Fabrication of Chitosan-Alginate /Carboxymethyl Cellulose Microcarriers for Delivery of Bioactive Agents

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CERTIFICATE

This is to certify that the dissertation work entitled **"Fabrication of Chitosan-Alginate/Carboxymethylcellulose Microcarriers for Delivery of Bioactive Agents"** submitted by Suborna Chatterjee (Roll. No. 601604007) in partial fulfillment for the award of degree of Master of Technology in Biotechnology from Thapar Institute of Engineering and Technology, Patiala, Punjab, is the record of the candidate's own independent and original research work carried out under our supervision and guidance. The matter embodied in this dissertation has not been submitted in part to any other University/Institute for the award of any degree or diploma in India.

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DECLARATION

I hereby declare that the work being presented in the dissertation report entitled **"Fabrication of Chitosan-Alginate/Carboxymethylcellulose Microcarriers for Delivery of Bioactive Agents"** submitted by me for the award of the degree of **Master of Technology** in Department of Biotechnology, TIET University, Patiala is true and original record of my own independent and original research work carried out under the supervision of Dr. Shekhar Agnihotri. Further, I declare that no part of this dissertation has been submitted to any other University/Institute for the award of any degree in India or abroad.

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LIST OF SYMBOLS AND ABBREVIATION

%	Percentage
µl	Micro liter
µm	Micrometer
Ag	Silver
$Ag(NH_3)^2$	Silver diamino ion
Ag^+	Silver ions
$AgNO_3$	Silver nitrate
AgNPs	Silver nanoparticles
Ag^o	Silver atoms
$CaCO_3$	Calcium carbonate
CFU	Colony Forming Units
DLS	Dynamic light scattering
CMC	Carboxymethyl cellulose
DD	Degree of deacetylation
Gm	Grams
hrs.	Hours
kDa	Kilo Dalton
L-b-L	Layer by layer
Mg	mili grams
MgO	Manganese oxide
Ml	Milliliter
mM	Micromolar
Mw	Molecular weight
NaCl	Sodium chloride

Nm	Nanometer
NP	Nanoparticle
ºC	Degree centigrade
OD	Optical density
PBS	Phosphate buffered saline
PEC	Polyelectrolyte complex
pH	Potential of hydrogen
Rpm	Revolutions per minute
SEM	Scanning electron microscope
spp	Species
TEM	Transmission electron microscopy
TiO_2	Titanium oxide
UV	Ultra-violet
w/v	Weight by volume
ZnO	Zinc Oxide

ABSTRACT

Recent advancements in developing an efficient system for delivery of bioactive particles viz. enzymes, therapeutic peptides and even nanoparticles are gaining importance nowadays. Particularly, microcarriers developed using biopolymeric systems are currently under investigation so as to deliver the required material with high efficiency, facilitating their controlled degradation-driven release without posing toxicological implications. Studies have been done in the recent past to develop an efficient carrier system employing biopolymers. The conventional approaches followed for their fabrication are either expensive or may cause toxicity, which restricts their widespread application.

The main objective of this research was to emerge with a cost effective yet efficient template system for proficient delivery of various active ingredients ranging from enzyme to bactericidal silver nanoparticles. Microcarriers with a unique combination of biopolymeric system employing chitosan, alginate and carboxymethyl cellulose was synthesized through layer by layer assembly onto which α-amylase was immobilized through *in-situ* and *ex-situ* immobilization and silver nanoparticles were synthesized separately and surface immobilized. Immobilization of 73% and 75% of the highest feed amount was achieved by microcarriers based on chitosan-alginate complex and CMC-alginate complex respectively through surface immobilization. Through *in-situ* immobilization 83% of the feed amount was immobilized into the core particles. Total amount of enzyme immobilized through *in-situ* immobilization was 9 times higher than that immobilized through surface immobilization. Efficient controlled release profile was at pH7 was observed. Cumulative release of 96 % till day 6 was achieved for microcarriers with surface immobilized enzyme, whereas cumulative release of 40% and 25% was obtained by microcarrier of biopolymeric combination chitosan-alginate and CMC-alginate respectively with *in-situ* immobilized enzyme. On the basis of final concentration of biopolymer utilized to form biopolymeric layering, immobilization and release efficiency; microcarriers with chitosan-alginate biopolymeric layering illustrates better immobilization efficiency and release profile. As 0.1mg/ml concentration of chitosan with high molecular weight and 85% DD has more functional groups and chain length when

compared to 1mg/ml Carboxymethyl cellulose. Better release profile of chitosan-alginate complex is due to increased porosity of the complex at pH7.

The resulting microcarriers with biopolymeric combination of chitosan-alginate were utilized as a carrier for biogenically synthesized silver nanoparticles Biopolymeric microcarriers coated with silver nanoparticles were combined with chitosan solution to form bionanocomposite (BNC) film. The silver nanoparticles coated microcarriers as well as bionanocomposite films illustrated good antibacterial activity against diverse bacterial strains, i.e. against *E.coli*(gram negative) and *B.substilis*(gram positive).BNC film was used for the preservation of grapes under double layer (grape wrapped in film and placed in sealing bag) and single layer (grape wrapped only in film) preservation conditions. Under both the preservation conditions; grapes wrapped BNC film had better physical appearance than then negative and positive controls till day 7 .Conclusively, these preliminary findings suggest this co-polymeric system to be a versatile carrier which has a dual role for the controlled release of bioactive molecules without affecting its efficacy.

CHAPTER 1

INTRODUCTION

The latest innovations in trans-disciplinary research have witnessed the role of bioactive compounds in developing value-added biochemical products. Several bioactive agents' viz. peptides, enzymes, proteins, antioxidants and lipids have been identified from diverse sources and by definition, they are inherently adorned with therapeutic benefits, nutritional value and protective effects for humans and animal healthcare (Mohamad *et al.,* 2015). Over a span of last 10 years, there has been wide spread use of bioactive agents various domains of biotechnology ranging from environment, medicine and food applications and have gained considerable success. As a result, a standard cascade of studying these bioactive agents, right from exploring their biological activity to their actual delivery to target individuals including the selection of a carrier, their clinical relevance, biocompatibility, standardization, quality control, mechanisms of action and their possible molecular interactions is currently sought for translational research in modern medicine. One of the major limitations that impeded the practical usage of active agents in free form is their high sensitivity towards external environment and physicochemical factors. Reportedly, bioactive agents are susceptible to degradation and many times, there occurs a substantial loss bioactivity if not used under favorable conditions. Hence, it is a matter of great concern to focus on the design and development of efficient carrier systems for localized delivery of bioactive agents through novel immobilization strategies (Leitgeb *et al.,* 2016).

While electing an ideal delivery vehicle the major features of carrier system are to provide an optimum release profile for a significant duration of time, preventing degradation of bioactive agent from the environment and allowing the movement of bioactive agent from the semi permeable matrix (Hernández *et al.,* 2010). These properties have been obtained by using polymers for the fabrication of carrier systems. Polymers belonging to natural and synthetic origins have been used as an immobilization matrix for bioactive agents and have been successful in delivery of bioactive agents as therapeutics. Owing to their well-defined microstructure environment, tunable biodegradability and stimulus sensitivity, polymers as their parental and hybridized form have been implemented for the supply of drugs and therapeutic proteins into biomedical applications.

However, the current scenario demands the exploitation of polymers with the properties of biodegradability and biocompatibility enabling customization to suit specific delivery needs. To facilitate this issue bioplastics and biopolymers have been brought to use which has illustrated better results.Exploiting biopolymers alone in the fabrication of carrier system are restricted due to the deficiency of mechanical strength and physiochemical properties (Kadam *et al.,* 2005).To enhance the mechanical strength as well as physiochemical properties, blending of bioplastics along with natural polymers have been successfully implemented (Sionkowska, 2011).However, introduction of bioplastics incur an added cost to the complex structure and hence become uneconomical under practically relevance conditions (Srikanth Pilla, 2011).

The conventional solution to the problem in question has been fabrication of carrier system with polymers from natural origin using crosslinkers. Chitosan-alginate carrier systems have been fabricated using crosslinkers like formaldehyde, glutaraldehyde and genipin (Bhattarai *et al,* 2010, Mi *et al,* 2001). By forming crosslinkages between functional groups of the polymers they have been noted to impart good mechanical strength to the system. But these agents are expensive and may possess toxic implications to environment and on consumption. Several other crosslinking techniques such as photo crosslinking, thermal crosslinking and chemical crosslinkers have also been exploited, but they also posses limitation of high cost and equipment sophistication along with weak crosslinking ability (Bhattarai *et al,* 2010). Thus the commercial viability of a carrier system would require fabrication of delivery vehicle using economical raw materials which should lead to increased efficiency of immobilisation of bioactive agents without compromising on its efficacy.

The proposed solution is to reduce the utilization of crosslinking agents and synthetic polymers is to employ property of interaction between oppositely charged polymers to form polyelectrolyte complexes (Hamman .J.H, 2010) for the fabrication of microcarriers. For a rigid support, the core-shell template for the synthesis of microcarriers is well suited. Microcarrier has been widely studied as an immobilization and encapsulation matrix with their size in the range for 1-1000µm. Their application has been seen in surface immobilization of doxorubicin (Zhao *et al.,* 2007) for cell internalization (Ribeiro *et al.,* 2018). They have been exploited as carrier system for various types of materials such as proteins, food materials, pesticides, herbicides and enzymes (Campos *et al.,* 2013). An

essential advantage to using biopolymeric microcarriers is its biocompatibility and biodegradability, high surface area to volume ratio for immobilizing bioactive material via in-situ and ex-situ processes. For a core-shell microcarrier the core provides a firm base or template for subsequent formation of layers of biopolymeric polyelectrolyte complex which in turn, maintains the shape of the microcarrier throughout the process (Zhao *et al.*, 2007).

The main purpose of the current work is to study the impending benefits of biopolymer as a microcarrier template for immobilization of bioactive agents. The structure of microcarrier is based on core-shell template employing chitosan, alginate and carboxymethylcellulose 'biopolymeric-triplets' to form immobilization matrix through layer by layer approach. Bioactive agents such as α-amylase enzyme is immobilized both at the surface through *ex-situ* as well as within the core via *in-situ* approaches. The efficacy of microcarrier support for enzyme immobilization will be evaluated at various test conditions so as to understand the extent of immobilization, loading efficiency, stability parameters, and release kinetics of bioactive loaded microcarriers. Bioactive agents of inorganic origin viz. silver nanoparticles are also formed through biogenic reduction method using amla extract, which were subsequently immobilized onto microcarrier's surface and embedded into biopolymeric film for its potential application as coating material.

The overall aim of this work is presented below;

- Developing a new method or tool for the fabrication of microcapsules using biopolymeric-triplet and immobilizing enzyme alpha amylase via *in-situ* and ex-situ approaches.
- Studies on comparing various microcarriers system fabricated with different micro-structures on the basis of immobilisation efficiency of enzyme and its release profile
- Evaluation of the antibacterial efficacy of AgNP-loaded microcapsules against model test strains, *E.coli* (MTCC 791) and *Bacillus substilis.*
- Establishing the potential of AgNP-loaded microcapsule film to enhance the shelf life of fruit (grapes).

CHAPTER 2

REVIEW OF LITERATURE

2.1) Microsphere and Microcapsule as Carrier System

Microcarriers in a simple term can be defined as a carrier system in the micro range which can be exploited as a transporter for the delivery of molecules that have impact on biological system. Microcarrier has been further sub divided as microsphere and microcapsule. Microspheres comprises of uniform blend of raw material and active compound, whereas the term microcapsules is used for microparticles with a central space (core) where the active material is immobilized. The core may be gas, liquid or even solid. The core may or may not be removed by a different solvent system or material. Depending upon the core structure microcapsule can be divided into two categories: solid microspheres, where the core of the microcapsule is kept intact and hollow microspheres where the core of the microcapsule is removed (Campos *et al.,* 2013). Microspheres can be employed as multiparticulate drug carrier system prepared to obtain delivery of the immobilized material at a predetermined rate, improved stability, availability of the compound to specific target site. Their fabrication comprises of polymeric or combination of other polymers from natural, semi synthetic and synthetic polymeric origin. Microspheres are morphologically in powder form with particle dimension in the range of 1-1000 μm. Variety of techniques have been applied for fabrication of microspheres offers numerous options to influence the drug administration features as well as improve the therapeutic potential of a specified bioactive compound (Kadam *et al.,* 2015). Microcapsules have been seen as a promising carrier system since it provides unlimited scope for the combinations of biopolymeric layers along with core and shell template (Krishna *et al.,* 2014). The reasons for the increase in demand for microcarriers in recent years is mainly due to its property to increase the stability of the bioactive molecules by providing an shield from the reaction environment, sustained release of the loaded material, modulating release profile, converting bioactive agents from liquid state to powdered form, reducing material's toxicity and alterations at the site of absorption through appropriate surface modifications (Kadam *et al.,* 2015).

So far, several practices have been developed for the microsphere sysnthesis such as spray drying, double emulsion method, solvent evaporation, single emulsion method, phase separation coacervation technique, solvent extraction, spray congealing, also the quassi emulsion solvent diffusion. Although these methods have their own advantages, majority of them are limited by their expensive and time consuming features. As a general procedure, the microspehere are formed through encapsulation of bioactive material in the void spaces while the formation of sphere. This ultimately renders the microsphere fragile and susceptible to breakage by shear force (Kadam *et al.*, 2015). Whereas the methods used for the preparation of microcapsules such as coacervation process, ionotropic gelation, pan coating, matrix polymerization offers a wide scope for the development of a new technique which could be a combination of the other basic techniques for the preparation of microcapsules. These methods are not sophisticated which renders them inexpensive but may be time consuming (Krishna *et al.*, 2014). Apart from their ease of preparation they have been studied as a potential candidate for immobilization and encapsulation of bioactive agents.

By substantially reducing the fabrication complexity of microcarriers, Lu et al. (2012) successfully immobilized lyzozyme on the surface of microcarriers formed by the combination of calcium carbonate and carboxymethylcellulose. Authors concluded microcarrier as an economical approach to immobilize enzymes with high loading efficient and proficient release profile in the presence of NaCl. Increasing ionic strength of desorption solution lead to increased desorption of lyzozyme from the surface. Complete desorption was obtained in the presence of 0.2M NaCl. To increase the strength and structure stability of microcarriers, Zhao et al. (2007) reported a pristine technique for the synthesis of microcapsule as drug delivery vehicle, by fabrication of hollow chitosan-alginate multilayer microcapsules for loading of doxorubicin .The fabrication of microcapsule was based on the alternate layering of alginate polymeric and oppositely charged chitosan onto carboxyl-methyl cellulose (CMC)-$CaCO_3$ core elements in and assembly of layers. The deposition of adequate layers was subsequently cross-linked with glutaraldehyde and the core was decomposed by disodium ethylene-diamine-tetra-acetic acid. Dissolution of core maintained the structure of microcarriers throughout the process of immobilization of doxorubicin on the surface. Apart from their ease of preparation, L-b-L particles have been studied as a potential candidate for immobilization and encapsulation of bioactive agents. In a similar work Riberio

et al. (2018) demonstrated biomedical applications of particles showing internalization of cells in the hollow compartment of the microcapsules. By substantially reducing the fabrication complexity of microcarriers Lu et al. (2012) successfully immobilized lyzozyme on the surface of microcarriers formed by the combination of calcium carbonate and carboxymethylcellulose. The conclusion made through the results signifies that microcarriers can be employed as an economical approach for immobilization of enzymes.

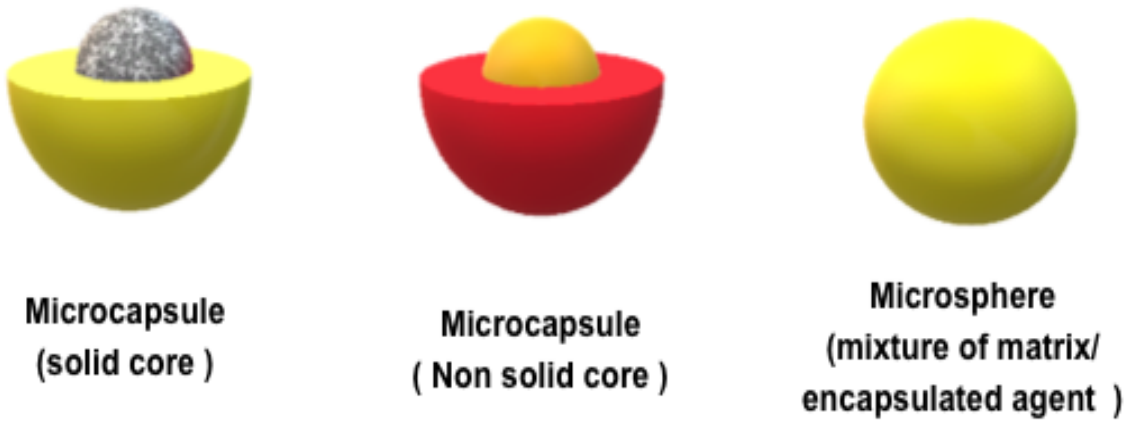

Figure 1: Graphical representation of microcarriers

2.2) Polymer as Immobilization Matrix

Polymers have been exploited as an immobilization matrix due to their unique properties of prolonging drug availability, favorably alter biodistribution, and enable hydrophobic drug administration, transportation of drug to its ordinarily inaccessible spot of action and makes drug accessible in response to stimuli (Gandhi *et al.*, 2012). They are been known to a play a vital part in the fabrication of carrier system due to above mentioned properties which help a site specific controlled release of bioactive agents. The origin of polymers has been categorized into synthetic as well as natural. Synthetic polymers which are been utilized in the carrier system is termed as bioplastics due to their property of biodegradability and biocompatibility. Natural polymers are acquired from renewable resources such as microorganism, plants and living sources.

2.2.1) Biodegradable Polymers

Biodegradable polymers are those materials which degrade in the presence of organisms present in the ecological system or enzymes at optimum temperature, moisture and oxygen availability, which results in their cleavage into short polymeric chains without any toxic implications or environmentally harmful byproducts (Chandra *et al.*, 1998).Biopolymers can be categorized according to their origin: polymers extricated from biomass directly (eg. polysaccharides, proteins),chemically synthesized polymers using renewable heterogeneous sources of petroleum (i.e. poly lactic acid) and biomass, and polymers obtained from renewable sources (eg. Chitosan, starch) given in table 1.

Table 1: Classification of biopolymer

Types of Polymer	**Examples**	
Polyester	Polyhydroxyalkanoates	Polylactic acid
Proteins	Silks	Polyaminoacids
	Collagen/gelatin	Casein
	Elastin	Resilin
	Soy	Wheat gluten
	Adhesive	Serum albumin
Polysaccharide (bacterial)	Cellulose	Dextran
	Xanthan	Polygalactosamine
	Curdlan	
Polysaccharide (fungal)	Phullulan	Yeast glucans
Polysaccharide (plant/algal)	Cellulose	Alginate
	Starch	Carrageen
	Agar	Pectin
Polysaccharide (animal)	Chitin	Hylouranic acid
Lipid	Acetoglycerides	Waxes
	Surfectants	Emulsan

2.2.2) Synthetic Biopolymers

These are developed by ring-opening polymerization (ROP) and condensation methods or by introducing natural components along with other synthetic polymers as precursors for the synthesis of biodegradable polymers. During synthesis processes, their properties can be

modulated so as to meet the desired requirements (mechanical strength, rigidity, flexibility, degradation rate) as per the scope of applications (Francis *et al.*, 2013). polyamides, polyesters, poly(amide-enamine)s, polyurethanes (PUs)and polyureas, poly(anhydrides) (PAs, poly (lactic acid)), poly(p-dioxanone), co-polymers of poly(glycolic acid), and co-polymers of trimethylene carbonate also the glycolide have been utilized in a many of the clinical applications such as carrier systems, resorbable sutures, and orthopedic fixation maneuvers such as rods, pins, as well as screw (Gunatillake *et al.*,2013). This excellent property of flexibility and biodegradability comes along with increased cost of production. Biopolymers procured from petroleum resources are resistant to disintegration into simple units through natural metabolism. The biodegradation of such polymers are facilitated through addition of additives; one of the methods to vitiate polyolefins is the antioxidants incorporation in the polymeric chains. Presence of antioxidant in the polymers reacts under UV; which initiates disintegration through photo-oxidation. The biodegradability extent of such systems cannot be confirmed as there are specific requirements for the degradation to take place. Whereas aliphatic polyesters have hydrolysable ester bonds which make them biodegradable. PGA, poly (glycolic acid) is simple linear aliphatic polyester that is prepared chemically through ring inaugural polymerization of a cyclic lactone, glycolide. Even after being endowed with excellent mechanical strength and flexibility its applications in the field of biomedical sciences are restricted due its truncated solubility along with the disadvantage of degradation at high rate yielding acidic products. PLA, poly (lactic acid) is chemically acquired from polycondensation of L - or D -lactic acid or through ring opening polymerization of a cyclic dimer of lactic acid, lactide. Biodegradability and physical properties of PLA can be delimited by introduction of hydroxy acids comonomeric components or by L- and D-isomer racemization (Vroman *et al.*, 2009). There is requirement of sophisticated chemicals and infrastructure for the synthesis as well as degradation of such polymers (S.Pilla, 2011).

2.2.3) Natural Polymers

Natural polymers are obtained from various life forms available on earth. The reactive sites present on the surface of natural polymers participate in crosslinking, conjugation with ligand, and various other modifications. Presence of such reactive sites makes these polymers an ideal candidate as carriers for extensive range of bioactive molecules. Natural polymers

can be categorized as polysaccharide and protein-based polymers on the grounds of their origin. Polysaccharide based polymers like chitosan, dextran, starch, cellulose, alginates are acquired from renewable origin such as animal, plant and microbial extracts. These polymers exhibit diverse structures and properties. Due to broad molecular weight range, presence of significant figure of reactive groups, and diverse structural composition they can be modified easily and efficiently according to the demand and requirement (Liu *et al.*,2008). Polysaccharides from natural origin are extremely stable in vivo; they are nontoxic, and degrade under biological conditions. Nature of polysaccharides is hydrophilic and binds with biological tissues through non-covalent bonds. This interaction is because of the phenomenon of mucoadhesion. Presence of reactive groups on the molecular structure of such as hydroxyl, carboxyl, and amino groups are responsible of the phenomenon of mucoadhesion (Akash *et al.,* 2015). Protein-Based Polymers are also been expansively used due to their abundance, availability, minimal or negligent toxicity, ease of modification, versatile routes of administration and their complex heterogeneity (Elzoghby *et al.,* 2012) The most recurrently used proteins as carrier system for deliverance of therapeutic proteins are collagen, elastin, albumin, silk and gelatin. It is a great challenge to conserve the stability of protein-based polymers, however various approaches has been put forward to prevent the deprivation of protein-based polymers (Kaul *et al.*, 2002).The hurdles associated with natural polymers are categorized on the basis of performance, processing, and cost. Several characteristics, such as brittleness, high gas and vapour permeability, lower heat distortion temperature and poor resistance to processing operations have strongly limited their industrial applications (Scott 2000).

Natural polymers have ample advantages when compared to synthetic polymers because of their renewable and natural origin, abundance which makes it easily available and economical; it also offers liberty to chemical modify these polymers (Akash *et al.,* 2015). Usually, the definite configuration of synthetic polymers demonstrates precise and tunable disintegration kinetics as well as mechanical properties (Hamidi *et al.,* 2008). However, when compared to natural polymers such as protein, the later offer numerous advantages over synthetic polymers. They can be easily disintegrated into peptides by digestive enzymes whereas accumulation of synthetic polymers in the body above a certain molecular weight may take place and result in toxic byproducts (Elzoghby *et al.,* 2012). They also display

several drug loading mechanisms through covalent bonding, electrostatic attractions, and hydrophobic interactions. There are ample possibilities for modification of the polymeric surface due to the presence of several functional moieties which enables specific targeting of the drug to the site of action. Likewise polysaccharides are also susceptible to enzymatic digestion (Mizrahy *et al.,* 2012). Polysaccharides is advantageous over synthetic polymers such as far as prolonging circulation time is concerned as these do not degrade at a rapid rate(Heinze *et al.,* 2005). Biopolymer from natural sources are been widely worked on due to the various advantages they posses ,the most important being that the origin is renewable and the processing cost is reduced. Their degradation can take place under enzymatic condition and does not produce toxic byproducts upon degradation

Chitosan Biopolymer

Chitosan is a tremendously promising biopolymer that is obtained mainly from sea food processing wastes. It is a single polycation of natural origin and possesses number of valuable properties (Philippova *et al.,* 2012). The most noteworthy properties of the polymer are being nontoxic, biocompatible, and biodegradable as chitosan does not accumulate niether within the body nor in the environment (Uragami *et al.,* 2006). It was found that chitosan shows haemostatic, bactericidal, fungicidal, antitumoral, anticholesteremic and immunoadjuvant properties and has sedative effect on the central nervous system. Chitosan is widely used in the manufacturing of weight loss drugs due to its property of binding to fat present in food and removes them from the body. However, its most significant application is its use as a drug carrier. The positive charge of chitosan facilitates its penetration through cellular membranes and epithelial dense layers, providing good adhesion to mucous coatings and antimicrobial properties. Chitosan can be used for gene delivery owing to its ability to bind oppositely charged DNA. Chemical modification of chitosan through incorporation of corresponding substituent can lead to the introduction of new properties. The incorporation of hydrophobic substituents into the molecular chain of chitosan has been as a very promising approach to absorb drugs poorly soluble in water; these modifications can widen the areas application of chitosan as a drug carrier (Philippova *et al.*, 2012).

It has been reported to have antimicrobial activity yet the exact mechanism of its action is not completely explored. Several reasons for its antimicrobial mechanisms have

been hypothesized. Chitosan attacks on the external surface of bacteria; reason for the antimicrobial property is the positively charged amino group on the chitosan surface which binds with negatively charged surface of microbial cell membranes leading to agglutination which causes outflow of proteinaceous and intracellular constituents of the microbial cells. Chitosan selectively binds to trace metals and thereby inhibiting the production of cellular toxins and microbial growth, thereby acting as a chelating agent. It also activates several defense mechanisms in the host tissue by acting as water binding agent leading to inhibition of various enzymes (Dutta *et al.,* 2009).

Alginate Biopolymer

It is a naturally obtained polymer which is produced by processing (kelp) brown algae. Several distinctive properties of this polymer have signified its application as matrix for immobilization and liberation of a range of biological agents. Alginate polymer belongs to a category of linear unbranched polysaccharides which consist of varying units of α-l-guluronic acid residues and 1, 4′-linked β-d-mannuronic acid. They vary broadly in composition and sequence due to their arrangement pattern, these residues are placed as blocks along the chain. Ionic crosslinking of alginate can be through addition of divalent calcium ions in aqueous solution that forms linking bridges between units of alginate by interacting with monovalent sodium ions. The comparatively placid gelation procedure has enabled proteins, animal and microbial cells and DNA to be integrated within the matrices composed of alginate along with preservation of complete biological activity. Alginate has a property of bioadhesivity which makes it a prospective for the development of microcarriers for delivery of bioactive agents to mucosal tissues. Alginate being hydrophilic polymer renders the material non-immunogenic and biocompatible, this have led to its elevated use as a protein carrier system. Therefore, has been extensively used as drug excipient, base for taking dental impressions, and as wound dressing material in pharmaceutical industry (Gombotz *et al.,* 2012).

Carboxymethylcellulose (CMC) Biopolymer

Cellulose is a natural polymer present in abundance but is completely insoluble in water introduction of carboxymethyl group have been known to develop the property of water solubility in cellulose. Introduction of carboxymethyl group into the cellulose molecule is

easy; it leads to the elimination of the hydrogen bonds that render the parent molecule soluble. Their easy accessibility, flexibility, non-toxicity and cost have increased their application in numerous fields (Batelaan *et al.,* 1992).Controlled drug release was investigated using CMC. The polymer has been used as a carrier for erythomycia (model drug) (Emregül *et al.,* 1996).CMC can be blended with polymer with other polysaccharides by mixing in the state of aqueous solution .Properties of such products like mechanical strength depends on the formation of hydrogen bonding, which can be controlled by blending ratio. CMC has been put to use in numerous tissue engineering and drug delivery applications. Apomorphine; bioactive compound used to control motor responses in Parkinson's disease, has been fruitfully integrated into carboxymethylcellulose powder formulation that presented a persistent release in the nasal route, and its performance was improved in comparison to delivery vehicle based on starch. CMC has also found its successful application in gastrointestinal drug delivery.

2.3) Cross-Linking of Natural Polymers

Depending on the kind of reactive groups present on polymer surface appropriate crosslinking agents have been used to form linkages between the polymer surfaces to form a copolymer. This approach has been followed to impart required mechanical strength and desired properties to the composite material. Crosslinking agents such as genipin (Mi *et al*. 2001), sulphuric acid, tartaric acid, mallic acid, citric acid and succinyl acid (Bodnar *et al*.2005) and glutarldehyde (Tiyaboonchai *et al.,* 2003) have been used substantially. Mi and coworkers (1999) fabricated chitosan microsphere with substantial porosity for the controlled release of antigen of Newcastle disease vaccine. These microspheres were prepared by means of wet phase-inversion method which involved structural modification chitosan microsphere by changing the pH value of aqueous tripolyphosphate (TPP) solution. Three types of functional groups: carboxyl group, quaternary ammonium groups as well as hydrophobic acyl groups were introduced which facilitated the immobilization of antigen of vaccine into the pores of porous chitosan microspheres. It was observed that these chemical manipulations has a strong control on the loading efficiency and rate of release of antigen under study .With substantial increase in research employing biopolymers as an immobilization matrix many new and

novel techniques were reported for microcarrier formation. Indomethacin entrapped microcapsules were fabricated through complex coacervation of chitosan-carboxymethylcellulose followed by crosslinking of polymers with glutaraldehyde. Tiyaboonchai et al., (2003) investigated the implications of concentration and pH variation of chitosan solution, amount of glutaraldehyde and crosslinking time on the physicochemical properties and drug release of these microcapsules. Usage of cross linking agents imposes various possibilities regarding their acceptance towards a particular application. Glutaraldehye is known to be toxic to gastrointestinal tract (Public Health Statement for Glutaraldehyde), whereas genipin is expensive and other crosslinking such as citric and tartaric acid does not possess very strong crosslinking ability.

2.4) Polyelectrolyte Complex

Polyelectrolyte complexes are formed between ionically oppositely particles. Electrostatic interaction takes place between oppositely charged polymers to form a complex. This approach averts the utilization of cross linking material and compounds of chemical origin thus substantially trimming down the possible causes of toxicity. This conception of complex formation between chitosan and DNA has broadly been investigated in the development of carrier system for oral vaccination and gene therapy (Lankalapalli *et al.,* 2009). The polyelectrolyte complex formation takes place in three steps (figure 2).Initial step is the formation of primary complex which takes place due to Coulomb forces, followed by intracomplexes formation. During this step, formation of new bonds takes place and distortions in the polymeric chains are rectified. Third and final step is the formation of intercomplex aggregates which involves the secondary complexes aggregation primarily through hydrophobic interactions (Kokufuta, 1979)

Tapia *et al.*, (2004*)* studied prospect of using combination of PECs (polyelectrolyte complexes) from both alginate –chitosan complex as well as carrageenan-chitosan complex for controlled delivery systems. Different release profiles for diltiazemclorhydrate were obtained by altering the polymeric matrix system and procedure utilized in combining these polymers into a copolymeric formulation .It is reported that alginate-chitosan systems are

considered to be more effective in prolonged release of intended agent when compared to chitosan-carrageenan systems by considering the swelling behavior. It has been studied that chitosan-alginate polyelectrolyte has better mechanical strength along with biocompatibility (Hamman, 2010).To enhance the mechanical strength of Chitosan-tripolyphosphate beads, these where coaugulated in presence of gelatin. This greatly increased the mechanical strength of the structure by ten times and improved loading efficiency (Shu *et al.*, 2000). Wavering of surface charge leads to the formation of uninterrupted assembly of positively and negatively charged materials establishing a great liberty to decide the number of layer sand layering sequence thus removing use of crosslinker for bond formation.

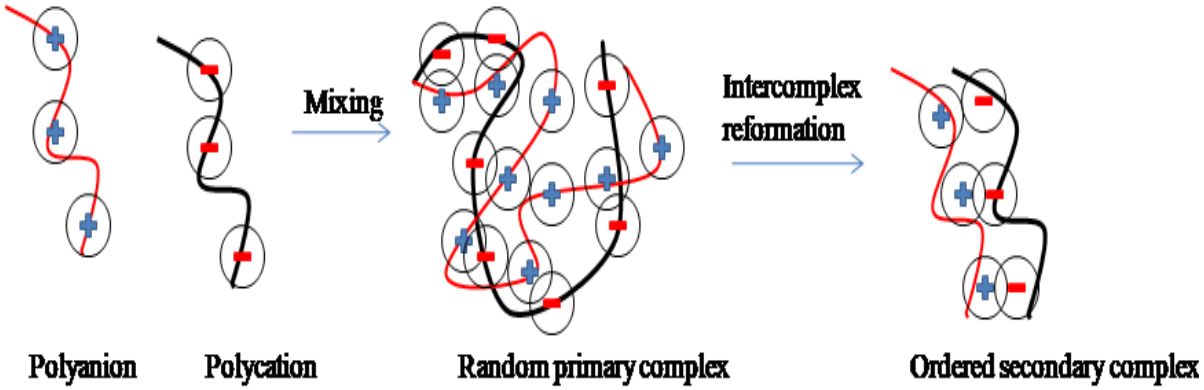

Figure 2: Mechanism of formation of polyelectrolyte complex

2.5) Methods for Fabrication of Microcapsule

The simple assembly mechanism with bare minimum requirement of apparatus has gained rapid popularity within various research societies. The regular assemblies of a variety of materials including polymers, inorganic substances and biomaterials have been made efficient and cost effective due to the simple yet efficient approach. Fabrication of biopolymeric layers with alternate ionic charge results in a continuous assembly due to electrostatic interactions or ionic interactions between polymers. This approach offers liberty to decide the number of polymeric layers to be formed and their sequence (figure 3). Interactions responsible for the L-b-L assemblies to form are not confined only to electrostatic

interactions. Various sorts of interactions like hydrogen bonding, metal coordination, covalent bonding, complex formation through charge transfer and bio-specific recognition have been demonstrated. In current scenario, exploitation of combination of naturally obtained polymers to form biodegradable polyelectrolyte such as: positively charged chitosan, dextran amine, gelatin A ,protomine sulfate along with negatively charged dextran sulfate, sodium alginate, chondroitin sulfate, heparin, gelatin B and hylouranic acid have been encouraged for the fabrication of carrier system (Ariga *et al.*, 2011).

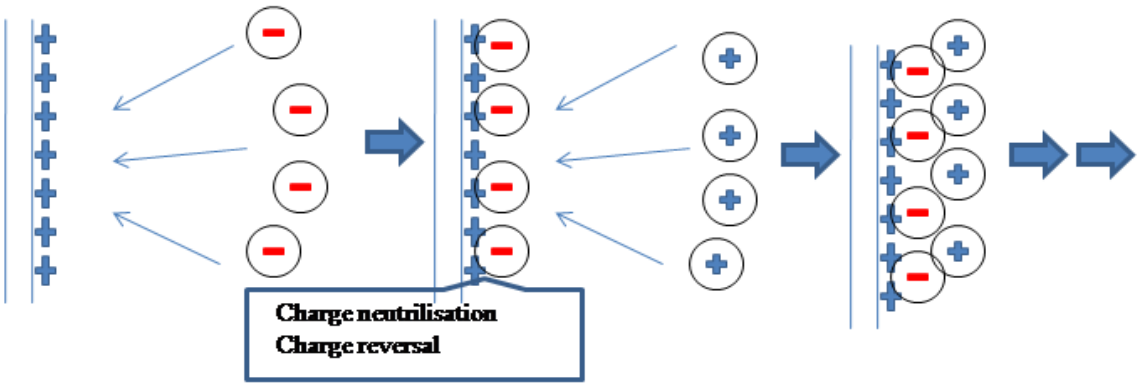

Figure 3: Graphical representation of layer by layer assembly by formation of polyelectrolyte complex

The fabrication of microparticles based on core –shell templates have been exploited a long time but less frequently .Work by Lopez et al.,(1997)was based on fabrication of multicore microparticle for drug delivery was based on encapsulation of chitosan micropaticles into cellulosic polymeric solution to form the final microparticle structure with chitosan core. The conclusion made through this study signified that it can be a potential candidate in terms of controlled release of drug and maintaining the activity of the subject. Following a similar pattern of core-shell template a novel technique for the fabrication of microcapsule was reported. The fabrication was based on alternate assembly of oppositely charged alginate and chitosan deposited onto (CMC)–doped $CaCO_3$ core particles (Zhao *et al.,* 2007). Cross-linking using glutaraldehyde strengthened this layer and the disintegration of the cores was done through disodium ethylenediaminetetraacetic acid (EDTA). Revelation of their applications is one of the most critical yet less explored areas so far. Most of its applications ranging from drug delivery, growth of artificial cells, to participating in catalysis, depend

strongly on the internal volume. Loading efficiency and release rate of preferred substances into or from these microcapsules becomes a crucial point in this perspective. Till date, diverse procedures and approaches have been formulated for loading and release of the substance by deliberate modification of the capsule interior or wall properties by altering components microenvironment such conditions as pH, salt, temperature, solvent system and chemical reaction mechanism (De Geest *et al.,* 2006). A similar work by Reberio et al., (2018) has been reported to internalize cell in the center space of microparticles fabricated through alternate assembly of oppositely charged polymers. Layer-by-layer assembled hybrid polymeric composite microcapsules have been established to be a prospective carrier system for various areas of application due to its versatility with respect to ability to bind to diverse molecules, targeted delivery, and controlled release options. These shells can hold a range of molecules and other molecules with different water solubility. The performance of these microcarriers is categorized either as steady release by diffusion or burst release through forced remote activation with change of temperature, salt concentration, pH, redox potential and intensity of ultrasonic vibrations (Heinze *et al.,* 2005). The pattern of release is a function of microenvironment; changes in the conditions can transform the release pattern from controlled release to burst release, vice versa. The main reason behind formation of layers is the chemical interactions that take place due to the opposite charges of these polymers which causing their binding to each other. Chitosan and carboxymethycellulose being polycationic and alginate being polyanionic undergoes charge neutralization upon their interaction driven by electrostatic mechanism.

2.6) Polymer - Protein Interaction

Selection of biodegradable polymers for protein and peptide drug delivery is firmly dependent on the interactions between proteins and polymeric materials, which appears to vary with attributes of protein and polymer under study. The deciding criteria for the combinations relies on the following points : (i) a crucial aspect to be considered in concern to diffusion the characteristics is molecular weight of the protein (ii) isoelectric point (PI) of the protein governs the charge-charge interactions between protein-polymer and protein-protein (iii) cysteines on the protein participates in the formation of intermolecular disulfide

bonds in between protein and polymer, (iv) chemical modification of polymeric material renders the amino acid sequence of protein susceptible to conformational modification , (v) interaction with polymeric materials may be enhanced or prevented by presence or absence of carbohydrates on the protein sequence (vi) hydrophobicity of a protein governs the extent of interactaction with hydrophobic sites on a polymer. Carrier system needs to be tested independently with protein of interest to specifically understand protein-polymer interactions. These exchanges play an significant role in illustrating the rate and extent of degradation of the biopolymeric system which influences the rate of release of the protein and their overall stability (Gombotz *et al.*, 1995).

2.7) Mechanism of Controlled Release

She et al., (2010) investigated the mechanism for protein accumulation and release in their work related to the fabrication of polyelectrolyte multilayer microcapsules for the delivery of bioactive material. The inference made suggested that release profile strongly depends on the kinetics responsible for protein adsorption by the capsule shell. Mechanism responsible for the release of protein from biopolymeric multilayer microcapsules was studied by encapsulating the protein into the microcapsules using two different approaches. First being "preloading" via coprecipitating tetramethylrhodamine isothiocyanate -labeled bovine serum albumin onto calcium carbonate particles followed by multilayer assembly of polymers and second approach was "postloading" of tetramethylrhodamine isothiocyanate-labeled bovine serum albumin into preformed empty capsules. Alginate, dextran sulfate and polyarginine were used for the formation of multilayer assembly. On the basis of various factors which influence release mechanism such as: the effects of capsule shell components and its thickness, mechanism for protein release , method of protein encapsulation, volume of the surrounding medium, and frequency of medium refreshment was stipulated. The most important criteria which determines the release mechanism is the efficiency of multilayer polyelectrolyte shells to accumulate and entrap protein molecules. An intense polymeric network in the shell of the capsule acts as barrier thereby causing hindrance in the release of encapsulated molecule. The driving force for the discharge of "preloaded" molecules is high concentration gradient between the capsule shell and surrounding medium along with

changes in pH and salt concentration which ultimately leads to increased permeability of polyelectrolyte multilayers.

In “preloading” process the protein by and large gets trapped in and between the inner surfaces of the polymeric layers. In contrast, in the “postloading” process, molecules are entrapped on the external surface of the polymeric layers and material diffuses toward center of the capsule. Hence, the accumulation takes place over and in between the outer most layers of microcarrier. Therefore rapid release rate of “postloaded” tetramethylrhodamine isothiocyanate -labeled bovine serum albumin through polyelectrolyte microcapsules is probably because of deliverance of protein moieties trapped on the external surface. In the situation of “preloaded” capsules, a significant amount of tetramethylrhodamine isothiocyanate -labeled bovine serum albumin molecules is sited in the central regions of the shell. The shell acts a mechanical barrier posing restriction to the easy diffusion of molecules out of the capsules. They concluded that irrespective of the approach followed for protein encapsulation, either ‘‘preloading” or “postloading”, thickness and composition of capsule shell performs a chief function in estimating the amount of protein molecules encapsulated and their release pattern. Release of protein molecules accumulated in the external layers of the capsule shell is faster than from the inner core. Hence, this offers liberty to customize the release profile to obtain sustained release of proteins in predefined concentration by modifying the shell composition and thickness.

Their degradation mechanism is also very much responsible for the release of encapsulated bioactive agents into their surrounding medium. The main phenomenon through which biodegradable polymers degrades is through erosion and degradation. During application, surface eroding polymer loose material from the exterior only. They tend to reduce in size smaller but maintain their original geometric shape. Whereas degradation and erosion of bulk eroding polymers is not confined to surface. Hence, the size of the structure remains constant for a considerable duration of time during its application (Göpferich *et al.*, 1996).

2.8) Bionanocomposites in food preservation

Interest has been growing in development of biopolymers and pioneering nano technologies that can efficiently diminish the dependence of food packaging on synthetic polymers. Bionanocomposites provides prospect for the exploitation of light weight biodegradable composites with high performance as an alternate to conventional non-biodegradable plastic packaging materials based on petroleum sources. Bio-nanocomposites endowed with antimicrobial property are extremely helpful in reducing the escalation of pathogenic microorganisms post-processing thereby substantially increasing shelf life of food as well as maintaining product quality. Among the most commonly used inorganic nano-antimicrobial agents for fabricating biopolymeric composites, silver nanoparticles (AgNPs) are being exploited to the highest extent. The benefits of silver has been acknowledged in the many field of application over a considerable period of time because of its strong antimicrobial action against a broad spectrum of fungi, viruses, and bacteria

2.8.1) Biocomposite: Combination of Natural Material

Overuse of plastics and synthetic polymers have raised alarming situation due their non-biodegradability. These tend to accumulate in the soil when discarded and not recycled. Environmental concerns related to its sustainability has been the driving force which has initiated substantial research in the direction of improving the application of green materials in through the development of biocomposite (Mantia *et al.*, 2011). The focus is to trim down the economical strain and ecological burden by diversified usage of agricultural products and various other renewable resources (Farag MM. 2008). Studies on combination of natural polymers such as : polysaccharide ,protein , fiber , polymer obtained from microbes i.e. Poly(hydroxyalkanoate) (PHA) and biodegradable synthetic polymers (bioplastics) have been performed to assess their potential against conventional plastics and polymers .Typical techniques such as injection molding, extrusion, direct long-fiber thermoplastic (D-LFT), pultrusion, compression molding and filament winding have been used to fabricated green polymeric composites .Biocomposite have found its applications in various field, such as automobiles, packaging and building industries. Study by Jandas et al., (2012) was based on investigating the potential application of PLA/banana fiber biocomposite fabricated by melt-

mixing and compression molding. Inference of the primary results indicated that the features of PLA/banana fiber composites possess promising potential to be used in packaging applications. Song et al., (2012) focused on the mechanical attributes, the thermal properties and viscoelastic behavior of hemp-PLA composites. The impact and tensile properties of PLA resin reinforced with twill structure fabric were found to be 15% and 10% elevated than the plain woven respectively. Thermal expansion Coefficient of fabricated hemp fabric composites decreased considerably from 70 × 10-6 m/ºC to 10 × 10-6 m/ºC with increasing fiber volume fraction from 6 to 20%, this indicates that the composites have great potential for application in fields where parts experience a wide range of temperature changes, such as automobile and aerospace applications.

2.8.2) Nanotechnology

Nanotechnology is defined as science and engineering involved in the designing, synthesis, characterization, and application of materials and devices with smallest functional organization, at least in one dimension on the nanometer range (Saini *et al.,* 2010). Nanotechnology, a multidisciplinary branch of science has gained a lot of interest in research areas like chemistry, biochemistry, medical, environment remediation and material science (Joo *et al.,* 2006). Extensive research is going on to apply nanomaterial for environment remediation, including improvement in the current manufacturing procedures to reduce pollution and creating alternative energy sources which are more economical. The art of manipulating matter at its atomic and molecular level for creation of novel properties and applications might appear to be a modern concept but craftsmen of the past have been controlling matter at most minuscule levels.

Richard Feynman gave a visionary talk "*There's Plenty of Room at the Bottom*", in 1959 and introduced the concept of ''nanoscience'' in it (Feynman 1960). The word ''nanotechnology'' was introduced by Norio Taniguchi in 1974 and defined it as the ''production technology which helps to obtain ultra-small sizes of approximately 1 nm with an ultrahigh precision'' (Taniguchi 1974). Development of nanotechnology took a pace after the invention of Scanning Tunneling Microscope in 1981 and Atomic Force Microscope in 1986. These tools opened the doors of ''nanoworld'' for scientists by revolutionizing imaging

system which makes the manipulation of objects easier (Schaming *et al.,* 2015). The unconscious application of nanoparticles can be dated to ancient era when people used silver utensils as water storage vessels for water disinfection and purification purpose and for regulars to boost immunity towards diseases (Melaiye *et al.,* 2005). In India there was a tradition to feed children in utensil made of silver. It was hypothised that it boosts the immune system and cure infections and diseases. The time when there was no solid proof of such hypothesis yet the results were observed to be successful.

2.8.3) Concept of Nano-Biocomposite

It is an immerging area in the field of hybrid material where biopolymers are combined with inorganic or organic material in nano dimension (Hitzky *et al.,* 2005). Substantial research efforts are being made to develop biopolymer based nanocomposites which would display properties of biodegradability, biocompatibility, improved physical strength, better thermal stability, and gas-barrier properties (Alexandre *et al.,* 2000). Their application has been diversified into various fields of medicine, life science, packaging and food preservation. Polysaccharides such as starch have been used as biopolymers to which in general natural or synthetic clay minerals, organically modified clay minerals have been incorporated providing a network bridge in between the moieties. Montmorillonite and cloisite are the usually employed silicates acting as nanocharges produces a reinforcing effect to improve the mechanical strength of polysaccharide (M. Darder *et al.*2007).Incorporation of silver nanoparticles in bioplymeric matrices have been used in increasing shelf life of food material*(* Costa *et al.,* 2012).Nanospheres of Silica-based bionanocomposites processed by means of spray-drying techniques have also been envisaged as a system for drug delivery. Another application of bionanocomposites has been seen in the field of biomedical application is their use as drug delivery system for the controlled release of drugs and DNA. Substantial studies have been initiated in the development of materials by combining biomaterial as chitosan, exhibiting ion-exchange ability and nanomaterial to fabricate bio-nanohybrid materials. Diverse inorganic solids and their combinations have been developed as matrices to protective the activity and structural conformation of immobilized enzymes.(M. Darder *et al.2007)*

2.8.4) Silver Nanoparticles Synthesis

Silver nanoparticles have most commonly been synthesized through the most conventional method i.e. chemical reduction of silver nitrate in water or organic solvents. Commonly used reductants are sodium borohydride, ascorbate, citric acid, and elemental hydrogen. The methods results in formation of silver nanoparticles (Ag NPs) as stable colloidal dispersions with particle diameter in range of nanometers (Lee *et al.,* 1982). The mechanism behind the formation of silver nanoparticles follows two steps. Initially, reduction of various complexes with Ag^{+} ions leads to the formation of silver atoms (Ag^{0}), which is followed by agglomeration into oligomeric clusters. These clusters eventually lead to the formation of colloidal Ag particles (Kapoor *et al.,* 1994)

Silver nanoparticles synthesized through green approach have advantages over conventional chemical methods. The chemicals used in the conventional approach have a detrimental effect on environment by causing toxicity. These chemicals are strong reducing agents and cannot be employed when the need of silver nanoparticles is for of consumption and medical applications. The traces of these agents can also cause toxic immune response. Green synthesis methods include mixed-valence polyoxometallates, Tollens, polysaccharide, irradiation, and biological. Previous studies have explained the application of various green sysnthesis methods as follows: In the mixed-valence polyoxometallates method water has been used as an environmental-friendly solvent. Solutions of silver nitrate containing glucose and starch solutions resulted in starch protected Ag NPs, which could be incorporated into medical applications. Tollens process involves the reduction of $Ag(NH_3)^{2+}$ by saccharides leading to the formation of silver nanoparticles with particle sizes in the range of 50–200 nm and different shapes. Ag NPs synthesis by irradiation of Ag+ ions occurs in the absence of a reducing agent. Extracts obtained from microorganisms such as fungus and algae act both as reducing as well as capping agents during the Ag NPs synthesis thereby providing stability and maintaining narrow size range. The reduction of Ag+ ions takes place due to the heterogeneous mixture of biomolecules present in extracts such as enzymes, proteins, amino acids, vitamins and polysaccharides that is environment friendly, yet follows a chemically complex procedure of action. Plant extracts from live alfalfa broth, the broths of lemongrass, extract of geranium leaves, extract from leaves and fruit of amla tree, bael and pineapple extracts have served as green reducing agents for Ag NP synthesis (Sharma *et al.,* 2009)

2.8.5) Silver Nanoparticles as Antimicrobials

The most essential role of a polymer in food packaging is to provide an aseptic environment to the growth of microorganisms. Conventional method for food preservation after pasteurization, sterilization and blanching is addition of preservatives. Recent developments in food packaging aims to develop material which could provide a sterile environment in single step, that is by incorporating inorganic antimicrobial agents into the biopolymer matrix. Antimicrobial action of some nanocomposites materials has been acknowledged and actively applied in the sector of food packaging. Silver nanoparticles are efficient because of its elevated ratio surface by volume and improved reactivity of the surface enabling them to kill microorganisms much more meritoriously than the micro- or macro-scale counterparts. Commonly used antimicrobial resources to prepare nanocomposite with antimicrobial role include metal ions such as silver, gold, platinum, copper and metal oxide such as ZnO, MgO, TiO2 (Franci *et al.,* 2015). It is one of the widely exploited nanomaterials used for the fabrication of innovative packaging materials. This is mainly due to its unique properties of catalytic, electric, thermal stability, in addition to its antimicrobial action against a broad spectrum of bacteria, viruses, and fungi (Carlson *et al.,* 2008). It has been hypothised that silver ions interact with negatively charged bio-macromolecular components of the microbial cells such as disulfide/sulfhydryl groups of enzymes and nucleic acids, thereby leading to structural changes in the cell wall and membranes resulting in disruption of metabolic processes followed by cell death (Franci *et al.,*2015). It has also been suggested that presence of free radicals facilitate the antimicrobial mechanism of silver. Accumulation of silver ions in the cytoplasmic membrane of bacterial cells causes a considerable increase in permeability and cell death (Gombotz *et al.,* 1995). (Figure 4)

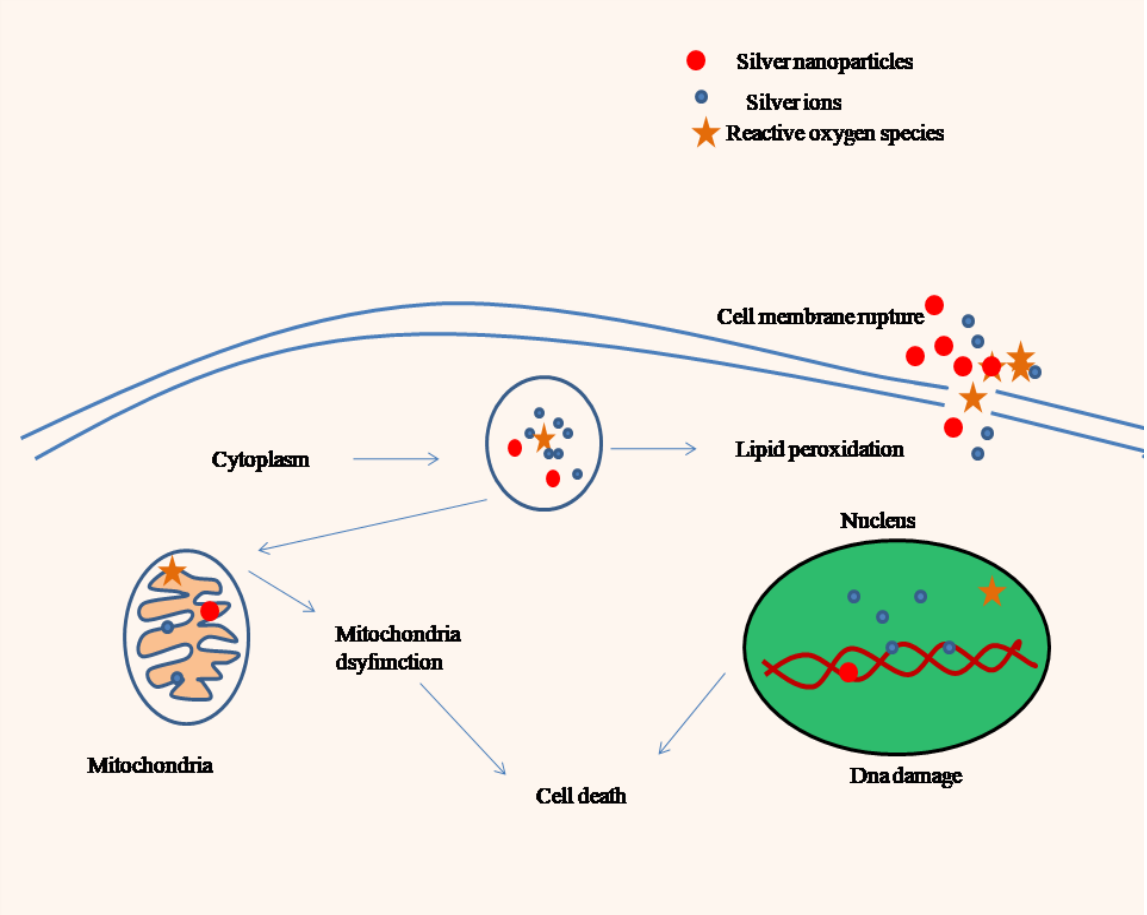

Figure 4: Antimicrobial action of silver nanoparticles

2.8.6) Applications of Nano-silver loaded Bionanocomposites

Costa and coworkers fabricated coating material comprising of calcium alginate with silver montmorillonite nanoparticles .They investigated effects of active coating and film barrier properties on shelf life of fresh-cut carrots my monitoring sensory changes and microbiological growth during the storage period. Results highlighted considerable differences in microbial populations between active coated and control samples. Samples with active coating had microbial cell load below the predetermined microbial threshold and were better preserved from a sensory point of view (Costa *et al.*, 2012). Fayaz and coworkers biosynthesized silver nanoparticles using extract from *Trichoderma viride* culture and incorporated them in sodium alginate films to study its effect on the preservation of vegetable and fruit. Analysis using TEM (transmission electron microscopy) revealed the development

of polydispersed nanoparticles. Bionanocomposite film demonstrated significant antibacterial activity against test strains. Results signified that the film substantially increased the shelf life of carrot and pear when compared to control on the basis of weight loss and soluble protein content of the sample (Fayaz *et al.,* 2012). Whereas migration of silver ions from composites of polylactide prepared by solution-casting and melt-mixing/compression moldings were compared by Fernandez et al., (2010). Through the results it was signified that all sort of PLA-silver zeolite composites released silver ions and it can be concluded that silver migration and antimicrobial activity are sensitive to the approach followed to fabricate PLA films. Khalaf and coworkers investigated the antimicrobial effect of pullulan edible films embedded with nanoparticles of silver and zinc oxide in preservation of turkey deli meat. The results demonstrated that bionanocomposite films with silver NPs were more active than those containing zinc oxide NPs. Gram-positive bacteria *L.Monocytogenes* and *S. Aureus* were used to test the antimicrobial activity of the films for durations of 7 weeks at varied storage temperatures (4°, 25°, 37°, and 55 °C). The best results for storage in pullulan edible films with silver NPs were obtained at 4°C. Substantial decrease in the population of both bacteria was observed in food samples packaged with pullulan film with AgNPs and oregan oil (Khalaf et al., 2013).Similar work by Morsy et al. (2014) demonstrated the activity of AgNPs incorporated into pullulan films to control growth of pathogens such as *S. Aureus* and *L. Monocytogenes* on meat and poultry products. Study by Incoronato and coworkers demonstrated the use of Agar hydrogel as hosting matrix for silver montmorillonite (Ag-MMT) nanoparticles to prolong the shelf life of Fior di Latte cheese. The results confirmed the antimicrobial effect of Ag-MMT matrix on the growth of *Pseudomonas* spp (Incoronato *et al.,* 2011). Starch has also been used as an edible film. A nanostructured starch based film containing clay and AgNPs proposed by Abreu et al., (2015) have demonstrated exceptional antimicrobial results along with improved mechanical and gas barrier properties. .In their native forms, starches are organized into granules and show poor mechanical properties and high water affinity, addition of AgNPs easily form complexes with the large number of hydroxyl groups of the biopolymer, thereby improving the properties of biopolymer.

2.9) Degradation of Polymers

Breaking down of chitosan into simple monomeric units can be commenced in the presence of enzymes which hydrolyse glucosamine-N-acetyl-glucosamine, glucosamine-glucosamine, and N-acetyl-glucosamine-N acetyl glucosamine linkage .Degradation of Chitosan is in vertebrates is hypothised to predominantly dependent on the action of lysozyme as well as enzymes produced by other bacteria present in the colon (Kean *et al.,* 2010).Rate as well as extent of chitosan biodegradability in living organisms is function of degree of deacetylation (DD) (Yang *et al.,* 2007). With increasing DD a sharp decrease in the degradation rate is reported. This phenomenon is due to dense network formation in between functional groups. Lysozyme has been reported to efficiently degrade chitosan to 50% acetylated chitosan with 66% loss in viscosity after incubating the polymers in phosphate buffer of pH 5.5 at 37 °C for 4 h (Onishi *et al.,* 1999).Pectinase isozyme from *Aspergilus niger* has also been recorded to digest chitosan at low pH providing decreased molecular weight chitosan (Kittur *et al.,* 2003).The type of crosslinker used for providing mechanical stability to the film influences the degradation rate; usage of glutaraldehyde as a crosslinking agents forms robust bonds in between functional groups. The intensity if bond strength formed through crosslinking by glutaraldehyde is far a greater than tripolyphosphate. Molecular weight of chitosan also plays major part in deciding the degradation rate of chitosan, high (310–600 kDa) and medium (190–310 kDa) Mw chitosan tend to degrade slowly when compared to low molecular weight chitosan (Verheul *et al.,*2009).

Degradation of alginate has been speculated to follow these possible pathways depending upon environmental conditions such as: disintegration of the alginate takes place through the exchange of gelling calcium ion with sodium ions. Hydrolysis of alginate has through acidic and alkali treatments have been reported. Agents such as peroxides oxidizes gels, it not only helps to accelerate the process but also weakens the gel leading in a ring opening reaction of the polymer (Holme *et al.,* 2003). Polymers including alginates undergo “free-radical depolymerization” or “oxidative-reductive depolymerization.” In this process, free radicals in living systems is generated by water molecules or molecular oxygen generates which acts on the polymeric chains leading to changes in the structural conformation and bond disruption. Additional exposure to gamma radiation hastens this process and can be used to improve the rate of degradation (Jeon *et al.,* 2009).It have been reported that

Alginates undergoes proton catalyzed hydrolysis which is dependent on time, pH, and temperature when exposed to gamma radiations. Exposure of crosslinked alginate matrix carrier system to low pH can cause reduction in molecular weight of alginate which results in faster degradation of the polymer (Haug *et al.*, 1963).

Studies have been performed to analyze the degradation pattern of carboxymethylcellulose. Various methods employed used for degradation of carboxymethylcellulose ranges from enzymatic degradation to use of physical treatment such as ultrasound and thermal degradation to the break the complexity of the structure to decomposable substituents. Endoglucanases from *Humicola insolens* and *Trichoderma reesei* have been exploited in the degradation of carboxymethylcellulose by Karlsson et al., (2001). The pattern of degradation was explained to be dependent on the substituted and unsusbstituted glucose units which could be hydrolysed by the enzyme. In another study carboxymethylcellulose was degraded by giving thermal treatment to the polymer upto 600ºC. The Thermogravimetric analysis of the product indicated weight loss in the two distinct zones. The preliminary weight loss was due to the existence of minimal amount of moisture in the sample and the succeeding weight reduction was due to the loss of CO_2 from the polysaccharide. Decarboxylation of COO^- group present in CMC at specified temperature signifies elevation in the rate of weight loss with increase in temperature (Biswal *et al.,* 2004). Degradation of carboxymethylcellulose has also been illustrated through ultrasonic vibrations. The conclusions made through this study signified that the intensity of ultrasonic vibrations and time of treatment played an important role in reducing the viscosity of the polymers thereby reducing the molecular weight to a substantial extent. (Mohod *et al.*, 2011)

CHAPTER 3

MATERIAL AND METHOD

3.1) Material Required

Chitosan (purity 99%; deacetylation degree 85%; pH 5-6; MW 100,000–300,000) was procured from Nano Wings Pvt. Ltd., Telangana (India) while silver nitrate was obtained from Sigma-Aldrich (India). Alginate and α-amylase were obtained from Hi Media Pvt. Ltd (Mumbai, India). Sodium salt of CMC was obtained from Sigma-Aldrich. For antimicrobial studies, the active slants of bacterial strains *E. coli* and *Bacillus subtilis* were obtained from Department of Biotechnology, TIET, Patiala. Double deionized water was used thoroughly for all the experiments related to nanomaterial synthesis and biological studies.

3.2) Preparation of Microcarriers

Fabrication of Core

The core of microcarriers was followed as per the previous study (Ribeiro *et al.,* 2018) with substantial modifications. For synthesis of the core, 0.2g of calcium chloride (36mM) and 0.2g of sodium carboxymethycellulose (15mM)was mixed together in 50ml of distilled water and stirred for 30 minutes on magnetic stirrer until completely dissolved. Solution of sodium bicarbonate of 37.8mM was prepared by adding 0.2g sodium bicarbonate to 50ml of distilled water and stirred until the solute is mixed completely. This was poured into the solution of calcium chloride and sodium carboxymethylcellulose under the steering condition and was left on stirrer for next one hour. The resultant solution was centrifuged for 10 minutes at 10,000 rpm, supernatant was discarded and pellet was dried in hot air oven at 45˚Cfor 24 hours to remove excess water and stored in the form of powder. Size estimation of the core particles was performed through DLS.

Formation of Biopolymeric Layer

The biopolymeric layering on the core was done in two combinations, which is via chitosan-alginate and carboxymethylcellulose-alginate co-polymeric system.

a. Biopolymeric layers of Chitosan and Alginate

Chitosan solution of 0.1 mg/ml concentration was prepared by dissolving chitosan in 2% acetic acid on a shaker at 35˚C overnight. Alginate solution of 1mg/ml concentration was prepared by dissolving alginate powder in 0.5M NaCl solution. For the coating of core with chitosan as the first layer, required amount (40 mg) of previously formed core particles were taken and mixed with 400µl, 0.1 mg/ml chitosan solution and was agitated over vortex for 15 minutes. Afterwards, the mixed solution was centrifuged for 10 minutes at 10,000 rpm. While the supernatant was discarded, pellet was again rinsed with 400 µl, 0.5M NaCl followed by DI water twice. The second layer of alginate was formed by adding 400 µl alginate solution to the chitosan-coated core particles and was again agitated over vortex for 15 minutes, centrifuged and washed as mentioned above. The procedure for multilayer (layer by layer assembly) formation was executed by introducing the coated microparticles in chitosan and alginate solution in alternative manner.

b. Biopolymeric Layers of Carboxymethylcellulose and Alginate

Sodium carboxymethylcellulose solution of 1mg/ml concentration was prepared in deionized water under stirring condition until dissolved. Alginate solution of 1mg/ml concentration was prepared by dissolving alginate powder in 0.5M sodium chloride solution. Required amount (40 mg) of the core particles formed was taken in an eppendorf. For the formation of first biopolymeric layer on the core 400 µl of 1mg/ml cmc solution was added and incubated for 15 minutes on vortex, after which the solution was centrifuged for 10 minutes at 10,000 rpm and the supernatant was discarded followed by washing of the pellet with 400 µl of 0.5M sodium chloride and distilled water twice. The second layer was of alginate which was fabricated by adding 400 µl alginate solution to the carboxymethylcellulose coated core particles and incubated on vortex for 15 minutes and then centrifuged and washed. The procedure for multilayer (layer by layer assembly) formation was executed by introducing the coated microparticles in carboxymethylcellulose and alginate solution in alternative manner. The size and structure of the microcarriers was characterized through SEM at different magnifications.

3.3) Immobilization of α-Amylase on the Microcarrier

Surface immobilization of alpha amylase

For immobilization of α-amylase onto the biopolymeric microcarriers, 200 µl of α- amylase of concentrations 0.5mg/ml, 1 mg/ml and 2mg/ml was added to the required amount (10mg) of coated microcarrier respectively and incubated for 24 hours on shaker incubator at 35ºC. This was followed by centrifugation for 10 minutes at 10,000 rpm at the end of 24 hours. The supernatant for removed and stored for assessing the amount of α-amylase immobilized onto the surface of microcarriers. The pellet was air dried to remove the remaining moisture and stored at 4ºC until used.

In-Situ Immobilization of Alpha Amylase

For *in-situ* immobilization, α-amylase was prepared in two concentration *i.e.* 0.5 mg/ml and 1 mg/ml. As a typical procedure, 0.2g of calcium chloride (36mM) and 0.2g of carboxymethycellulose (15mM) was mixed together in 50ml of distilled water and stirred for 30 minutes on magnetic stirrer to which α-amylase was added in the concentration of 0.5mg/ml and 1mg/ml in two different sets and stirred for 30 min on magnetic stirrer. Afterwards, 0.2g of sodium bicarbonate (37.8mM) was added to 50ml of distilled water and stirred until dissolved completely. After mixing calcium chloride with CMC and α-amylase, sodium bicarbonate solution was added under stirring condition and left on stirrer for 1 hour. The resultant solution was centrifuged for 10 min at 5,000 rpm. Supernatant was removed and stored to assess the quantity of enzyme immobilized in the core particles. The procedure was repeated in an interval of 1day for 3 days. Every time, the sample was agitated for 5 minutes, centrifuged and the assay was performed. The sample was ultimately centrifuged for 20 minutes at 5000 rpm, the supernatant was discarded and the pellet was air dried. These core particles were layered in the same manner as the above-mentioned microcarriers were fabricated.

3.4) Assays for Enzyme Immobilization

Immobilization of α-amylase was quantified using DNS assay for both immobilization strategies. As a substrate for the assay, 0.1% starch was prepared by dissolving 0.1g of starch

in 100 ml of distilled water on a hot plate under stirring condition until completely dissolved. The reaction mixture comprised of 800µl of 0.1% starch solution onto which 200µl of the supernatant was added followed by mixing the solution by inversion. The mixture was incubated at room temperature for 30 minutes. After which, 1ml of DNS solution was added to reaction mixture and further incubated at 90ºC water bath for 15 minutes. The absorbance of the mixture solution was recorded at 540 nm using a spectrophotometer. A standard graph was plotted for the varying concentration of α-amylase keeping the substrate concentration constant for assessing the amount of enzyme immobilized by both the approaches.The amount of enzyme immobilized was calculated by the following formula:

$$X = \frac{A_0 - A_s}{A_0}$$

Where X=amount of enzyme immobilized; A_o= absorbance of initial enzyme conc.;

A_s= absorbance of supernatant

Release profile for immobilized α-amylase from the microcarriers was performed by suspending the immobilized microcarier in PBS of pH 7 and 6 respectively. Different pH was employed to quantify their effect on the release profile of the immobilized enzyme from the microcarriers. For this study, the required amount (10mg) of microcarrier was suspended in 500 µl of PBS solution maintained at different pH and left on shaker incubator at 35ºC. After this, 200µl aliquot was taken in time intervals and 200µl of fresh PBS was added every time an aliquot was taken to maintain the final volume. The released enzyme was assayed through DNS using 0.1% starch solution as a substrate.

3.5) Synthesis of Silver Nanoparticles

For biogenic synthesis of silver nanoparticles, amla fruits were cut into fine pieces and boiled in distilled water for 5 mins. The extract was filtered using muslin cloth; extract was stored at 4ºC until used. Silver nanoparticles were synthesized using 1mM of silver nitrate solution was dissolved in deionized water in the dark. To 1ml of silver nitrate solution taken in an eppendorf tube covered with aluminium foil, 500 µl of amla extract was added and vortexed for 15 minutes. After which it was incubated in dark until brownish orange color change was observed. The solution was centrifuged at 10000 rpm for 5 minutes, the supernatant was removed and the pellet was dissolved in 200 µl of deionised water. Formation of silver

nanoparticles was validated by assessing the reduction in periodic time intervals varied from 2 hours to 24 hours. Estimation of the nanoparticles formation with the reduction of silver nitrate through both the approaches was done by UV-visible spectrophotometer. The absorbance peak was measured in the range of 390-430 nm. The intensity of the peak formed signified the extent of reduction of silver nitrate.

3.6) Immobilization of Silver Nanoparticles on Microcarriers

Required amount of (20mg) of microcarrier was taken in an eppendorf to which 200 µl of silver nanoparticle solution was added and left for immobilization on a shaker for 24 hours. The resultant was centrifuged at 10000 rpm for 5 minutes, the supernatant was removed and the pellet was air dried and stored as powder.

3.7) Silver Nanoparticle Immobilized Microcarrier Embedded Chitosan Film

Chitosan solution was prepared by dissolving 2g of chitosan in 100ml of 2% acetic acid under stirring condition at 35 ºC overnight. The solution was filtered using a fine pore sieve to remove the undissolved flakes and impurities. To 20ml of 2% chitosan solution, 500 µl of silver nanoparticle coated microcarrier solution was mixed and vortexed for 5 minutes. The mixture was poured in a petridish, spread evenly and kept in hot air oven at 37º C for 24 until the film started to peel from the petridish

3.8) Antibacterial Assays of Nano-Silver Loaded Microcarriers

Antimicrobial activity of silver nanoparticle coated microcarriers was tested against *E.coli* and *B.substilis* through well diffusion method. Overnight grown bacterial culture was multiple times in PBS buffer and was diluted to OD of unity at 600 nm which is equivalent to ~10^9 CFU/ml. For test concentration, 1 OD pellet was redissolved in PBS and serially diluted in PBS to obtain a cell concentration of 10^5which was spread on nutrient agar plate. With the help of a well borer, wells were made on the agar plate. Required amount of coated microcarrier (5mg) was mixed with PBS and 100 µl of the sample was placed in the wells and plates were incubated at 37ºC overnight. The diameter of zone of inhibition was measured to estimate the antimicrobial activity.

Antimicrobial activity of silver nanoparticle coated microcarriers was also tested against *E.coli* and *B.substilis* through spread plate method. Overnight grown bacterial culture was

pelleted at the OD of 1 at 600 nm. The pellet was dissolved in PBS and serially diluted in PBS to obtain a cell concentration of 10^5 which was spread on nutrient agar plate. Required amount of coated microcarrier (5mg) was mixed with PBS and 100 µl of the sample was spread on a small area of the agar plate .The plates were incubated at 37ºC overnight. The diameter of zone of inhibition was measured to estimate the antimicrobial activity. Antimicrobial activity of silver nanoparticle coated microcarriers embedded films was also tested against *E.coli* and *B.substilis* under similar conditions.

3.9) Preservative Effects of Nano-Silver loaded Microcarriers

Grapes (green) were taken as test sample for evaluating the applicability of AgNP-loaded microcarrier chitosan film for food preservation. Grapes sample was prepared by washing them with distilled water and drying them using paper towel. The grapes and films were disinfected prior with ethanol wiping (30%) followed by exposure to UV rays for 30 minutes before packaging. After this, grapes were covered with the nanobiocomposite film were subjected two preservation conditions ie placed in a plastic self sealing bag as well as in open. The samples were placed at room temperature along with two controls. As a positive control, grape was placed in the sealing bag devoid of nanobiocompsite film. While grape was also kept in open air without being placed in any sealing bag as negative control. The surface of the sample was observed for changes over time and every alternate day the sample was removed from the bag to measure its weight

CHAPTER 4

RESULTS

4.1) Synthesis and Characterization of Microcarriers

Fabrication of microcarrier was starting with synthesis of core particles on to which the biopolymeric layers would be formed. Compositions of core consisted of combination of calcium carbonate and sodium carboxymethyl cellulose, this mixture was stirred onto magnetic stirrer on varied time intervals to obtain an optimum size. The size of core was estimated through DLS. Figure 5, the change in polydispersity with time can be observed clearly. After 30 minutes of stirring, the size of the particles varied in the range of 430- 1500 nm in different frequencies with an average size of 832 nm. Whereas after 1 hour of stirring, size of the particles varied in the range of 200- 1150 nm in different frequencies with an average size of 510 nm. The size of the particles did not reduce further even after stirring for more than an hour. Core particle obtained after 1 hour of stirring was used for the formation biopolymeric layers based on two biopolymeric combination (chitosan- alginate and sodium carboxymethyl cellulose – alginate). The purpose of the calcium carbonate as a component of core is to provide a firm base for the layers to form whereas role of sodium carboxymethyl cellulose provides a platform for the spontaneous loading of positively charged molecules (Zhao *et al.,* 2007).

Biopolymeric layers in the combination of chitosan-alginate and sodium carboxymethyl cellulose-alginate were formed onto the core particles respectively in layer by layer assembly. Information regarding the size, structure and surface morphology were obtained by subjecting the core particles and biopolymeric microcarriers to SEM analysis at different magnifications (Figure 6).SEM images at 10,000X magnification revealed the structural distribution of the core particles as well as biopolymeric microcarriers. The shape of both the samples ranged from being spherical to oval and some being cuboidal. The size of the biopolymeric microcarrier varied in range of 1-3µm which was determined by analysing the sample at higher magnifications i.e. 30,000X. Comparison between the SEM images at of core particles (image B) and biopolymeric microcarriers (image D) at 30,000X was made to study the difference in surface morphology of the samples. In image B, sharp groves and rough surface

characteristics of core particles can be observed. Whereas in image D the surface of biopolymeric microcarriers appears to be smoother than the surface of core particles and the sharp appearance of the grooves is diminished. The diminished appearance of rough groves and sharp edges signifies that the biopolymeric layers have been formed successfully over the core surface.

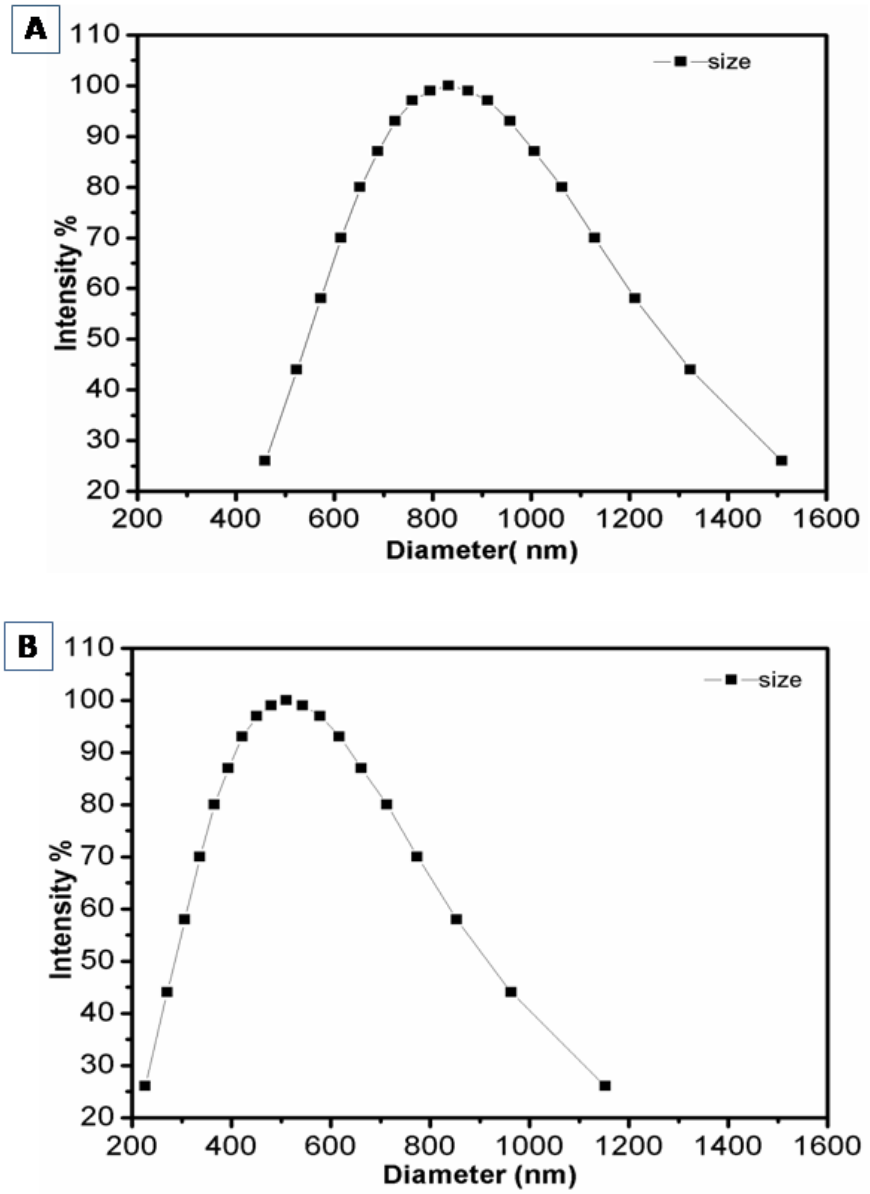

Figure 5: Size distribution by intensity of core particles at A) 30 min and B) 1 hour

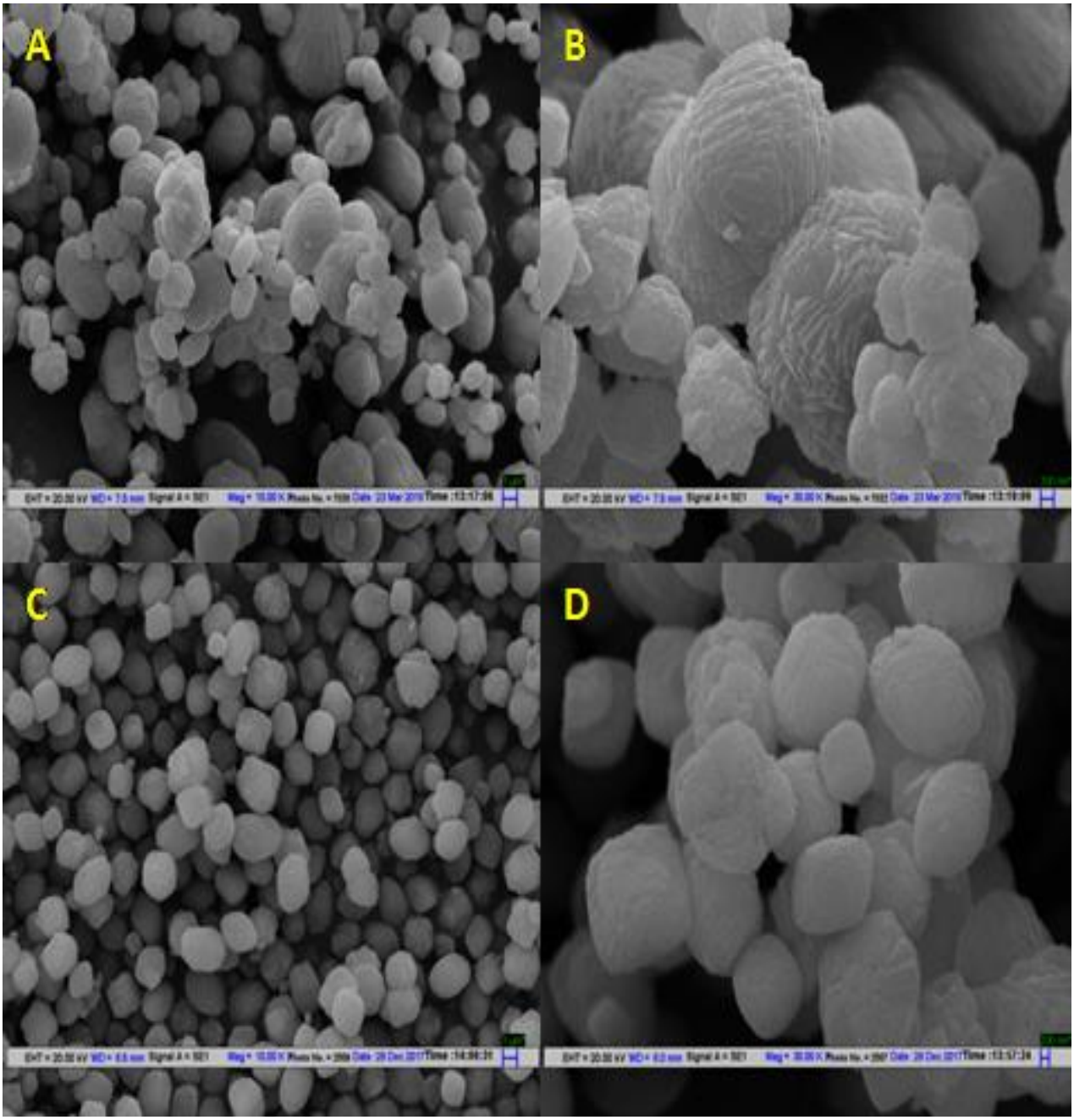

Figure 6: SEM images at different magnification of Core particles at magnification A) 10,000X B) 30,000X and Biopolymeric microcarrier at magnification C) 10,000X, D) 30,000X

The formation of core-shell template of the microcarrier was confirmed by analysing the cross section of the microcarrier through SEM (figure 7). The cross-section of the microcarrier was achieved by sliding microcarrier in between butterpaper with minimal

sliding force. Distinct core and shell region was observed, the core being at the centre and biopolymeric layering as shell around the core.

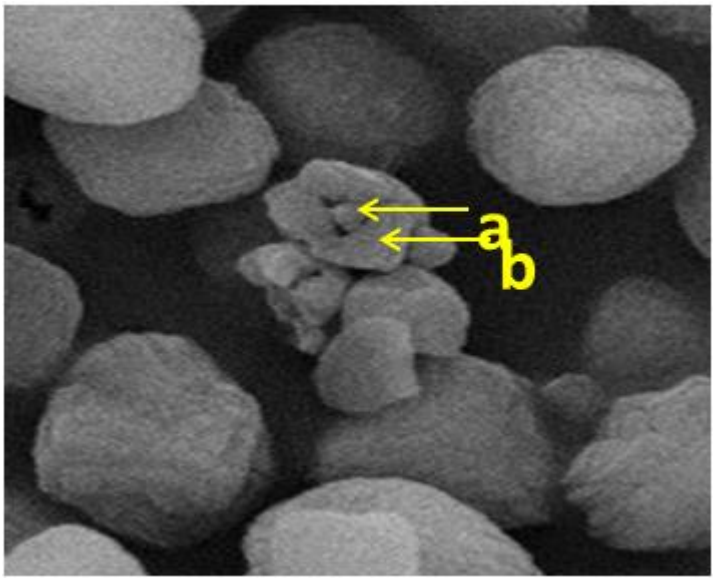

Figure 7: SEM image of biopolymeric microcarrier representing core and shell template, a) core and b) shell

The number of biopolymeric layers was varied in order to obtain maximum loading efficiency. The number of layers were varied in succession and their loading efficiency was determined (Table 2). Each layer is a combination of two biopolymers, i.e. 1 layer consist of one layer of chitosan along with another layer of alginate, similar was for another combination of sodium carboxymethyl cellulose and alginate. Table 2 represents the increase in immobilisation efficiency with the increasing biopolymeric layers. Microcarriers with single biopolymeric layer showed low immobilisation efficiency, it was same for both the biopolymeric combinations. Microcarriers with 2 layers showed an increase in immobilisation efficiency to 9-10%. Substantial increase in immobilisation efficiency of 70-75% was observed in microcarriers with 5 biopolymeric layers. The number of biopolymeric layers could not be increased more than 5 for the biopolymeric combination of chitosan-alginate as the microcarriers started to stick to each other and walls of the eppendorf. Their sticking to each other made them form a solid pellet which restricted further fabrication of layers.

The shell formation is due to the interaction between combinations of polymers due to opposite surface charges leading to formation of polyelectrolyte complex. This eliminates requirement of chemical cross linkers in order to form bonds between biopolymers. Sodium carboxymethyl cellulose present in the core particles being polyanionic interacts with polycationic chitosan leading to the formation ionic bonds between them. This phenomenon is responsible for the formation of ionic bonds between alginate and chitosan as well (Hamman, 2012). Sodium carboxymethyl cellulose and alginate interacts due the formation of glycosidic linkages between two polysaccharides along with ionic interaction.(Han et al 2017) The layers of biopolymers were formed alternatively which led to the formation of polyelectrolyte complex and did not require any cross-linkers for the inter bond formation

Table 2: Represents the increase in immobilisation efficiency along with increase in number of biopolymeric layers

Number of layers	Immobilisation efficiency	
	Chitosan-alginate	**CMC-alginate**
1	low loading	Low loading
2	10%	9%
3	25%	22%
4	55%	50%
5	75%	70%

4.2) Immobilisation of α- amylase

4.2.1) Surface Immobilisation of α- Amylase on Microcarriers

For the Immobilisation of α- amylase two approaches were followed i.e. in-situ immobilisation and surface immobilisation. For Surface immobilisation of enzyme, the biopolymeric microcarriers with different combination of biopolymeric layers were incubated in different concentration of α-amylase enzyme aliquots in incubator shaker at 37ºC for 24

hours to facilitate efficient loading of enzyme onto their surface. The efficiency was estimated by measuring the residual enzyme present in the supernatant. The immobilisation efficiency at different concentration was plotted for both the biopolymeric combinations. (Figure 8) A gradual increase in the immobilisation efficiency was observed with increasing enzyme concentration. The immobilisation efficiency of the microcarrier with biopolymeric combination chitosan- alginate increased from 65 to 75% with increase in concentration of enzyme. Whereas the immobilisation efficiency of microcarrier with biopolymeric combination of sodium carboxymethyl cellulose – alginate varies from 64–74% with increasing enzyme concentration.0.3mg of enzyme was immobilised onto the microcarrier surface at feeding amount of 4mg of enzyme for 10mg of microcarrier the gradual increase in the immobilisation percentage with the increasing feeding concentration is due to accumulation effect. (Zhao *et a*l., 2007)

On the basis of immobilisation efficiency, microcarriers with biopolymeric combination of chitosan –alginate is much more efficient. 0.1mg/ml concentration of chitosan was utilized for the formation of layers along with alginate whereas in the combination of sodium carboxymethyl cellulose – alginate the layering concentration of sodium carboxymethyl cellulose was 1mg/ml, keeping the concentration of alginate same in both the combination. DDA and MW of chitosans are important for its physical and biological properties (Yuan *et al*., 2011). Studies have reported that higher degree of deacetylation of chitosan results in increased charge density on the molecule thereby increasing the adhesion properties. The increase in adhesion properties leads to slow release of encapsulation agents and also lowers the degradation rate of chitosan (Aranaz *et al*.,2009) Chitosan being used of high molecular weight and 85% degree of deacetylation have longer chains and more functional group which have the ability to bind more amount of enzyme even when used at low concentration where as sodium carboxymethyl cellulose at higher concentration binds to lesser amount of enzyme than chitosan, this makes chitosan much more efficient candidate to be used as an immobilisation matrix. Apart from the presence from function

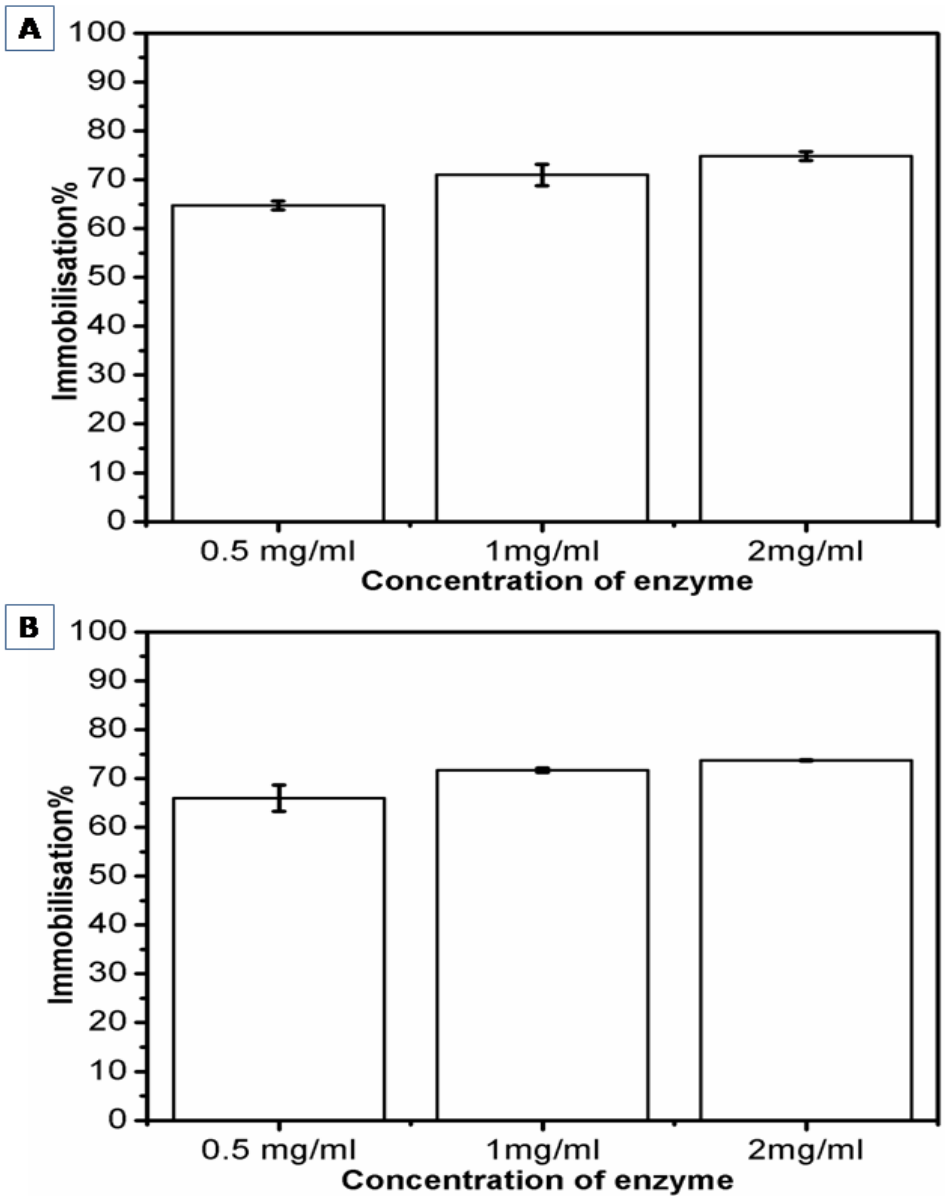

Figure 8: Enzyme immobilisation efficiency of microcarrier of biopolymeric combination A) Chitosan- alginate and B) Sodium carboxymethyl cellulose –alginate

The surface immobilised microcarriers were subjected to SEM analysis at different magnifications to study the surface characteristics and confirm the loading of enzyme (Figure 9). The difference in surface morphology was observed by comparing the SEM images of the enzyme immobilized biopolymeric microcarrier with biopolymeric microcarrier without enzyme being immobilised. In image B, the biopolymeric microcarriers have been analysed at magnification 50,000X. Slight groves and roughness on the surface can be observed where as

in image D, smooth cloudy appearance over the surface of the microcarriers was observed .The difference in the surface morphology forms the basis of inference that there has been successful immobilisation of enzyme onto the microcarrier surface.

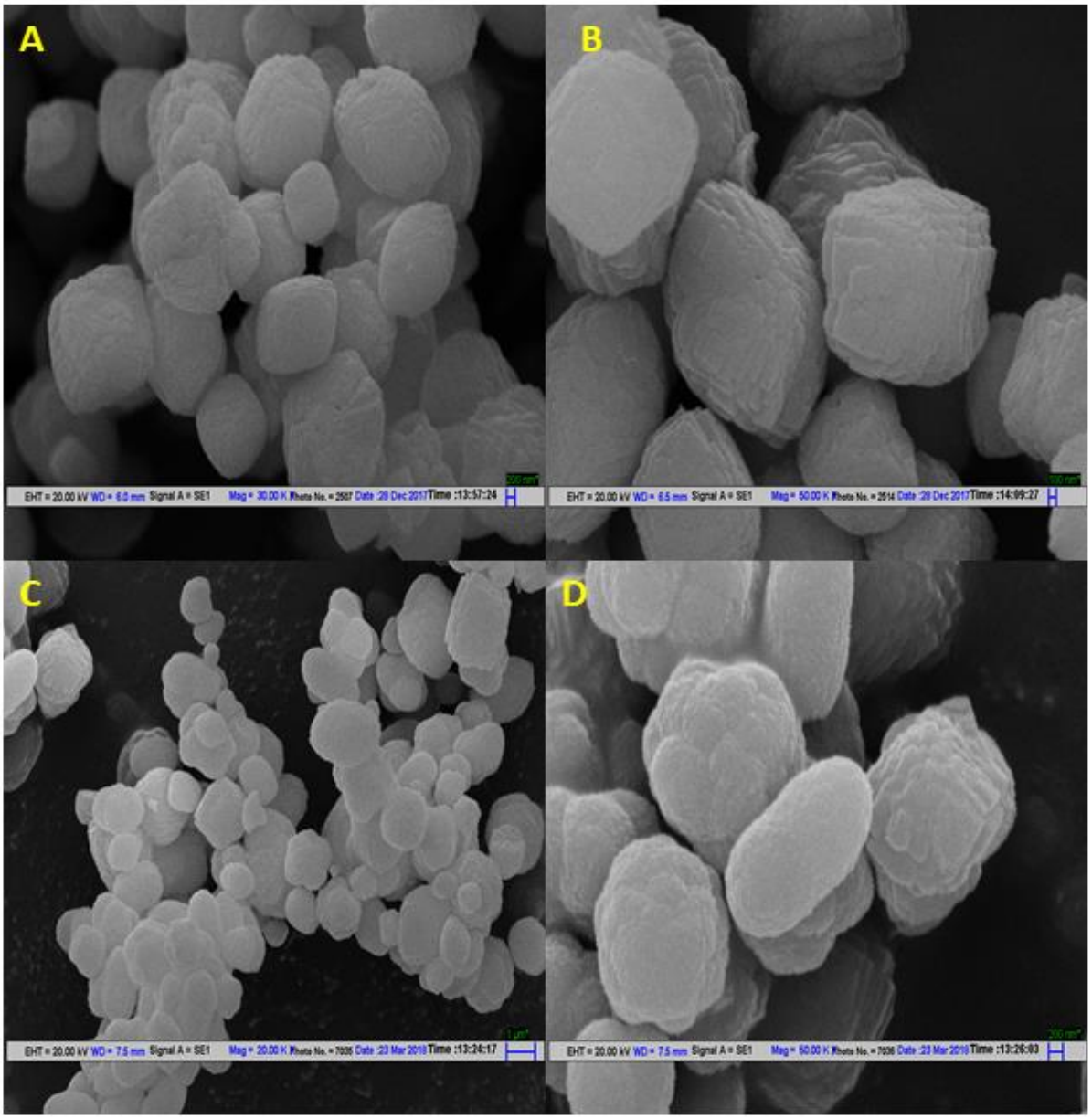

Figure 9: SEM images of biopolymeric microcarrier at magnification A) 30,000X and B) 50,000X and Enzyme immobilised biopolymeric microcarrier at magnification C) 30,000X and D) 50,000X

4.2.2) *In-Situ* Immobilisation of α- Amylase into Core Particles

For in-situ immobilisation of α- amylase, the enzyme was added along with calcium carbonate and sodium carboxymethyl cellulose during the fabrication of the core particles. Immobilisation efficiency in the core particle was optimised by assessing the immobilisation percentage of enzyme by varying the enzyme concentration, time of incubation and incubation conditions. The amount of enzyme immobilised onto microcarriers was estimated at certain intervals through DNS assay with 0.1% of starch as substrate. Addition of α-amylase during the core fabrication allowed the enzyme to form bonds with sodium carboxymethyl cellulose due to opposite charge interaction which leads to the entrappement of amylase into core particles during its synthesis. Conditions for the immobilisation were varied to study the variation in immobilisation efficiency (Zhao *et al*., 2007). Core particles after fabrication were left in the solution for 3 days on a shaker to allow maximum immobilisation of enzyme. Figure 10 represents the graph showing increase in immobilisation efficiency with increasing days of incubation. At the end of 3 days of incubation, 83% immobilisation percentage was achieved for the enzyme concentration 0.5mg/ml and 80.45 % for the enzyme concentration 1mg/ml. To obtain the maximum immobilisation percentage the conditions for the fabrication of core particles were changed to magnetic stirring from being kept on shaker for 24 hours. Aliquots were taken at different time intervals to estimate the percentage of enzyme immobilised. In the first 2 hours the immobilisation percentage of 60-64 % was achieved which increased to 78% after 4 hours of stirring which remained constant even after 24 hours of incubation. From the data obtained it could be inferred that fabrication of enzyme immobilised core particle with substantial immobilisation could be achieved when kept at stirring for 24 hours than being kept on shaker for 3 days , thereby substantially reducing time to obtain optimum immobilisation efficiency (figure 11). A total amount of 1.4 mg and 2.7 mg of enzyme was immobilised into the 10mg of core particles at feeding concentration 0.5 mg/ml and 1mg/ml respectively.

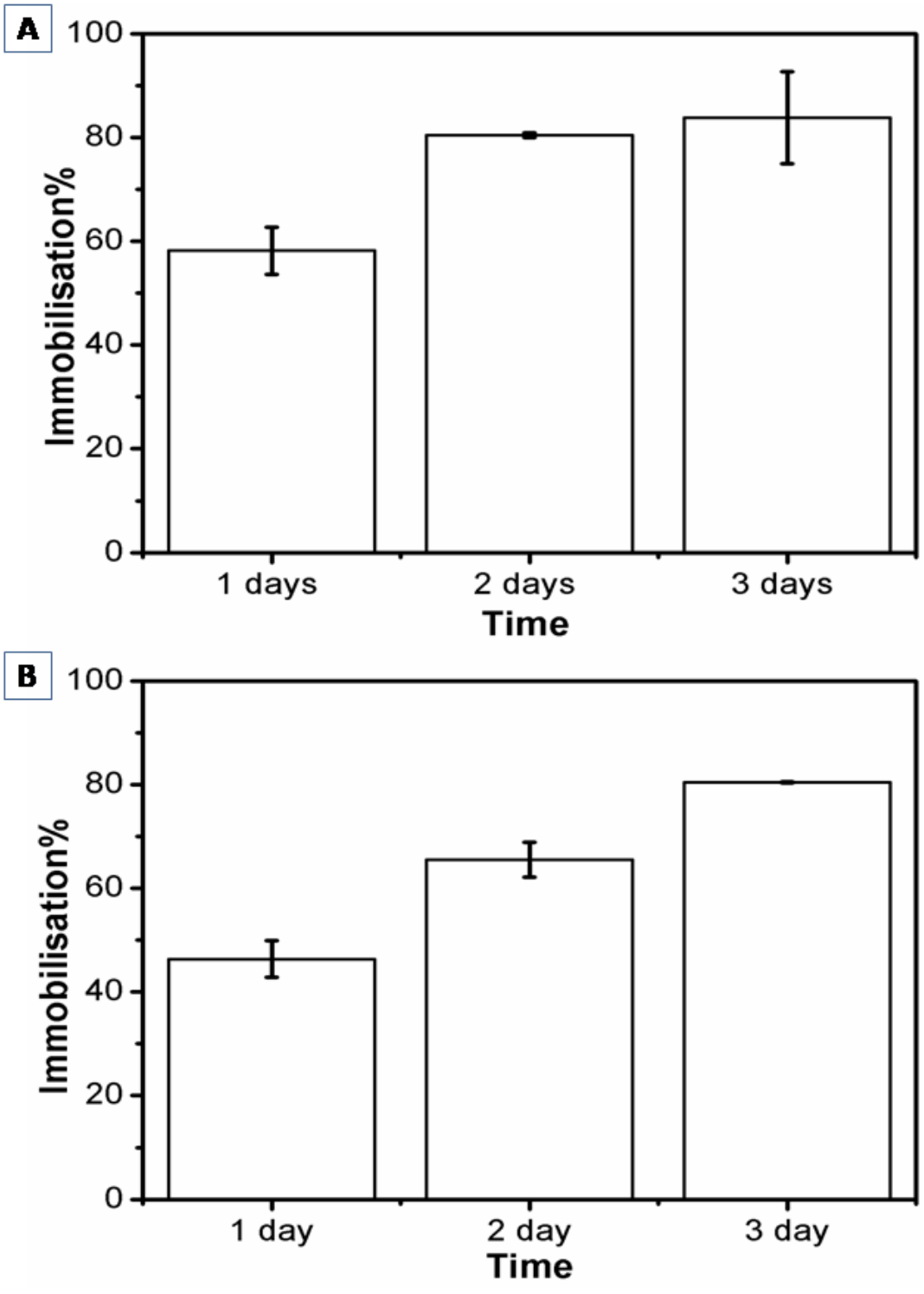

Figure 10: Immobilisation percentage of different α-amylase concentration 1) 0.5mg/ml and 2) 1mg/ml into core particles.

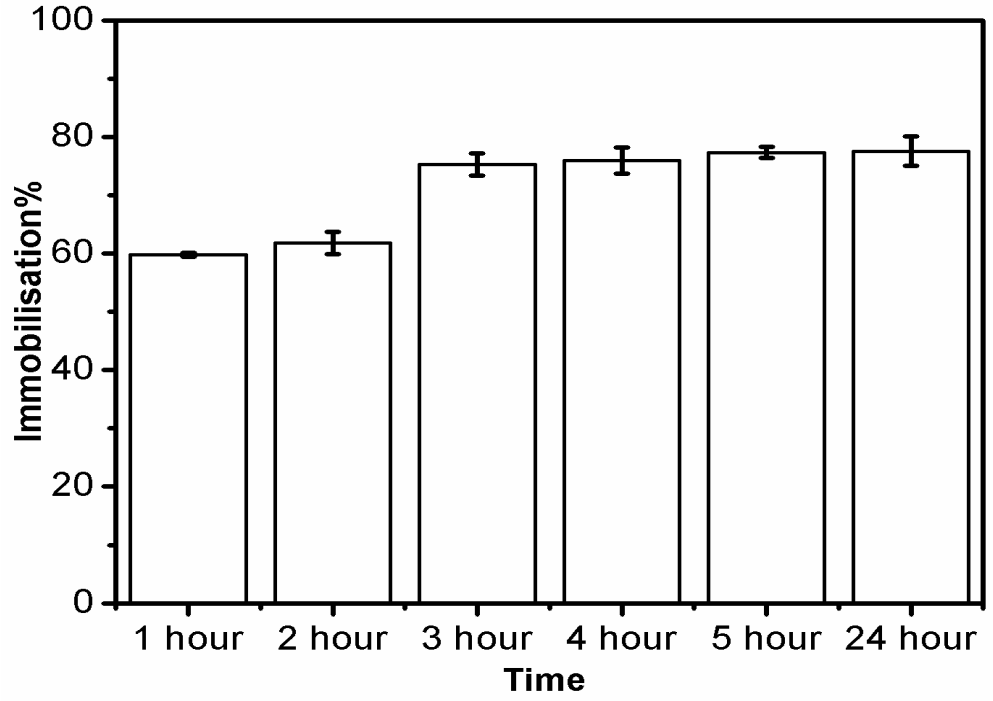

Figure 11: Percentage of enzyme immobilised in core when kept at stirring.

Information regarding the size, structure and surface morphology were obtained by subjecting the core particles and α-amylase immobilised core particles biopolymeric microcarriers to SEM analysis at different magnifications (Figure 12).SEM images at 10,000X magnification revealed the structural distribution of the core particles as well as α-amylase immobilised core particles. The shape of both the samples ranged from being spherical to oval and some being cuboidal. The size of the biopolymeric microcarrier varied in range of 2-3μm which was determined by analysing the sample at higher magnifications i.e. 30,000X. Comparison between the SEM images at of core particles (image B) and α-amylase immobilised core particles (image D) at 30,000X was made to study the difference in surface morphology of the samples. In image B, sharp groves and rough surface characteristics of core particles can be observed. The edges of rhomboidal structures in core particles have sharp clean edges. Whereas in image D the surface of α-amylase immobilised core particles appears to be smoother than the surface of core particles with different structural appearance than core particles. Fibril like structure has been observed over the surface and in between two particles. The difference in surface and structural appearance

leads to the inference that there has been successful immobilisation of α-amylase during the synthesis of core particles.

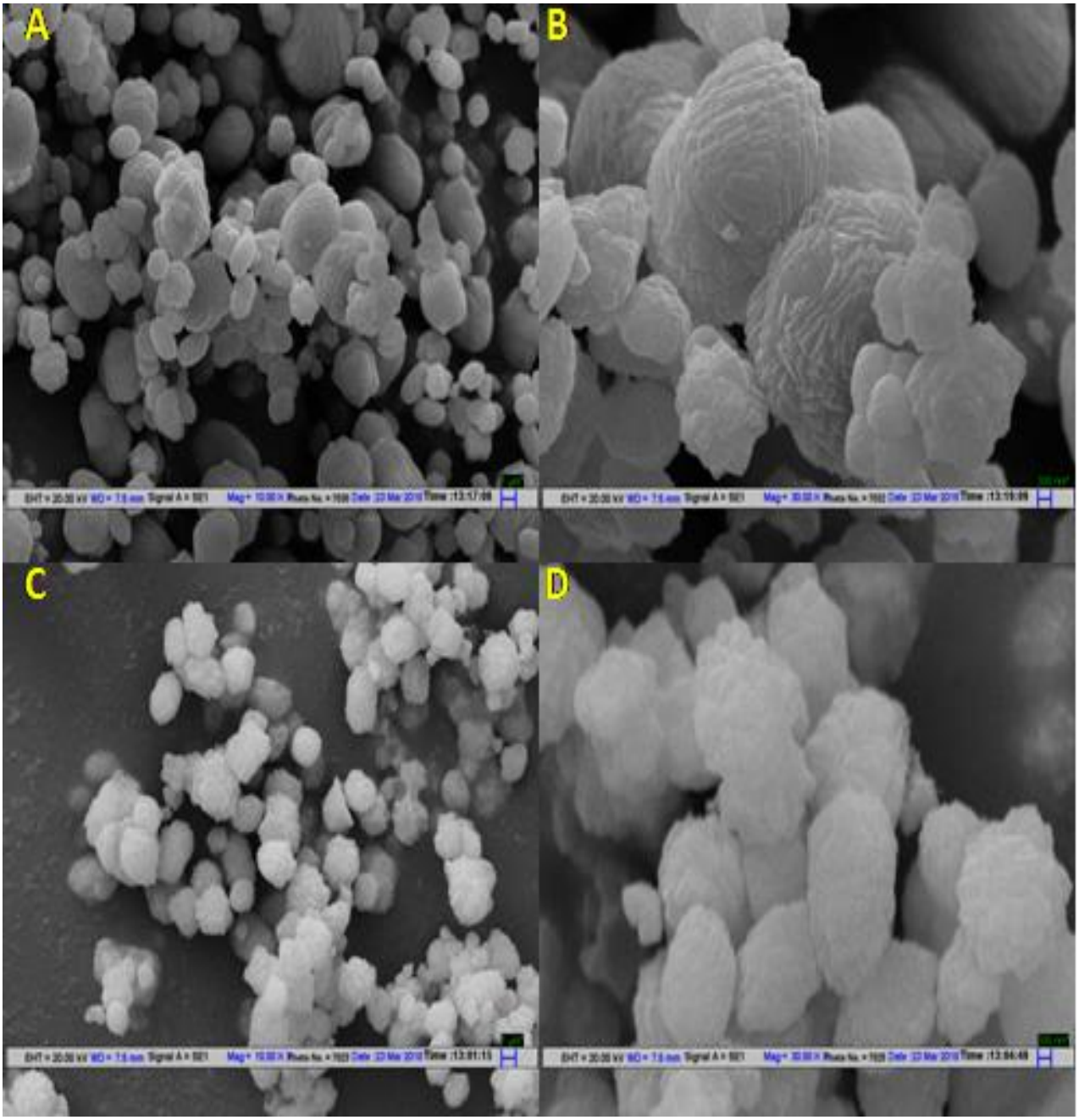

Figure 12: SEM images at different magnification of Core particles at magnification A) 10,000X B) 30,000X and α-amylase immobilised core particles at magnification C) 10,000X, D) 30,000X

4.3) Release Profile

The swelling and degradation behaviour of biopolymers varies extensively depending upon the molecular weight, type of functional groups present and bong strength. To study these parameters microcarriers fabricated with different biopolymeric combinations were used for *in-situ* immobilisation of enzyme as well as surface immobilisation of the enzyme. After which their release profile was noted over a period of time to gather information related to its release pattern with changing environmental parameter. The purpose was to conduct a comparison between following methods for immobilisation of enzyme and the combination biopolymeric layers on the basis of release pattern. Release profiles of these microcarriers were studied by dispersing them in PBS solution of pH7 and pH6, placed on shaker at 37ºC. Aliquots were taken at certain time intervals to estimate the amount of enzyme released by DNS assay taking 0.1% starch as substrate.

4.3.1) Release Profile at pH7

Figure 13 and figure 14 represents the release profile microcarriers of both biopolymeric combinations with surface immobilised α- amylase as well as in-situ immobilised enzyme at pH 7. Linear release profile was observed with increasing time for both the biopolymeric combinations. Microcarriers with biopolymeric combination chitosan-alginate with surface immobilised enzyme illustrated a cumulative release percentage of 100% on day 6, where as for the microcarriers with biopolymeric combination carboxymethyl cellulose –alginate was 97% on day 6. Microcarriers with in-situ immobilised enzyme shows controlled release of the enzyme. The cumulative release percentage of enzyme from microcarriers with biopolymeric combination chitosan-alginate was 45 % till day 10, where as for the microcarriers with biopolymeric combination carboxymethyl cellulose –alginate was 27%. In the case of surface immobilised enzyme complete release of enzyme was obtained due to following reasons: the availability of enzyme on the surface, the biopolymeric complex forms bonds on the basis of charge –charge interaction which can dissociate over time due to change in microenvironment and lead to the release of the total immobilised enzyme. Where as in the case of in-situ immobilised enzyme , the enzyme is present in the core and the substrate or the enzyme have to permeate through the biopolymeric layers which forms a barrier and restrict the release of enzyme or bulk permeation of the substrate into the microcarriers. The swelling characteristic

of chitosan is higher than sodium carboxymethyl cellulose making it more porous, due to which there is higher release of enzyme than the later combination. This can be reason behind higher release of enzyme in the both the methods of immobilisation from the microcarrier with biopolymeric combination of chitosan-alginate

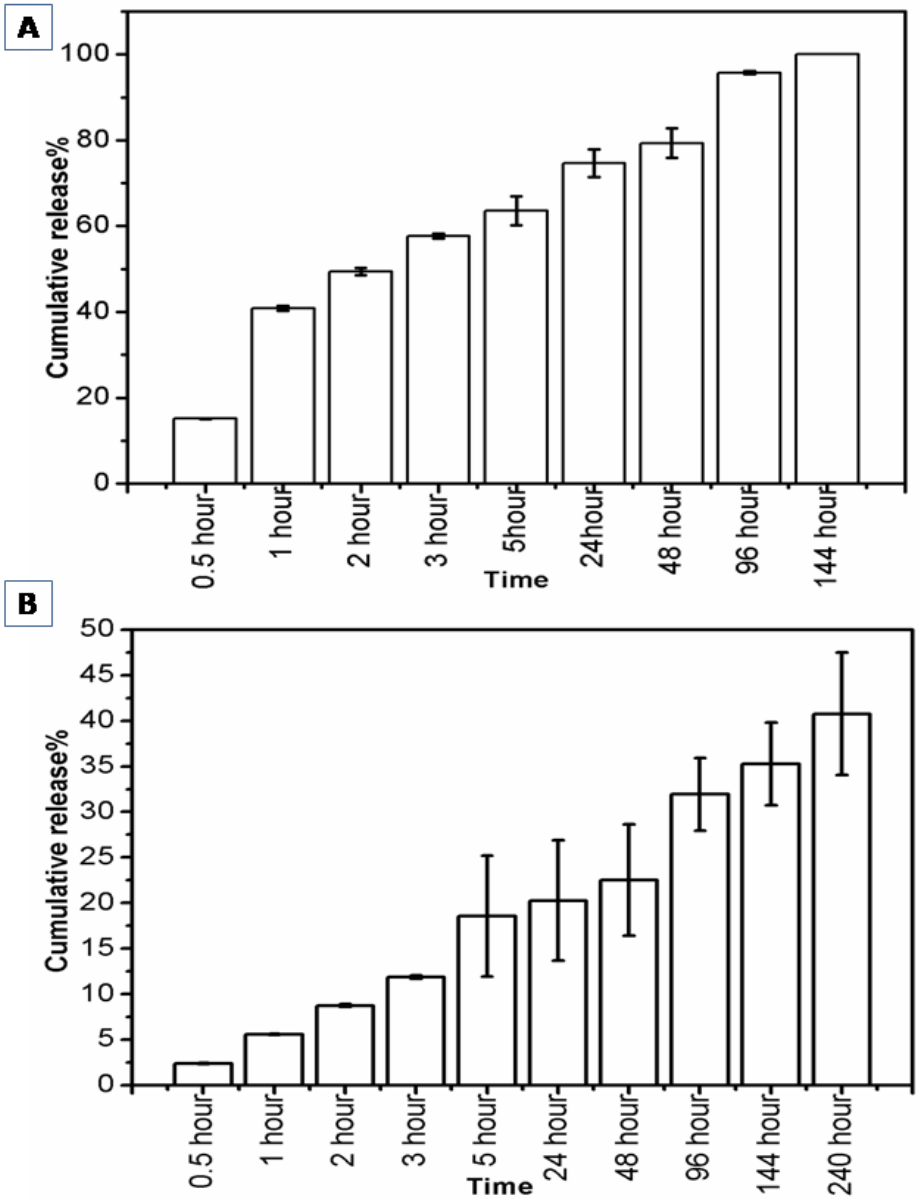

Figure 13: Cumulative release profile of microcarrier with chitosan-alginate biopolymeric combination at pH7, where graph 1) represents release profile of surface immobilised enzyme and 2) represents release profile of *in-situ* immobilised enzyme

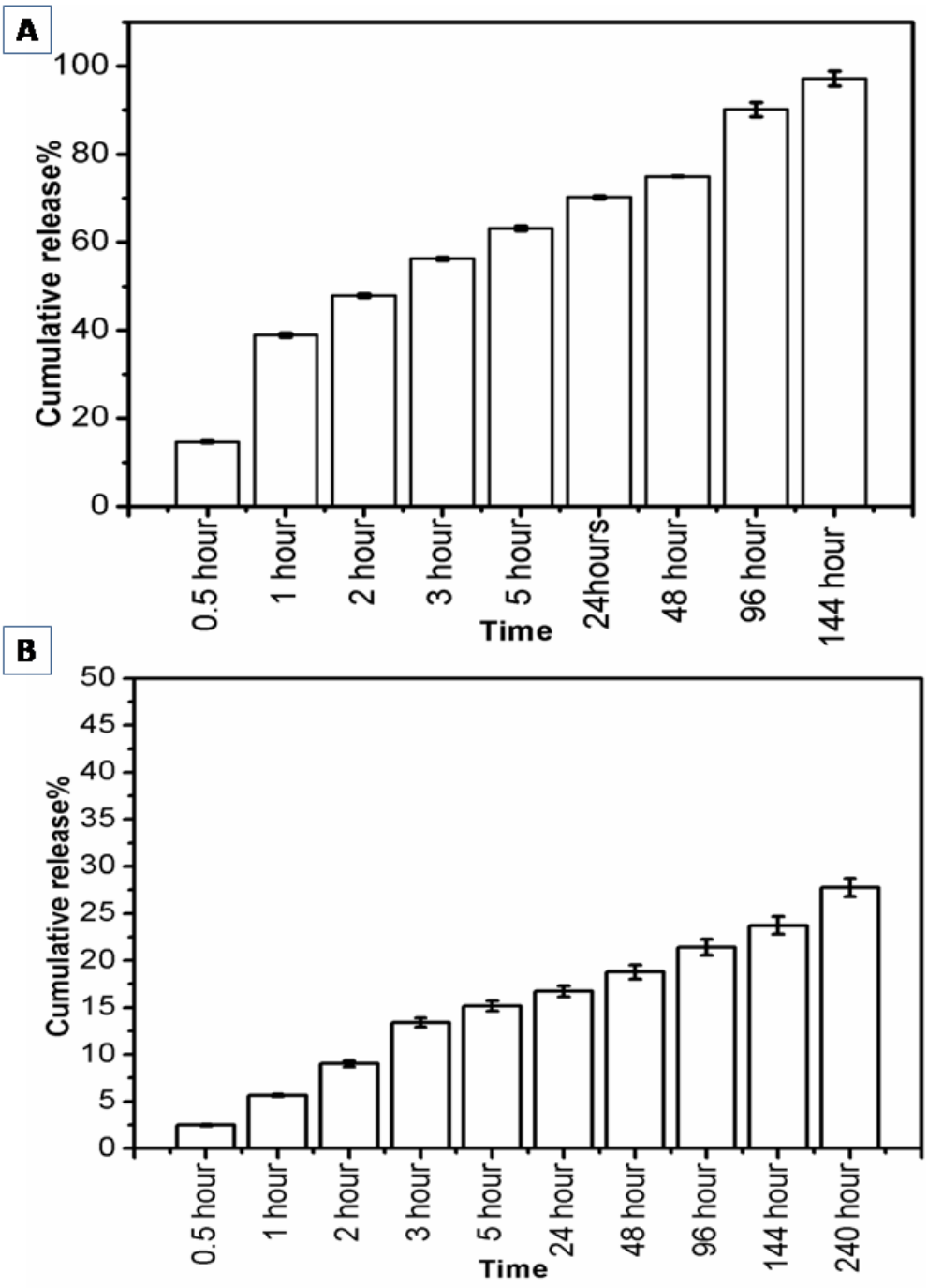

Figure 14: Cumulative release profile of microcarrier with Carboxymethylcellulose-alginate biopolymeric combination at pH7, where graph A) represents release profile of surface immobilised enzyme and b) represents release profile of *in-situ* immobilised enzyme

4.3.2) Release Profile at pH 6

Figure 15 and figure 16 represents cumulative release profile of microcarrier with chitosan-alginate and carboxymethylcellulose-alginate biopolymeric combination at pH6 respectively. Burst release profile was observed with increasing time for both the biopolymeric combinations. Microcarriers with surface immobilised enzyme with biopolymeric combination of chitosan-alginate and of sodium carboxymethyl cellulose –alginate illustrated a cumulative release percentage of enzyme from microcarriers was 86% in 1 hour. Microcarriers with *in-situ* immobilised enzyme shows marginal controlled release of the enzyme. The cumulative release percentage of enzyme from microcarriers with biopolymeric combination of chitosan-alginate was 49.5%, where as for the microcarriers with biopolymeric combination of sodium carboxymethyl cellulose –alginate was 51% in 3 hours. The acidic ph of the microenvironments is the primary reason for the burst release of enzyme in both the combination and methods of immobilisation as the interaction between the drug and biopolymers weakens at lower pH. Chitosan, alginate and carboxymethyl cellulose are all susceptible to disintegration at acidic conditions. The bonds being ionic can easily dissociate at lower pH leading the disintegration the complex and becoming more porous (Zhao et al., 2007).

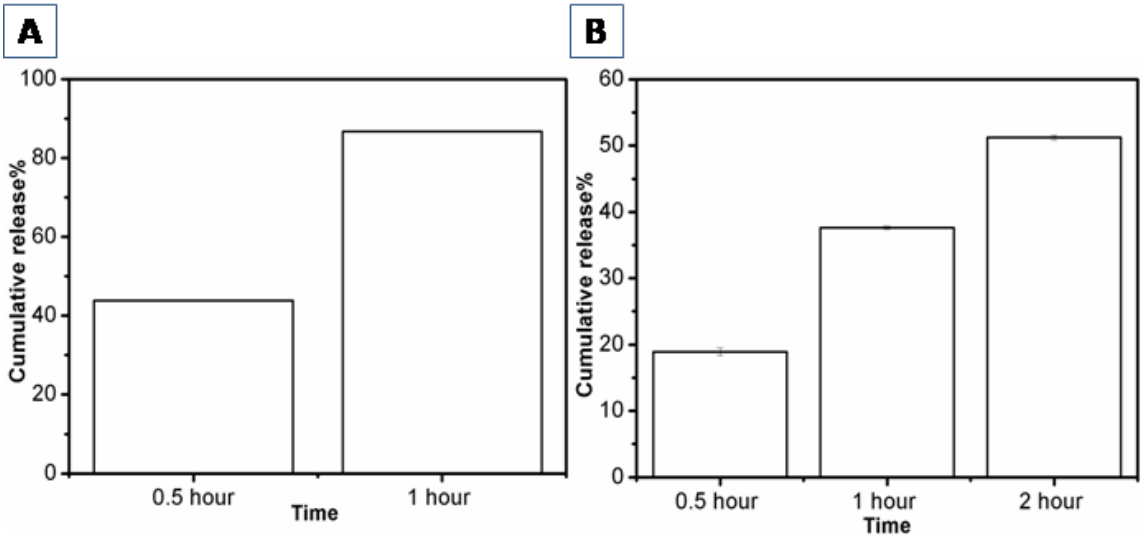

Figure 15: Cumulative release profile of microcarrier with chitosan-alginate biopolymeric combination at pH 6, where graph A) represents release profile of surface immobilised enzyme and B) represents release profile of *in-situ* immobilised enzyme

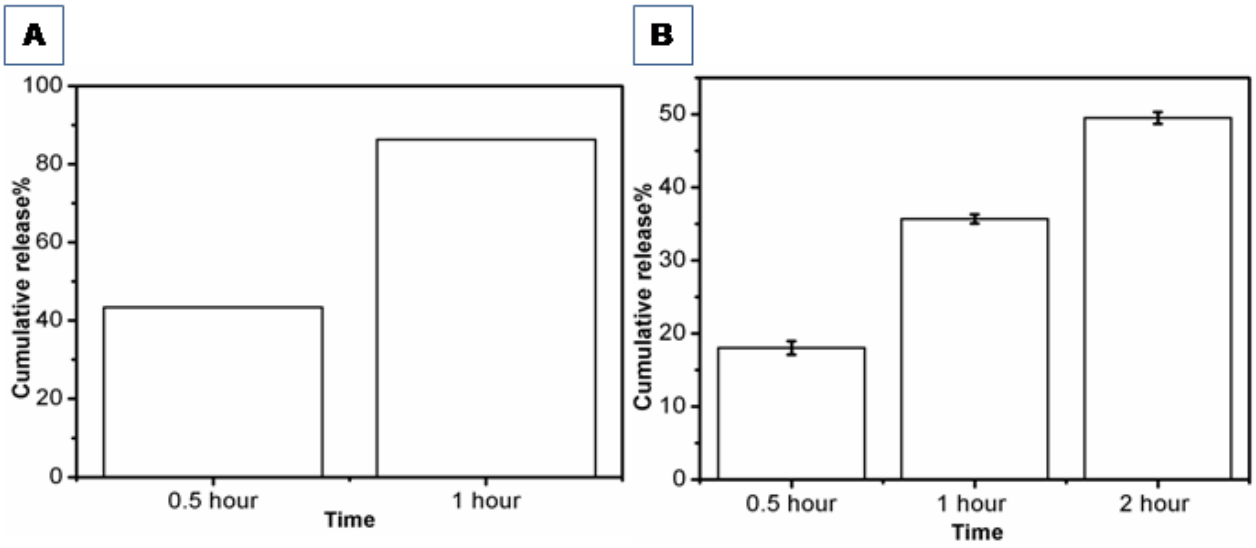

Figure 16: Cumulative release profile of microcarrier with sodium carboxymethylcellulose-alginate biopolymeric combination at pH6, where graph A) represents release profile of surface immobilised enzyme and B) represents release profile of *in-situ* immobilised enzyme

By studying the immobilisation pattern and release profile of microcarrier with different biopolymeric combination at different pH, it can be inferred that microcarrier fabricated with biopolymeric combination of chitosan –alginate illustrates optimum results and can be a potential candidate as an immobilisation matrix. Among the methods of immobilisation of enzyme, in-situ immobilisation of enzyme imparts the property of controlled release of enzyme over a longer period of time when compared to surface immobilisation. In-situ immobilisation restricts the bulk permeation of enzyme and substrate through the microcarriers. This leads to the prolonged applicability of in-situ immobilised enzyme microcarriers. Combination of microcarrier with chitosan-alginate biopolymeric layering along with the in-situ immobilisation of enzyme can be a potential candidate for immobilisation of enzyme for various applications.

4.4) Synthesis of Bionano-Composite for Food Preservation

4.4.1) Biogenic Synthesis of Silver Nanoparticles

1 mM silver nitrate solution was reduced with crude amla extract to form silver nanoparticles. Gradual change in the colour of silver nitrate solution was observed upon addition of amla extract with increasing time. The change in colour was the primary sign which signified that silver nitrate have started to reduce leading to the formation of silver nanoparticles (Figure 17).

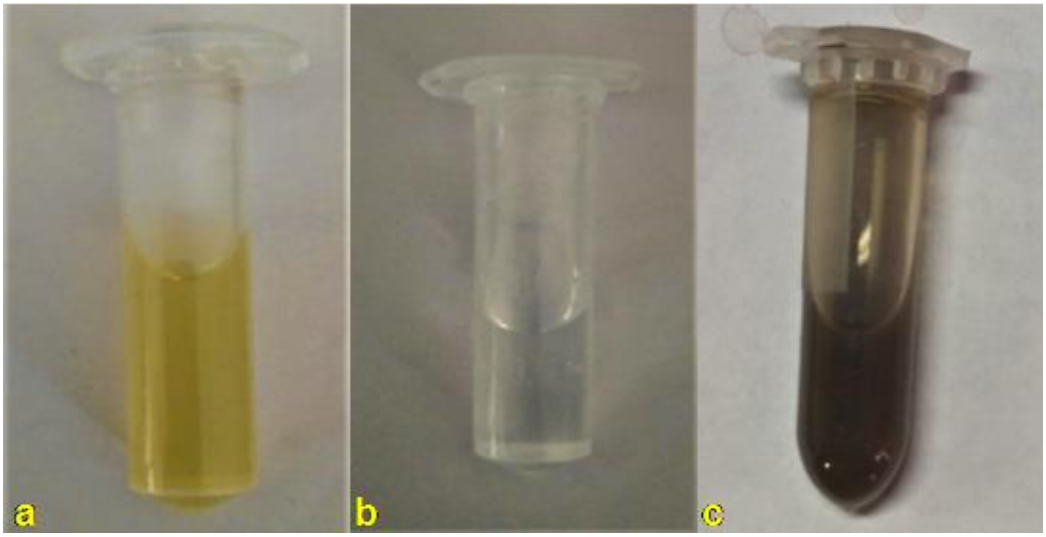

Figure 17: Solution of a) fruit extract, b) silver nitrate and c) silver nanoparticles

Amla is known to be a rich source of vitamin C which signifies that it stores abundant amount of ascorbic acid apart from other phenolic compounds .Ascorbic acid has been reported earlier to acts a reducing agent of silver nitrate. Therefore the presence of ascorbic acid in amla extract leads to the reduction of silver nitrate to silver nanoparticles. The colour intensifies with time due to the increasing amount of silver nanoparticle formation in the solutions (Mishra *et al*., 2009)The first change in the colour of the solution was observed after 1 hour which was brownish-orange after which the colour intensified gradually with time . The appearance of brownish–orange color was due to the excitation of surface Plasmon vibrations and provides a suitable spectroscopic signature to designate the formation of silver nanoparticles (Prathna et al., 2011).At the end of 24 hours, the colour of the solution turned to dark brown signifying the saturation of solution with silver nanoparticles visually

(figure 18). To confirm the formation of silver nanoparticles and estimate its intensity, aliquots of sample was taken after every hour and subjected to UV-visible spectrophotometer analysis.

Figure 18: Change in colour due to reduction of silver nitrate by fruit extract at a) 1 hour b) 3 hours, c) 5 hours and d) 24 hours

The minimun time required for the synthesis of silver nanoparticles was 1 hour with chsnge in colour from pale yellow to brownish orange. Peak at 391.5 nm wavelength was observed in the the UV spectral anaylsis of the amla extract reduce silver nitrate solution. According to literatute the wavenlength range in which silver nanoparticles show abosorance is 390-450 nm. Thus formation of peak in the givem range confirms the synthesis of silver naboparticles.(Figure 19 A)The increasing peak intensity with time signifies the increase in the concentration of silver nanoparticles in the solution .UV spectral analysis done after every 1 hour showed shift of band. At 2nd, the absorbance waving was 420 nm followed by 423 nm in the 3rd hour and 427 nm in the 4th hour. The spectral position of plasmon band of absorption as well as its width determines size and shape of metal nanoparticles. The spectrum can exhibit a red shift or blue shift depending upon the shape, particle size, state of aggregation and the surrounding medium. (Prathna et al., 2011)According to Henglein, the surface Plasmon band shifts towards blue end when electrons are donated to the particles and to the red end when holes are injected to the clusters (Henglein, 1993). The shift of peak from 391.5nm to 427 nm signified red shift which is due to clustering of silver nanoparticles with

increasing contact time. Aggregation of metal sols normally occurs through two mechanisms: cluster–particle aggregation and cluster–cluster aggregation. Cluster–particle aggregation is phenomenon where aggregates are formed primarily with the addition of single nanoparticle to a growing cluster. Whereas in the cluster–cluster aggregation, clusters of all sizes assimilate into larger clusters (Moskovits et al., 2005).

Size of silver nanoparticle was estimated through DLS which reveals the range of sizes and their frequency of occurance (figure 19,B). The size of the silver nanoparticles varies from 8.7-40 nm present in different frequencies. The average size of the silver nanoparticles is 19.3 nm. Due to polydispersity of silver nanoparticles, different sizes absorb at different wavelength there by illustrating a broad peak when analysed through UV visible spectrophotometer.

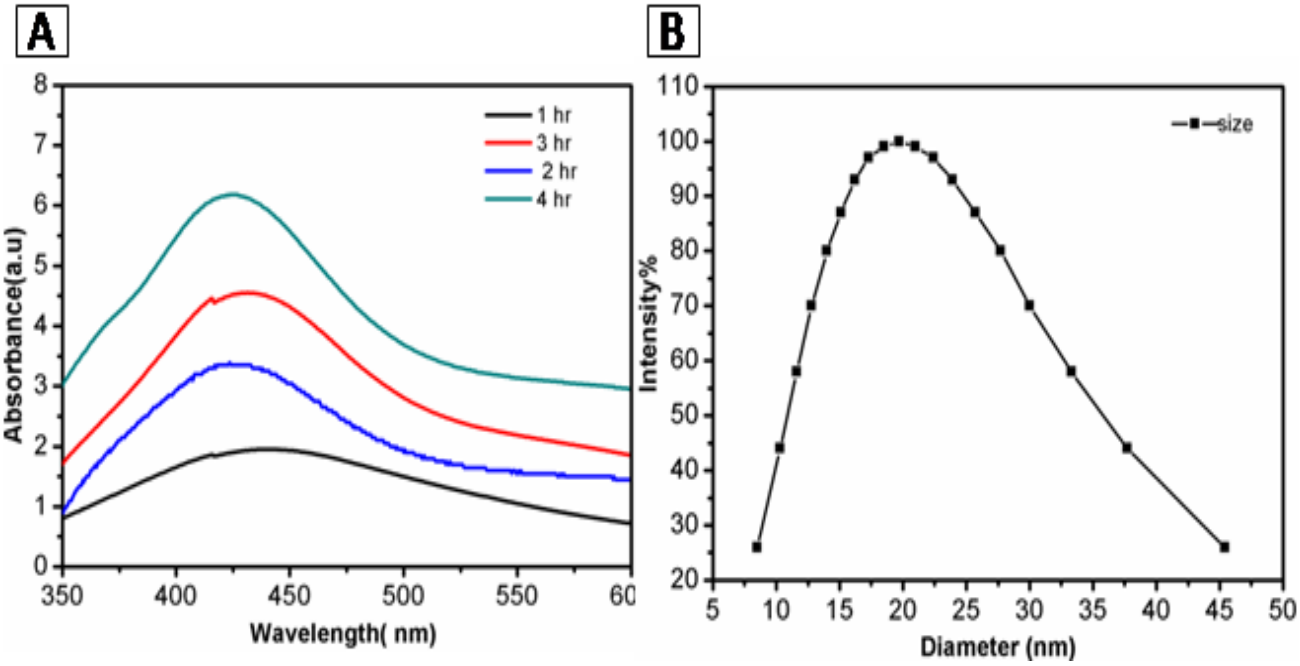

Figure 19: (A) UV-Visible spectra of silver nanoparticle synthesis at various intervals and (B) size distribution of silver nanoparticles estimated through DLS

4.4.2) Fabrication of Bionanocomposite Film

The silver nanopartcile solution was centriguged to obtain the nanoparticles , which were then immobilised onto biopolymeric microcarrier surface and embedded into chitosan solution to form a bicomposite solution .The bicomposite solution was transformed intofilms via solution

casting method at 37ºC without using any chemical crosslinking agent ,the obtained films were placed in open air to condition them. In figure 20,A it can be observed that the obtained film is transparent in apperance and when subjected to mechannical starin through physical means such as folding it multiple times it retained its integrity. In figure 20 B&C, comparison has been made between picture of a graphic image and the same image covered with the film to assess its transparency. It can be observed that the graphic image can be seen clearly without any hindrance.

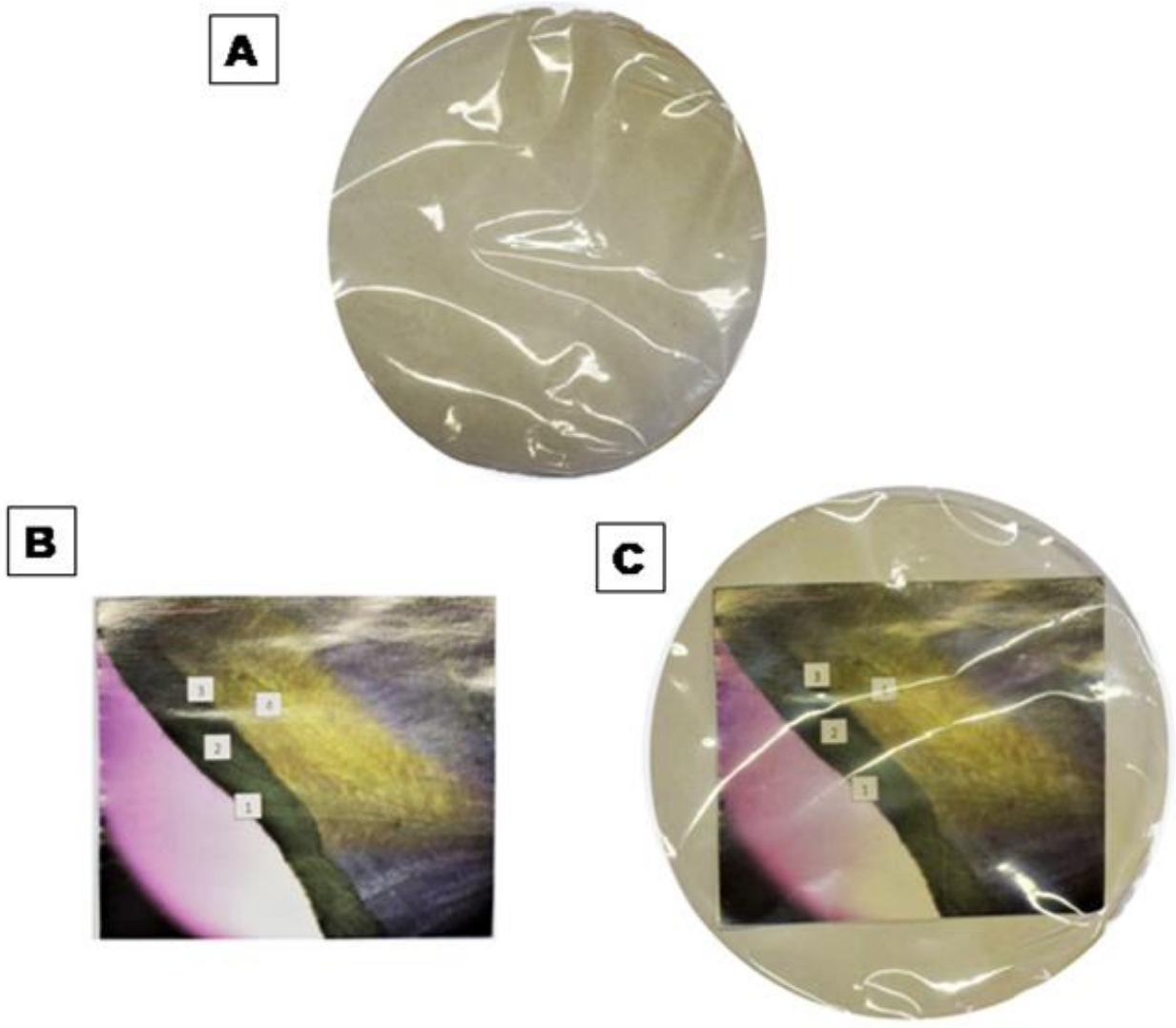

Figure 20: Digital images of A) Silver nanoparticles coated microcarrier embedded chitosan film, B) graphic image and, C) graphic image covered with bionanocomposite film

4.5) Antibacterial Assay

To assess the bactericidal activity of the silver nanoparticles coated microcarrier and bionanocomposite film antibacterial assay was performed against gram negative bacteria *E.coli* and gram positive bacteria *B.substilis*. Well diffusion method and spread plate method was adopted to assess the antibacterial activity of silver nanoparticles coated microcarriers and bionanocomposite films were placed over the inoculated plates to assess its bactericidal activity. In figure 21 and 22 , the zone of inhibition can be clearly observed . The aqueous extract of amla has been reported to have anti-pyretic laxative and tonic properties as well as antibacterial properties (Mishra et al., 2009). In the figures it has been observed that the areas under the control film as well bionanocomposite film have no bacterial growth. This signifies that chitosan alone can show bacteriostatic activity. Chitosan attacks on the external surface of bacteria; reason for the antimicrobial property is the positively charged amino group on the chitosan surface which binds with negatively charged surface of microbial cell membranes leading to agglutination which causes outflow of proteinaceous and intracellular constituents of the microbial cells. Chitosan selectively binds to trace metals and thereby inhibiting the production of cellular toxins and microbial growth, thereby acting as a chelating agent (*Dutta et al.*, 2009).Due to the increased size of silver nanoparticles after centrifugation a well formed zone of inhibition could not be obtained but still there are zones near the well were inhibition was observed. Due to the increased size of nanoparticles their diffusivity decreased due to which prominent zone of inhibition could not be observed. To further confirm the antibacterial activity drops of the silver nanoparticles coated microcarrier suspended in PBS solution was spread over the inoculated plate and incubated for 24 hours. The resulting image shows zone of inhibition at spots were the drops of solution was placed thereby affirming the antibacterial activity of the coated microcarriers. Silver nanoparticles are known to have antibacterial activity It has been hypothised that silver ions interact with negatively charged bio-macromolecular components of the microbial cells such as disulfide or sulfhydryl groups of enzymes and nucleic acids, thereby causing structural changes in the cell wall and membranes resulting in disruption of metabolic processes followed by cell death (Franci *et al.*,2015). It has also been suggested that presence of free radicals facilitate the antimicrobial mechanism of silver. Accumulation of silver ions in the cytoplasmic membrane of bacterial cells causes a considerable increase in permeability and cell death (Gombotz *et al.*, 1995)

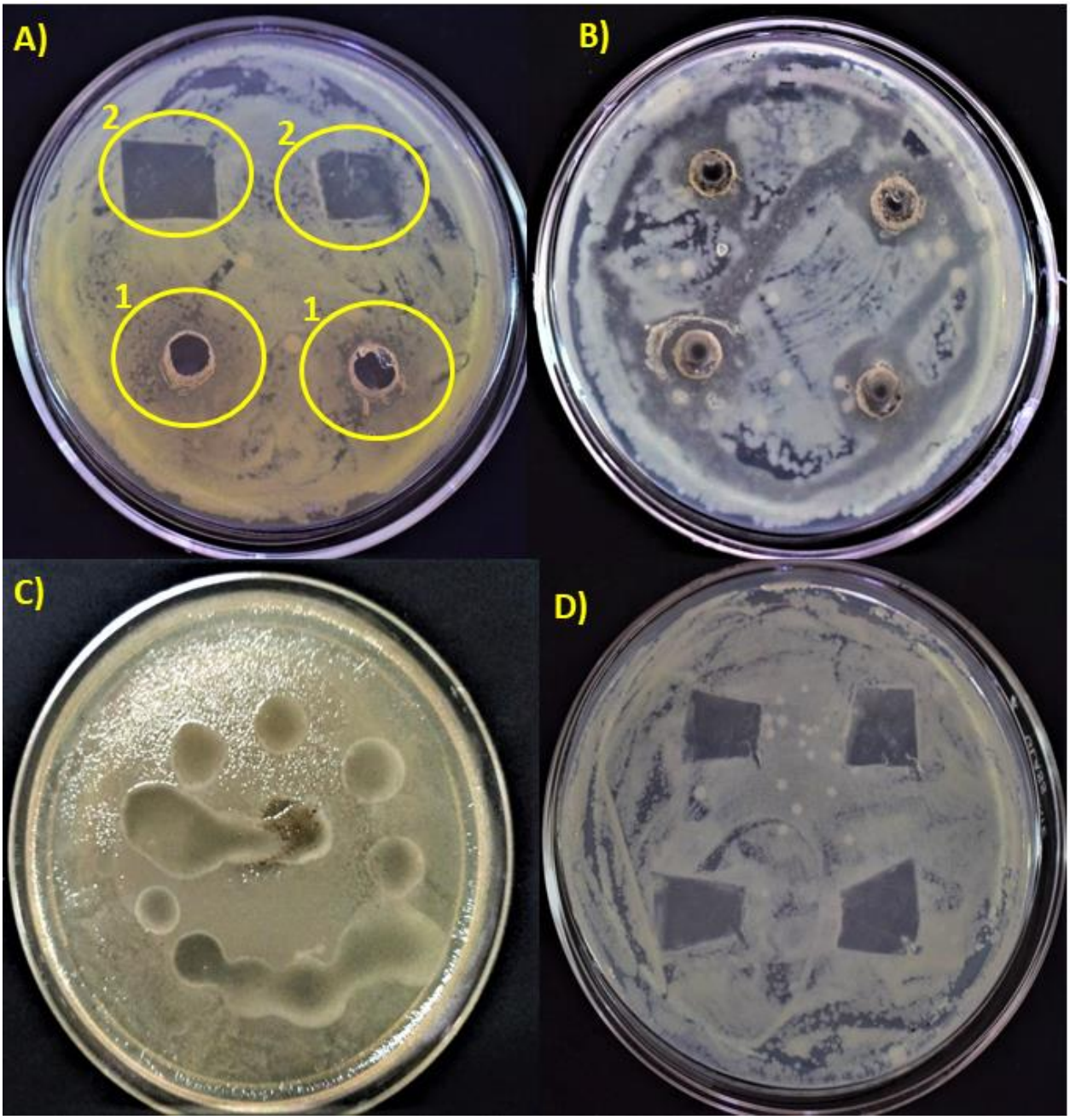

Figure 21: Results for the Antibacterial assay against *E.coli* of A) 1- Amla extract, 2- chitosan film, B&C) silver nanoparticles coated microcarrier (well diffusion and spread plate) and D) silver nanoparticles coated microcarrier embedded film

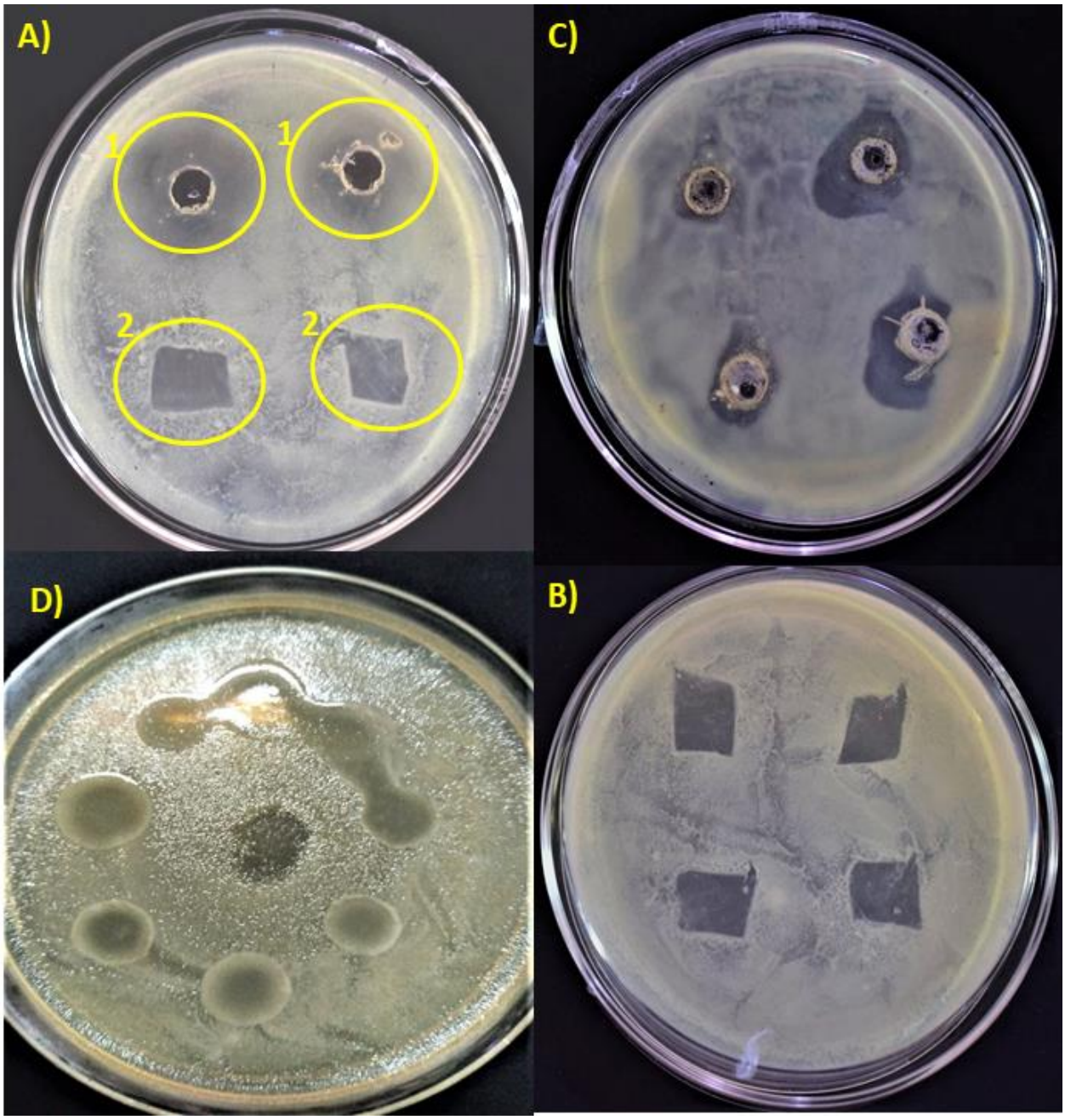

Figure 22: Results for the Antibacterial assay against *B.substilis* of A) 1- Amla extract, 2-chitosan film, B&C) silver nanoparticles coated microcarrier (well diffusion and spread plate) and D) silver nanoparticles coated microcarrier embedded film

4.6) Preservation Studies

Preservation studies were conducted by subjecting grapes wraped in bicomposite film to two different storage conditions. The first condition was placing the grape wraped in biocomposite film and chitosan film respectively in self sealing plastic bag followed by incubation at room temperature and grape without any wrapping placed in a plastic self sealing bag was set as control(figure 23,1).Second condition was the grape wraped in biocomposite film and chitosan film respectively in open at room temperature and grape placed in open was set as control(figure 23,2). The physical apperance and weight changes in the grapes placed in both the conditions were observed till day 7.

Figure 23: Conditions for preservation studies 1) Double layered packaging and 2)Single layered packaging

4.6.1) Double Layer Preservation

Figure 24 and figure 25 represents the water loss percentage and gradual change in physical apperance of the gape friut respectively over a period of time when preserved under first condition ie. the grapes being packed in film and then in plastic bag. In figure 24 it can be observed that the water loss percentage was more in the case of grape wraped in films and packed in plastic bag when compared to control. There was strong increase in weight loss for the first 2 days which later stabilised and increased comparatively slowly. The loss of water is due to natural process of the catabolism in fresh friuts and is atrributed to the process of

respiration and other senesence related metabolic processes during storage.(fayaz *et al.*,2009). Reason for increased water loss in the case of grape wraped in films and packed in plastic bag may be due the formation of double protectetive layer. This may have caused the entrapment of heat inside the packaging leading to incraesed water loss when compared to control. The control has a single layer packaging which gave room for the escape of heat through surface cooling of plastic material where as the chance of heat escaping was minimal in case of double packaging.

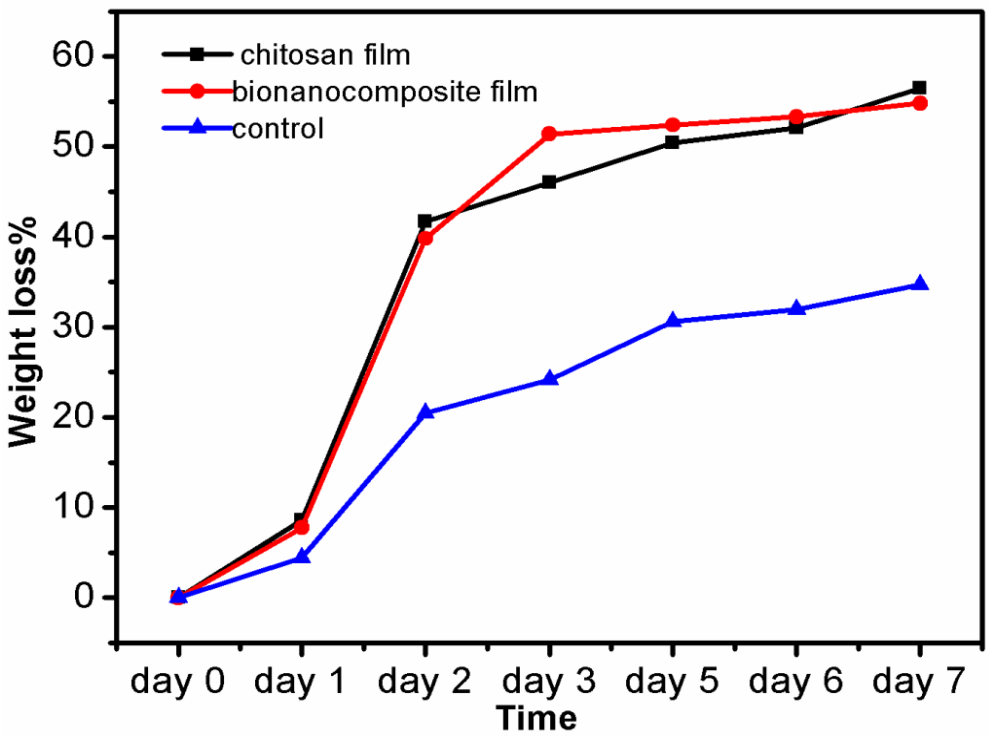

Figure 24: Weight loss percentage of fruit in double layered packaging condition

In figure 25, it can be observed that for the first two days the grapes looked fresh and green. The fisrt change in the surface apperance was observed on day 3 in the samples wraped in bionanocomposite and chitosan film. There was slight shrinkage of the grape skin which can be corelated to dratsic water loss because ofdouble protective layering apart from which there was no change in the colour of the grapes . On day 5 it was noted that chitosan and

biocomposite films started to get soft in texture reason being absorbing the mositure caused by water loss and a slight amount of sticky fluid started to appear at the tip of the fruit that was conditioned to double packaging. Due to accumulation of moisture inside the packaging the deterimental effects on the fruit accelearted. On day 6 the surface of grapes started to have more brown spots along with chnages of colour from green to orangnish green marking the start of detoriation of fruit when compared to control. On day 7 the friut packed in chitosan film along with plastic sealing bag was observed to have detoriated completely where as the control and grape packed in bionanocomposite film along with plastic bag did not perish completely. It can be concluded that bionanocomposite film which has silver nanoparticles microcarrier embedded contributes in increasing the shelf life of the fruit significantly.

4.6.2) Single Layer Preservation

Figure 26 and figure 27 represents the water loss percentage and gradual change in physical apperance of the gape friut respectively over a period of time when preserved under second condition ie. the grapes being packed in film and placed in open air.in figure 26 , strong increase in water loss for the intial 2 days is observed which later stabilised and increased comparatively slowly. The loss of water is due to natural process of the catabolism in fresh friuts which is an atrribution to the process by respirational and other senesence related metabolic processes during storage.(Fayaz *et al.*,2009). Grape packed in bionanocomposite film was observed to have minimum water loss when compared to chitosan film and control. The bionanocomposite film and chitosan film wraped graped showed lesser water loss percentage than control when compared to the results of water loss percentage under first condition. The reason being that single layer packaging gives room for the escape of heat and moisture from the film surface thereby not causing accumulation of moisture and heat inside the packaging.

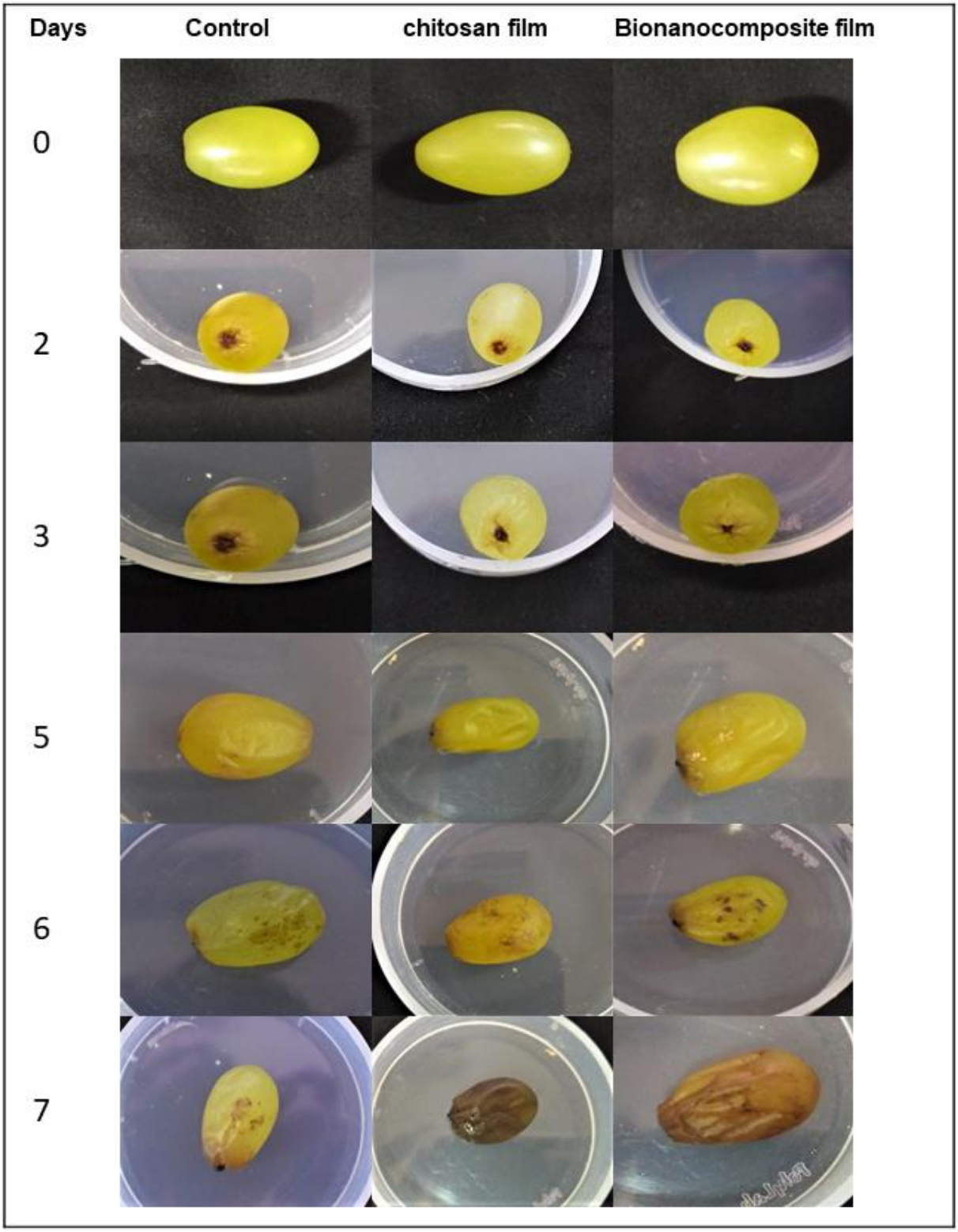

Figure 25: Changes in physical appearance of fruit over time when subjected to double layered packaging

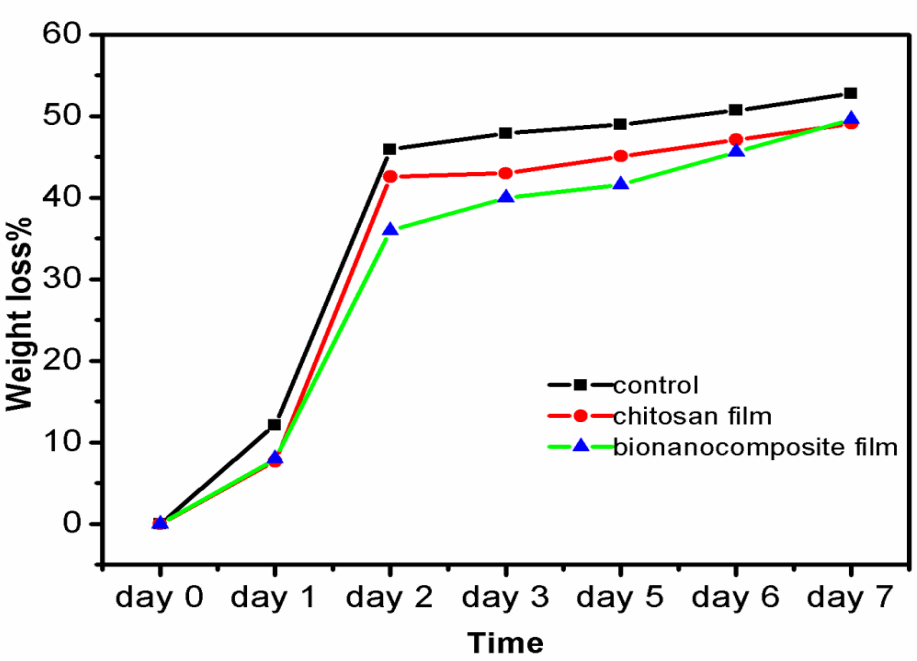

Figure 26: Weight loss percentage of fruit in single layered packaging condition

In figure 27, the difference in the physical appearance of control, grape wrapped in chitosan film and grape wrapped in bionanocomposite film can be easily observed. Control started to show signs of spoilage on day 2 where as the ones packed in chitosan and bionanocomposite film was still bright green and fresh. The grapes wrapped chitosan and bionanocomposite film continued to have green appearance with slight surface shrinkage till day 4 when compared to control. On day 5 the colour of the grapes packed in films started to change towards greenish orange but were better in appearance than control. The control turned brown in colour with spots over its surface. Complete shrinkage of control was marked on day 7 whereas the grapes wrapped in films was observed to have lesser extent of shrinkage and were still not completely perished. The grape packaged in bionanocomposite film was better in appearance than the grapes packaged in chitosan film and control at the end of day 7. It can be concluded that bionanocomposite film which has silver nanoparticles coated microcarrier embedded contributes in increasing the shelf life of the fruit significantly. Silver nanoparticles known to have antimicrobial effect over a wide range of microorganism along with chitosan which has surface adhesion property can be a potential candidate in the field of food preservation.

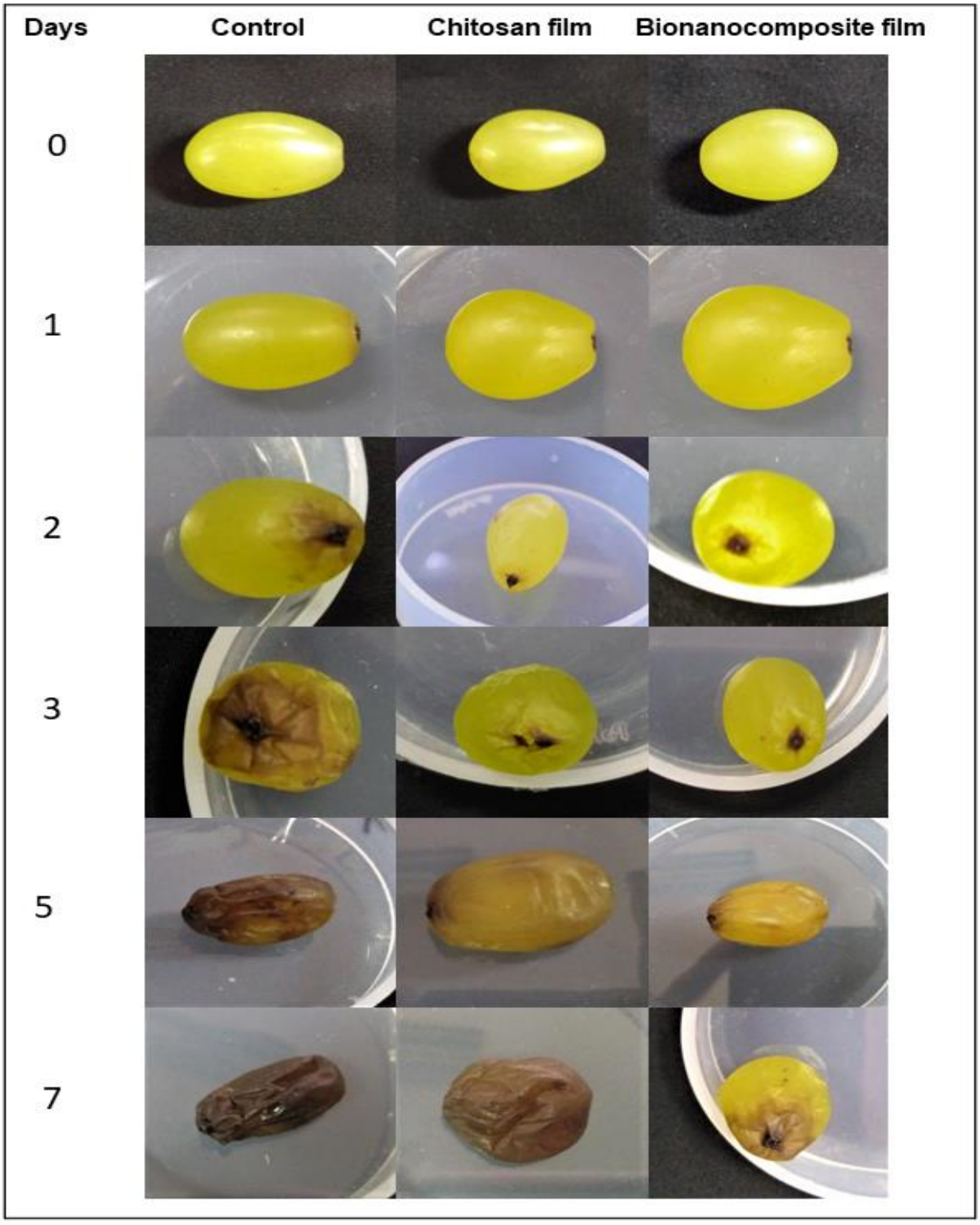

Figure 27: Changes in physical appearance of fruit over time when subjected to single layered packaging

CONCLUSION

Biopolymer-based biodegradable food packaging materials and enzyme immobilization matrix could potentially replace non-biodegradable plastics and use of synthetic biopolymers for the fabrication of support matrix for enzyme immobilization respectively. However, poor mechanical strength of these biodegradable materials limits their widespread use. Techniques, developed to improve the properties of these biodegradable materials, include modification of biopolymers by blending with other biopolymers from natural origin which would make it possible to commercialize these biopolymers based materials. Blends of biopolymers with other biodegradable polymers are the easiest way to prepare polymers with "tailor-made" properties (functional physical properties and biodegradability).The major challenge is to reduce production and material cost of these biopolymers to make them cost-effective against synthetic polymers. To overcome this challenge, there is a need to improve biocomposite formulation and the processing method to produce these at a lower cost. There is also a need for better understanding of microcarrier structure formation and interaction between the polymer and bioactive molecules and their patter of release. More research is also needed to utilize different types of novel crosslinkers, which would not be toxic at any point of time during its course of existence to human or environment

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