 ± 1 (1.447)^{g}	2.33 ± 0.5 (0.360)^{c}	5.0 ± 1 (0.693)^{cd}
T_1S_2 (T_1 + 2.5% NA +3.0% $CaCl_2$ + 0.75% SM)	30.0 ± 1 (1.477)^{f}	4.0 ± 0 (0.602)^{ab}	5.66 ± 1.15 (0.748)^{abcd}
T_1S_3 (T_1 + 2.5% NA +3.5% $CaCl_2$ + 0.75% SM)	40.0 ± 1 (1.602)^{d}	4.33 ± 0.57 (0.634)^{ab}	6.33 ± 0.57 (0.800)^{abc}
T_1S_4 (T_1 + 3.0% NA +2.5% $CaCl_2$ + 0.75% SM)	34.33 ± 1.15 (1.536)^{e}	3.0 ± 1 (0.460)^{bc}	5.33 ± 0.57 (0.725)^{cd}
T_1S_5 (T_1 + 3.0% NA +3.0% $CaCl_2$ + 0.75% SM)	50.66 ± 0.57 (1.705)^{b}	5.0 ± 1 (0.693)^{a}	8.66 ± 1.52 (0.933)^{ab}
T_1S_6 (T_1 + 3.0% NA +3.5% $CaCl_2$ + 0.75% SM)	55.33 ± 1.52 (1.743)^{a}	6.0± 1 (0.774)^{a}	9.33 ± 1.52 (0.966)^{a}
T_3S_7 (T_3 + 2.5% NA +2.5% $CaCl_2$ + 0.75% SM)	30.0 ± 1 (1.477)^{f}	1.33 ± 0.57 (0.100)^{d}	2.66 ± 0.57 (0.418)^{ef}
T_3S_8 (T_3 + 2.5% NA +3.0% $CaCl_2$ + 0.75% SM)	35.0 ± 1 (1.544)^{e}	2.33 ± 0.57 (0.360)^{c}	4.66 ± 1.52 (0.651)^{cde}
T_3S_9 (T_3 + 2.5% NA +3.5% $CaCl_2$ + 0.75% SM)	47.33 ± 2.08 (1.675)^{c}	4.66 ± 1.15 (0.661)^{a}	5.0 ± 0 (0.699)^{cd}
T_3S_{10} (T_3 + 3.0% NA +2.5% $CaCl_2$ + 0.75% SM)	36.0 ± 1 (1.556)^{e}	2.33 ± 0.57 (0.360)^{c}	3.66 ± 2.08 (0.519)^{def}
T_3S_{11} (T_3 + 3.0% NA +3.0% $CaCl_2$ + 0.75% SM)	54.0 ± 1 (1.732)^{a}	4.33 ± 0.57 (0.634)^{ab}	6.0 ± 2 (0.761)^{abc}
T_3S_{12} (T_3 + 3.0% NA +3.5% $CaCl_2$ + 0.75% SM)	55.0 ± 1 (1.740)^{a}	6.0 ± 0 (0.778)^{a}	8.0 ± 3 (0.881)^{abc}
C_1S_{13} (KB + 3.0% NA +3.0% $CaCl_2$ + 0.75% SM)	30.33 ± 1.15 (1.482)^{f}	4.33 ± 1.52 (0.619)^{ab}	2.33 ± 1.52 (0.301)^{f}
C_1S_{14} (KB + 3.0% NA +3.5% $CaCl_2$ + 0.75% SM)	35.33 ± 3.21 (1.547)^{e}	5.0 ± 1.73 (0.678)^{a}	5.0 ± 1 (0.693)^{cd}
CD(0.05)	0.027	0.183	0.234

*NA- Sodium alginate, $CaCl_2$- Calcium chloride, T_1- Mannitol + PVP + CMC + Tween-80, T_3- Mannitol + PEG + CMC + Tween-80, C_1- Control, KB-King's B broth, SM- Skim milk. MAP – Month after production. Values are arranged in Mean ± SD. Log transformations are given in parentheses. In each column figures followed by same letter do not differ significantly according to DMRT.

Table 4.22 Survival rate of *P. fluorescens* after secondary multiplication in alginate beads

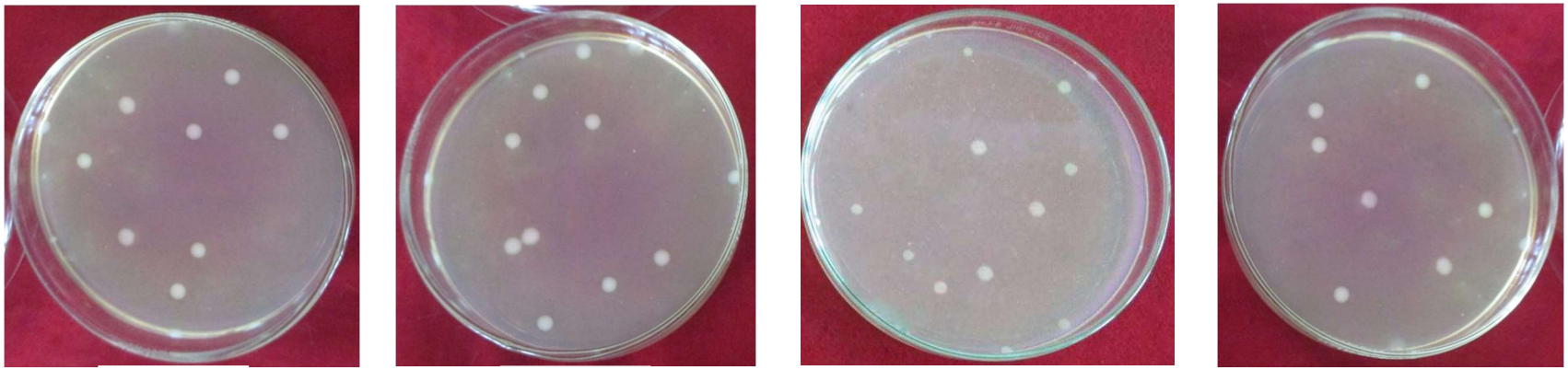
Treatments	Population of bioagent (X 10^{38} cfu g^{-1})
T_1A_1 (T_1 +2.5% NA+ 2.5% $CaCl_2$)	24.0 ± 1.0 (1.398)^{g}
T_1A_2 (T_1 + 2.5% NA +3.0% $CaCl_2$)	39.67 ± 3.05 (1.608)^{de}
T_1A_3 (T_1 + 2.5% NA +3.5% $CaCl_2$)	42.33 ± 1.52 (1.637)^{cde}
T_1A_4 (T_1 + 3.0% NA +2.5% $CaCl_2$)	31.0 ± 1.0 (1.505)^{f}
T_1A_5 (T_1 + 3.0% NA +3.0% $CaCl_2$)	45.33 ± 1.52 (1.666)^{abc}
T_1A_6 (T_1 + 3.0% NA +3.5% $CaCl_2$)	48.33 ± 2.08 (1.693)^{ab}
T_3A_7 (T_3 + 2.5% NA +2.5% $CaCl_2$)	38.0 ± 2.64 (1.590)^{e}
T_3A_8 (T_3 + 2.5% NA +3.0% $CaCl_2$)	41.33 ± 2.08 (1.626)^{cde}
T_3A_9 (T_3 + 2.5% NA +3.5% $CaCl_2$)	43.67 ± 2.08 (1.650)^{bcd}
T_3A_{10} (T_3 + 3.0% NA +2.5% $CaCl_2$)	38.0 ± 1.0 (1.591)^{e}
T_3A_{11} (T_3 + 3.0% NA +3.0% $CaCl_2$)	46.0 ± 3.0 (1.672)^{abc}
T_3A_{12} (T_3 + 3.0% NA +3.5% $CaCl_2$)	50.33 ± 5.03 (1.709)^{a}
C_1A_{13} (KB + 3.0% NA +3.0% $CaCl_2$)	24.0 ± 4.0 (1.394)^{g}
C_1A_{14} (KB + 3.0% NA +3.5% $CaCl_2$)	24.67 ± 1.52 (1.409)^{g}
CD(0.05)	0.050

*NA- sodium alginate, $CaCl_2$- calcium chloride, T_1- mannitol + PVP + CMC + tween-80, T_3- mannitol + PEG + CMC + tween-80, C_1- control, KB-King's B broth, SM- skim milk. MAP – Month after preparation. Values are arranged in Mean ± SD. Log transformations are given in parentheses. In each column figures followed by same letter do not differ significantly according to DMRT.



T_1S_6 T_3S_{12} T_3S_{11} T_1S_5

Plate 4.10a Survival of entrapped bacteria in alginate + skim milk beads at 0 MAP (x 10^{32} cfu g^{-1})



T_1S_6 T_3S_{12} T_3S_{11} T_1S_5

Plate 4.10b Survival of entrapped bacteria in alginate + skim milk beads at 2 MAP (x 10^{32} cfu g^{-1})

From the above discussed parameters, it was observed that alginate beads have higher shelf life compared to alginate amended skim milk beads and beads after secondary multiplication. At higher concentration of sodium alginate (3%) and calcium chloride (3% and 3.5%), the beads were perfectly spherical in shape with larger diameter, higher bead weight, higher yield of production and holds higher water content inside the beads which led to longer survival of bioagent inside the beads. The swelling and shrinkage percentage was also found low which lead to slow and constant release of entrapped bioagent from the beads. Moreover, higher bacterial entrapment were observed inside the beads produced from treatment combinations containing 3.0 per cent sodium alginate with 3.0 per cent calcium chloride and 3 per cent sodium alginate and 3.5 per cent calcium chloride. As both the combinations could entrap equal and sufficient number of *P. fluorescens* inside the alginate beads, treatments amended with 3.0 per cent of sodium alginate and calcium chloride were used for *in vitro* inhibition studies against major soil borne plant pathogens. The best two bead combinations used for *in vitro* inhibition studies are as follows:

B-1: T_1 (mannitol + PVP + CMC + tween -80) + sodium alginate (3%) + $CaCl_2$ (3%)
B-2: T_3 (mannitol + PEG + CMC + tween -80) + sodium alginate (3%) + $CaCl_2$ (3%)

4.5 *In vitro* INHIBITION OF FORMULATION AGAINST MAJOR SOIL BORNE PLANT PATHOGENS

The antagonistic potential of two best treatment beads selected above were evaluated against six major soil borne pathogens *viz., Fusarium oxysporum, Phytophthora nicotiana, Pythium aphanidermatum, Sclerotium rolfsii, Rhizoctonia solani* by dual culture method and *Ralstonia solanacearum* by filter paper disc method. The cultures of plant pathogenic fungi and bacteria were collected from the Department of Plant Pathology, College of Horticulture, Vellanikkara. The results of the *in vitro* inhibition of the bead formulations of *Pseudomonas fluorescens* against growth of the above mentioned plant pathogens are recorded in Table 4.23.

The results revealed that the bead formulation B-1 showed cent per cent inhibition under *in vitro* against *Pythium aphanidermatum*. This was closely followed by *Phytophthora nicotiana* (72.22 per cent), *Ralstonia solanacearum* (70.36 per cent) and *Fusarium oxysporum* (27.77 per cent). Whereas, no inhibition was observed against *Sclerotium rolfsii* and *Rhizoctonia solani* which revealed that the formulation B-1 was not able to suppress these pathogens. Similarly, bead formulation B-2 showed cent per cent inhibition against *Pythium aphanidermatum*. However, the inhibition percentage against *Phytophthora nicotiana* and *Ralstonia solanacearum* of B-2 formulation was 77.77 and 74.07 per cent respectively. It was observed that like B-1 formulation, B-2 also showed no inhibition on the growth of *Sclerotium rolfsii* and *Rhizoctonia solani* under *in vitro* conditions (Plate 4.11 and Plate 4.12).

Cost incurred for production of 1 Kg of bead, which can be applied to 333 tomato plants in three split doses @ 1.0g plant^{-1} was calculated. A total of 1500 ml of sodium alginate- additive mixture was required to produce one kilogram bead. For the same, mannitol (4.5 g), PVP (3.0 g), CMC (1.5 g), tween-80 (0.75 ml), PEG (0.375 g), peptone (3.0 g), K_2HPO_4 (0.22g), $MgSO_4$ (0.22g), glycerol (1.2 ml), sodium alginate (180.0g) and $CaCl_2$ (27.0g). The cost of these ingredients (input costs) include Mannitol (INR 724 Kg^{-1}), PVP (INR 782 Kg^{-1}), CMC (INR 1096 Kg^{-1}), Tween-80 (INR 1510 Kg^{-1}), PEG (INR 1099 Kg^{-1}), peptone (INR 2466 Kg^{-1}), K_2HPO_4 (INR 1560 Kg^{-1}), $MgSO_4$ (INR 1240 Kg^{-1}), glycerol (INR 742 Kg^{-1}), sodium alginate (INR 2124 Kg^{-1}) and $CaCl_2$ (INR 700 Kg^{-1}). Further, the bottles used for packing amounts to INR 14.16 per piece where four bottles of 300 mL capacity were required. So, the production cost occurred for preparing 1 Kilogram of beads was estimated to INR 282.39 Kg^{-1} bead.

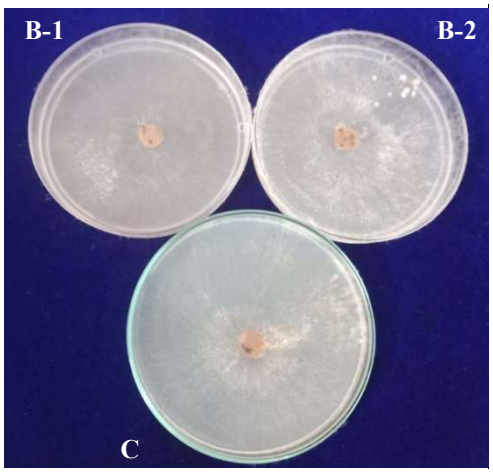
The cost of talc based formulation required to treat 333 tomato plants in three split doses @ 20.0 g L^{-1} was approximately INR 900.00. Thus, the benefit cost ratio was around 3.1:1.

Table 4.23 *In vitro* evaluation of alginate beads of *Pseudomonas fluorescens* against major soil borne pathogens

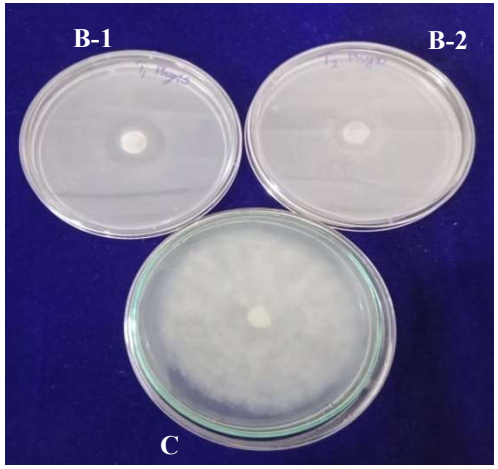
Treatment No.	Per cent inhibition						CD (0.05)
	Fusarium oxysporum	***Phytophthora nicotiana***	***Pythium aphanidermatum***	***Sclerotium rolfsii***	***Rhizoctonia Solani***	***Ralstonia solanacearum***	
B-1.	27.77 ± 0 (5.317)^{d}	72.22 ± 0 (8.528)^{b}	100.00 ± 0 (10.025)^{a}	0.00 ± 0 (0.707)^{e}	0.00 ± 0 (0.707)^{e}	70.36 ± 1.60 (8.418)^{c}	0.069
B-2.	30.55 ± 2.78 (5.569)^{c}	77.77 ± 0 (8.847)^{b}	100.00 ± 0 (10.025)^{a}	0.00 ± 0 (0.707)^{d}	0.00 ± 0 (0.707)^{d}	74.07 ± 3.20 (8.634)^{b}	0.225

*Values are arranged in Mean ± SD. In each row figures followed by same letter do not differ significantly according to DMRT. Transformed values are given in parenthesis.

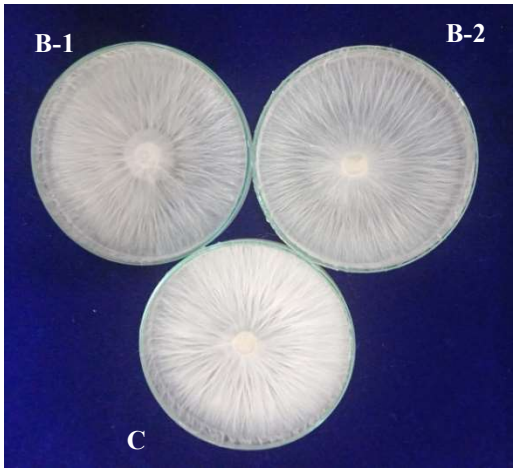
B-1: T_1 (mannitol + PVP + CMC + tween -80) + sodium alginate (3%) + $CaCl_2$ (3%)
B-2: T_3 (mannitol + PEG + CMC + tween -80) + sodium alginate (3%) + $CaCl_2$ (3%)



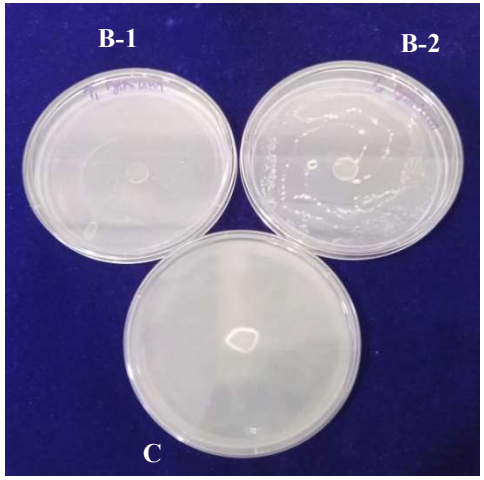
(i) *Rhizoctonia solani*



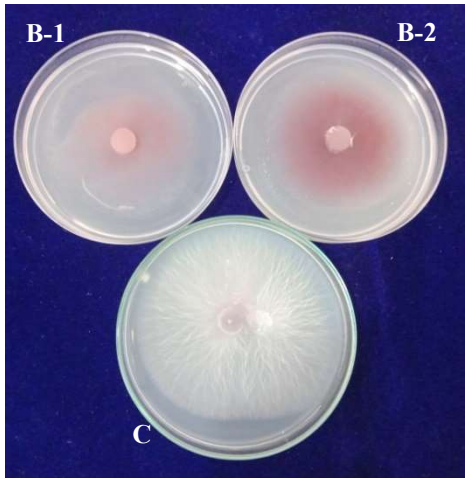
(ii) *Phytophthora nicotiana*



(iii) *Sclerotium rolfsii*



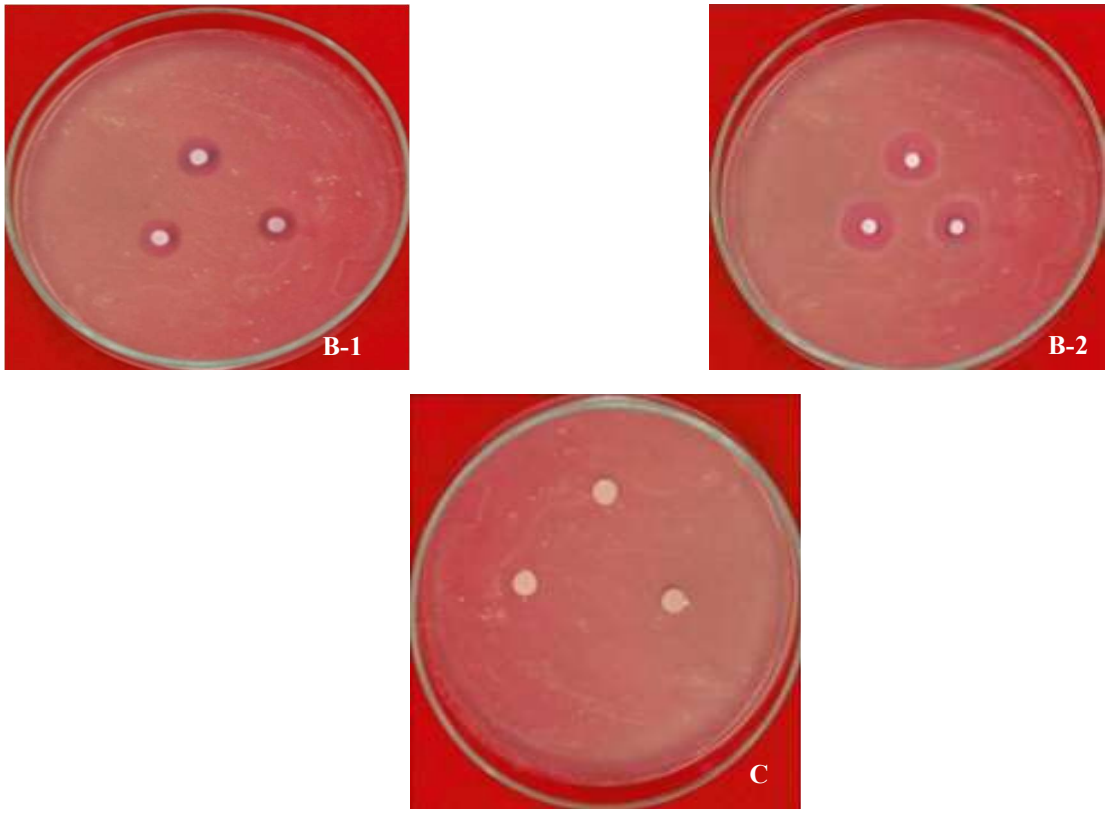
(iv) *Pythium aphanidermatum*



(v) *Fusarium oxysporum*

Plate 4.11 *In vitro* evaluation of alginate beads of *Pseudomonas fluorescens* against fungal pathogens

B-1: T_1 (mannitol + PVP + CMC + tween -80) + sodium alginate (3%) + $CaCl_2$ (3%);
B-2: T_3 (mannitol + PEG + CMC + tween -80) + sodium alginate (3%) + $CaCl_2$ (3%);
C: control.



(vi) *Ralstonia solanacearum*

Plate 4.12 *In vitro* evaluation of alginate beads of *Pseudomonas fluorescens* against *R.solanacearum*

B-1: T_1 (mannitol + PVP + CMC + tween -80) + sodium alginate (3%) + $CaCl_2$ (3%)

B-2: T_3 (mannitol + PEG + CMC + tween -80) + sodium alginate (3%) + $CaCl_2$ (3%)

C : Control

DISCUSSION

5. DISCUSSION

Plant disease management depends on the indiscriminate use of fungicides or chemicals which lead to the development of toxic residues, environmental contamination, fungicide resistance, carcinogenic and mutagenic effects in humans, plants and animals (Rajavel, 2000). Biological control in this paradox is an alternate innovative approach for management of plant diseases, considered as eco-friendly and cost effective system under commercialization of agriculture. In this context, *Pseudomonas fluorescens*, is one such biocontrol agent (BCA) which engrossed the major domain of biocontrol research due to their unique antagonistic properties against wide range of plant pathogens together with their plant growth promoting activity (Walsh *et al*., 2001; Haas and Defago, 2005; Jayraj *et al.,* 2007; Morales *et al*., 2010; Özyilmaz and Benlioglu, 2013). Inoculation of plants with bio-control agents has been practiced for several decades to control plant diseases and also to enhance crop yield, but it is not widely popular as compared with synthetic pesticides as they exhibit inconsistent performance in practical agriculture which results in limited commercial application of microorganisms as biocontrol agents (Mathre *et al.*, 1999; Harman, 2000; chaube *et al.*, 2004).

The success of potential biocontrol activity depends on the development of appropriate formulations preventing rapid degradation and extending the shelf-life of introduced microorganisms. Various formulations available are powder, liquid and granular formulations where such carrier based inoculants produced in India generally faces problems like poor shelf life, high chances of contamination, bulk sterilization problem, unpredictable field performance and sometimes unavailability of good carrier materials. For further improvement in formulation technology, preparation of alginate bead formulation of the beneficial bacteria is found to be a better alternative compared to powder or liquid formulations as these alginate formulations temporarily protect the higher population density of bacteria inside beads for longer period of time from the soil environment, protects from microbial competition and release them gradually in to

the plants for plant root colonization (Bashan *et al*., 2002). Hence the present investigation was conducted to develop an alginate based formulation of *Pseudomonas fluorescens* for the management of soil borne pathogens.

5.1 STANDARDIZATION OF MEDIA WITH ADDITIVES AND ITS EFFECT ON SHELF LIFE OF *P. fluorescens*

The additive combination of T_1 (KB broth + mannitol + PVP + CMC + tween -80) (1.33 x 10^8 cfu ml^{-1}) and T_3 (KB broth + mannitol + PEG + CMC + tween-80) (1.66 x 10^8 cfu ml^{-1}) was found effective in maintaining the viable population density of *P. fluorescens* after nine months of storage compared to control (0.00 x 10^8 cfu ml^{-1}) (Fig. 5.1a and 51b). The results are in accordance with the findings of Vidhyasekaran and Muthamilan (1995) observed that addition of CMC as adhesive recovered a population of more than 10^7 cfu g^{-1} in the preparation of different *P. fluorescens* based formulations upto 240 days of storage. Similar observations were reported by Navi (2004) that additives such as glucose and mannitol served as additional carbon sources for cell proliferation, thus increased the shelf life of bacteria in liquid formulations. Supplementation of PEG 8000 (5 to 20%)and Tween 80 (1%) as one of the additive combination in powder formulation effectively enhanced the conidial germination and dispersion of *Penicillium oxalicum* and controlled tomato wilt to a greater extent.

The results obtained by Tittabutr *et al.* (2007) are in line with the present study that addition of polyvinyl pyrrolidone (PVP) as additive in liquid inoculant formulations of *Bradyrhizobium* were found effective, as PVP slowed down the drying process of the inoculant due to their adhesive properties compared to gum arabic, polyvinyl alcohol (PVA), polyethylene glycol (PEG) and cassava starch. Lahlali and Jijakli (2009) observed that cell suspension of *Candida oleophila* supplemented with PEG as one of the additive effectively controlled *Penicillium expansum* as PEG act as the protectant against desiccation thus, enhancing the long term survival of the bioagent.

The efficacy of *Pseudomonas fluorescens* EPS62e in the biocontrol of *Erwinia amylovora* was improved by the nutritional enhancement of glycine and Tween-80 to the basal broth (Cabrefiga *et al*., 2011). Also stated that tween 80 act as detergent and

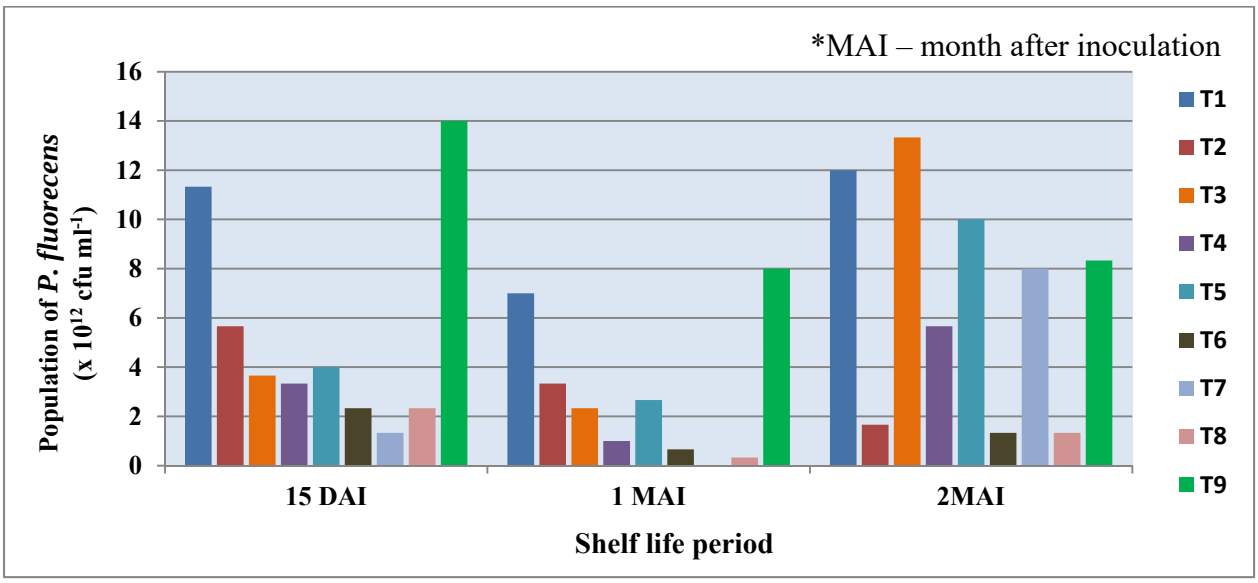


Fig 5.1a Population of *P.fluorescens* in King's B broth upto 2 months of storage period

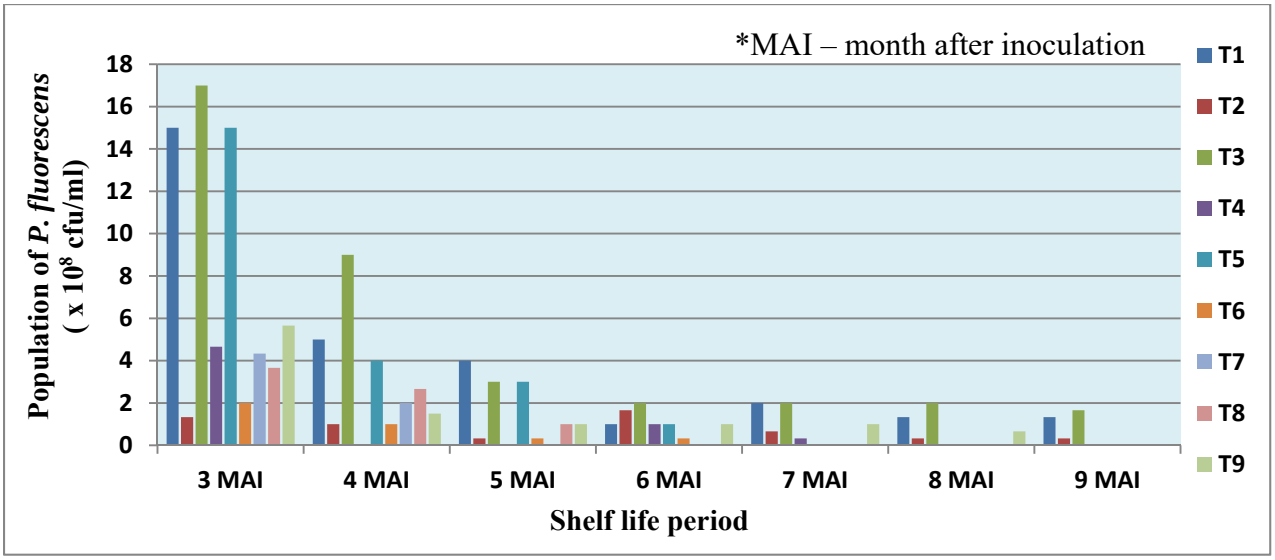


Fig 5.1b Population of *P.fluorescens* in King's B broth at monthly intervals (3rd to 9th MAI)

enhances bacterial dispersion in plant surfaces and helps to reduce surface tension at the liquid-solid interface, facilitating air distribution. Alamraj *et al.* (2013) also observed that liquid formulations amended with PVP (2%), CMC (0.1%) and polysorbate 20 (0.025%) maintained higher survival of *Bacillus megaterium var. phosphaticum* (5.6 x 10^7 cfu ml^{-1}), *Azospirillum* (1.9x10^8 cfu ml^{-1}) and *Azotobacter* (3.5x10^7 cfu ml^{-1}) respectively after 480 days of observation which is in agreement with the present study where the treatment combination containing PVP and CMC promoted the shelf life of the bacteria. Segarra *et al*. (2015) also observed that bio pesticide formulation prepared from the additive combination of corn steep liquor, lingosulfonate, and polyethylene glycol (PEG) improved the survival of *Lysobacter capsici* AZ78 cells living on grapevine leaves as PEG effectively protect the cells against desiccation from under field conditions.

The results are also in conformity with Trivedi *et al*. (2016) who reported that survival of *Rhizobium* sp. was higher in nutrient broth supplemented with PVP (2.5%) + glycerol (2%) till 720 days of storage. Biradar and Santhosh (2018) also observed higher survival of *P. fluorescens* in treatment combination containing PVP as one of the additives (King's B broth + 2% PVP + 0.3% Xanthan gum + 0.5% Tween-20 + 0.2% potassium sorbate) even after six months followed by PEG and CMC against *Fusarium* wilt of tomato under greenhouse conditions.

5.2 STANDARDIZATION OF DIFFERENT PARAMETERS FOR BEAD PREPARATION

5.2.1 Concentration of sodium alginate and calcium chloride

An attempt was made to standardize the protocol for mass production of alginate beads in order to develop an effective formulation of the same. Therefore, five different concentrations of sodium alginate and four different concentrations of calcium chloride solution were used to standardize the formation of alginate beads. It was noticed from the result that lower concentration of either sodium alginate (1.0%, 1.5% and 2.0%) or calcium chloride (2.0%) resulted in improper bead formation. Uniform,

spherical and rigid beads were formed with higher concentration of sodium alginate (2.5% and 3.0%) and calcium chloride (2.5%, 3.0% and 3.5%) respectively. Therefore, beads formed from 2.5 per cent and 3.0 per cent concentration of sodium alginate and 2.5, 3.0 and 3.5 per cent of calcium chloride solution respectively was selected for further studies. The present study is in conformity with the findings of Vij *et al.* (2001) and Mondal *et al.* (2002) where they reported maximum survival rate and excellent encapsulation efficiency of beads at 3, 3.5 and 4 per cent sodium alginate in 100 mM, 90 mM and 90 mM $CaCl_2$ solution respectively which was found optimum for production of isodiametric and compact beads. This study also supports the findings of Mandal *et al.* (2010) where they observed that alginate beads prepared using 3.0 per cent w/v $CaCl_2$ and higher alginate concentration showed more rigid gel network due to more crosslinking resulting in greater sustained release characteristics. Several other workers also found that the beads produced using sodium alginate (3.0%) and 100 mM $CaCl_2$ are firm, clear, compact and isodiametric in nature compared to other combinations (Sarmah *et al*., 2010; Ali *et al*., 2012; Iqbal *et al.*, 2019).

5.2.2 Gelation and curing of beads

The gelling rate was assessed during particle formation and was determined from weight changes of the beads in calcium chloride solution. For this average weight of five randomly selected beads of 28 different treatments were analysed at five different gelation period *viz*., 30, 45, 60, 75 and 90 min. It was observed that after 60 min of gelation period, average weight of beads were gradually maintained in treatment combination supplemented with sodium alginate (3.0%) and calcium chloride (3.0% and 3.5%) respectively in both alginate and alginate amended skim milk beads compared to other treatments presented in (Fig 5.2 and 5.3). The present study is in conformity with the findings of Trevors *et al*. (1993), alginate beads when allowed to cure for one hour attained complete gelation. The results were also comparable with Tous *et al.* (2014) where they reported that complete curing is the time at which the weight of the alginate bead became stable.

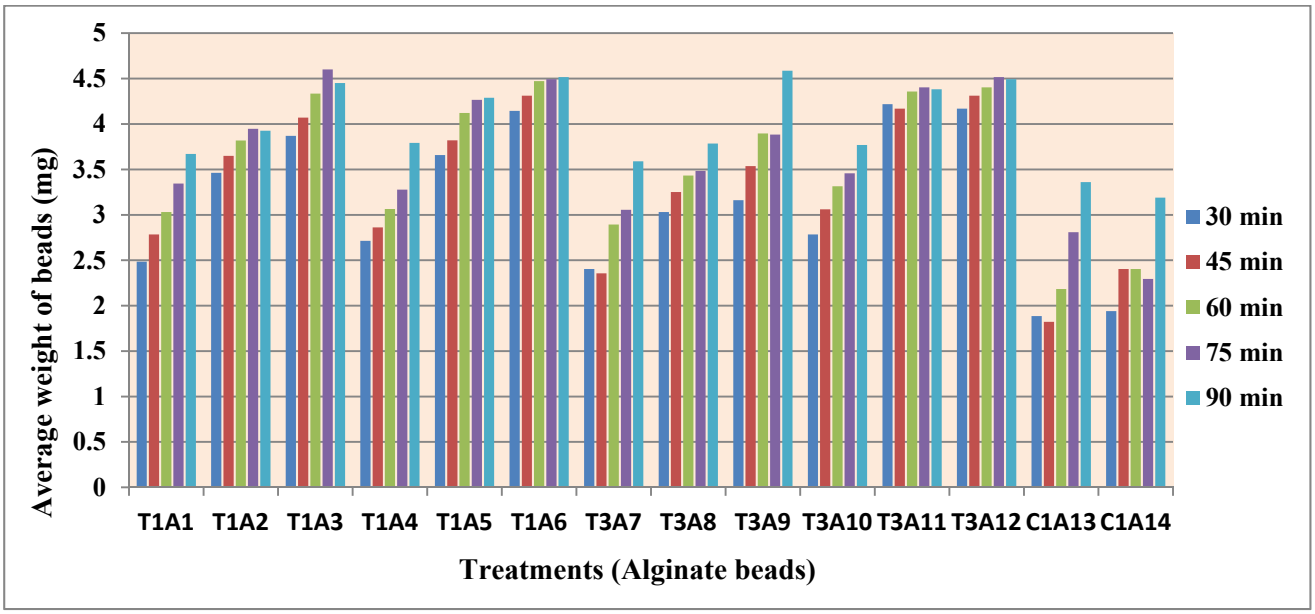


Fig 5.2 Mean weight of Alginate beads at different gelation period

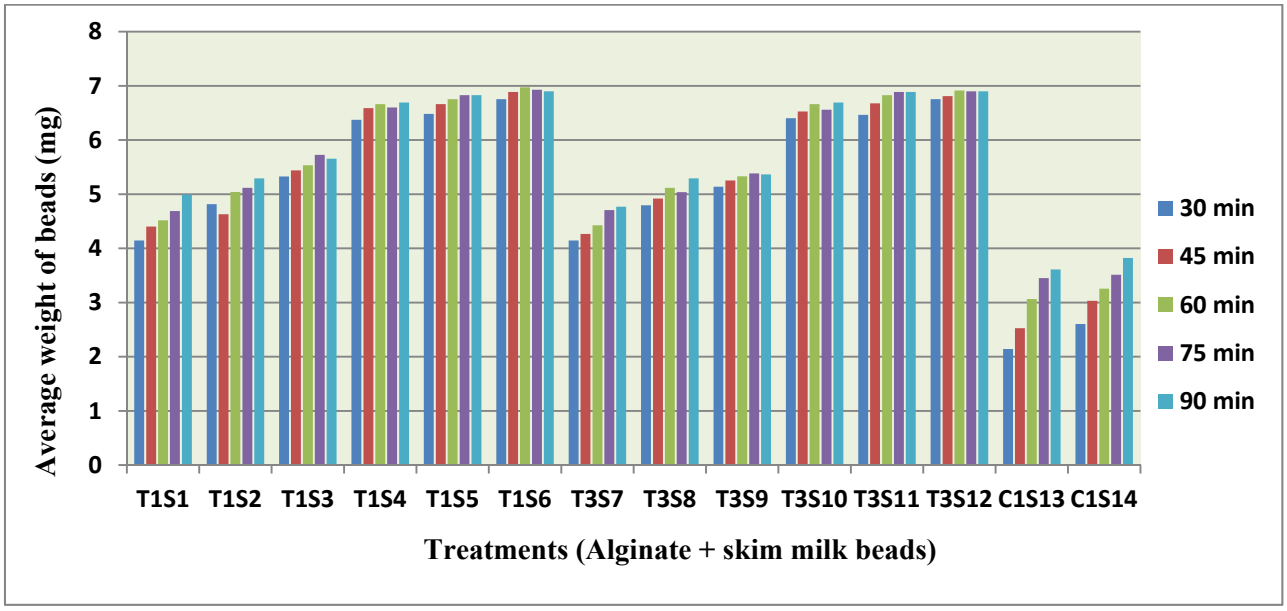


Fig 5.3 Mean weight of Alginate + Skim milk beads at different gelation period

5.2.3 Determination of cell leakage

Next point of investigation is to determine the cell leakage. In the present study, it was observed that higher cell leakage in $CaCl_2$ solution was observed in control beads followed by treatment beads prepared from lower concentration of sodium alginate (2.5%) and calcium chloride solution (2.5%) in both alginate and alginate amended skim milk beads. From the Fig 5.4, it was observed that beads prepared from higher concentration of sodium alginate (3.0%) and calcium chloride (3.5% and 3.0%) the cell leakage/cell wastage was lower in the cross linking solution which is evident in treatment T_1A_3 (2.0×10^6 cfu ml^{-1}) followed by T_3A_{12} (2.33×10^6 cfu ml^{-1}) of alginate treatments and T_1S_6 (1.0×10^6 cfu ml^{-1}) of alginate + skim milk treatment during bead formation which in turn led to higher bacterial entrapment in beads and vice versa. The results obtained in the present study are in agreement with the findings of Gao *et al.* (2017) where they observed that increase in concentration of sodium alginate from 1 - 3 per cent (w/v) and calcium chloride from 0.3 M to 0.4 M, that leads the formation of rigid beads, enabling minimal cell leakage into the calcium chloride solution and maximal production yield. However, contradictory to the present findings, Bokkhim *et al*. (2018) observed highest leaked cell density in higher concentrations of calcium chloride (0.5 M) whereas minimal leakage was noticed at 0.2 M $CaCl_2$ concentration. This variation in our study might be due to the effect of additives supplemented in the medium for preparation of beads.

5.2.4 Standardization of optimum height of air column

For developing a formulation, proper shape of alginate bead is essential which depends on the distance between dripping tip and surface of $CaCl_2$ solution (air column). From the results it was observed that spherical beads were obtained from 9 - 25 cm of collecting distance, whereas tailed beads (non-spherical) were produced from 1 - 7 cm of collecting distance. Optimum height of air column maintained during bead preparation was from 9 - 15 cm. The results are comparable with the findings of Bokkhim *et al.* (2018) where they observed that a dropping height of 10 cm was used

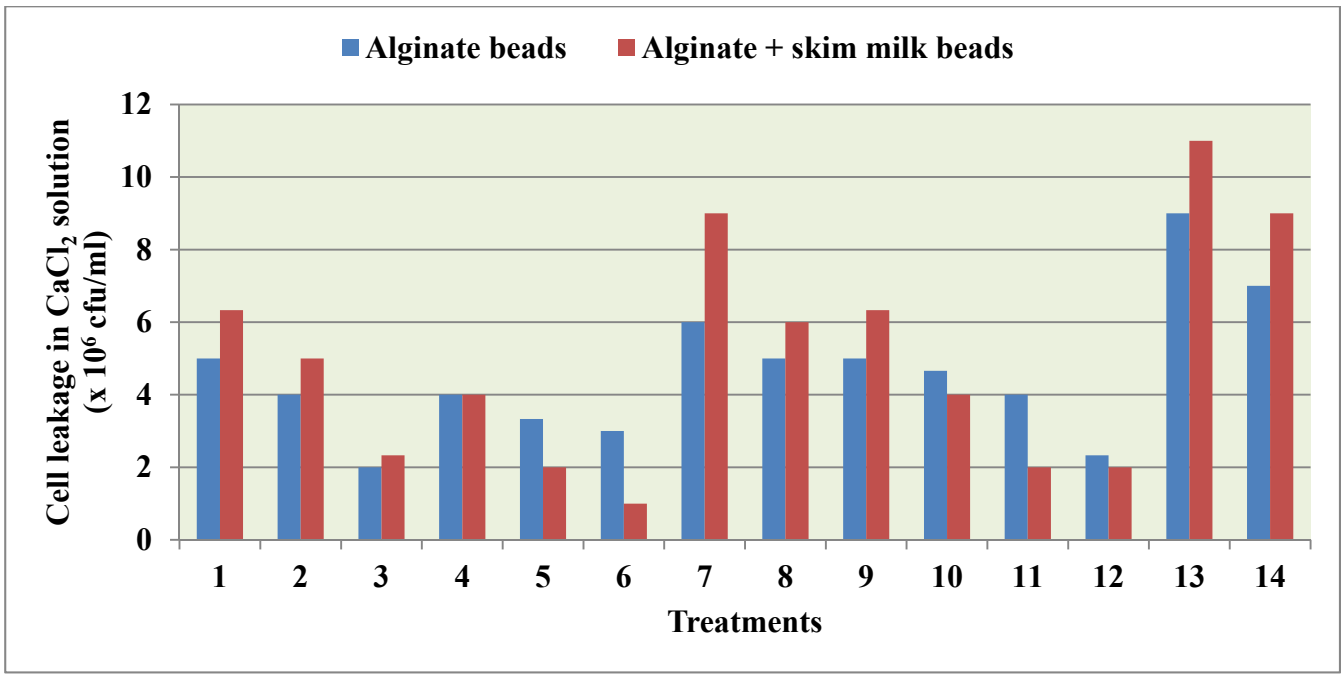
to form spherical beads. A study conducted by Al-Hajry *et al*. (1999) noticed that non spherical beads reduced the strength of the bead as compared to spherical beads. Similar findings were observed by Woo *et al.* (2007) as breakage and cracking in non-spherical and tear shape beads, resulting in the uneven release of the encapsulated material. Likewise, Chan *et al*. (2009) reported that the shape transition of the alginate droplet with increasing falling distance was distinguished into three phases *viz*., tear shape, egg shape and spherical shape. They also observed that an increase in the collecting distance lead to the formation of spherical beads. In addition, Lee *et al.* (2013) reported that spherical beads possessed mechanical and chemical stability compared to non-spherical beads. On the other hand, non-spherical and tear shaped beads led to more physical damage which resulted in the bursting of the encapsulated material.

5.3 CHARACTERIZATION OF MICROENCAPSULATED BEADS

Various parameters such as bead size, shape, weight, number of beads per gram, bead yield, water content, swelling and shrinkage percentage to be estimated for developing effective formulation

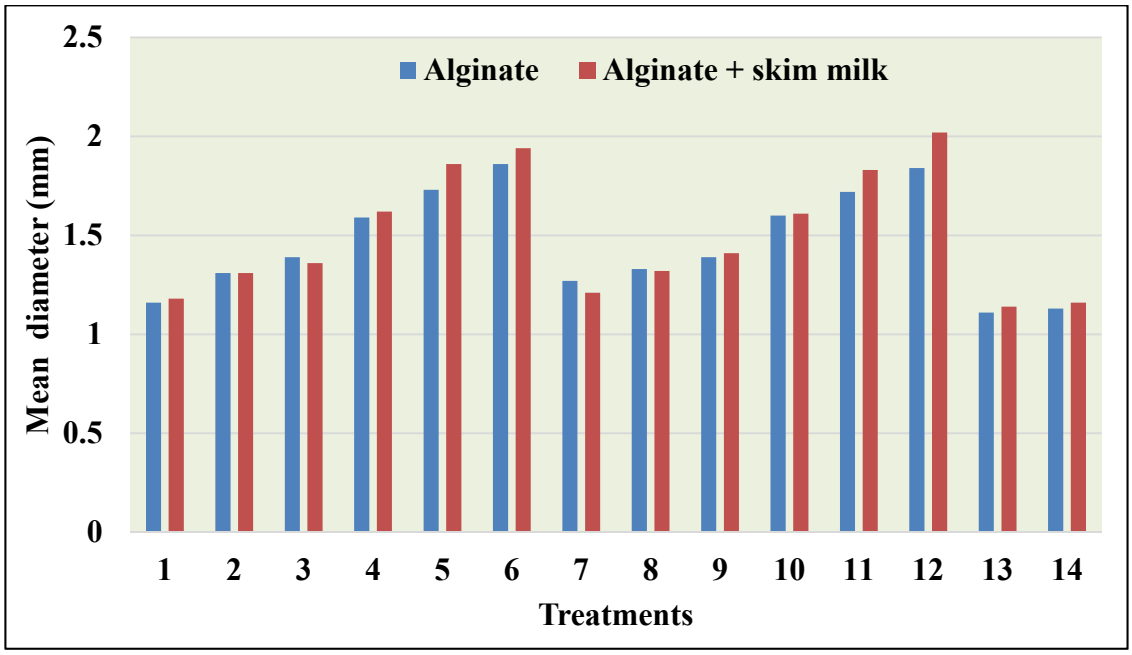
5.3.1 Bead size and shape

In the current study, the diameter and shape of alginate beads was determined through stereomicroscope (Fig 5.5). Results indicated that average diameter of the bead increased with increase in sodium alginate concentration (3.0%) and calcium chloride concentration (3.0% and 3.5%) respectively in both alginate and alginate amended skim milk beads compared to other treatments. It was also noticed that increased concentration of sodium alginate (3.0%) and calcium chloride (3.0% and 3.5%) produced uniform spherical beads compared to lower concentration of sodium alginate and calcium chloride solution. Moreover, the size of the bead depends on the viscocity of the sodium alginate solution and the opening through which with alginate solution passed into calcium chloride solution for bead preparation. The results is in accordance with Das and Senapati, (2007) where they observed that an increase in the alginate concentration of 2.5 - 4 per cent resulted in high degree of crosslinking which lead to the development of larger microbeads and greater drug entrapment inside the beads.



1-T_1A_1, T_1S_1; 2- T_1A_2, T_1S_2; 3- T_1A_3, T_1S_3 ; 4- T_1A_4, T_1S_4; 5- T_1A_5,T_1S_5; 6- T_1A_6, T_1S_6; 7- T_3A_7, T_3S_7; 8- T_3A_8, T_3S_8; 9- T_3A_9, T_3S_9; 10- T_3A_{10}, T_3S_{10} ; 11- T_3A_{11}, T_3S_{11}; 12- T_3A_{12}, T_3S_{12}; 13- C_1A_{13}, C_1S_{13}; 14- C_1A_{14}, C_1S_{14}.

Fig 5.4 Population of *P. fluorescens* in $CaCl_2$ solution in various treatment combinations as a measure of cell leakage



1-T_1A_1, T_1S_1; 2- T_1A_2, T_1S_2; 3- T_1A_3, T_1S_3 ; 4- T_1A_4, T_1S_4; 5- T_1A_5,T_1S_5; 6- T_1A_6, T_1S_6; 7- T_3A_7, T_3S_7; 8- T_3A_8, T_3S_8; 9- T_3A_9, T_3S_9; 10- T_3A_{10}, T_3S_{10} ; 11- T_3A_{11}, T_3S_{11}; 12- T_3A_{12}, T_3S_{12}; 13- C_1A_{13}, C_1S_{13}; 14- C_1A_{14}, C_1S_{14}.

Fig 5.5 Mean diameter of microbeads of various formulations

Similar findings were reported by Soni *et al.* (2010) where the size of the bead increased (7.6 - 22.35 µm) with increase in alginate concentration from 2 - 8 per cent. Similarly, Kaur *et al.* (2018) reported that increase in sodium alginate concentration (from 1 - 3 %) increases the bead size based on the fact that sodium alginate binds more calcium chloride by cross linking.

According to Bashan (1986a) and Bashan and Gonzalez (1999), the most common experimental formulation for bacterial inoculants is production of macro beads with a diameter ranging from 1 - 4 mm which is used either for agricultural or environmental use which is in agreement with the present study, where the bead size of various treatments ranged from 1.27 - 1.86 mm in alginate beads and 1.28 - 2.02 mm in alginate amended skim milk beads. Similar studies were noticed by Ivanova *et al*. (2005) where they reported that survival of bacteria was enhanced by 36 per cent in beads where the size ranged from 1 to 5 mm, which is in conformity with the present findings. Likewise, Sankalia *et al*. (2005) reported that the bead size was influenced by viscosity of the alginate solution and the opening through which sodium alginate solution is allowed to pass for bead preparation. Keshavaraz *et al*. (1996) also reported that the size of alginate beads can also vary according to the instrument being used for the preparation of alginate beads. They observed that the use of different types of equipment for making alginate beads such as pressurized multi nozzle, resonance, rotating nozzle ring produced a bead diameter of 0.5 - 1.2 mm, 1.0 - 2.0 mm, 0.5 - 1.0 mm and 1.6 - 6.4 mm respectively.

5.3.1.1 *Sphericity factor (SF)*

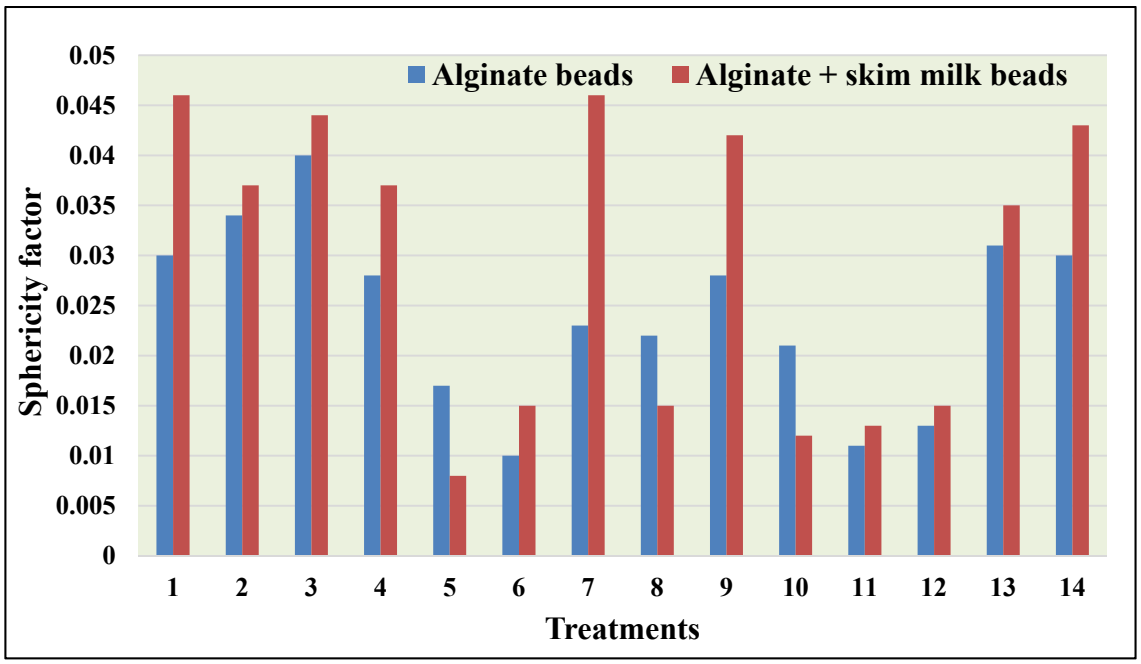
Sphericity factor is an efficient dimensionless shape indicator to study the shape of alginate drops. In this study, shape of the bead was estimated by sphericity factor by using stereomicroscope (Fig 5.6). Previous studies by Chan *et al*. (2009) found that sphericity factor varied from zero for a perfect sphere to less than 0.05 which were considered as spherical beads and this is in agreement with the present study, where all the beads formed from different treatments were generally spherical (SF < 0.05). The perfectly spherical beads of sphericity factor near to zero were formed from high

alginate (3.0%) and calcium chloride (3.0% and 3.5%) concentration after gelation possessed SF of 0.01 in both alginate and alginate amended skim milk treatments. In the earlier studies, Jeong *et al.* (2020) reported that the sphericity of beads gradually improved when the viscosity of sodium alginate solution increased from 1.2 - 3.6 per cent which is in accordance with present finding that the perfect sphere of the beads are obtained at higher sodium alginate (3.0%) concentration.

5.3.2 Bead weight and number of beads per gram of formulation

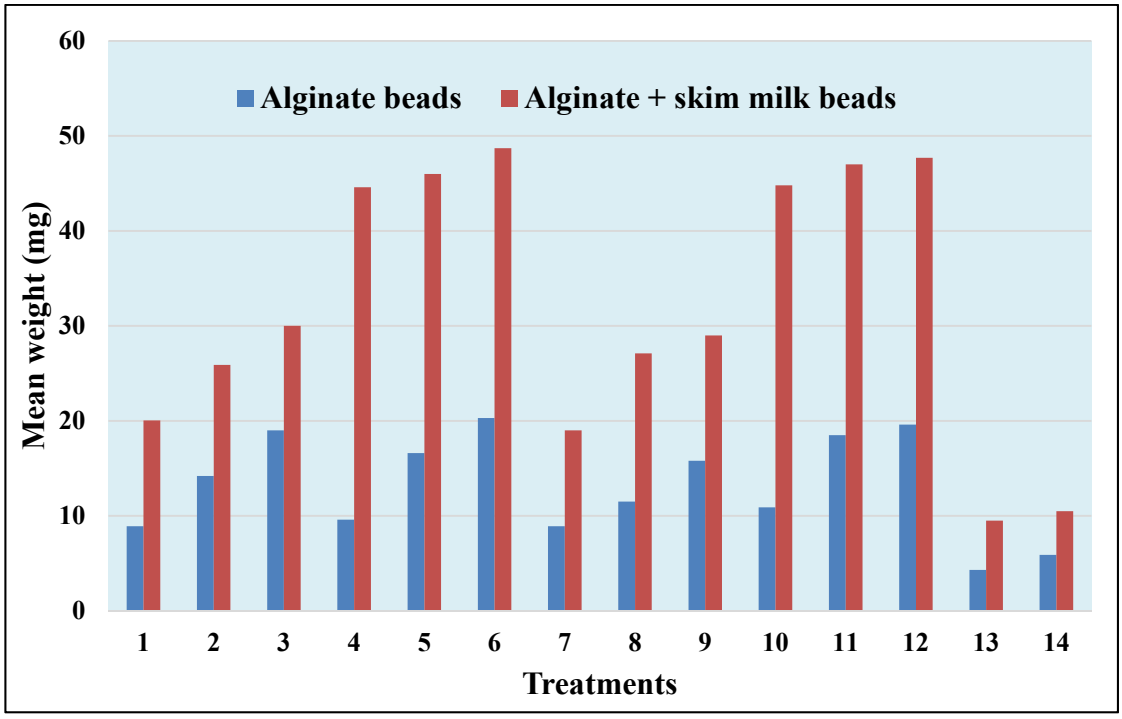
It was desirable to check the weight of bead and number of beads per gram of formulation to ascertain the optimum concentration of sodium alginate and calcium chloride required for the bead formation. Results revealed that weight of beads ranged from 8.9 - 20.3 mg in beads prepared from alginate alone whereas, 20.5 - 48.7 mg for the beads produced from alginate amended with skim milk (Fig 5.7). Maximum bead weight was obtained when the beads were produced from the combination of 3.0% sodium alginate with 3.0 and 3.5% calcium chloride respectively in both alginate and alginate skim milk beads. This study also supports the findings of Yotsuyanagi *et al*. (1987) where they reported that increase in initial alginate concentration of 1-4 per cent significantly increases the bead weight from 15.6 - 18.7 mg because of increased bead density. Similarly, Zago *et al*. (2019) reported that weight of alginate beads ranged between 17 - 38 mg. where they also stated that supplementation of additives during bead preparation caused variation in bead weight and diameter.

It was noticed that as the concentration of sodium alginate increased, lower bead numbers constituted per gram of beads. Higher number of beads were obtained for one gram of beads in lower concentration of sodium alginate (2.5%) and calcium chloride (2.5%). In the present study, it was observed that higher number of beads of 112 were noticed in T_1A_1 and 53 in T_3S_7 which constituted one gram in alginate and alginate skim milk formulation. Whereas, minimum bead number of 49 was recorded in T_1A_6 and 20 in T_3S_{11} and T_1S_6 where these were obtained in 3.0 per cent sodium alginate and 3.5 per cent calcium chloride respectively in both formulations. Control beads (without additives) showed highest bead number of 168 in alginate beads (C_1A_{14})



1-T_1A_1, T_1S_1; 2- T_1A_2, T_1S_2; 3- T_1A_3, T_1S_3 ; 4- T_1A_4, T_1S_4; 5- T_1A_5,T_1S_5; 6- T_1A_6, T_1S_6; 7- T_3A_7, T_3S_7; 8- T_3A_8, T_3S_8; 9- T_3A_9, T_3S_9; 10- T_3A_{10}, T_3S_{10} ; 11- T_3A_{11}, T_3S_{11}; 12- T_3A_{12}, T_3S_{12}; 13- C_1A_{13}, C_1S_{13}; 14- C_1A_{14}, C_1S_{14}.

Fig 5.6 Sphericity factor of microbeads of various formulations



1-T_1A_1, T_1S_1; 2- T_1A_2, T_1S_2; 3- T_1A_3, T_1S_3 ; 4- T_1A_4, T_1S_4; 5- T_1A_5,T_1S_5; 6- T_1A_6, T_1S_6; 7- T_3A_7, T_3S_7; 8- T_3A_8, T_3S_8; 9- T_3A_9, T_3S_9; 10- T_3A_{10}, T_3S_{10} ; 11- T_3A_{11}, T_3S_{11}; 12- T_3A_{12}, T_3S_{12}; 13- C_1A_{13}, C_1S_{13}; 14- C_1A_{14}, C_1S_{14}.

Fig 5.7 Mean weight of microbeads of various formulations

and 106 in alginate + skim milk beads (C_1S_{13}) compared to the remaining treatments (Fig 5.8). From the results, it was observed that increase in sodium alginate and calcium chloride concentration led to higher bead weight which led to reduced bead number that constituted per gram of alginate beads and vice versa.

5.3.4 Bead yield

The percentage yield of all treatment batches were calculated and the yield was found to be in the range of 38.35 - 74.62 per cent in batches of beads produced from alginate alone whereas, 41.17 - 74.13 per cent bead yield was obtained in batches of beads prepared from alginate amended with skim milk (Fig 5.9). Data revealed that more than 60 per cent yield of production was obtained in beads produced from 3.0 per cent sodium alginate and 3 and 3.5 per cent calcium chloride respectively in alginate and alginate amended skim milk formulations. The results were comparable with Prasad *et al.* (2012) who observed that size of all the beads produced from 3.0 per cent sodium alginate was found spherical and uniform and possesses higher percentage yield of approximately 80 per cent. Reddy *et al.* (2012) reported that the percentage yield and entrapment efficiency are proportionate to each other. Higher the percentage yields, higher will be the entrapment efficiency which is in agreement with the present study where the entrapment efficiency was higher at increased yield of production. A similar study was conducted by Patel *et al*. (2016) who reported that bead yield was increased from 24.9 - 69 per cent which was observed when bead prepared from increased concentration $CaCl_2$ solution (2-3.75%).

5.3.5 Swelling ratio of alginate beads

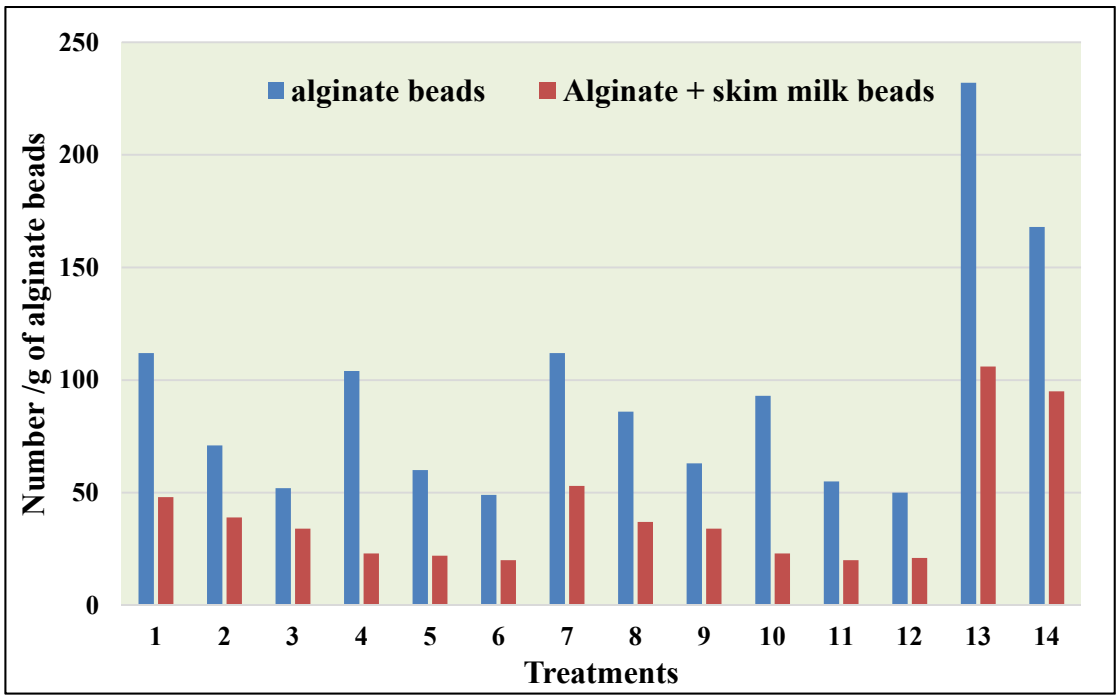
The swelling behaviour of alginate beads in two different medium *i.e*., in phosphate buffer and sterile water is illustrated in Fig 5.10a and 5.10b. A dynamic weight change of beads was observed in phosphate buffer medium compared to sterile water. The results of the study revealed that the beads prepared from treatment combination containing 3.0 per cent (w/v) alginate in 2.5 per cent solution of calcium chloride increased the swelling ratio in both phosphate buffer and water medium as

shown in Fig 5.10a and 5.10b. in both formulations. The probable reason behind this may be due to the per cent water uptake of alginate increases with increase in concentration. This is in congruence with the findings of Sathali and Varun (2012) who reported that increased swelling rate was due to the presence of hydrophilic groups on the polymer chain which interacts with the water molecule and hydrates the chain which contributes to a greater degree of swelling. At the same time, reduced swelling percentage of beads was observed in both phosphate buffer and sterile water in treatment combinations prepared from higher concentrations of cross linking agent (3.0 and 3.5% of calcium chloride) in both formulations. The findings of Patel *et al.* (2016) also supports our study as they reported that swelling ratio directly related to drug release from the beads and they also stated that the increase in calcium chloride concentration from 2 - 3.75 per cent led to decline in swelling index. This can be explained by the fact that a strong gel is formed at high concentrations of calcium chloride which resulted in slow penetration of dissolution medium into the matrix.

Yotsuyanagi *et al*. (1987) also reported that the dried alginate bead swelled to its original size in 1 hour at pH 7.0 phosphate buffer and then it gradually disintegrated and dispersed over several hours. They observed that no or minimum swelling was observed in pH 1.6 KC1-HCl buffer and distilled water which in accordance with the present as swelling percentage was found higher in phosphate buffer and minimum in sterile water. Moreover, Isıklan *et al*. (2008) reported that sodium alginate beads amended with poly (Nvinyl-2-pyrrolidone) could improve swelling degree, owing to the increase of hydrophilicity of the polymeric matrix, which is in line with the current study in which alginate beads containing PVP as one of the additives (T_1) showed slightly increased swelling ratio in both buffer and sterile water medium.

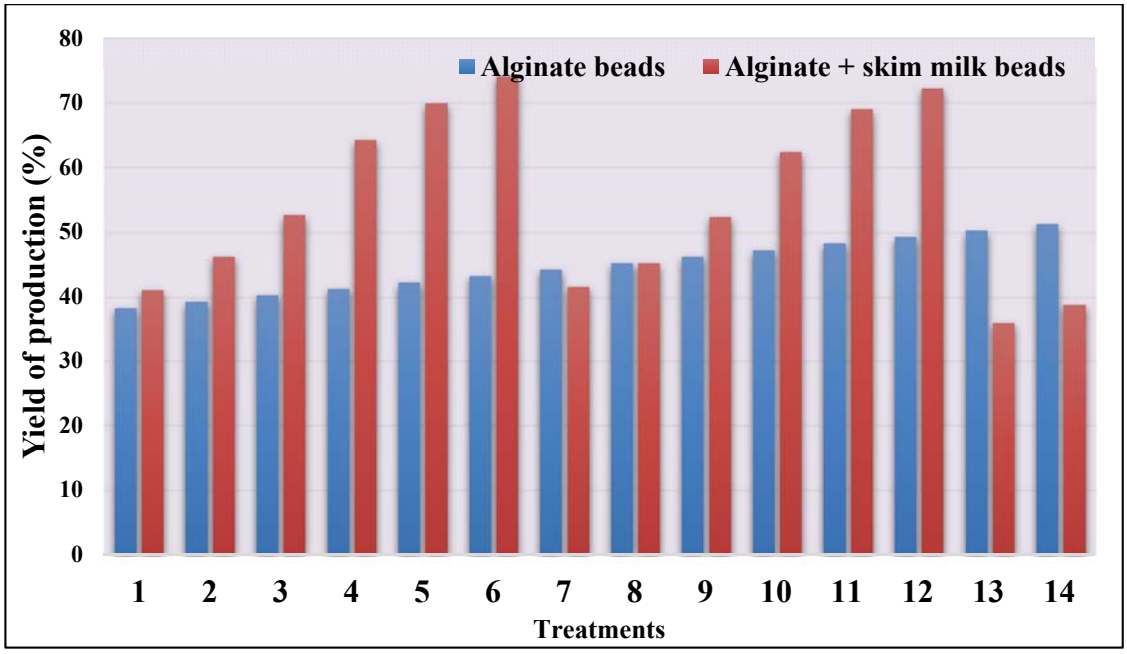
5.3.6 Water content in beads

The mean water loss from the beads was estimated to determine the percentage of water withheld by the beads. From the results shown in Fig 5.11, it was noticed that highest water content was observed in beads prepared from higher concentrations of sodium alginate (3.0%) and calcium chloride (3.5%) which was



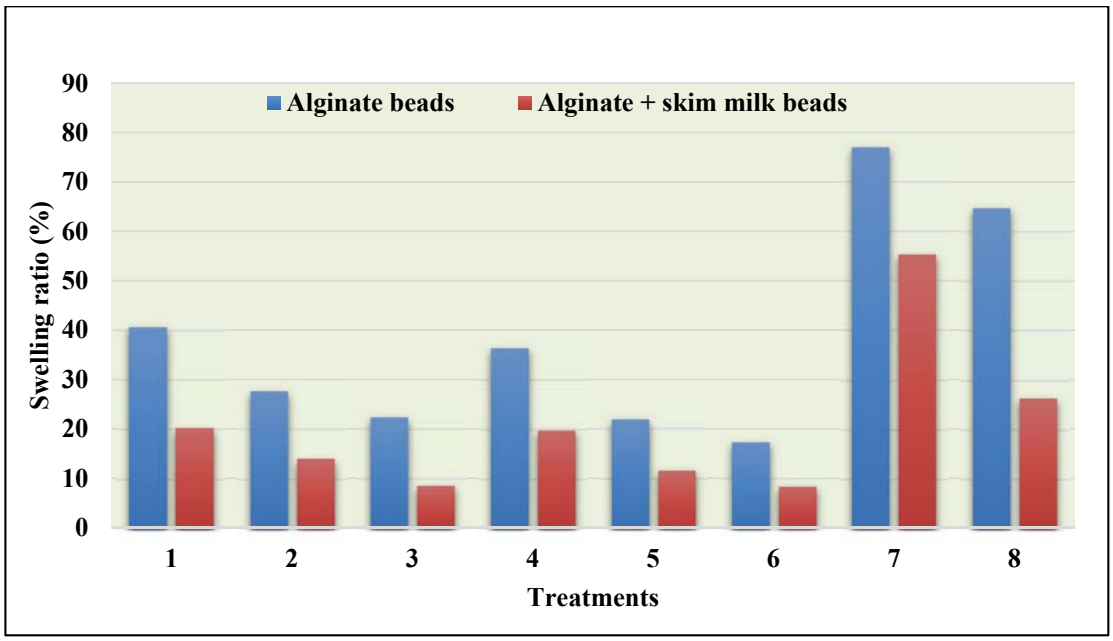
1-T_1A_1, T_1S_1; 2- T_1A_2, T_1S_2; 3- T_1A_3, T_1S_3 ; 4- T_1A_4, T_1S_4; 5- T_1A_5,T_1S_5; 6- T_1A_6, T_1S_6; 7- T_3A_7, T_3S_7; 8- T_3A_8, T_3S_8; 9- T_3A_9, T_3S_9; 10- T_3A_{10}, T_3S_{10} ; 11- T_3A_{11}, T_3S_{11}; 12- T_3A_{12}, T_3S_{12}; 13- C_1A_{13}, C_1S_{13}; 14- C_1A_{14}, C_1S_{14}.

Fig 5.8 Number of beads constituted per gram of Alginate and Alginate + skim milk beads of different treatments



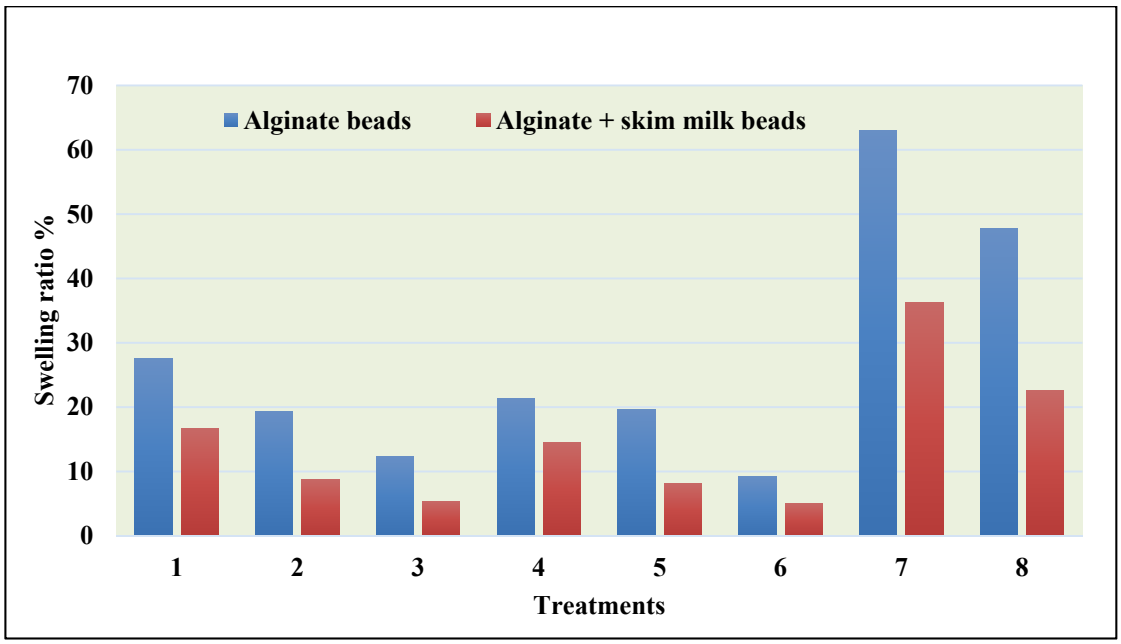
1-T_1A_1, T_1S_1; 2- T_1A_2, T_1S_2; 3- T_1A_3, T_1S_3 ; 4- T_1A_4, T_1S_4; 5- T_1A_5,T_1S_5; 6- T_1A_6, T_1S_6; 7- T_3A_7, T_3S_7; 8- T_3A_8, T_3S_8; 9- T_3A_9, T_3S_9; 10- T_3A_{10}, T_3S_{10} ; 11- T_3A_{11}, T_3S_{11}; 12- T_3A_{12}, T_3S_{12}; 13- C_1A_{13}, C_1S_{13}; 14- C_1A_{14}, C_1S_{14}.

Fig 5.9 Bead yield of Alginate and Alginate + skim milk beads of different treatments



1- T_1A_1, T_1S_1; 2-T_1A_2, T_1S_2; 3-T_1A_3, T_1S_3; 4- T_3A_4, T_3S_4; 5-T_3A_5, T_3S_5; 6-T_3A_6, T_3S_6; 7- C_1A_7, C_1S_7; 8-C_1A_8, C_1S_8

Fig 5.10a Swelling percentage of micro beads of different treatments in 0.2 M of phosphate buffer



1- T_1A_1, T_1S_1; 2-T_1A_2, T_1S_2; 3-T_1A_3, T_1S_3; 4- T_3A_4, T_3S_4; 5-T_3A_5, T_3S_5; 6-T_3A_6, T_3S_6; 7-C_1A_7, C_1S_7; 8-C_1A_8, C_1S_8

Fig 5.10b Swelling percentage of microbeads of different treatments in Sterile water

noticed in treatments T_1A_3 (65.29%) followed by T_3A_6 (61.96%) in alginate beads and T_3S_6 (44.18%) followed by T_1S_3 (39.21%) in alginate + skim milk beads. These treatments were followed by beads prepared from sodium alginate (3.0%) and calcium chloride (3.0%) where maximum water content was noticed in treatments T_1A_2 (54.50%) followed by T_3A_5 (48.20%) in alginate bead treatments and T_3S_5 (21.50%) and T_1S_2 (21.27%) in alginate + skim milk beads which are statistically on par with each other. All these above treatments enabled the survival of bacteria for longer period inside the beads compared to the other treatments whereas, the control beads showed reduced water content in the beads. The present study is in accordance with the findings of Patel *et al.* (2016) who observed that maximum water content (89.0%) was noticed in beads supplemented with higher concentration of calcium chloride solution (3.75%). A study conducted by Tous *et al.* (2014) also reported that higher the water content resulted in higher encapsulation efficiency inside the beads.

5.3.7 Shrinkage percentage of beads

It was estimated to determine the percentage of water expelled from the beads during gelation by measuring the diameter of beads immediately after preparation and also after drying process. It is obvious from the present study that reduced shrinkage percentage was observed at higher calcium chloride concentrations (3.5% followed by 3.0%) which is in line with the findings of Huang and Lin (2017) reported that alginate beads in low cross linker concentrations (1.0%) had minor mobility restrictions which resulted in higher shrinkage (25% greater shrinkage) compared to those in high calcium chloride concentrations (10%). Data revealed that shrinkage percentage ranged from 127.56 - 114.41 per cent in alginate beads and 127.10 - 114.74 per cent in alginate + skim milk beads whereas, control (without additives) showed higher shrinkage percentage compared to the remaining treatments (Fig 5.12).

It was noted that reduced shrinkage percentage was observed in treatments containing higher concentration of sodium alginate (3.0%) and calcium chloride solutions (3.5% and 3.0%). Martinsen *et al.* (1999) opined that the physical properties of beads depended strongly on the concentration of the polymers and beads with the highest weight possessed higher mechanical strength, lowest shrinkage and led to

higher stability which is most advantageous for the cell immobilization. Similarly, Rassis *et al.* (2002) reported that reduced shrinkage percentage from 62.7 - 11.0 per cent was observed with increasing sodium alginate concentration from 1 - 2.5 per cent amended with different fillers or additives. A study conducted by Abdellatif *et al.* (2016) reported that less shrinkage percentage was observed with bigger size of beads which is again in line with the present study where as the bead size was higher in treatments, reduced shrinkage percentage was observed in both alginate and alginate amended skim milk formulations.

5.4 EVALUATION OF SHELF LIFE OF *P. fluorescens* ENCAPSULATED BEADS UNDER *in vitro* CONDITIONS

5.4.1 Rate of release of bioagent from beads

The release profile of entrapped *P. fluorescens* from alginate and alginate + skim milk beads in phosphate buffer (pH 7.0) and sterile water was estimated at 30 mins, 1 h, 2 h and 24 h which is represented in Fig 5.13 and 5.14. From the data, it was observed that rate of release of bioagent from beads was higher in phosphate buffer compared to sterile water. This in in agreement with the findings of Patel *et al.* (2016) who reported that percentage release of drug from the beads was higher in 0.2 M phosphate buffer (pH 7.0). Similar studies by Dainty *et al.* (1986) have shown that high degree of swelling was observed when alginate beads are exposed to phosphate buffer at pH greater than 5.

The results of the study showed that in alginate beads, lower bacterial release was observed in treatments T_1A_3 (2.0×10^{16} cfu g^{-1}) supplemented with higher concentration of sodium alginate (3.0%) and calcium chloride (3.5% followed by 3.0%) respectively in phosphate buffer medium and in T_1A_6 (0.66×10^{16} cfu/g) in sterile water. Which, in alginate + skim milk beads, the beads completely dissolved in phosphate buffer medium which led to higher bacterial release in treatments T_1S_3 (41.66×10^{32} cfu g^{-1}) and T_3S_6 (44.66×10^{32} cfu g^{-1}) in phosphate buffer medium whereas, a slow and constant bacterial release was observed in sterile water T_1S_3 (13.33×10^{24} cfu g^{-1}) at the end of 24 h of dissolution studies.

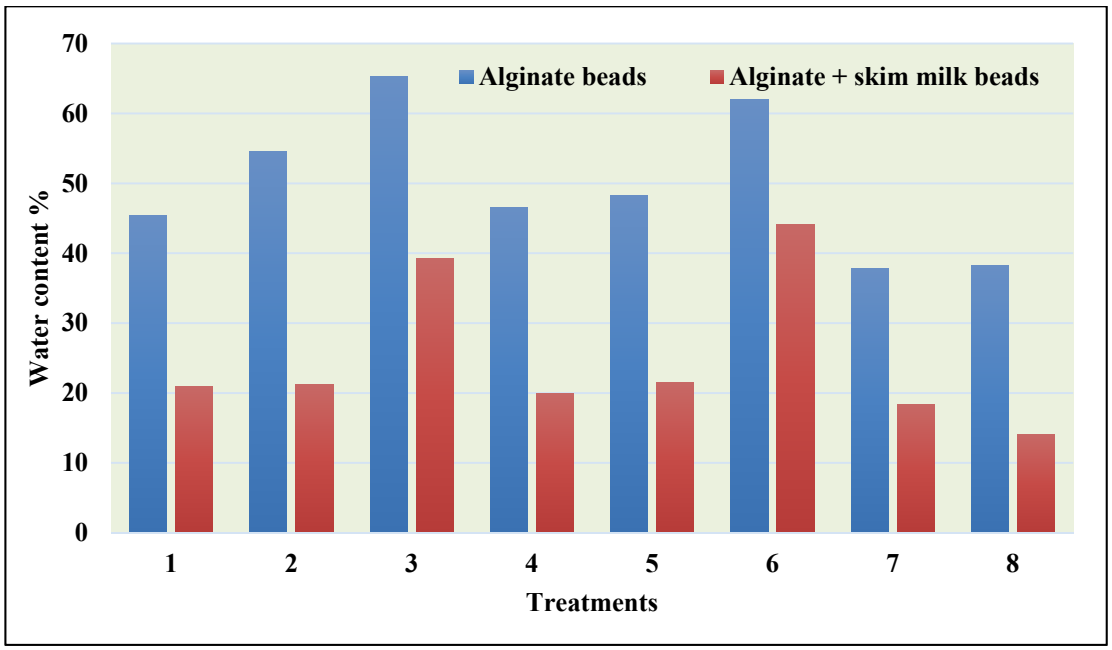


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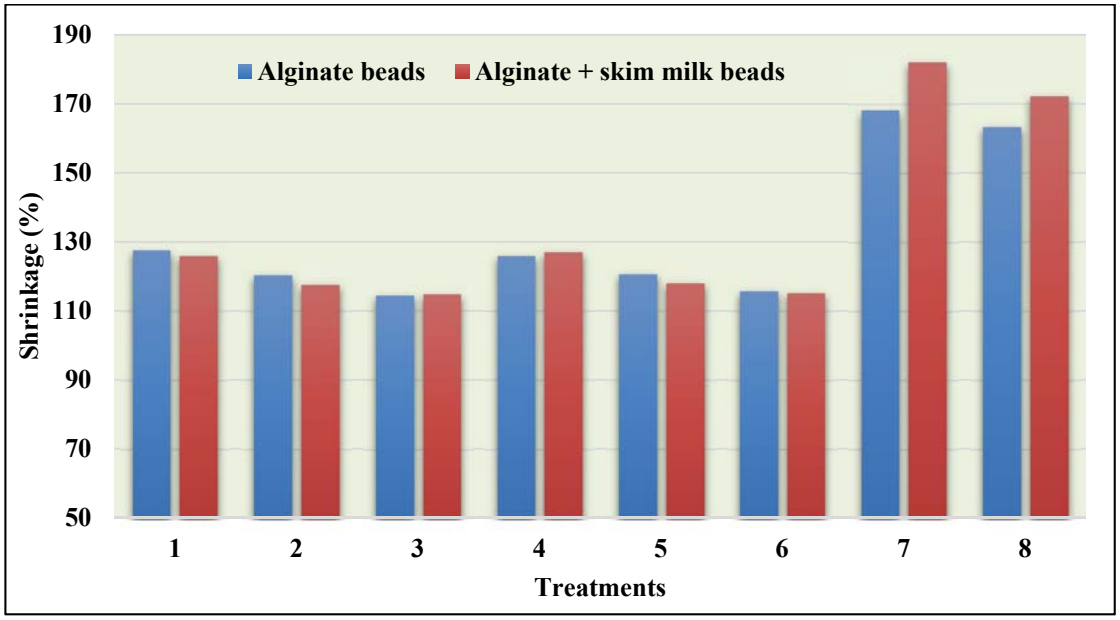
Fig 5.11 Percentage of water content in microbeads of different treatments



11- T_1A_1, T_1S_1; 2-T_1A_2, T_1S_2; 3-T_1A_3, T_1S_3; 4- T_3A_4, T_3S_4; 5-T_3A_5, T_3S_5; 6-T_3A_6, T_3S_6; 7-C_1A_7, C_1S_7; 8-C_1A_8, C_1S_8

Fig 5.12 Shrinkage percentage of microbeads of different treatments

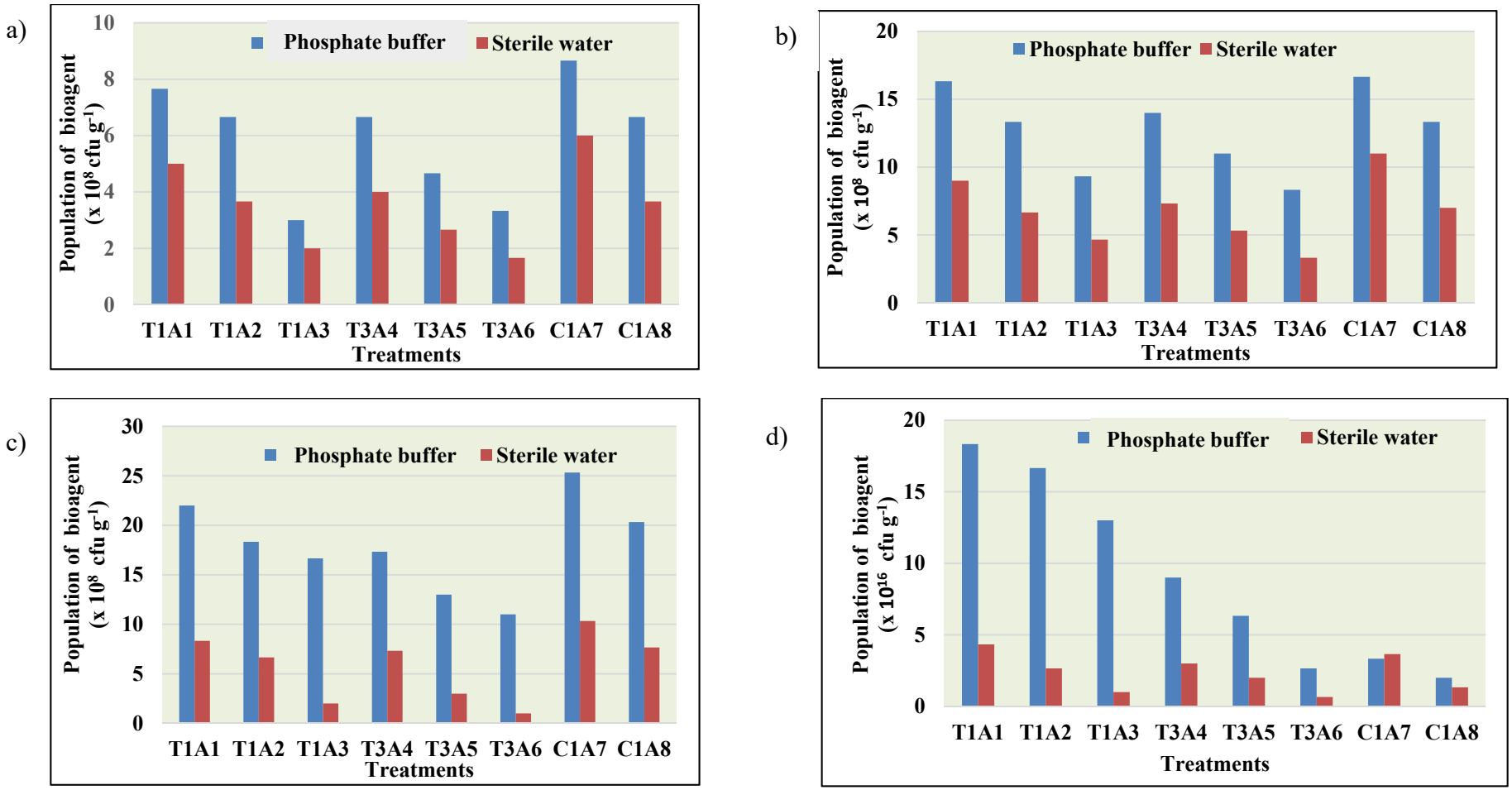


Fig 5.13 ***In vitro*** **release of bioagent from alginate beads in phosphate buffer and sterile water medium at a) 30 min dissolution b)1 h dissolution c) 2 h dissolution d) 24 h dissolution**

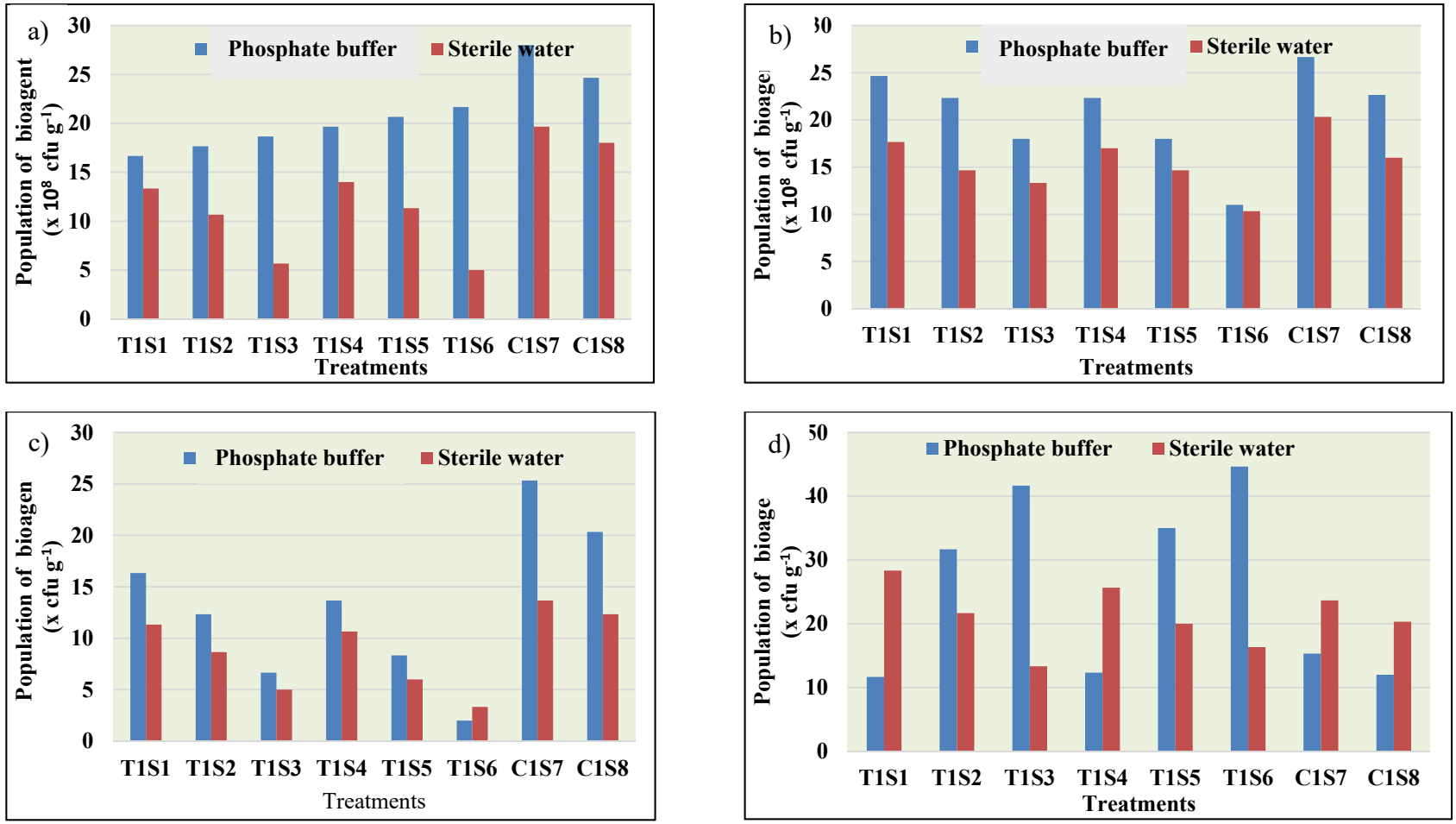


Fig 5.14 *In vitro* release of bioagent from alginate amended skim milk beads in phosphate buffer and sterile water medium at a) 30 min dissolution b)1h dissolution c) 2h dissolution d) 24h dissolution

The results are comparable with the studies of Mandal *et al*. (2010) who observed that rate of release of bioagent is lower in higher concentration of sodium alginate (>2.5%) and calcium chloride (3.0%) solution. They also reported that the release of drug from alginate beads is dependent on the penetration of the dissolution medium into the beads, swelling and dissolution of the alginate matrix and the dissolution of the drug subsequent to leaching through the swollen matrix. Patel *et al*. (2016) also reported that the rigid bead produced by higher alginate polymer mixture reduced gel erosion by decreasing the calcium ion diffusion from the cross-linked calcium alginate matrix. Several other studies have also shown that alginate beads amended with CMC, pectin etc promotes mechanical strength to the beads, robustness and enhance the drug release properties (Kim *et al.*, 2012 and Fontes *et al.*, 2013). Moreover, supplement of additives like mannitol, PVP, PEG, CMC and tween-80 during bead preparation also have a positive effect on the rate of release of biocontrol agents compared to control beads.

5.4.2 Population density *P. fluorescens* per bead

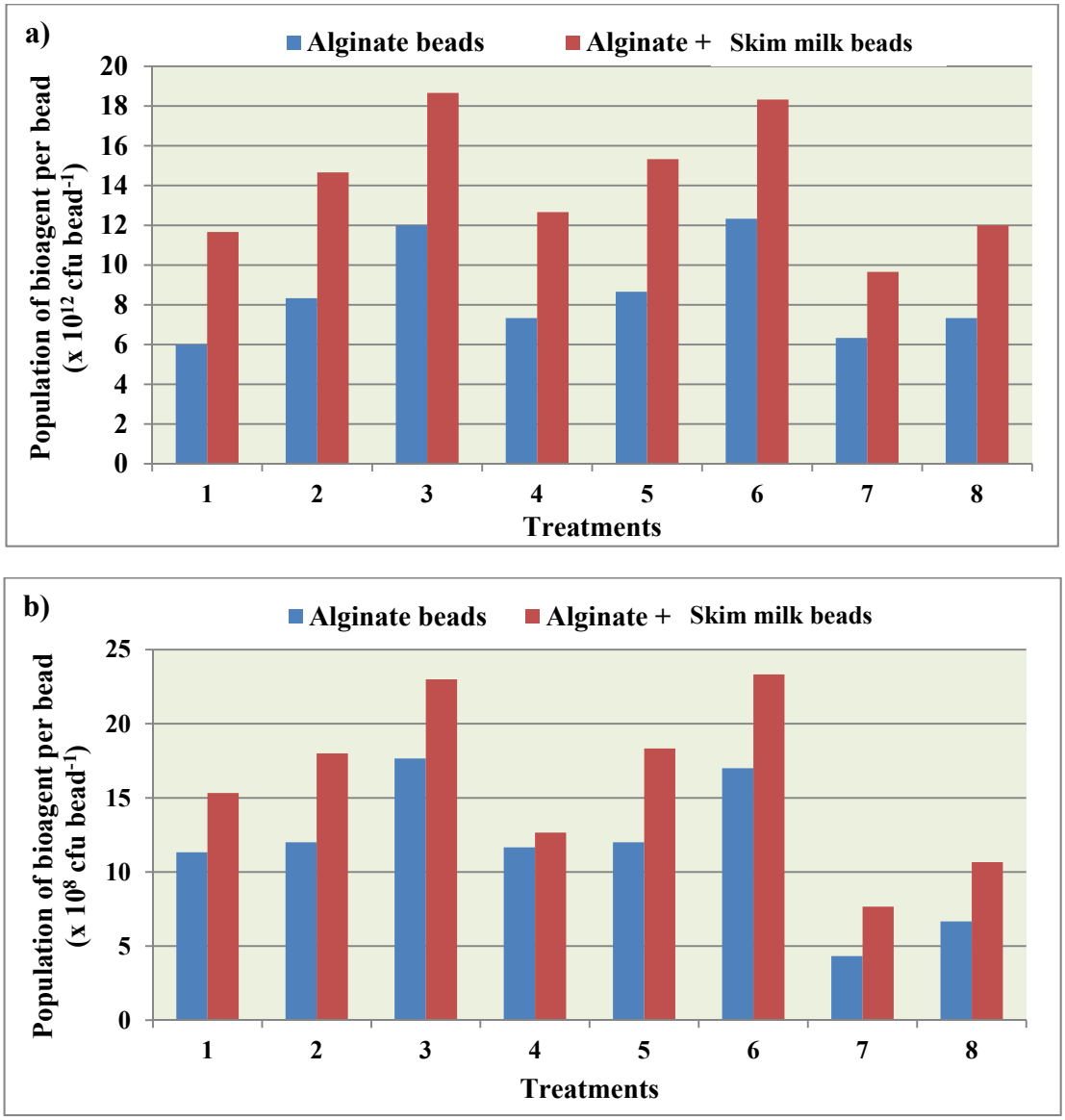
The population of *P. fluorescens* entrapped in one bead was estimated by serial dilution and plating technique and is depicted in the Fig 5.15. From the data, it was observed that alginate amended skim milk beads dissolved in 24 h whereas, alginate beads took 48 h to dissolve in buffer solution. Observations revealed that higher bacterial entrapment was noticed in alginate and alginate amended skim milk beads prepared from 3.0 per cent sodium alginate and 3.0 and 3.5 per cent calcium chloride compared to other combinations. Higher bacterial entrapment of alginate bead was observed in treatments T_1A_3 (17.66 x 10^8 cfu/bead) and T_3A_6 (17.0 x 10^8 cfu/ bead), while, in alginate + skim milk bead, higher bacterial release was recorded in treatments T_3S_6 (23.33x 10^8 cfu/bead) and T_1S_3 (23.0 x 10^8 cfu/ bead) one month after bead production. However in control treatments it was noticed that, the alginate beads dissolved much faster than alginate amended skim milk beads. The results are in accordance with the findings of Bashan (1986a) where he observed alginate beads without skim milk showed rapid solubilization within 30 min compared to alginate

amended skim milk beads. The variation in solubilisation in control and other treatments might be due to the presence of additives during bead preparation.

5.4.3 Evaluation of shelf life of entrapped bioagents in alginate and alginate + skim milk beads

For commercial use, a microbial inoculum should be supported by an appropriate formulation preventing a rapid decline of introduced microorganisms and with an extended shelf life. In the present investigation, alginate and alginate amended skim milk beads are prepared and the population of *P. fluorescens* in both beads were evaluated at monthly intervals by serial dilution and plating technique where the results are presented in Fig 5.16a, 5.16b and 5.16c. It was observed that in general, the shelf life of alginate beads was higher compared to alginate amended skim milk beads because skim milk amended alginate beads got dehydrated and lost their quality after one month of preparation. The study was in line with the findings of Szczech and Maciorowski (2016) who reported that supplement of skim milk during bead preparation containing *Burkholderia cepacia*, *Bacillus* sp. and fungus *Trichoderma virens* reduced quality of the beads. Moreover, they also reported that skim milk did not influence the viability of entrapped microorganisms and their release in soil. The findings of Bashan *et al.* (2002) also revealed that alginate beads produced with and without skim milk were capable to release the bioagent at the rate of 10^5 cfu g^{-1} and 10^7 cfu g^{-1} respectively. Studies of Trivedi *et al.* (2005) are also in line with the present investigation who reported that maximum colonise of *Bacillus subtilis* and *Pseudomonas corrugate* were recovered from alginate beads followed by alginate beads supplemented with skim milk formulations even after 180 days of storage. Archana and Brahmaprakash (2014) also observed that survival rate of *Azotobacter chroococcum, Acinetobacter sp* and *Pseudomonas fluorescens* was higher in alginate formulations followed by alginate +skim milk, alginate + charcoal and alginate +lignite formulations.

From the Fig. 5.16a, 5.16b and 5.16c it is very clear that the population of bacteria inside alginate beads decreased gradually. Earlier studies by Bashan (1998)



*MAP –Month after production

1- T_1A_1, T_1S_1; 2-T_1A_2, T_1S_2; 3-T_1A_3, T_1S_3; 4- T_3A_4, T_3S_4; 5-T_3A_5, T_3S_5; 6-T_3A_6, T_3S_6; 7-C_1A_7, C_1S_7; 8-C_1A_8, C_1S_8

Fig 5.15 Population of *P. fluorescens* released per bead at a) 0 MAP b) 1 MAP

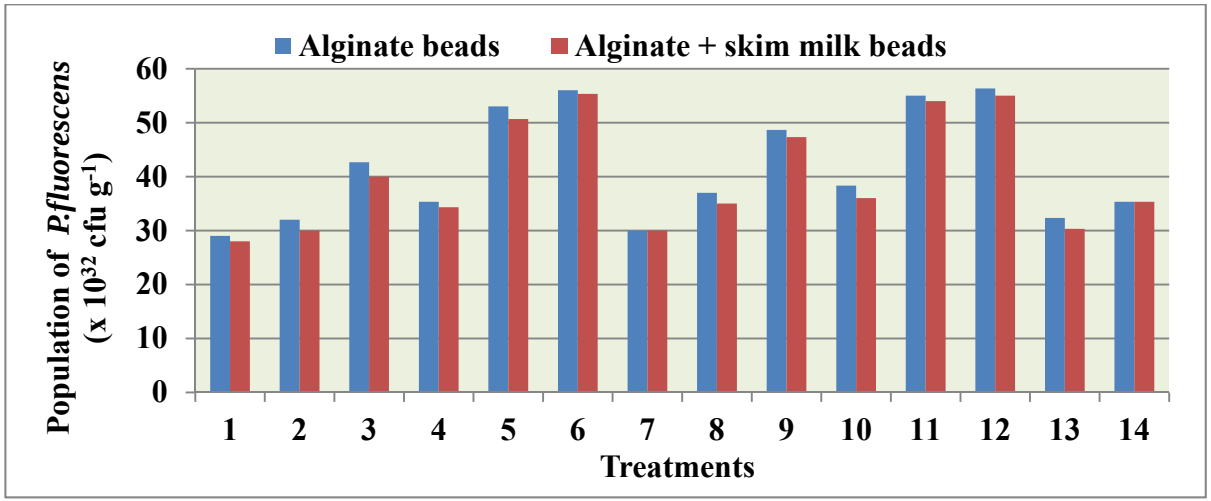
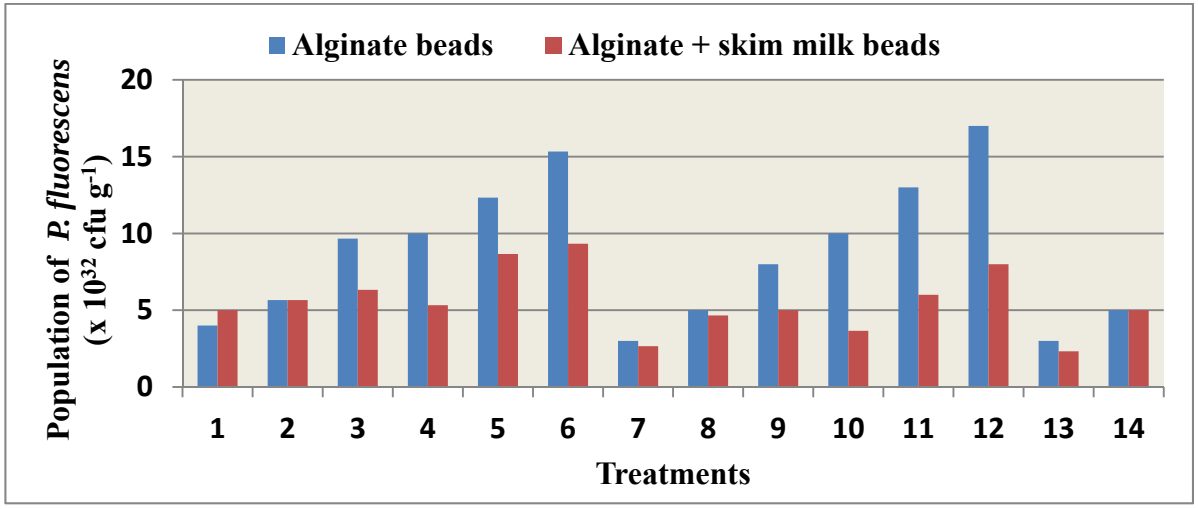
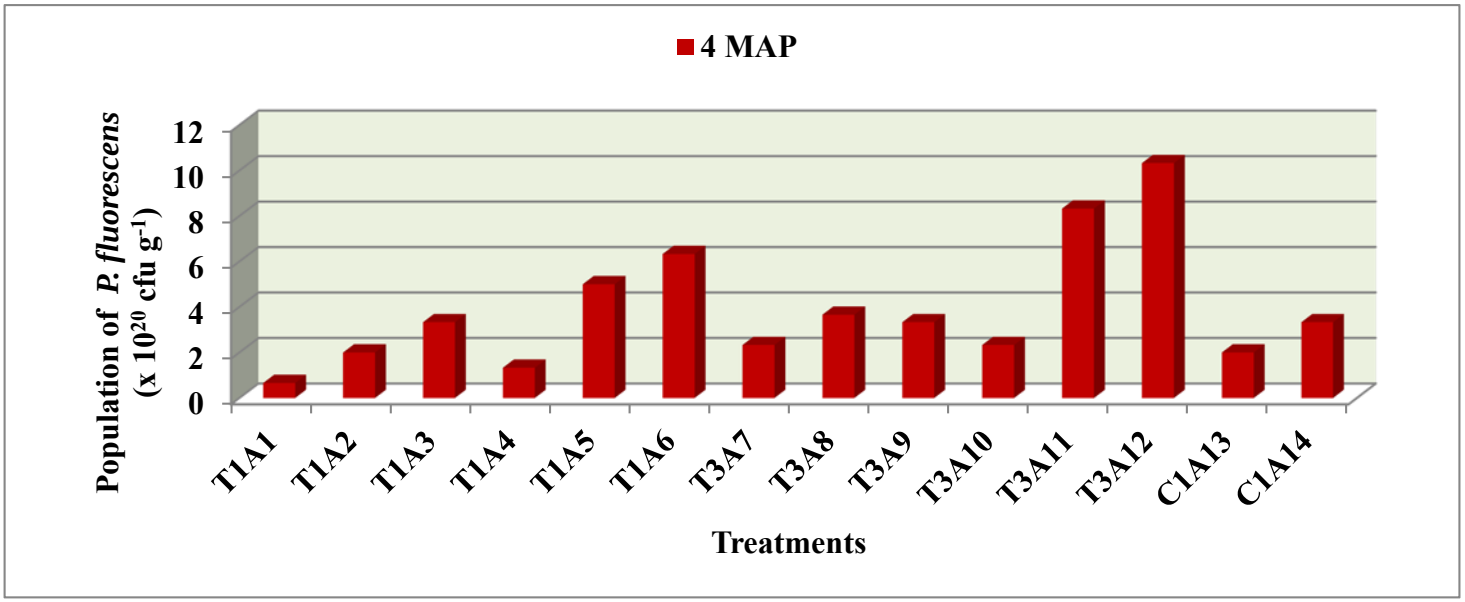


Fig 5.16a Population of *P. fluorescens* in alginate and alginate + skim milk beads at monthly intervals at 0 MAP



1-T_1A_1, T_1S_1; 2- T_1A_2, T_1S_2; 3- T_1A_3, T_1S_3 ; 4- T_1A_4, T_1S_4; 5- T_1A_5,T_1S_5; 6- T_1A_6, T_1S_6; 7- T_3A_7, T_3S_7; 8- T_3A_8, T_3S_8; 9- T_3A_9, T_3S_9; 10- T_3A_{10}, T_3S_{10}; 11- T_3A_{11}, T_3S_{11}; 12- T_3A_{12}, T_3S_{12}; 13- C_1A_{13}, C_1S_{13}; 14- C_1A_{14}, C_1S_{14}.

Fig 5.16b Population of *P. fluorescens* in alginate and alginate + skim milk beads at monthly intervals at 2 MAP



*MAP- Month After Preparation

Fig 5.16c Population of *P. fluorescens* in alginate beads at monthly intervals at 4 MAP

and Sivakumar *et al*. (2014) also reported that the population of the inoculated bacteria declines progressively over time, preventing the accumulation of a bacterial pool in the rhizosphere sufficient to promote beneficial effects. These findings are also in line with the present study where the initial population of bacteria in the alginate beads were significantly higher when compared with the population of microorganisms after four months of storage.

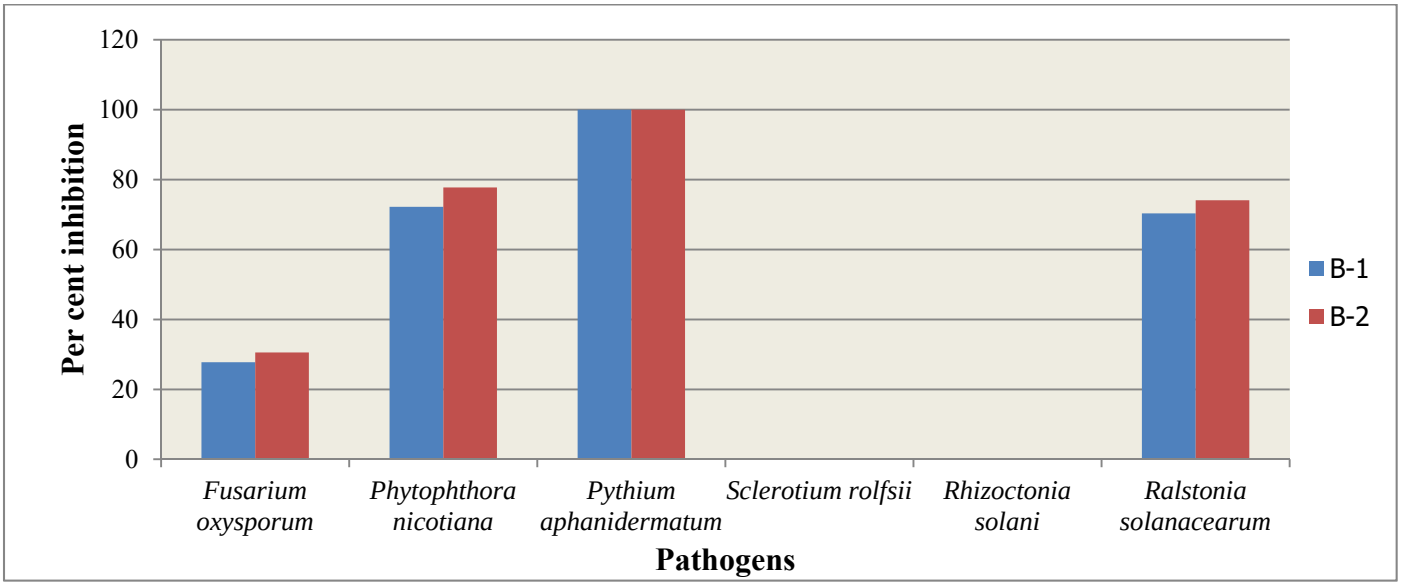
In this study, alginate beads prepared from sodium alginate (3.0%) and calcium chloride (3.5% and 3.0%) respectively holds higher bacterial population in treatments T_3A_{12} (10.33 x 10^{20} cfu g^{-1}) followed by treatments T_3A_{11} (8.33 x 10^{20} cfu g^{-1}), T_1A_6(6.33 x 10^{20} cfu g^{-1}) and T_1A_5 (5.0 x 10^{20} cfu g^{-1}) after four months of preparation which is in accordance with the findings of El-Kamel *et al*. (2003) where they observed that drug loading was found to be directly proportional to polymer concentration. Similar reports by Galiana *et al*. (1994) observed that alginate gel network supported the bacterial densities in stable numbers even after five months of storage. Sangeetha and Stella (2012) also noticed that higher population of *P. fluorescens* (63.89 x10^8 cfu g^{-1}) was maintained in alginate beads followed by pressmud, vermiculite and lignite based carriers after six months of storage. Likewise, Sivakumar *et al*. (2014), reported that high viability and continuous release of cells from the bead for one week was noticed and encapsulated bacteria maintained its population for five months with minimal cell loss at storage

The investigation on shelf life also revealed that the control beads (without additives) showed reduced population of *P. fluorescens* inside the beads compared to other treatments which is supported by the findings of Schoebitz and Belchi (2016) where they observed that alginate gel matrix has the capability for extending the shelf life of microbial strain under biotic and abiotic stressful conditions especially when the beads are supplemented with nutritional additives during encapsulation which in turn maintained the viable population of the bioagent for a longer period.

4.5 *In vitro* inhibition of formulation against major soil borne plant pathogens

Nowadays, the application of biocontrol agent is encouraged in agriculture because of their potential to increase crop production in an environmental friendly way. However, the major factor determining the success of the developed formulation of a biocontrol agent is dependent on the microorganism present in the formulation which are to be effectively delivered into the environment to control wide range of plant pathogens (Prasad and Rangeshwaran, 2000). Hence, *in vitro* studies are inevitable so as to study the interaction between the biocontrol agent and the plant pathogens. After the development of the formulation, the study was continued further to evaluate two best bead formulations against soil borne pathogens like *Fusarium oxysporum, Phytophthora nicotiana, Pythium aphanidermatum, Sclerotium rolfsii and Rhizoctonia solani*. From the data, it was observed that the fungal pathogen *Pythium aphanidermatum* was effectively controlled (100 per cent) by both the bead formulations (B-1 and B-2) (Fig 5.17). The results were confirmed by the reports of Russo *et al*. (2001) who observed that alginate microbeads of *P. fluorescens* F113 LacZY inhibited the hyphal growth of both *Pythium ultimum* and *R. solani* under *in vitro* conditions. The results obtained were also comparable with the findings of Muthukumar *et al.* (2010) who reported that talc based formulation of *P. fluorescens* reduced the mycelial growth of *Pythium aphanidermatum* (26mm) under *in vitro* conditions. Wiyono *et al*. (2008) also reported the antagonistic activity of *P. fluorescens* B5 formulated in bentonite, diatomaceous earth and peat with 60 per cent moisture significantly reduced the *in vitro* growth of *P. ultimum* var. *ultimum* in dual culture method.

It was also noticed that alginate beads of *P. fluorescens* prepared from two best treatments (B-1 and B-2) inhibited the mycelial growth *Phytophthora nicotiana* by 72.22 and 77.77 per cent followed by inhibition of *Ralstonia solanacearum* by 70.36 and 74.07 per cent and *Fusarium oxysporum* by 27.77 and 30.55 per cent respectively. The results were comparable with the findings of Gallardo *et al*. (1989) where they reported that application of liquid suspension of *Pseudomonas* strain BC-8 inhibited the incidence of *R. solanacearum* under *in vitro* conditions. Niranjana *et al.* (2009) where



B-1: T1 (mannitol + PVP + CMC + tween -80) + sodium alginate (3%) + $CaCl_2$ (3%)
B-2: T3 (mannitol + PEG + CMC + tween -80) + sodium alginate (3%) + $CaCl_2$ (3%)
C : control.

Fig 5.17 *In vitro* evaluation of alginate beads of *Pseudomonas fluorescens* against soil borne pathogens

they reported that talc and sodium alginate formulations of mass multiplied *P. fluorescens* were effective against *Fusarium* wilt under *in vitro* and greenhouse conditions. Chawla and Gangopadhyay (2009) reported that the farm yard manure, vermicompost, and mustard cake based formulation *P. fluorescens* showed maximum inhibition of mycelial growth of *Fusarium oxysporum* f. sp. *cumini* was under *in vitro* conditions.

Manikandan *et al*. (2010) observed the inhibitory effect of the liquid formulation of *P. fluorescens* Pf1 on the growth *F. oxysporum* f. sp. *lycopersici* with a least mycelial growth of 54 mm in dual plate method. Likewise, Zegeye *et al.* (2011) tested the biocontrol potential of cell suspensions of strains *Pseudomonas fluorescens*-Bak150 which inhibited the mycelial growth of *Phytophthora infestans* by 88 per cent under *in vitro* conditions. Similarly, Al-Waily *et al*. (2018) observed higher inhibition percentage of 55.55 per cent against *F. solani* with powder formulation of *P. fluorescens* upto six months of storage.

Moreover, Singh and Jagtap (2017) reported that the antagonistic bioagent *Pseudomonas fluorescens* resulted in maximum inhibition of the *Ralstonia solanacearum* with an inhibition zone of 24.33 mm under *in vitro* conditions. Soesanto *et al.* (2019) reported that granular formulation of *P. fluorescens* P60 bacteria inhibited the growth of *R. solanacearum* under *in vitro* conditions.

However, in the present study, no inhibition on the growth of *Sclerotium rolfsii* and *Rhizoctonia solani* was observed under *in vitro* conditions. The results obtained were in contrast with the findings of Russo *et al.* (2005) who observed that application of alginate beads of *P.fluorescens* strain 134 promoted antifungal activity against *Rhizoctonia solani*. Similar studies by Dhanya and Adeline (2014) also noticed that alginate beads of *P. fluorescens* showed higher percentage inhibition of *Rhizoctonia solani* (39.2%) compared to that of control plate under *in vitro* conditions. The present study is also in agreement with the findings of Manu (2012) who observed that *P. fluorescens* did not show any inhibition of mycelial growth of *Sclerotium rolfsii* as the pathogen grew over the bioagent. Sab *et al.* (2014) also reported that *Pseudomonas*

fluorescens showed the least mycelial inhibition of *Sclerotium rolfsii* compared to *Trichoderma harzianum* under *in vitro* conditions.

The recurring cost involved in the production of 1 Kg bead of *P. fluorescens* is INR 282.39 where a benefit cost ratio of 3.1:1 was calculated over talc based formulation.

Hence, recollecting the results of the present study, alginate beads encapsulated *P. fluorescens* were prepared from the King's B broth amended with adjuvants of two best treatments (T_1- mannitol +PVP +CMC +tween-80 and T_3- mannitol +PEG +CMC +tween-80) respectively. Based on the parameters standardised, it was clear that alginate beads were more effective than alginate amended skim milk beads and beads after secondary multiplication. Moreover, alginate beads formed each with 3.0 per cent concentration of sodium alginate solution and calcium chloride with 60 min of curing time and 9-15 cm of falling distance produced perfectly spherical beads having a bead diameter of above 1.70 mm and the bead weight ranged from 16.6-18.5 mg having higher bead yield, reduced swelling and shrinkage percentage which holds higher water content that facilitates higher survival and slow release of the bioagent from the beads for a longer period (four months) of time. From the *in vitro* antagonistic study, it is clear that alginate based formulation (B-1 and B-2) proved to be promising as they inhibited soil borne pathogens *viz*., *Pythium aphanidermatum* (100%) followed by *Phytophthora nicotiana* (72.22 and 77.77%) *Ralstonia solanacearum* (70.36 and 74.07%) and to some extent inhibition of *Fusarium oxysporum* (27.77 and 30.55%). However, no inhibition was observed on the growth of *Sclerotium rolfsii* and *Rhizoctonia solani* under *in vitro* conditions.

It is very well known that among the various carriers, alginate beads have been extensively used in laboratory studies for inoculum formulation and are compatible with industrial scaling up. One important advantage for immobilized microbes is the good storage capacity for practical application. Furthermore, another advantage of the microbeads is the possibility to produce them dry where the bacteria are inactive but alive and such dry preparations are preferable since their activities are needed only after

seed germinations and the decomposition of beads. A minor disadvantage of microbead production, however, is that the entrapping procedure kills a large number of the bacteria because of crosslinking of the alginate- calcium complex with the bacterial cell wall. However, it was proved that encapsulated bacteria better survive in soil than free cells. Cells are gradually released from the capsules by matrix degradation; therefore they are more protected against destructive factors than not coated microorganisms. But, there is little known about the influence of microcapsules on the pathogens population in soil and hence further studies are needed. Moreover, the study should be complemented with multilocational field trials to prove the efficacy of the developed formulation.

In conclusion, a simple is being proposed in this study to produce microbeads useful for application of *Pseudomonas fluorescens* to agricultural plants which is less bulky, biodegradable, has an ease of handling and is non-toxic in nature. Moreover, this formulation enables slow and controlled release of the biocontrol agent and thus, maintains a bacterial population for relatively longer period. However, further research should be conducted to study its shelf life and performance under field conditions.

SUMMARY

6. SUMMARY

Biological control is an eco-friendly and cost effective approach for management of plant diseases in agriculture. Inoculation of plants with microorganisms has been practiced for several decades. *Pseudomonas fluorescens* is one among the most commonly used biocontrol agent against wide range of plant pathogens. The success of microbial inoculant technology is dependent on the development of suitable formulations which has extended shelf life, protects bacterial cells against many environmental stresses and its effectiveness against plant pathogens. However, only few methods of inoculation were used like the application of bacteria in liquid broth or dried bacterial cultures on seeds, use of various organic inoculants and peat based formulations. In order to overcome the disadvantages of these formulations, there is a need for development of an alternate formulation which results in extended shelf-life and controlled microbial release from the formulation thus, enhancing their application efficacy. In this context, the present research work is therefore focused on 'Alginate based encapsulation of *Pseudomonas fluorescens* for management of soil borne pathogens' with an objective to prepare sodium alginate bead based formulation of *Pseudomonas fluorescens* and to evaluate its efficacy under *in vitro* conditions.

1. *Pseudomonas fluorescens*, the reference culture of KAU was used as the biocontrol agent for preparation of alginate beads. To improve the shelf life of alginate beads, King's B broth was supplemented with adjuvants *viz*., sugar source (mannitol and trehalose), wetting agent (PVP and PEG), adhesive (CMC and liquid paraffin) and surfactant (tween-80)

    - The broth amended with selected additives was inoculated with a loopful of *P. fluorescens* and were incubated at room temperature for 48h and the treatments were subjected to serial dilution and plating technique at monthly intervals upto nine months for enumeration of *P. fluorescens* in order to evaluate the best additive combination improving the shelf life of *P. fluorescens*.
    - After nine months of observation, maximum population of *P. fluorescens* was recorded in treatment T_1 (1.33×10^8 cfu ml^{-1}) and T_3 (1.66×10^8 cfu ml^{-1}).

Hence, the best two treatments T_1 (mannitol +PVP +CMC +tween-80) and T_3 (mannitol +PEG +CMC +tween-80) were selected for the preparation of beads.

- The beads were prepared from the above selected treatments as per the protocol of Bashan *et al*. (2002) with modifications. The beads were prepared in three different batches *viz*., beads from alginate alone, beads from alginate amended with skim milk and beads after secondary multiplication.

2. Different parameters were analysed for mass production of alginate beads in order to develop an effective formulation

    - Spherical and rigid beads were formed with alginate alone and alginate amended skim milk at 2.5 and 3.0 per cent concentration of sodium alginate with each of 2.5, 3.0 and 3.5 per cent concentration of calcium chloride solution.
    - The gelation period in $CaCl_2$ solution was standardised at 60 min for both alginate and alginate amended skim milk beads.
    - Shape of the beads depends on the distance between dripping tip and surface of $CaCl_2$ solution (air column). Spherical beads were obtained when optimum height of air column was maintained from 9 to 15 cm during bead preparation.
    - Diameter and shape of beads were determined using a stereomicroscope. Maximum diameter and perfectly spherical alginate and alginate amended skim milk beads were produced at increased concentration of sodium alginate (3%) and calcium chloride (3.5% and 3.0%) solution.
    - Maximum bead weight and minimum bead numbers per gram of bead were obtained when the beads were produced from the combination of 3.0 per cent sodium alginate with 3.0 and 3.5 per cent calcium chloride respectively in both alginate and alginate skim milk formulations.
    - Bead yield of more than 60 per cent was obtained in beads produced from 3.0 per cent sodium alginate and 3.0 and 3.5 per cent calcium chloride respectively in alginate and alginate amended skim milk formulations.

- Higher concentration of sodium alginate (3.0%) and calcium chloride (3.5% and 3.0 %) during bead preparation showed reduced swelling percentage, holds higher per cent of water content inside beads and showed lowest shrinkage percentage which resulted in slow release of the entrapped bioagent from the formulation.

3. Shelf life of *P. fluorescens* encapsulated in alginate alone, alginate amended skim milk beads and beads after secondary multiplication were evaluated.
    - Rate of release of bacteria from alginate and alginate + skim milk beads was higher in phosphate buffer compared to sterile water under *in vitro* conditions.
    - Alginate amended skim milk beads completely dissolved in phosphate buffer medium after 24 h of dissolution. However, slow and constant bacterial release was observed from alginate beads compared to alginate amended skim milk beads in phosphate buffer medium.
    - The release-profile of beads prepared from higher concentrations of sodium alginate (3%) and $CaCl_2$ (3% and 3.5%) in treatments depicted a slower rate and lower extent of bacterial release compared to other treatments.
    - The two best treatments T_1 (mannitol + PVP + CMC + tween-80) and T_3 (mannitol + PEG + CMC + tween-80) in alginate, alginate + skim milk beads and beads after secondary multiplication were selected and were monitored upto four months of storage.
    - Maximum population of *P. fluorescens* was observed in alginate beads prepared from sodium alginate (3%) and calcium chloride (3.5% and 3.0%) respectively in treatments T_3A_{12} (10.33×10^{20} cfu g^{-1}) followed by treatments T_3A_{11} (8.33×10^{20} cfu g^{-1}), T_1A_6 (6.33×10^{20} cfu g^{-1}) and T_1A_5 (5×10^{20} cfu g^{-1}) respectively after four months of preparation.
    - Alginate + skim milk beads produced from sodium alginate (3.0%) and calcium chloride (3.0% and 3.5%) holds higher bacterial population in treatments T_1S_6 (9.33×10^{32} cfu g^{-1}) followed by T_1S_5 (8.66×10^{32} cfu g^{-1}) and T_3S_{12} (8.0×10^{32} cfu g^{-1}). However, shelf life of alginate + skim milk beads were poor compared to alginate beads as they were subjected rapidly to

dehydration and hence got deteriorated as it lost its entire spherical structure after one month of preparation.

- After secondary multiplication of alginate and alginate amended skim milk beads, there was a two fold increase in bacterial population entrapped inside alginate beads compared to beads without secondary multiplication. But it was noticed that the alginate amended skim milk beads dissolved in the basal broth during secondary multiplication. Whereas, alginate beads started dehydrating within one week after preparation. Hence, beads after secondary multiplication were not used for further studies.
- Alginate beads have higher shelf life compared to alginate amended skim milk beads and beads after secondary multiplication. At higher concentration of sodium alginate (3%) and calcium chloride (3% and 3.5%), the beads were perfectly spherical in shape with larger diameter, higher bead weight, higher yield of production and holds higher water content and bacterial entrapment inside the beads and lower swelling and shrinkage percentage which led to slow and constant release of entrapped bioagent from the beads. As both the combinations could entrap equal and sufficient number of *P. fluorescens* inside the alginate beads, treatments amended with 3.0 per cent each of sodium alginate and calcium chloride were used for *in vitro* inhibition studies against major soil borne plant pathogens.

4. The antagonistic potential of two best treatment beads selected above B-1: T_1 (mannitol + PVP + CMC + tween -80) + sodium alginate (3%) + $CaCl_2$ (3%) and B-2: T_3 (mannitol + PEG + CMC + tween -80) + sodium alginate (3%) + $CaCl_2$ (3%) were evaluated against six major soil borne pathogens *viz*., *Fusarium oxysporum, Phytophthora nicotiana, Pythium aphanidermatum, Sclerotium rolfsii, Rhizoctonia solani* by dual culture method and *Ralstonia solanacearum* by filter paper disc method.

    - The bead formulation B-1 showed cent per cent inhibition under *in vitro* against *Pythium aphanidermatum*. This was closely followed by *Phytophthora nicotiana* (72.22 per cent), *Ralstonia solanacearum* (70.36 per cent) and *Fusarium oxysporum* (27.77 per cent).

- Similarly, the bead formulation B-2 showed cent per cent inhibition against *Pythium aphanidermatum*. However, the inhibition percentage against *Phytophthora nicotiana*, *Ralstonia solanacearum* and *Fusarium oxysporum* of B-2 formulation was noticed as 77.77, 74.07 and 30.55 per cent respectively.
- It was observed that both the formulations B-1 and B-2 did not show any inhibition on the growth of *Sclerotium rolfsii* and *Rhizoctonia solani* under *in vitro* conditions.

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APPENDICES

APPENDIX-I

Composition of King's B media (pH 7.2) used for serial dilution

Peptone	:	20.0 g
Glycerol	:	10.0 ml
$MgSO_4.7H_2O$	:	1.50 g
K_2HPO_4	:	1.50 g
Agar	:	20.0 g
Distilled water	:	1000 ml

Composition of Potato dextrose agar used for dual plate method

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

ALGINATE BASED ENCAPSULATION OF *Pseudomonas fluorescens* FOR THE MANAGEMENT OF SOIL BORNE PATHOGENS

By
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ABSTRACT OF THE THESIS

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ABSTRACT

Biological control, an eco-friendly and cost effective approach for plant disease management in agriculture has been practiced for several decades. *Pseudomonas fluorescens*, one such biocontrol agent is used to combat many phytopathogens. For commercial use, microbial inoculum should be supported by an appropriate formulation preventing a rapid decline of introduced microorganisms and extending their shelf-life. Various formulations available in the market are powder, liquid and granular formulations where such carrier based inoculants which generally faces problems like poor shelf life, high chances of contamination, bulk sterilization problem, unpredictable field performance and sometimes unavailability of good carrier materials. In order to overcome the disadvantages of these formulations, microencapsulation is one such alternate viable option prepared by using sodium alginate as a polymer which results in extended shelf-life and controlled microbial release from the formulation thus, enhancing their application efficacy. Hence, the present study was undertaken in the Department of Plant Pathology, College of Horticulture, Vellanikkara to develop alginate based formulation of *Pseudomonas fluorescens* for the management of soil borne pathogens.

P. fluorescens, the reference culture of KAU was used for preparation of alginate beads. To improve the shelf life of alginate beads, King's B broth was enriched with adjuvants *viz.,* sugar source (mannitol and trehalose), wetting agent (PVP and PEG), adhesive (CMC and liquid paraffin) and surfactant (tween-80) in nine different treatments and was evaluated at monthly intervals. After nine months of observation, maximum population of *P. fluorescens* was recorded in treatments T_1 (mannitol +PVP +CMC +tween-80) (1.33×10^8 cfu ml^{-1}) and T_3 (mannitol +PEG +CMC +tween-80) (1.66×10^8 cfu ml^{-1}) compared to control which were selected for the preparation of beads.

The beads were prepared from the above selected treatments as per the protocol of Bashan *et al*. (2002) with modifications. The beads were prepared in three

different batches *viz*., beads from alginate alone, beads from alginate amended with skim milk and beads after secondary multiplication. Various parameters were standardised in order to prepare effective bead formulation. Beads from both alginate and alginate amended skim milk formulations produced from higher concentration of sodium alginate (3%) and calcium chloride (3.5% and 3.0%) solution with 60 min of curing time and 9 to 15 cm of falling distance produced perfectly spherical beads with maximum diameter of above 1.70 mm, higher bead weight of above 16.6 mg with more than 60 per cent bead yield. Such beads showed reduced swelling percentage which holds higher per cent of water content inside beads and lowest shrinkage percentage that facilitates higher survival and slow release of the bioagent for a longer period of time.

Shelf life of *P. fluorescens* encapsulated in alginate beads alone prepared from two best treatments T_1 (mannitol + PVP + CMC + tween-80) and T_3 (mannitol + PEG + CMC + tween-80) showed a higher shelf life compared to alginate amended skim milk beads and beads after secondary multiplication. Higher bacterial entrapment were observed in alginate beads prepared from sodium alginate (3%) and calcium chloride (3.5% and 3.0%) respectively in treatments T_3A_{12} (10.33×10^{20} cfu g^{-1}) followed by treatments T_3A_{11} (8.33×10^{20} cfu g^{-1}), T_1A_6 (6.33×10^{20} cfu g^{-1}) and T_1A_5 (5×10^{20} cfu g^{-1}) respectively after four months of preparation.

The alginate bead combinations B-1: T_1 (mannitol + PVP + CMC + tween -80) + sodium alginate (3%) + $CaCl_2$ (3%) and B-2: T_3 (mannitol + PEG + CMC + tween -80) + sodium alginate (3%) + $CaCl_2$ (3%) were selected for *in vitro* evaluation studies against major soil borne pathogens and it was noticed that these formulations inhibited soil borne pathogens *viz*., *Pythium aphanidermatum* (100%) followed by *Phytophthora nicotiana* (72.22 and 77.77%) *Ralstonia solanacearum* (70.36 and 74.07%) and to some extent inhibition of *Fusarium oxysporum* (27.77 and 30.55%). However, no inhibition was observed on the growth of *Sclerotium rolfsii* and *Rhizoctonia solani* under *in vitro* conditions. Hence the study has clear by demonstrate a protocol to produce microbeads of *P. fluorescens* which are less bulky, non-toxic, biodegradable and enables slow and

controlled release of the biocontrol agent and thus could maintain a bacterial population for a relatively longer period.