

Fabrication of Nano-Silver loaded Microcarriers in Chitosan based Films for Potential Application in Fruit Preservation

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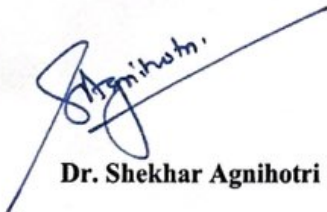
Department of Biotechnology

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August, 2019

CERTIFICATE

This is to certify that the dissertation work entitled **"Fabrication of Nano-Silver loaded Microcarriers in Chitosan based Films for Potential Application in Fruit Preservation"** submitted by **Ms. Shubhangi Jain** (Roll No.301701026) in partial fulfillment for the award of degree of **Master of Science** 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 the supervision and guidance of **Dr. Shekhar Agnihotri**. 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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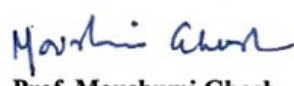
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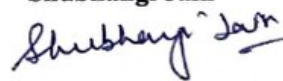
DECLARATION

I hereby declare that the work being presented in the dissertation report entitled "**Fabrication of Nano-Silver loaded Microcarriers in Chitosan based Films for Potential Application in Fruit Preservation**" submitted by me for the award of the degree of **Master of Science** in Department of Biotechnology, TIET, 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.

Place: Patiala

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Dated: 9.8.19

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LIST OF SYMBOLS AND ABBREVIATION

Abbreviations	Full Form
μl	microliter
μm	micrometer
Ag	Silver
Ag ⁺	Silver ions
AgNO ₃	Silver nitrate
AgNPs	Silver nanoparticles
CaCO ₃	Calcium carbonate
CFU	Colony forming units
CH	Chitosan
CMC	Carboxymethyl cellulose
CaCl ₂	Calcium chloride
DD	Degree of deacetylation
DLS	Dynamic Light Scattering
FTIR	Fourier Transform Infrared Spectroscopy
Gly	Glycerol
kDa	Kilo Dalton
L-B-L	Layer By Layer
mM	micromolar
Mw	Molecular weight
NaCl	Sodium chloride

NPs	Nanoparticles
OD	Optical density
Rpm	Revolutions per minute
Ag-MMT	silver modified montmorillonite
AAO	Artemisia annua oil
WVP	Water Vapor Permeability
PEC	Polyelectrolyte Complexes
NCP	Nanocomposite based Packaging
EDTA	Ethylenediamine-tetraacetic acid
Alg	Alginate
SEM	Scanning Electron Microscope
Na-Alg	Sodium alginate
TSC	tri-sodium citrate
DPPH	2, 2-diphenyl-1-picrylhydrazyl
RH	Relative humidity
Q	swelling ratio
PBS	Phosphate buffer saline

ABSTRACT

A new strategy for fabricating bionano-composite films consisting of chitosan (CH), glycerol (Gly) and silver nanoparticles (AgNPs) is reported in this study. The layer by layer (L-B-L) approach was employed to synthesize microcarriers consisting of alternate layers of CH and alginate (Alg). These microcarriers acted as template for *in-situ* synthesis of AgNPs in an eco-friendly manner. The synthesized microcarriers were characterized using DLS and SEM. The size of core particles analyzed through SEM was found to be 10 μ m. DLS results indicated that the existence of L-B-L assembly, as with each layer the hydrodynamic diameter of the microcarriers was increased. CH films with and without nanosilver laden microcarrier particles were produced by casting method. The addition of AgNPs into the CH matrix leads increase in translucency and darker appearance of the composite films. Obtained films revealed strong interactions between film matrixes as per FTIR studies. The UV-Vis spectra showed that all nanocomposite films blocked the UV light in 450 to 800 nm range. The moisture content of the films ranged from 12.24% to 20.93%. The bionanocomposite films containing 0.2wt% Ag, showed an increase in antioxidant activity by 70% as compared to the pristine CH films. The percentage swelling of films decreased with increase in silver (Ag) concentration. The blended film demonstrated excellent antibacterial activity against *E.coli*, *Enterococcus aerogenes*, and *Staphylococcus aureus* bacterial strains. The films showed contact inhibition, which enhanced with increasing Ag concentration. These films were not only restricted to biomedical purposes, but they were found to be equally efficient as an antimicrobial packaging material (fruits). Studies demonstrated that the nanocomposite films extended the shelf life of cherry fruits for 17 days under refrigerated conditions. Conclusively, this study is an attempt to extend our understandings for developing antimicrobial packaging to circumvent the wastage or spoilage of fruits and vegetables.

Keywords : Silver nanoparticles, Films, Food packaging, Microcarriers, Antimicrobial

CHAPTER 1

INTRODUCTION

Almost 37,000 tons of edible agricultural and farm products produced on global scale are lost because of improper handling, lack of storage facilities, and non-compatible packaging. These perishable products are prone to spoiling without proper protection in typical conditions, causing food waste and economic loss. Thus, it is vital to extend product shelf life by protecting food. To avoid the spoilage of raw food products, food packaging is considered as an efficient approach. Food packaging helps in preventing food materials via eliminating direct contact with contaminating agents by refining air surrounding food and limiting moisture availability. Over last few decades, various conventional approaches had been used to cover the raw food with materials such as glass, metal, paper, paperboard and plastic. Amongst them, plastic was preferred due to its functional properties, convenience, high barrier, mechanical properties, low weight and lower prices. The petrochemical-based plastics such as PET, PVC, PE, and PA have been widely utilized as food packaging materials. However, their use is being restricted now due to limited biocompatibility, non-recyclable and non-biodegradable nature. These non-degradable protections have led to waste disposal concerns, serious food borne diseases and environmental issues. Taking that into consideration, quest for easily biodegradable and highly biocompatible packaging films had been in research focus worldwide. Various biopolymeric materials such as cellulose, starch, chitosan, etc. have been explored widely for their film forming properties.

Biopolymers are organic molecules which are composed of covalently bonded repeating units of nucleotides, amino acids or monosaccharides. Biopolymers are derived from renewable resources and have been widely employed to produce packaging films include mainly proteins, lipids, and polysaccharides. The main film forming polysaccharides are mainly cellulose, chitosan, starch, pectin, alginate, and pullulan. They have intrinsic properties such as bioadhesiveness, crystalline/amorphous constitution, swelling, and viscosity appropriate for film synthesis. The economically feasible polysaccharide-based films have great applications in food product packaging.

The produced films have novel properties such as biodegradability, prevention from moisture, aroma loss, barrier to oxygen, and carbon dioxide. Amongst these CH had been broadly considered candidate in the preparation of biodegradable packaging films. CH is a natural cationic polymer obtained from crustacean shells. It is composed of non-symmetrically arranged β -linked D-glucosamine and N-acetyl-D-glucosamine. It has inherent properties of high molecular weight and solubility in acidic medium; it can be processed into gels, coatings, nanofibres, microparticles, nanoparticles, etc. CH exhibit application in food packaging as it has moderately increased hydrophobic nature which leads to high moisture barrier and water resistance. The shelf life of CH coated fruits can be enhanced in cold storages. However, the widespread application of pure CH is limited because of their poor mechanical property and not satisfactory antimicrobial capacity. To excel film limitations, they have been blended with the plant extracts, essential oils, nanoclays and nanoparticles (NPs). Amongst all these, NPs are being preferred over others as they can be easily blended into the CH films. The NPs belongs to new class where they are embedded into polymer material namely bionanocomposite, which had been introduced as a promising option.

Nanotechnology is a branch of science and technology that deals with lightweight materials of nano-range. It has shown active changes which can improve health and quality of life, with simultaneous decrease of ecological hazards. The different NPs have gained interest of industries due to their positive impact. It has potentially offered new composite materials and high-tech techniques to upgrade packaging functions which can enhance fruit shelf-life. The addition of nanofillers is reported to improve the water barrier properties, mechanical properties, thermal stability and other functional property of the nanocomposite films. Coatings have gained much interest as they can be blended with additives which help maintain texture, color, hydration of the food products and protect them from antimicrobial agents. Numerous NPs have been incorporated into biodegradable films as an additive which improves the property of the film such as Zn, Ag, Cu, and Au. Amongst wide range of NPs, Ag has been incorporated into CH films due to its high antibacterial properties, non-cytotoxicity, low volatility, stability at high temperatures, and high catalytic activity.

Nevertheless, developing awareness of these potential NPs has lead to acceptance of more suitable method for blending nano-silver into the CH films. Hence, immobilization method has been in practice for immobilizing nano-silver particles into the CH matrix. Immobilization technique is designed to restrict the movement of NPs out from the polymer matrix. It provides large surface area to volume ratio for metal NPs, prevents the core from oxidation or dehydration, agglomeration, enhances the shelf life, produce stable and dispersed NPs with controlled size, shape, and distribution. Though CH has great ionic interactions, but because of certain observed problem such as difficulty in creating multiple self layered assembly, it is coated with an acid resistant biopolymer such as sodium alginate (Na-Alg) which can bind suitably to CH. Alg which is obtained from seaweed is a natural polysaccharide composed of two uronic acids: β -D-mannuronic acid and α -L-guluronic acid. This novel biopolymer is non-toxic, low-cost, biodegradable, and biocompatible. Here, CH which is positively charged, covalently crosslinks with the negatively charged Alg under favorable biological and mild fabrication conditions to form Polyelectrolyte Complexes (PEC) via electrostatic interactions. Due to polyelectrolyte nature of CH and Na-Alg they can easily form L-B-L Assembly. They are proved as the resourceful carrier systems for (bio) macromolecules.

For further strength enhancement of the Ag incorporated CH films there is addition of plasticizer in order to obtain protein- and polysaccharides-based films. Plasticizers have intrinsic properties such as they increase mechanical strength, diminish deformation chances and increase the polymer chain flexibility. Some plasticizers commonly used are glycerol (Gly), sorbitol, glucose, and monoglycerides. Amongst all the plasticizers, Gly which is a natural plasticizer is used as it is non-toxic and easily blends with the CH. In the present study we have designed non-toxic Ag embedded CH films. They are used to cover the fruits and vegetables, which enhance the shelf-life. The purpose of Ag is to stop or retard the growth of unwanted pathogenic microorganisms. And these films are tested for their swelling study, moisture study, optical properties, antioxidant study, antimicrobial activity, Water Vapor Permeability. The AgNPs synthesis steps are characterized by DLS and zeta potential.

The overall aim of this work is presented below:-

- Fabrication of a cost effective yet efficient biopolymeric microcarriers as a support matrix for immobilization of bioactive agent
- One-step loading of AgNPs on the microcarriers as a core
- Synthesis and characterisation of microcarriers embedded CH films with improved mechanical and biocompatible properties
- Determining the industrial applicability via lab scale study of synthesised films on small fruits i.e. cherry for packaging applications

CHAPTER 2

REVIEW OF LITERATURE

2.1 Food Packaging

Deterioration of edible raw food products is correlated with desiccation of skin, color change and emergence of infection; which can be postponed by storing it at low temperature conditions. There are postharvest depreciations in nutritional quality of fruits and vegetables, mainly decrease in Vit-A, Vit-E, Vit-C, and polyphenol content. This is because of the physiological alterations which decrease the quality of nutritional benefits to the consumers. These changes occur during the food preservation period (Lin *et al.*, 2007).

2.1.1 Biodegradable Food Packaging

The fruit surface has many attached microbes which colonize rapidly and damages food products. To prevent the unwanted microbial growth, raw food materials have been covered with certain biopolymeric or non-biopolymeric coatings. Biopolymeric materials can be the potential replacement for non-biodegradable synthetic materials or traditional packaging materials as it reduces ecological hazards. (Garavand *et al.*, 2017; Leceta *et al.*, 2013; Rabnawaz *et al.*, 2017; Cutter *et al.*, 2006; Conca *et al.*, 1993). In recent years, biodegradable polymer coatings over food surface are most probably seen to preserve and maintain food quality as an alternative to non-degradable polymers (Mazzuca *et al.*, 2014; Anker *et al.*, 1996; Caner *et al.*, 1998; Gennadios *et al.*, 1990; Franssen *et al.*, 2003; Franssen *et al.*, 2002; Guilbert *et al.*, 1996; Greener *et al.*, 1994). These non-polluting low cost films limit the moisture transfer, respiration rate, oxidation, limits permeability to gases such as O₂, CO₂, water vapor, aroma, oil, maintains texture and extend shelf life (Kester *et al.*, 1986; J.H. Han *et al.*, 2000). Most abundant natural polysaccharides used for film formation are cellulose, pullulan, starch and CH (Valencia *et al.*, 2011; Dhall *et al.*, 2013; Elsabee *et al.*, 2013). In food related applications, water soluble polysaccharides act as thickeners, as in water they have high viscosity. Biopolymer liquid material can be casted onto the food by brushing, dipping, spraying,

and panning after that drying of the deposited layer. In past, CH often was used by spraying or coating on fresh fruit such as strawberries and tomatoes (Ghaouth *et al.*, 1992). The films are formed by dismantling of the polymer blocks and rejoining them into chains of polymer to form film matrix. The evaporated solvent material of food coating has various bonds such as hydrophilic, hydrogen bonds, and ionic crosslinking. Applications of edible films (Donhowe *et al.*, 1994; Conca *et al.*, 1993; Park *et al.*, 1993) has been well suited for commercialization.

In addition, these films can contain food additives or functional agents (antioxidants, antimicrobials), for better food spoilage prevention (Kester *et al.*, 1986). Many research papers are on CH coating the fruits such as litchi (Zhang *et al.*, 1997), longan (Jiang *et al.*, 2001), peach (Li *et al.*, 2001), mango (Rambabu *et al.*, 2019) and banana (Kittur *et al.*, 2001), grape (Romanazzi *et al.*, 2002) has enhanced their shelf life. The freshness of the food products been covered tells about the success of application of covering as well as its barrier and mechanical properties. The films made up of CH and oleic acid has been used to cover strawberry under cold conditions to maintain food nutrients. For the motive to increase fruit shelf-life, Holcroft *et al.*, (1999) had studied controlled and modified atmospheres along with their cold storage.

2.2 Natural polymers

Natural polymers are those polymers which are produced in correlation with phenomenon of natural growth cycles of living creatures. Therefore, they are recovered in greater amounts from renewable sources. Structurally, natural polymers are highly organized. Their intrinsic properties of biocompatibility and biodegradability, have made them being used for various applications. In addition, nature based polymers also offers ease of processing and chemical modifications. They exist as polyhydroxyalkanoates and polysaccharides (cellulose, starch, CH) (Campos *et al.*, 2013). Natural polymers can be derived from proteins (albumin, gelatin), carbohydrates (agarose, CH, Starch) and chemically modified carbohydrates (Poly-dextran, Poly starch)

2.3) Chitosan biopolymer

Amongst numerous natural biodegradable polymers, chitin can be converted into CH that can be used to form films. Chitin is used as raw material because of its easy availability and abundant nature after cellulose (Muxika *et al.*, 2017; van *et al.*, 2015). CH (poly b-(1-4) N-acetyl-d-glucosamine) is obtained from exoskeleton of crustaceans (crabs, shrimp and crayfishes) (Elsabee *et al.*, 2013; Mayachiew *et al.*, 2008; Van *et al.*, 2015) or from fungi cell wall such as *P. notatum*, *A. niger* (Knorr *et al.*, 1984; Tan *et al.*, 1996) by partial N-deacetylation of chitin, (Arbia *et al.*, 2013) which is a natural antimicrobial compound. According to a survey, 10^9 - 10^{10} tons of CH is produced per year. CH is a high molecular weight cation with chains of 2-deoxy-N-acetyl-D-glucosamine and 2-deoxy-D-glucosamine linked by b-1,4 glycosidic bonds formed by alkaline deacetylation at high temperatures (Muxika *et al.*, 2017; Abdou *et al.*, 2007).

CH can easily be dissolved in acidic solutions such as acetic, ascorbic acid, propionic acid. It has 2-amino-2-deoxy-D-glucose in the acidic state (Aljawish *et al.*, 2015; Babu *et al.*, 2013; Kaur *et al.*, 2014; Van *et al.*, 2015). CH has nature to be dissolved at pKa of less than 6.2. CH application to be formed into films or gels is due to its heavy weight and acidic nature (Jiang *et al.*, 2005; Lizardi *et al.*, 2016). The inherent properties of CH depend on factors such as molecular weight, chitin source, pH, deacetylation temperature and time. Typical activities include non-toxicity, renewability, mucoadhesiveness, antimicrobial activity (F. Devlieghere *et al.*, 2004), antitumor activity. Shahidi *et al.*, (1999) had studied the antimicrobial property of CH to protect plants. CH having molecular weight of 28 KDa to 1671 KDa has shown higher antimicrobial effects.

Kim *et al.*, (2003) had observed the polycationic nature to be responsible for antimicrobial behavior of CH. The antimicrobial action has been observed by change in cell permeability due to NH_2 factor of CH and the electronegative charges in the cell membrane (Tsai *et al.*, 2002; Liu *et al.*, 2004). This contact leads to seeping out of cellular substances (Papineau *et al.*, 1991; Sudarshan *et al.*, 1992; Fang *et al.*, 1994). Other mechanism leads to contact of diffused hydrolysis substances with bacterial genetic material, which inhibits mRNA synthesis (Sudarshan *et al.*, 1992) and other vital

enriched products (Cuero *et al.*, 1991; Devlieghere *et al.*, 2004). The *E. coli* cells attach to CH matrix at alkaline pH, which concludes that to check antimicrobial activity, NH_3^+ factor is not appropriate.

Park *et al.*, (2004) had studied that CH deacetylation value is not related to antimicrobial activity. Due to its unique physicochemical and biological nature it is used in organized industrial sector such as wastewater management, food packaging (Coma *et al.*, 2003; Coma *et al.*, 2002; Durango *et al.*, 2006; han *et al.*, 2004; Park *et al.*, 2004), cosmetics, photography, pharmaceutical and biomedical products (Campos *et al.*, 2013; Ruocco *et al.*, 2016; Rivero *et al.*, 2010), and in system of controlled release of medicines (in microcapsules). CH shows constrained capacity to discharge immobilized materials. Li *et al.*, (2008) had found a remedy to it, by trapping CH with Na-Alg layering. Qin *et al.*, (2006) had observed neutral pH inside the cell.

2.3.1 Chitosan Films

CH has great property to be casted into biodegradable edible films (Dhall *et al.*, 2013; Elsabee *et al.*, 2014; Shiekh *et al.*, 2013) to wrap food products for food packaging. It exhibits excellent biocompatibility, biodegradability, non-toxicity (Jayakumar *et al.*, 2005), barrier properties (Arvanitoyannis *et al.*, 1998, 1999; Bangyekan *et al.*, 2006; Mathew *et al.*, 2008) and selectively permeable to gasses (CO_2 and O_2). Many scientists have worked to improve shelf life of raw food products by covering them with CH (Kittur *et al.*, 2001; Dong *et al.*, 2004; Chien *et al.*, 2007; Meng *et al.*, 2008).

CH is approved for dietary applications in Japan, Italy and Finland (Kean *et al.*, 2010). CH is recommended safe as food additive by FDA in 2012, overall for humans as well as nature. Jiang *et al.*, (2001) and Bonilla *et al.*, (2012) had observed the CH films which limits the transpiration loss from raw food. The semi-permeable coating of CH as demonstrated several advantages on different fruits and vegetables (Suyatma *et al.*, 2005). CH film changes the environment around the fruit surface which limits its transpiration speed, reduces weight loss and ripening by reducing food moisture of fruits. The CH films have complex mechanism for water permeation. The WVT (Water Vapor Transmission) through hydrophilic material of films is inversely proportional to the water

vapor partial pressure. Wong et al., (1992), Butler et al., (1996), Caner et al., (1998) had observed that WVPR is high of CH films. It was observed that WVTR (Water Vapor Transmission Rate) through film matrix is directly proportional to storage time, film thickness (Kerch *et al.*, 2011; Poverenov *et al.*, 2014), its molecular weight, temperature of drying and inversely proportional to preservation at lower temperature. The use of pure CH films shows brittleness and low mechanical strength on practical applications (Liu *et al.*, 2017).

Alvarez et al., 2013 and Gol et al., 2013 had studied the properties of various concentrations of different acids and plasticizers along with preservation period of food covered with CH films. Amongst many acids, acetic acid is often used to dissolve CH to form films which have higher tensile strength but lower elongation capacity (Adila *et al.*, 2013; Kerch *et al.*, 2011). Butler et al., (1996) produced two concentrations of glycerin as plasticizer that are, 0.25 mL/g and 0.50 mL/g CH dissolved in acetic acid aqueous solutions. Increased molecular weight of CH increases the film strength. The blending of Gly (lipid materials) concentration into the CH film though decreases the tensile strength but increases the water vapor permeability (WVP) or moisture barrier properties and oxygen permeability (Zivanovic *et al.*, 2005; Amarante *et al.*, 2001).

Butler et al., (1996) had found that addition of Gly changes the barrier and mechanical properties of Gly added throughout preservation period. Baron et al., (2017) and Escamilla et al., (2017) had performed numerous efforts of blending CH with proteins to enhance its mechanical, barrier and functionality properties. CH has been combined with certain compatible polymers, minerals and proteins to develop coating with AgNPs which shows many useful properties. CH has broad spectrum of antimicrobial properties, due to which CH-based edible films have been used as potential “new economy” which are basically bio-based plastics (Spierling *et al.*, 2018). Chillo et al., (2008) had found that the CH films showed inflexible characteristics with high elastic modulus and limited elongation capacity.

2.3.2 Antimicrobial Property of Chitosan Film

Munoz et al., (2015) had studied various antimicrobial films formed from naturally originated biopolymers such as CH or Alg along with nano-silver particles is non-toxic and highly biocompatible. *E. aerogenes* has more antibacterial activity than *E.coli* and *S. aureus* on the CH matrix (Sudarshan et al., 1992). NVKV et al., (2011) had studied the occurrence of microbial zone of inhibition on CH blended with AgNPs matrix. Mahdi et al., (2012) studied the storage ability of PVC with AgNPs for crushed beef at low temperature. One week of examination lead to conclusion of its beneficial property against microbial survival.

Yoksan et al., (2010) had studied the antibacterial activity of CH blended with starch and AgNPs with ZOI. The reason for using CH as antimicrobial films is, the electronegative nature of bacterial cell membrane interacts with the positive charge of the CH causing the outflow of cellular material from microbial body (Durango et al., 2006). Research has been on *E. coli* discharge of Lactate dehydrogenase (LDH) along with glucose (Chung et al., 2008). Honarkar et al., (2009) had concluded that preservation becomes better, when CH film covers low pH food products. The microbial strength can be reduced by with time diffusion of antimicrobial agents from the film or through their direct contact (Lee et al., 2015). The bacterial cells have nature to form biofilm after some time of settling on polymer matrix. Donlan et al., (2001) had studied the direct relation of matrix non-smooth nature and reaction to water with the macromolecules in the film matrix which can be proteins or carbohydrates.

2.4 Plasticizer

New alternative to enhance the properties of biodegradable films, which are used to cover food products by adding different amounts of plasticizer. This can increase film applications. Normally, plasticizers decreases the tension inside the films, hence make them flexible. They can be added to degradable or non-degradable plastics (Mekonnen et al., 2013; Vieira et al., 2011). Gly is a famous plasticizer in food packaging industry, because of its stable intrinsic properties (Fernández et al., 2004). Gly has density of 1.26 g/cm. Suyatma et al., (2005) had studied that for CH film with PEG and Gly are

better than the glycols as plasticizer. Approximately, 20% (w/w) amount of it is suitable for months of preservation under low temperature conditions.

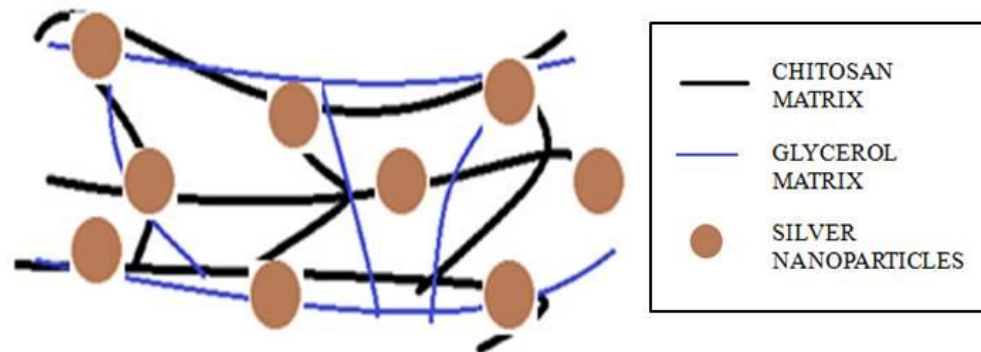


Figure 1: Graphical representation of the film matrix.

2.5 Fruit: Cherry

Cherry is a temperate, non-climacteric crop which provides vitamins, minerals and antioxidants in large amounts. Fresh cherries decay fast due to high temperature in summers which leads to maximum respiration range, alteration in biochemical characters, quality loss. Due to its sweet taste it is one of the most accepted edible fruit by consumers. So, it is important to increase the shelf life to make them available in all seasons by storing them under cold or favorable environment. To prevent their decay due to migration or other purposes they have been processed with other novel practices to keep them fresh at time of consumption. They are now being stored at low temperature with customized packaging in biodegradable semi-permeable films (Pertriceoni *et al.*, 2014).

2.6 Bionanocomposite in Food Packaging: Intelligent Packaging

Intelligent packaging is the name given to the packaging which maintains the food quality and appearance at best level which can be positively accepted by the consumer at larger rate (Kerry *et al.*, 2006; Metak *et al.*, 2013). Nanotechnology is fastly growing in food sector and several others in an increasing pattern (Carbone *et al.*, 2015). Neethirajan *et al.*, (2011) had observed that hundreds of organizations from the entire world are in thought of using nanotechnology to wrap food products in different ways. Tager *et al.*,

(2014) suggested that presently numerous engineered lighter NPs (AgNPs, nano-ZnO, nano-TiO₂, nanoclay, nano-TiN) are uniformly blended in food covering materials as bioactive agents (antimicrobial activity) (Duncan *et al.*, 2011).

Venugopal *et al.*, (2005) had studied various materials having NPs. Nanomaterials added to food product coverings are formed naturally or purposely supplemented with humanly created materials (Magnuson *et al.*, 2011). They can be added to packaging materials after being immobilized into polymer matrix. Bumbudsanpharoke *et al.*, (2015) has observed that nano-silver prevents the food from unpleasant smells. Food packaging with advanced nanotechnology can be done by “improved packaging”, in which NPs are put into the packing matrix before casting or by “active packaging”, in which food comes in direct contact with the NPs.

2.6.1 Silver Nanoparticles (AgNPs)

The common antimicrobial NPs are Cu, Zn, Au, Ag (Toker *et al.*, 2013). Rai *et al.*, (2009) and Martínez *et al.*, (2012) studied that Ag is best suited for antimicrobial properties (Domènech *et al.*, 2014). Agnihotri *et al.*, (2014) had found that approximately 30% of nano-based companies worldwide are working on Ag based NPs. Toker *et al.*, (2013) and Franci *et al.*, (2015) studied various methods of their formation. Youssef *et al.*, 2013 has observed that they are negligibly volatile with stability at high temperatures. Martínez *et al.*, (2012) suggested that Ag particles can be used in films by adding immobilized Ag or can be imbedded as such.

Agnihotri *et al.*, (2014) and Rai *et al.*, (2009) studied that decreased Ag particle (<10 nm) size shows maximum antimicrobial activity. Pelgrift *et al.*, (2013) and Chen *et al.*, (2008) had studied that AgNPs slow down membrane transport by instabiling ions, impairment of respiration by binding with mitochondrial DNA, interruption of energy transduction and/or cell lysis, and eventually cell death. The size, shape, surface charge, aggregation pattern and the way they are being used tells about its antimicrobial property. Pal *et al.*, 2007 and Panáček *et al.*, 2006 studied that amongst many random shapes of AgNPs, truncated shows better biocidal activity. Daima *et al.*, 2014 had studied that AgNPs kills bacteria by creating pores in their cell membrane, hence they loses their stability.

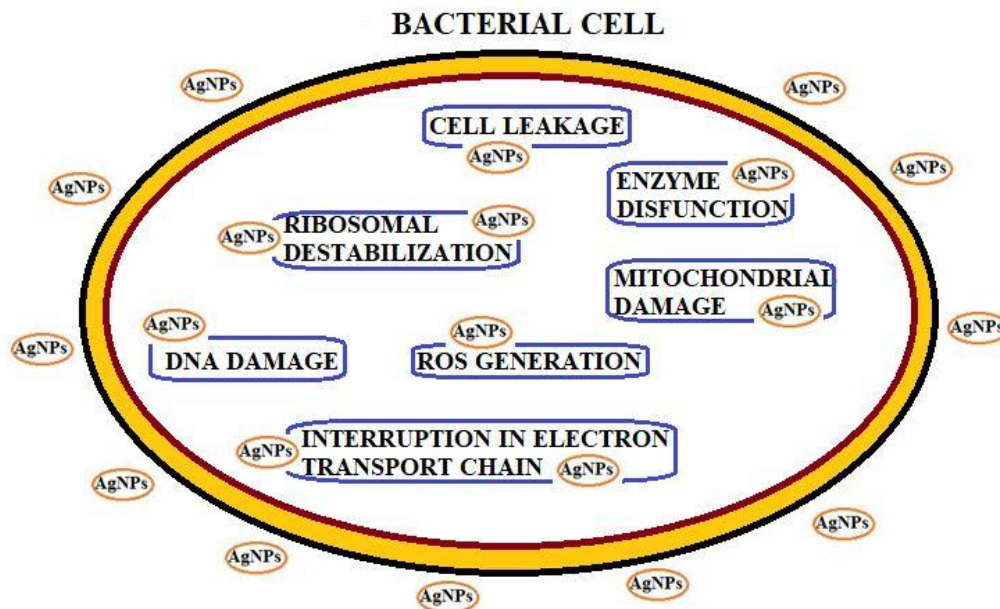


Figure 2: Graphical representation showing antibacterial property of AgNPs.

2.7 Microspheres and Microcapsules as Carrier System

The microspheres refer to round polymeric particles which are made up of throughout uniform material. The microcapsules refer to spherical polymeric particles which have one more types of particles (in core of microcapsule) of same or different material inside them. They are used to immobilize minute quantity of particles. The material used as core can be solid, liquid or gas. The biopolymer acts as a shield around the immobilized substance. The immobilized substances can be pesticides, drugs, proteins and others. These spherical particles have diameter in the micrometer range (mainly from 1 μm to 1000 μm).

Arshad et al., (1990) had stated that NPs are of nanometer range. These structures can be made from natural and synthetic materials. There are many different methods for their synthesis. Each method leads to formation of different size, their inherent chemistry and morphology. These structures are used for protection and controlled release of immobilized substances. The immobilized material get separated from external surrounding medium which prevents it from degradative reactions, change in organoleptic properties, effortless usage of toxic or other materials. Microspheres involve

various methods like single emulsion, double emulsion, spray drying solvent evaporation. Zhao et al., (2006) had studied its various properties to be used in drug carrier system, biosensor, etc. Nowadays, these highly stable particles are in industrial use, such as food packaging where they maintain freshness, morphology and properties of the food products (Joye *et al.*, 2014).

These particles are being in use in paints, explosions, films, medicines, pesticides, cosmetics, etc. Microparticles do however have some limitations such as in the case of not as aesthetically appealing of coatings/paintings or unusual surface of edible substances. Campos et al., (2013) had suggested that these particles can be widely used in different areas, hence for that more quality of knowledge and more research is required. Zhao et al., (2007) had studied methods of successfully loading various substance particles into micro structures by changing surface properties of the microcapsules.

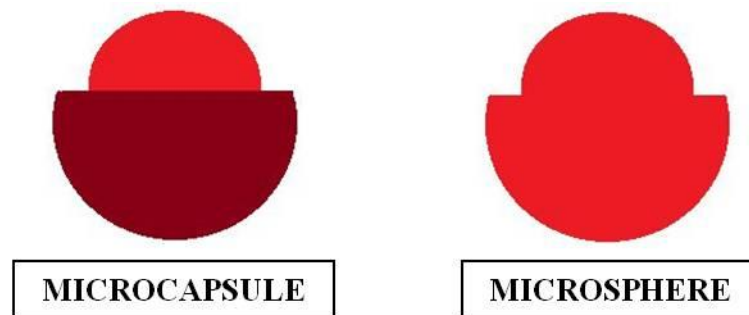


Figure 3: Graphical representation of microcarriers.

2.7.1 Alginate Biopolymer

Alg is a natural hydrophilic polysaccharide extracted from brown sea algae. It is made of β -D-mannuronic acid and α -L-guluronic acid which are alternatively linked together. Acevedo et al., (2012) has studied novel intrinsic properties which enable it to be used as thickening agent, suspension emergent, and formation of gel and stabilizer of emulsions. Li et al., (2008) had studied that the anionic nature of Alg, can bind to positively charged CH, which is also a polysaccharide through electrostatic interactions. These interactions under suitable conditions (biological or chemical) can form PEC. Seo et al., (2012) had

studied the antibacterial nature of coating of Alg-Ag on biomedical devices. Alg is widely being used for immobilizing NPs.

2.7.2 Carboxymethyl Cellulose (CMC) Biopolymer

CMC is a derivative of cellulose with high viscosity. This polysaccharide is abundant, cheap and has high shear stability. It is formed by its reaction with NaOH and $C_2H_3O_2Cl$. The properties of CMC depend on factors such as: molecular weight, number of carboxyl content per anhydroglucose unit, and the distribution of carboxyl substituents along the polymer chains. The sodium carboxymethyl groups (CH_2COONa) in the polysaccharide promotes the water solubility (Biswal *et al.*, 2004). For the formation of microcapsules, the layers of CH and Alg are being alternately deposited on to CMC as base which has better biocompatibility. The CMC is negatively charged substance which is used in industrial and health care aspects (Zhao *et al.*, 2006).

2.7.3 Polyelectrolyte Complexes (PEC)

The binding of oppositely charged complexes is done by electrostatic interactions. Here, these polyanion-polycation species interacts through various bonds such as Van der Waals, hydrogen bond, etc. The coming together of so many bonds among the polymers leads to formation of bulky complexes. Polymer charge density is inversely proportional to the percentage of polymer in the PEC. Here, one polymer solution is slowly added in a dropwise manner into the other solution. (Saether *et al.*, 2008).

Among polycations, CH is a highly non-toxic and has inherent mucoadhesive properties. CH structure has many pyranose rings which make it a rigid entity. The structural changes in the polymer due to bindind to form PEC, which changes the property of rigid nature into non-rigid structure of polymer. PEC of different characteristic can be obtained by changing the chemical characteristic of the component polymers, such as the molecular weight, flexibility, functional group structure, charge density, hydrophilicity and hydrophobicity balance, and stereo-regularity and compatibility, as well as the reaction conditions: the pH, ionic strength, concentration, mixing ratio, and temperature (Sankalia *et al.*; 2007).

Recent investigation has been done on binding Alg and CH through ionic interactions. The carboxylate entity of Alg and protonated amines of CH leads to formation of PEC. They are used to form membranes, capsule, fiber like structures, and scaffolds (Han *et al.*, 2010). Alginates which are anionic in nature form strong bonds with polycations which are more resistant due to presence of calcium chelators. They are utilized to form stabilized gels due to its nature of reduced porosity (Coppi *et al.*, 2001).

The complex of CH-Alg has pH values between 3.7 to 4.7. So it was considered that the two polymers i.e., CH and Alg would result in stronger complex with stability at lower pH and in media possessing calcium chelators (Coppi *et al.*, 2001). This CH/Alg matrix immobilization process can immobilize proteins, cells, DNA with proper stability of biological activities (Li *et al.*, 2008). The encapsulation of microcarriers can be done with two approaches: (i) loading with desired species of previously coated polyelectrolyte multilayer microcapsules; (ii) immobilization of template followed by subsequent coating with layers of PEC (She *et al.*, 2010).

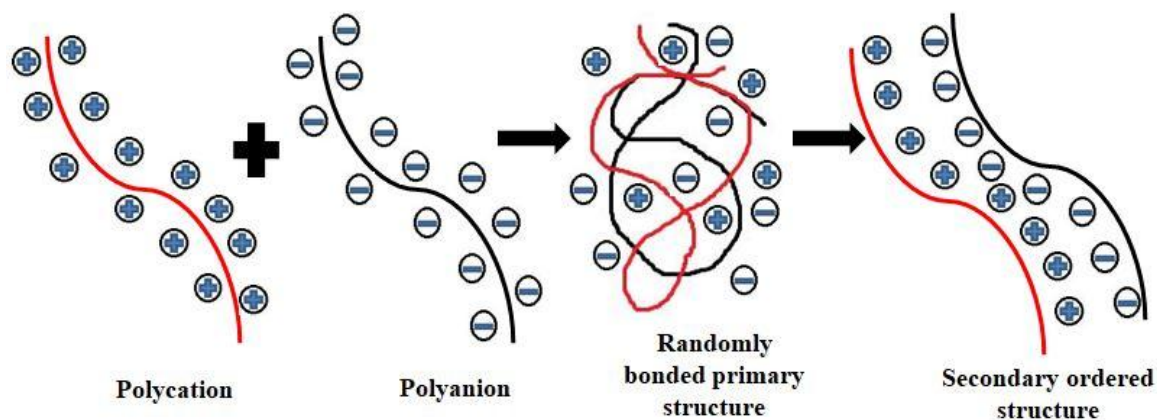


Figure 4: Mechanism of formation of PEC.

2.7.4 Fabrication of Microcapsule (L-B-L Assembly)

The self assembly of biomacromolecules to form microcapsule is a good choice to immobilize the metal NPs. This type of assembly prevents aggregation of immobilized material and provides large surface area to volume ratio. The assembly is created with biopolymers having considerable physicochemical properties and synthesized under

favorable conditions such as suitable pressure, temperature. In L-B-L Assembly, aqueous solutions of oppositely charged polysaccharides repeatedly adsorb on organic or inorganic spherical templates to form hollow microcapsules. This leads to alternate deposition of oppositely charged substances and polyelectrolytes. These microcapsules have large quantity of active compounds in the non-complexed state such as in the free form within the cavity of a capsule. They exhibit tremendous potential as their physicochemical and mechanical properties can be tailored easily leading to microcapsules with an unmet functionality.

The study of L-B-L assembly of CH and Alg in which CH is cationic and Alg is an anionic polysaccharide has been done. It is a simple technique which is of low cost, which produces multilayer assemblies. It has wide range of applications. It is a highly useful method which can modify every template surface despite of size and shape. Regardless of the source of a biopolymer as template, the multivalent interactions synthesize safe and stable microcapsules. The permeability of the microcapsule can be modified by changing the layer number which is adsorbed and the nature of the multilayer shell material. Amongst wide range of porous inorganic materials, calcium carbonate (CaCO_3) based colloidal template has been considered most suitable as it non-toxic, easy to synthesize and has some superior characteristics. The CaCO_3 particles were prepared by a precipitation reaction between calcium chloride and sodium carbonate under vigorous stirring.

The increased interest for CaCO_3 is directly proportional to low cost, easy availability, high mechanical stability, and large surface area to volume ratio, high efficiency, mild deposition conditions and biocompatibility. Moreover, CaCO_3 particles had been filled with macromolecules up to a size of about 40 nm. The template can be easily dissolved using ethylenediamine-tetraacetic acid (EDTA), organic solvents or acidic solutions. The CaCO_3 core was dissolved by adding a 0.2 M EDTA solution (pH 7.5) to multilayered assembly leading to complexation of the Ca^{2+} ions, which easily diffuses out through the polyelectrolyte coating (Ribeiro *et al.*, 2018; De *et al.*, 2006)

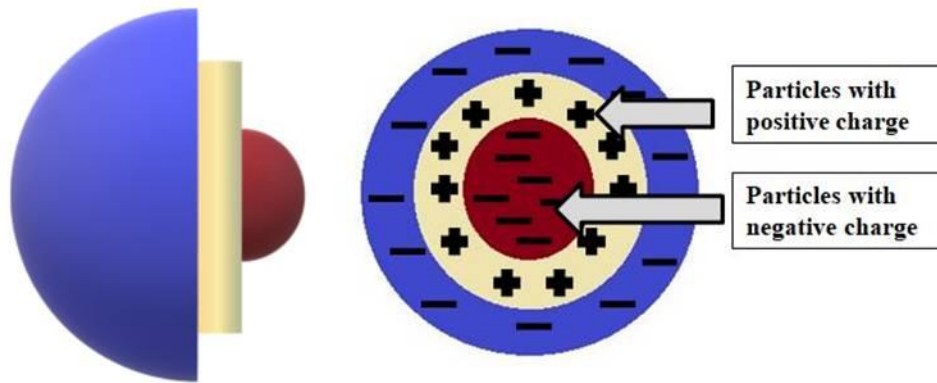


Figure 5: Graphical representation of two layered L-B-L Assembly

2.8 Research in Current Field of Application

Alvarez et al., (2013) studied the antibacterial nature of CH films and CH blended with enriched oils films. The test was done on modestly processed broccoli by covering it with the films. The films proved to be better against mesophilic, psychrotrophic bacteria, *E. coli* and *L. monocytogenes*.

Cui et al., (2017) proved activity *E. coli* O157:H7 on cherry tomatoes which were covered with CH films blended with agar and *Artemisia annua* oil (AAO) liposome. The results suggested that it was the best method to preserve them and protect them from bacterial action.

Costa et al., (2012) had suggested that the active coating is suitable for preserving modestly cut carrots. They were coated with Ag-MMT (silver modified montmorillonite) which has property to control microbial growth. This is proved to be a good method to preserve carrots for 70 days then uncovered carrots which decay in 4 days.

Hu et al., (2011) proved that NCP (Nanocomposite based Packaging) was efficient to stop the production of inhibiting ethylene, extend the organoleptic characteristics, decrease the degradation of nutritional components, prevent physiological changes, thus ripening of harvested kiwi fruit was delayed and shelf-life was increased. Therefore, nanocomposite films could provide new packaging strategies to retard the ripening and preservation of the quality of kiwi fruit.

Table 1: Current food packaging materials, their application on the fruit and their antimicrobial property

S.No	Material	Bioactive Agent	Microorganisms	Food Product	References
1	CH + essential oils (tea tree, rosemary, clove, lemon, & aloe vera)	bee pollen ethanolic extract of propolis pomegranate dried extract & resveratrol	<i>E. coli</i> <i>L. monocytogenes</i>	Broccoli	Alvarez <i>et al.</i> , 2013
2	CH	AAO	<i>Escherichia coli</i> O157:H7.	Cherry tomato	Cui <i>et al.</i> , 2017
3	CH	Nano-SiOx	Gram-positive food-borne pathogen	Chinese cherries	Xin <i>et al.</i> , 2017
4	Zein + essential oils (Cinnamon bark oil + mustard oil) + propylene glycol	nalidixic acid	<i>Salmonella typhimurium</i> LT2	Cherry tomato	Yun <i>et al.</i> , 2015
5	Calcium alginate	Ag-MMT	mesophilic and psychrotrophic bacteria, Enterobacteriaceae spp., Pseudomonas spp., yeasts and moulds	Fresh-cut carrots	Costa <i>et al.</i> , 2012
6	Polyethylene (PE)	nano-Ag, nano-TiO ₂ & montmorillonite	Nil	Kiwifruit	Hu <i>et al.</i> , 2011.

2.9 Characterizations Techniques

2.9.1 Dynamic Light Scattering (DLS)

In DLS, the size distribution of the biopolymeric particles which are diffused in solution is identified through light scattering at different intensities. The size of nanoparticles is measured through the phenomenon of Brownian motion. Due to slow movement the particles are observed to be larger than 3000 nm.

2.9.2 Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR spectra of all samples were recorded in the range of 4000–400 cm^{-1} . FTIR spectroscopy can be used to identify the interactions in the polymer matrix due to its sensitivity towards hydrogen bonds. In FTIR, the vibration spectra arise due to variations in chemical compositions (functional groups). It helps to determine the functional group, nature of the compound, and bonding amongst nanoparticles.

2.9.3 Scanning Electron Microscope (SEM)

It is a type of electron microscope that helps to scan and produce information about the sample surface with a focused beam of electrons. It can also tell about the size and composition of the sample.

CHAPTER 3

MATERIALS AND METHODS

3.1 Material Required

CH (purity 99%; deacetylation degree 85%; pH 5-6; Molecular weight 100,000-300,000) was procured from Nano Wings Pvt. Ltd., (Telangana, India) while NaOH pellets, CaCl_2 , silver nitrate (AgNO_3), tri-sodium citrate (TSC, $\geq 99\%$ pure), Gly and CMC was obtained from Sigma-Aldrich (India). Alg, nutrient broth & agar, phosphate buffer, glacial acetic acids, and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) were obtained from Hi Media Pvt. Ltd., (Mumbai, India). For antimicrobial studies, the bacterial strains of *E.coli*, *Enterobacter aerogenes*, and *Staphylococcus aureus* were obtained from NCL, Pune. Double deionized water was used thoroughly for all the experiments.

3.2 Preparation of Microcarriers

3.2.1 Synthesis of CaCO_3 Particles as Core Microstructure

A novel biomimetic mineralization strategy was adopted to form CaCO_3 microparticles using CMC and arginine as template and coupling agent, respectively. In brief, 48 ml 36 mM calcium chloride (CaCl_2) solution was mixed with 2 ml 10% (w/v) CMC solution and stirred at 600 rpm for 30 minutes. A freshly prepared 50 ml 7.8 mM sodium carbonate (Na_2CO_3) solution was rapidly poured into the CaCO_3 -CMC mixture while stirring was continued over different time duration (20-60 minutes) so as to achieve uniformity in the size of the microparticles. A 2 ml 25 mM arginine solution was introduced at last in the resulting mixture undergoing ultrasonication for 5 minutes. The prepared CaCO_3 microparticles were collected after centrifuging twice at 10,000 rpm for 10 minutes followed by vacuum drying (37 °C) for six hours. The dried powdered samples were stored in a sealed plastic vial till further use.

3.2.2 Preparation of Polyelectrolyte Solution

Polyelectrolyte solution of CH and Alg were prepared according to their behavior with respect to solution pH. For an aqueous CH suspension having $\text{pH} < \text{pK}_a$ of CH (~ 6.2), all primary amines of CH will be protonated (positively charged). On the other side, an Alg solution having $\text{pH} > \text{pK}_a$ ($\sim 3.4\text{--}4.4$) would exist as a polyanionic electrolyte due to the deprotonation of their carboxyl groups. Hence, the solution pH for working solution of CH (0.1 mg/ml, in 2% acetic acid solution) and Alg (1.0 mg/ml, 0.5 M NaCl solution) was fixed to 3.5 and 6.0, respectively.

3.2.3 Preparation of Hollow CH/Alg Multilayer onto CaCO_3 -CMC Core

The preparation for multilayered core-shell microspheres was executed through L-B-L assembly of CH and Alg in an alternative manner. Being negatively charged, CaCO_3 -CMC microspheres were coated with positively charged CH solution as the first layer. For this, 40mg of dried CaCO_3 -CMC microspheres were introduced to suitable volume, 0.1 mg/ml CH suspension (pH 3.5) and the mixture was incubated at room temperature for 15 minutes under gentle agitation. The resulting mixture was initially rinsed by adding suitable volume of deionized water and centrifuged (6000 rpm, 8 minutes) twice to remove the residual CH. This was followed by redispersion in suitable volume, 1 mg/ml Alg solution of the CH coated/ CaCO_3 -CMC core particles followed by incubation and washing steps. The above deposition cycle ($4 > n > 1$) was repeated till desired sets of CH/Alg layers were achieved on to CaCO_3 -CMC core microparticles. The core was subsequently removed by dissolving multilayered CH/Alg coated multilayered microparticles in 0.2 M EDTA (pH=7) solution for 15 minutes three times. The excess EDTA over microparticles was removed through washing in deionized water after each treatment. The hollow microparticles were recovered through centrifugation at 4000 rpm (3-4 minutes) in distilled water. All experiments were conducted in triplicates.

3.2.4 *In-Situ* Immobilization of AgNPs within Hollow CH/Alg Microspheres

The as-synthesized hollow CH/Alg microparticles were resuspended in a 20 ml, 25 mM AgNO_3 solution and sonicated intermittently for 5 minutes, four times over the entire

duration of 60 minutes of incubation. Excess AgNO₃ were removed by washing the microparticles using deionized water followed by collecting them using centrifugation at 4000 rpm for 5 minutes, thrice. Afterwards, for reduction of silver ions (Ag⁺) loaded microspheres, they were added dropwise into 25 mM TSC solution at 90 °C.

3.3 Preparation of CH-Ag Composite Films

Films were produced by mixing the suspensions of 2% w/v CH, 0.5% Gly and different concentrations of Ag loaded microcarriers (0.05wt%, 0.1wt% and 0.2wt%). The suspension was homogenized by vigorous vortexing, ultrasonication (100 W, 20 KHz) for 4 hours followed by overnight shaking. The antimicrobial films were formed by casting process, using circular moulds. The casted films were first dried at room temperature and then incubated at 40 °C for 4 hours. Thus, five types of films were obtained in accordance with composition is given in table 2. The film was easily peeled off from the smooth surface without any breakage at room temperature.

Table 2: Composition of the different CH films

Sample No.	Added amount of CH (ml)	Added amount of Gly (μl)	Added amount of Ag (mg)	Mass ratio Ag: CH (wt %)
1	8	40	0	0
2	8	40	0	0
3	8	40	4	0.05
4	8	40	8	0.1
5	8	40	16	0.2

3.4 Optical Properties

3.4.1 Film Thickness

The obtained uniform thickness of each film was measured using hand-held Digital micrometer, vernier caliper and also through the lab available microscope. The film thickness was measured from three different points using a hand-held digital micrometer with a precision of 0.001 mm and its average values were taken. The films were folded

four times to measure thickness by vernier caliper. For observing thickness through lab microscope, each type of the film was cut into thin size of 3 mm × 30 mm. And with the help of computer attached to microscope the thickness was determined.

3.4.2 Color

The various films were observed under proper light to determine the color of the films.

3.4.3 Opacity/ transparency

The films absorbance value was measured at 600 nm using a spectrophotometer. The sample films were taken in a standard of 10 mm × 20 mm and directly kept in a spectrophotometer test cell through which laser beam passes in vacuum medium. Opacity was calculated as,

$$O = \frac{\text{Abs at 600nm}}{X} \quad \text{Eq.1}$$

Where, O = opacity,

*Abs*600= absorbance value at 600 nm and X = film thickness (in mm).

Transparency of the film samples was expressed as,

$$T = \frac{\text{Abs at 660nm}}{\text{Thickness of film(mm)}} \quad \text{Eq.2}$$

3.5 Various Other Studies

3.5.1 Moisture Content

The moisture content of each film of 20 mm × 20 mm was calculated through its weight change, by keeping the various films at 105 ±0.5 °C till the weight becomes constant. The weight of each film sample was done by using digital balance. The moisture content was calculated at 25 °C, according to the following equation:

$$\text{Moisture content} = \left(\frac{M_W - M_d}{M_W} \right) \times 100 \quad \text{Eq.3}$$

Where, M_w is the weight of the films conditioned at 75 % of relative humidity at 105 ± 0.5 °C and M_d is the dry weight of the films. Test was conducted in triplicates and the average values were reported.

3.5.2 Water Solubility of Films

Samples of 20 mm × 20 mm were cut from each film and weighed carefully. The films were placed into beakers containing deionized water. The samples were constantly agitated for 1 hour at 100 rpm at room temperature. The remained pieces of the films after soaking were dried again in an oven at 105 ± 1 °C to constant weight. All the tests were conducted in triplicate and the means were reported. Film solubility (%) was calculated as follows:

$$\% \text{ Solubility} = \left(\frac{\text{Initial dry weight} - \text{Final dry weight}}{\text{Initial dry weight}} \right) \times 100 \quad \text{Eq.4}$$

3.5.3 Swelling Studies

The swelling property of the five different films was measured. All the films were first cut into equal 20 mm × 20 mm of size and their dry weight was taken. Then they were placed in 250 ml phosphate buffer (pH 7.4) and the water uptake was measured periodically by the weighing the film for approximately 9 hours. The film was blotted to remove excess water. The percent mass swelling was calculated using following expression:

$$\text{Percent mass swelling } (S_m \%) = \left(\frac{W_t - W_0}{W_0} \right) \times 100 \quad \text{Eq.5}$$

Where, W_t is the sample weight at time, t and W_0 is initial weight. The experiment was performed in duplicates and average values were taken for results. Also, swelling ratio (Q) of the films was calculated using an equation:

$$Q = \frac{W_s}{W_d} \quad \text{Eq.6}$$

Where, W_s is the weight of the swollen film at different time intervals and W_d is the weight of dry film.

3.6 Barrier Properties of the Films: Water Vapor Permeability (WVP)

The WVP of the five different films was determined carefully. For each sample, films were cut in circular form of the glass vial size. The films were then placed on glass vials in the vacuum desiccator having silica gel for 24 hours at 25 ± 2 °C, having 53 ± 1 % RH. The weight of the vacuumed films was taken. Then silica gel was removed from the vacuum desiccator and saturated solution of sodium chloride (NaCl) was placed inside it for 24 hrs. Then again weight of moisture gained films was taken. The weight change of film was determined carefully. This mechanism was performed thrice and average value was taken for further calculations. The film WVP was calculated as follows:

$$\text{Water vapor permeability} = \frac{(\text{Change in weight of the film}) \times X}{A \times \Delta p} \quad \text{Eq.7}$$

Where, A= area of film surface exposed, m^2

Change in weight = difference in weight before and after moisture gain (in gm)

X= thickness of each film, mm

Δp = differential partial pressure of water across the film, which is 3.202 KPa (at 25 °C)

With 75% of RH conditions

3.7 Antioxidant Activity

The antioxidant activity of the film was determined through ELISA reading of each film sample solution in triplicates at 517 nm. For reading 40 μL aliquot of the sample solution was mixed with 100 μL of methanol and 60 μL of DPPH (0.15 mM prepared in methanol). The positive control was made by adding 40 μL of quercetin in 100 μL methanol and 60 μL of DPPH. The negative control was made by adding 140 μL of methanol in 60 μL of DPPH. The reaction was performed in dark at 30 ± 2 °C for 30 minutes. For ELISA reading, 100 μL from each DPPH prepared sample was put in the ELISA plate carefully in triplicates and reading was taken at 517 nm. DPPH radical scavenging activity was calculated as follows:

$$\text{DPPH Scavenging Activity(\%)} = \left(\frac{A_{\text{DPPH}} - A_s}{A_{\text{DPPH}}} \right) \times 100 \quad \text{Eq.8}$$

Where, A_{DPPH} is the absorbance value of the methanol solution of DPPH and A_s is the absorbance value at of the DPPH sample solutions.

3.8 Antibacterial Assay of Nano-Silver Loaded Microcarriers in CH Film

The antimicrobial activity of Ag embedded in CH film was tested against *E.coli*, *Enterobacter aerogenes*, *Staphylococcus aureus*, through contact inhibition method. The bacterial strains were inoculated into the nutrient broth and kept for overnight growth at 120 rpm in incubator shaker at 37 °C. The OD (optical density) of the bacterial culture was set at unit absorbance at 600 nm by UV Visible spectrophotometer. The culture was centrifuged at 6000 rpm for 10 minutes and then the pellet was washed and redissolved in Phosphate buffer saline (PBS buffer). Appropriate dilution of bacterial culture was made in saline solution up until its concentration reached $\sim 10^9$ CFU/ml. Further working concentrations of 10^6 and 10^5 CFU/ml were made by serial dilutions in saline solution. These aliquots were taken (1 ml) into a previously sterilized spray bottle and were sprayed on nutrient agar plates from a significant distance. The plate was divided in four parts and the four different film samples of 10 mm $\times$ 10 mm were placed in each quadrant respectively. The plate was incubated in incubator shaker at appropriate temperature for 24 hours. In this experiment, CH/Gly film was taken as control and tests were performed in duplicates. This experiment was repeated with microorganisms namely, *E.coli*, *Enterobacter aerogenes* and *Staphylococcus aureus* at optimum conditions. The colonial growth on each film was observed.

3.9 Fruit Packaging

For packaging, fresh cherries were taken and each type of film was softly wrapped around the cherries. Packaged fruits were stored at 4 °C for two weeks. For assessing the shelf life of the fruit, five packed samples and one unpacked control were observed each day over the span of 17 days.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Characterization of Microcarriers

4.1.1 SEM

The information regarding the size, structure and surface morphology was obtained by subjecting the core particles and biopolymeric microcarriers to SEM analysis at different magnifications. SEM images at 10,000 X magnification revealed the structural distribution of the core particles as well as biopolymeric microcarriers. The shape of sample ranged from being spherical to oval and some being cuboidal can be observed in the figure 6. The size of the biopolymeric microcarriers varied in range of 1-10 μm which was determined by analyzing the sample at magnification of 10,000 X. At 10,000 X the characteristics of core surface is observed as clusters. The surface can be rough with sharp grooves or diminished with rough and sharp edged grooves.

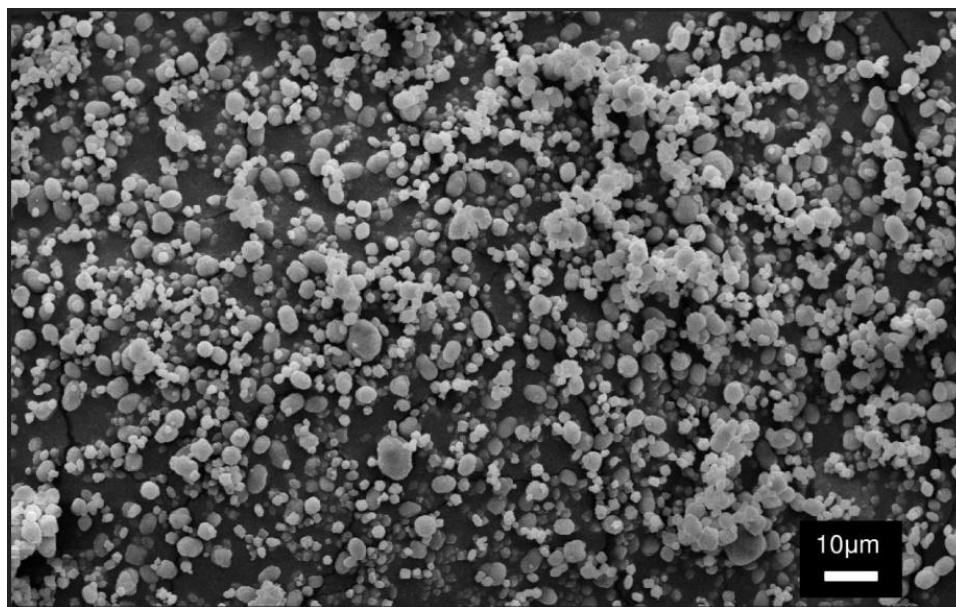


Figure 6: SEM image of core microparticles at 10,000 X.

4.2.2 DLS

The hydrodynamic diameter of calcium carbonate core was estimated through DLS. The obtained result as shown in figure 7, mean hydrodynamic diameter of calcium carbonate core was measured to be 1204.2 nm. The large size of core particles is due to their property of clustering together and forming an aggregation. The addition of L-B-L assembly of biopolymers on the base of core particles altered the mean hydrodynamic diameter of resulting particles.

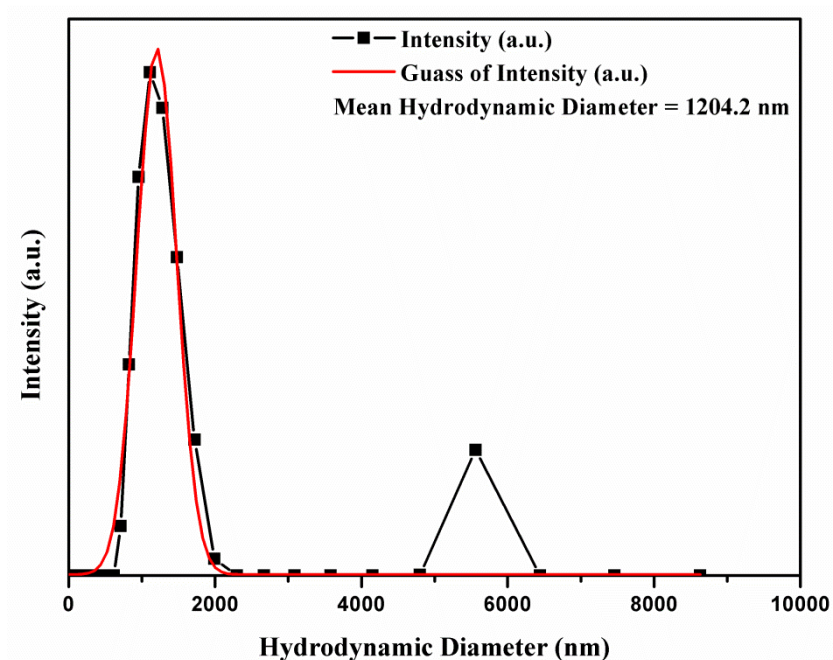


Figure 7: DLS plot of calcium carbonate core.

Figure 8, represents the hydrodynamic diameter of CH coated on negatively charged core particles. The core acted as the template on which CH biopolymer has been layered with uniform deposition. Due to this layering the average size of the particles increased to 1258.4 nm. Due to addition of Alg layer over CH layer, a double layered assembly was created. With the addition of one more layer, the increase in average particle size has been observed.

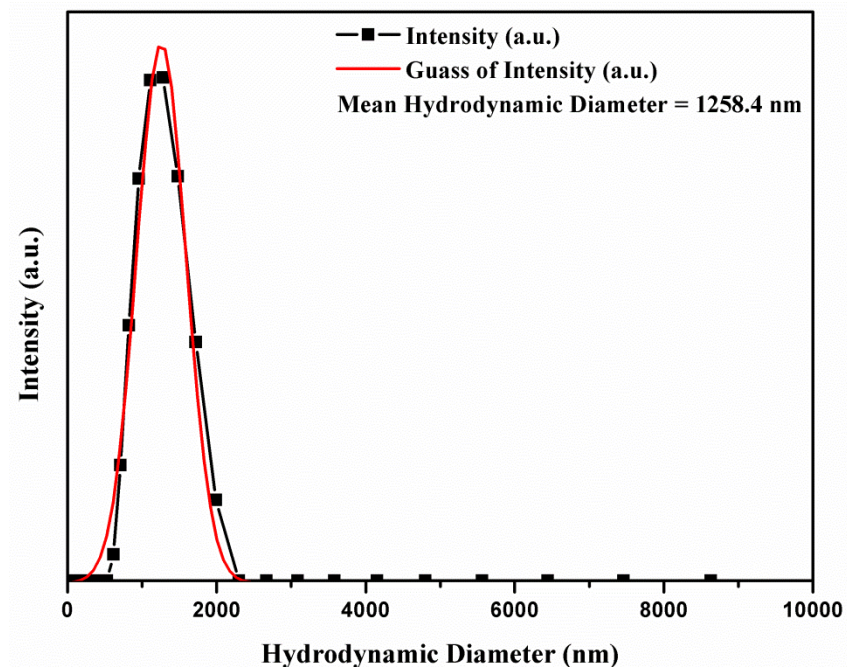


Figure 8: DLS plot of first CH layer over core.

The hydrodynamic diameter of the Alg coated particle observed is 1471.6 nm, which is shown in figure 9.

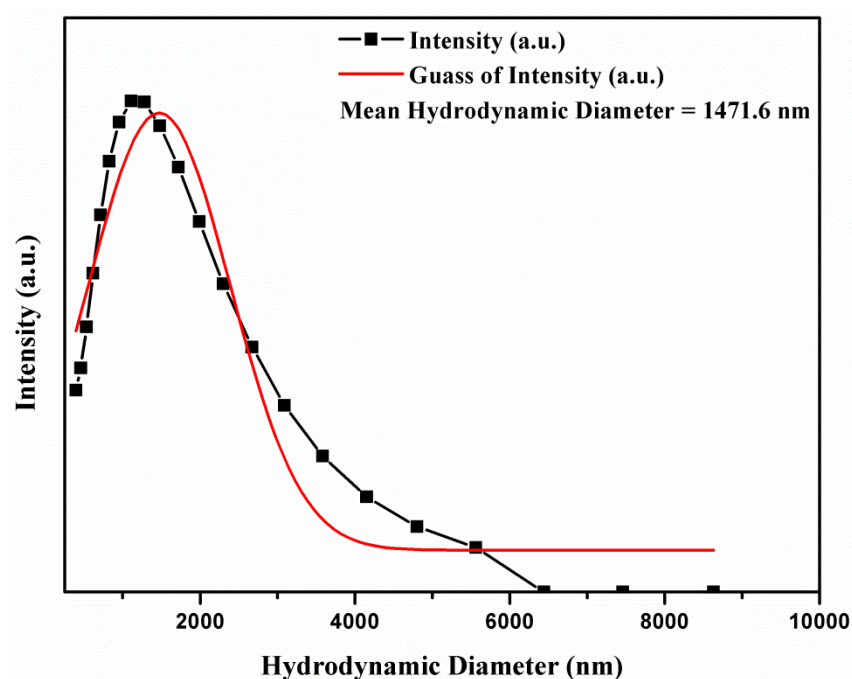


Figure 9: DLS plot of first Alg layer.

Figure 10 shows the hydrodynamic diameter of the third layer over template, which is of CH biopolymer. The size of the particle diameter increased to 1627.5 nm. As with each layer the diameter increased to a greater extend.

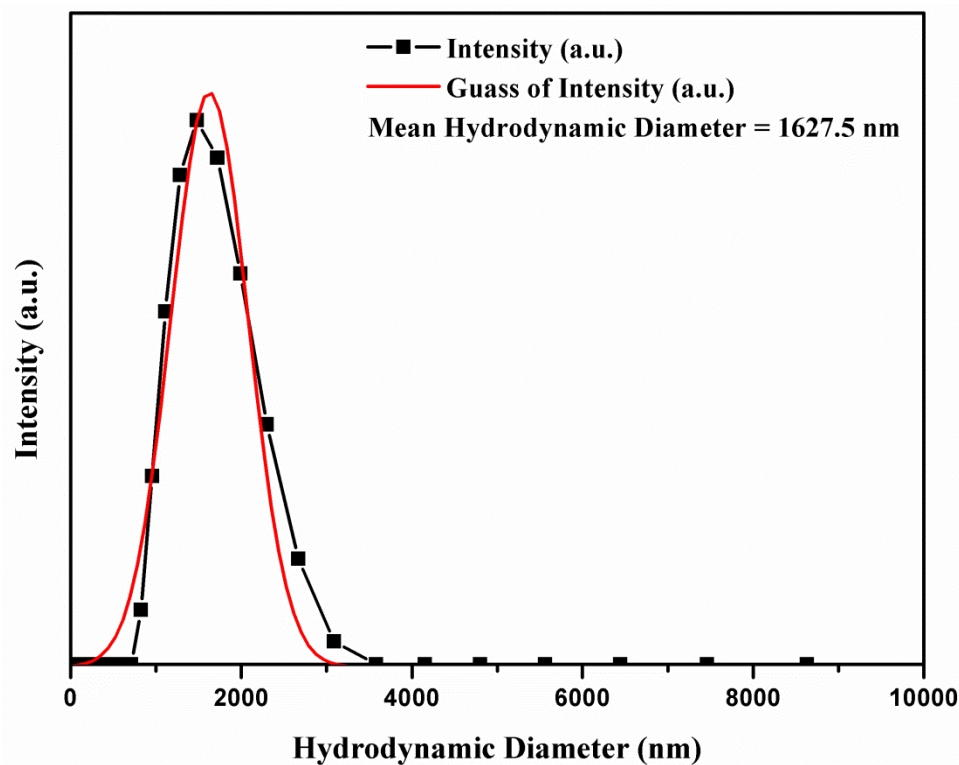


Figure 10: DLS plot of second CH layer.

Figure 11, shows the hydrodynamic diameter of second layer of Alg over second layer of CH. The average hydrodynamic diameter of second layer of Alg observed was 2953.8 nm. The figure 12 represents the hollow layered particle with size of 3074.9 nm. The core template which was layered four times was treated with EDTA to remove the core particles.

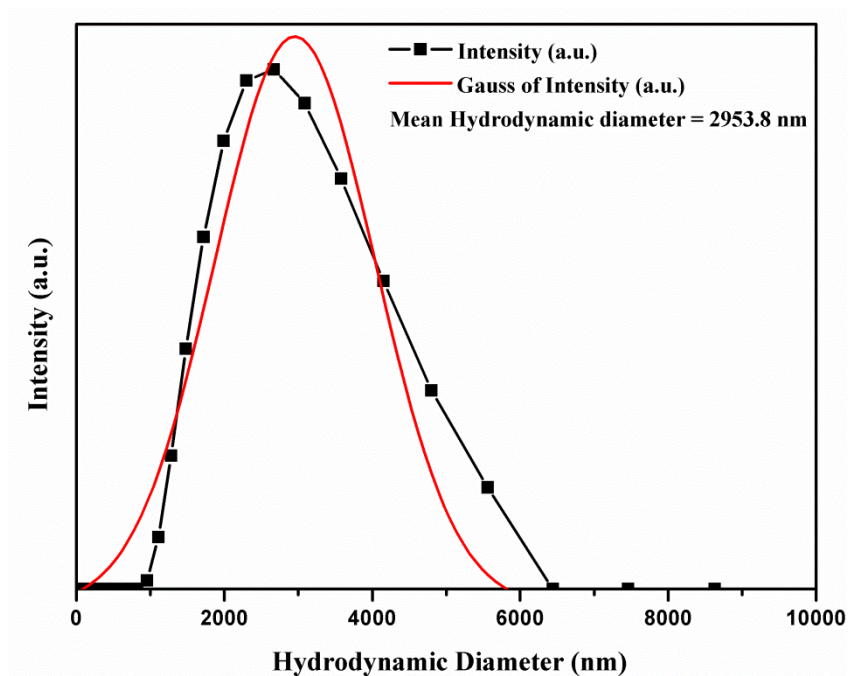


Figure 11: DLS plot of second Alg layer.

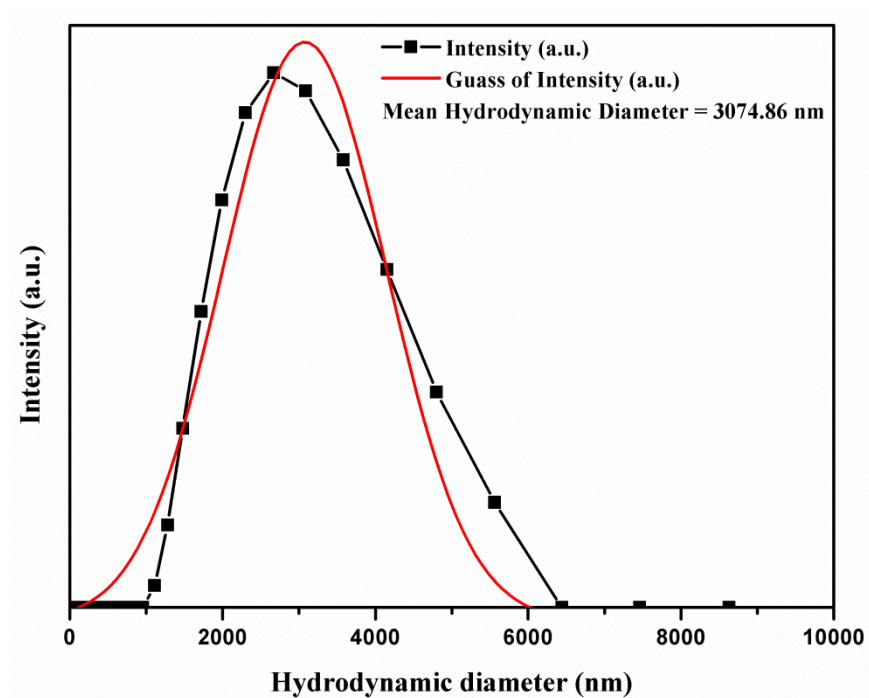


Figure 12: DLS plot of microcarriers after core removal.

4.2 Synthesis of *In-Situ* AgNPs

The in-situ reduction of Na_2CO_3 loaded microcarriers was evaluated initially via visual appearance. The gradual change in the color of microcarriers solution was observed upon addition of TSC with increasing time. The change in the color was the primary sign which signified that Na_2CO_3 has started to reduce, leading to the formation of AgNPs (Figure 13). Owing to the weak reducing efficacy of TSC at room temperature, high temperature (90°C) was attained for the reaction. At this temperature color change was evident within 5 minutes. After 10 minutes the color of the solution turned dark brown which signified the saturation of solution with AgNPs. The presence of TSC stabilized the NPs via organic capping.

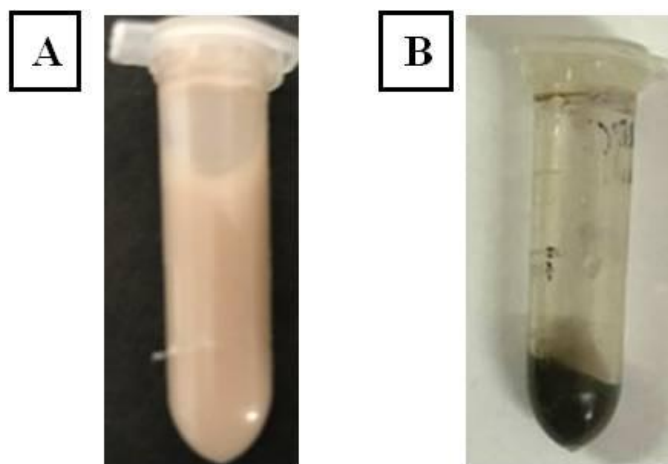


Figure 13: Visual appearance of (A) AgNO_3 loaded microcarriers before adding TSC, and (B) After 10 minutes of incubation at 90°C with TSC.

The reduction of particles with TSC to form AgNPs was observed. The immobilization process provides larger surface area to volume ratio. Due to which the immobilized nano-silver particles have average size of higher range that is 3610.3 nm.

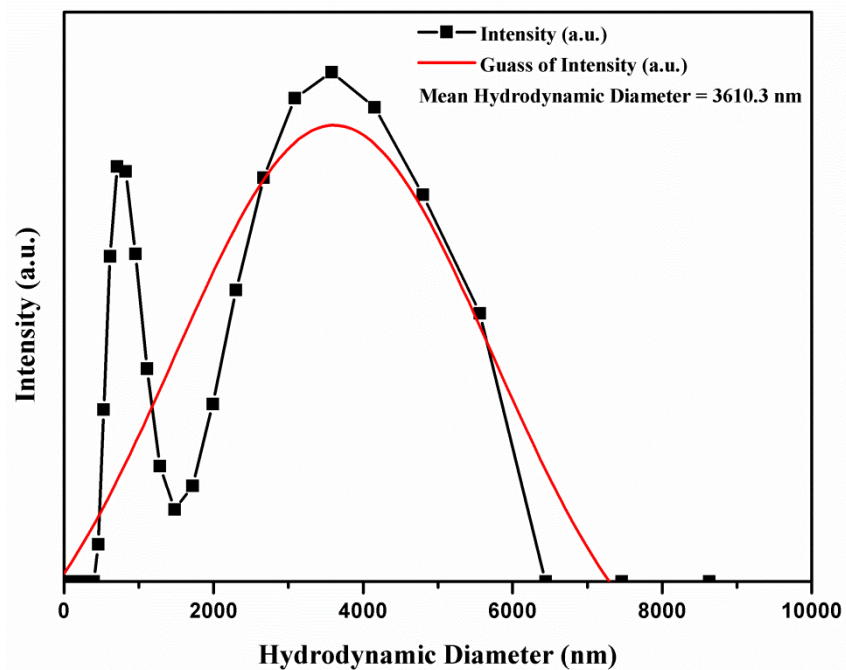


Figure 14: DLS plots of Ag loaded microcarriers.

4.3 Fabrication of Chitosan and Bio-Nanocomposite Films

It is possible to make only CH films without the plasticizer addition. The CH is dissolved in acetic acid which acts as a solvent for film formation. It has been observed that the obtained pure CH film (Figure 15, A) were slightly yellow in appearance. The yellow color is due to crosslinking between the amino groups in CH biopolymer. With increase in the thickness of the film, yellow color darkening can be easily observed. The diameter of the films is 13 cm. The addition of Gly in the CH film does not drastically change its morphology as it gets easily dispersed into the film. A slight change of color to transparency has been observed. Hence, provides good texture and matrix for the films. The plasticizer induces structural modifications of the CH network, leading to a less ordered but compact structure. It induces change in the number of hydrogen bonds in film. The figure 15, from C to E represents the ascending concentration of AgNPs. With the addition of higher concentration of AgNPs, the roughness of the film surface increased. The addition of Gly and Ag changes the both inter- and intra-molecular chemical bonding. The small size of AgNPs helps them to disperse properly in the film.

A



Film composition: 2% chitosan dissolved in 2% acetic acid

B



Film composition: 2% chitosan dissolved in 2% acetic acid + 0.5% Glycerol

C



Film composition: 2% chitosan dissolved in 2% acetic acid + 0.5% Glycerol + 0.05 wt% AgNPs

D



Film composition: 2% chitosan dissolved in 2% acetic acid + 0.5% Glycerol + 0.1 wt% AgNPs

E



Film composition: 2% chitosan dissolved in 2% acetic acid + 0.5% Glycerol + 0.2 wt% AgNPs

Figure 15: Digital images of various Bio-nano composite films and their compositions.

The AgNPs embedded films are yellowish-brown in color. With the increase in the concentration of AgNPs content the brown appearance increases in the film. All the different films were equally flexible. The films when subjected to mechanical strain through physical means such as folding it multiple times it regained its integrity. The presence of Gly increases the flexibility of the films because of the reduction of the intermolecular bonds between polymeric chains. Color attributes are of prime importance because they directly influence consumer acceptability.

4.4 Film characterization: Fourier Transform Infrared Spectroscopy

Figure 16, shows the FTIR transmittance spectra obtained from pure CH or CH/Gly with different amounts of AgNPs embedded into the films. The peak shows the presence of dipole movements in films. The band above 3000 cm^{-1} represents the O-H bond or N-H bonds.

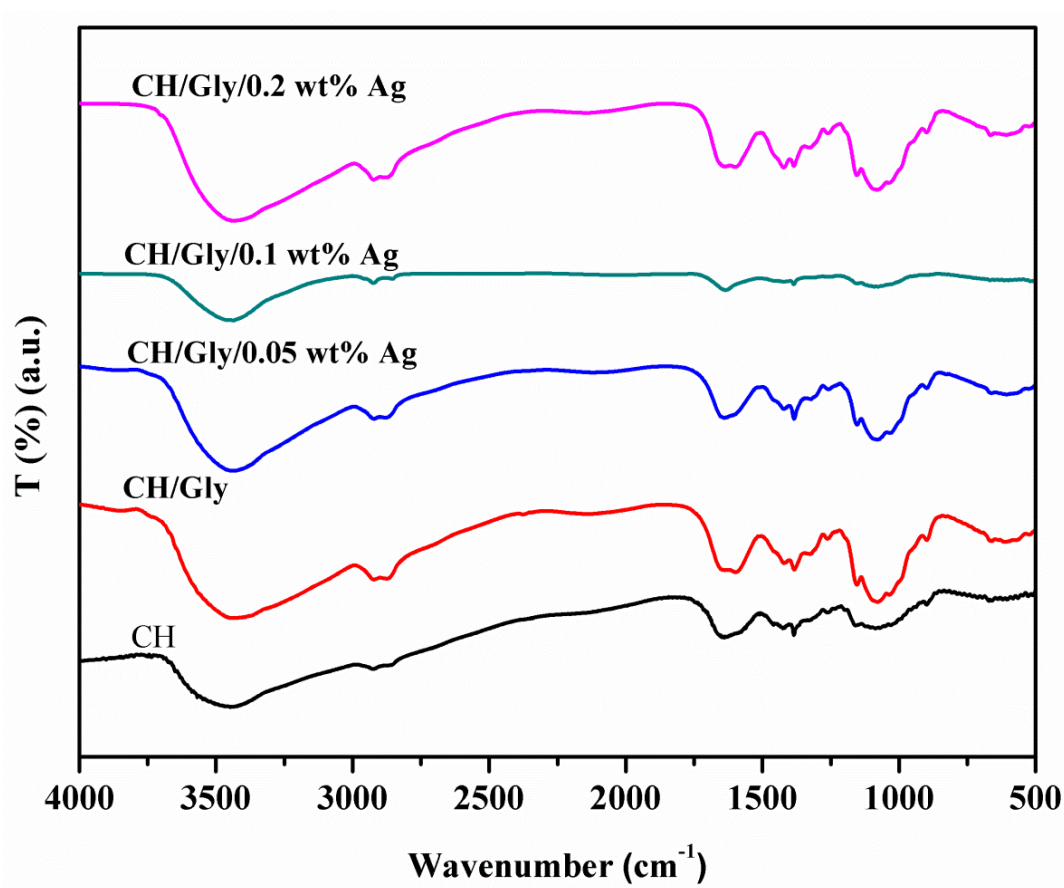


Figure 16: FTIR spectra of films with different compositions

The first band is at 3300 cm^{-1} which is due to O-H stretching vibrations present in the CH films. The peak at 3435 cm^{-1} is due to N-H stretch. The peak shift to higher intensity is observed with addition of Gly and nano-silver particles. The peak at or near wavenumber 2920 cm^{-1} is present due to Gly in films. The peaks of CH, CH/Gly and CH/Gly/0.05 wt% AgNPs are close to each other. The peak at 1625 cm^{-1} is due to C=O stretch and at 1382 cm^{-1} is due to C-N stretch in the film samples is plotted in graph. The absorption peak at 1078 cm^{-1} is assigned to C-O stretching.

4.5 Film Thickness

The films were casted and dried into the same mould with equal amount of CH under ambient conditions. Film thickness depends on the properties of the film forming materials. Table 3 shows the thickness of each type of film observed through different instruments. The average ranges from $11.75\text{ }\mu\text{m}$ to $15.82\text{ }\mu\text{m}$. The thickness of the films were observed to increase with increase in the AgNPs content as the surface area of mould was fixed which leads to more compact surface structure of the film. With higher amount of solid particles in the film, the amount of water content decreases and thickness increases. The increasing order of thickness is shown in figure 17.

Table 3: Thickness in μm of five different CH films

Type of CH film	Digital micrometer (μm)	Vernier caliper (μm)	Microscope (μm)	Average thickness of films (μm)
CH	15	8.5	11.75 ± 1.50	11.75 ± 1.50
CH/Gly	16	15	11.24 ± 1.91	14.08 ± 1.91
CH/Gly/0.05wt% AgNPs	19	10	14.5 ± 1.50	14.50 ± 1.50
CH/Gly /0.1wt% AgNPs	18	12.5	15.28 ± 1.70	15.26 ± 1.70
CH/Gly /0.2wt% AgNPs	15	18.24	14.22 ± 1.61	15.82 ± 1.61

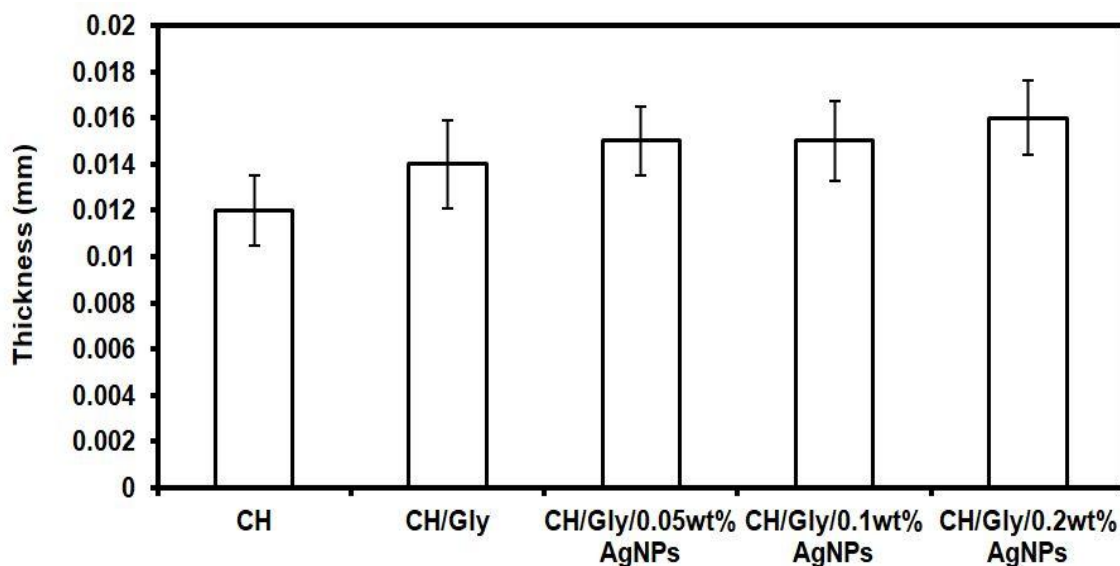


Figure 17: Thickness of various antimicrobial films

4.6 UV–Visible Absorption Spectra of Films

Transparency toward UV is one of the important factors considered for packaging materials to prevent bacterial growth or oxidation of food materials under UV light. The films were characterized through UV-visible spectroscopy in the range from 200 to 800 nm which is shown in figure 18. It is a simple and sensitive technique for the observation of NPs. All the different chitosan films were observed to have slight variation in their absorbance values. All the films blocked the UV light in 450 to 800 nm range which makes these films good for preservation of food, as most of the food get perished due to effects of UV light. Hence, these films can potentially decrease the UV light induced food oxidation. Films based on chitosan are known to have excellent barrier properties to UV light in the UV region, regardless of glycerol.

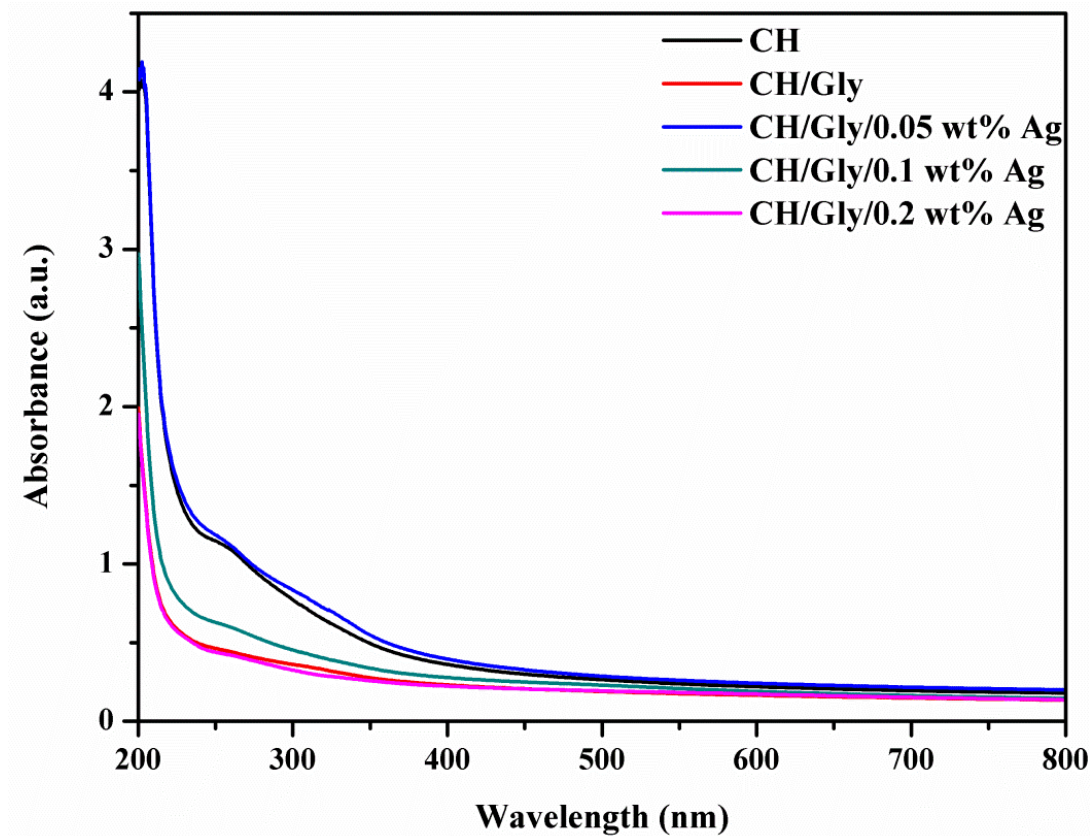


Figure 18: UV–Vis absorption spectra of five different types of CH films.

4.7 Opacity

The opacity of film is an indication of how much light can pass through it. The higher opacity signifies lower amount of light that can pass through the film matrix. The CH/Gly film is more transparent, and the opacity value of the 0.2 wt% nano-Ag/CH/Gly film is the highest of all films. The opacity of the film decreased with addition of lipid solution to pure CH. This phenomenon is probably due to light scattering from lipid droplets, distributed throughout the film network after formation. It has been observed that, opacity increased with increasing nano-silver concentration. The films opacity values ranges from 15.86 to 18.20 due to incorporation of AgNPs. The various opacity values of films are shown in table 4. The light transmittance of a package affects the rate of oxidation of lipids, and thereby food quality. Opacity of the film is an important index of appearance and consumer acceptance.

Table 4: Opacity of different CH films

Type of film	Absorbance (500nm)	Film thickness (mm)	Opacity (Abs ₅₀₀ /mm)
CH	0.194	11.75×10 ⁻³	16.51
CH/Gly	0.190	14.08×10 ⁻³	13.49
CH/Gly /0.05wt% AgNPs	0.230	14.50×10 ⁻³	15.86
CH/Gly /0.1wt% AgNPs	0.265	15.26×10 ⁻³	17.36
CH/Gly /0.2wt% AgNPs	0.288	15.82×10 ⁻³	18.20

Transparency is one of the measures of film quality from its appearance. The high values of opacity indicate lower transparency. The transparency analysis results were measured using UV spectrophotometer is shown in table 5. Transparency can be viewed as function of film color measured as a function of light transmittance. The results show that by increasing nano-silver concentration up to 0.2 wt%, transparency becomes least. The loss of transparency is due to scattering of light. The most transparent film of CH/Gly with transparency of 14.48. The transparency of a film is a desirable property as the consumer can clearly observe the food product while wrapped with the film.

Table 5: Transparency of different CH films

Type of film	Absorbance (660 nm)	Film thickness (mm)	Transparency (Abs ₆₆₀ /mm)
CH	0.183	11.75×10 ⁻³	10.54
CH/Gly	0.204	14.08×10 ⁻³	14.48
CH/Gly /0.05wt% AgNPs	0.173	14.50×10 ⁻³	11.93
CH/Gly /0.1wt% AgNPs	0.163	15.26×10 ⁻³	10.68
CH/Gly /0.2wt% AgNPs	0.155	15.82×10 ⁻³	9.80

4.8 Water Solubility of Films

Water solubility is an important factor to select the possible applications of biopolymer films. Maximum solubility is observed of the pure CH film and the least is observed is of

the CH with maximum Ag concentration (shown in table 6 and figure 19). With the increase of AgNPs content, there is decrease in the solubility of the films in the water. The diffusivity of water molecules could be increased as a function of the membrane free volume. Because of the interactions between the hydroxyl groups of CH and Gly by hydrogen bonding, hence, reducing the solubility of the film in water. Even though higher water solubility may have positive effects, such as enhancing the film biodegradability. Lower solubility represents stability of the films. Water solubility is a measure of the resistance of a film sample to water. CH films have a higher affinity for water compared with the powder, probably because protonated, rendering the films more hydrophilic than the powder itself.

Table 6: Water solubility of different CH films

Type of film	Initial weight (mg)	Final weight (mg)	Water Solubility (%)
CH	3.4	3.0	11.76 ±1.11
CH/Gly	6.9	6.7	2.89 ±0.95
CH/Gly /0.05wt% AgNPs	14.5	13	10.34 ±0.31
CH/Gly /0.1wt% AgNPs	6.5	6.2	4.61 ±0.67
CH/Gly /0.2wt% AgNPs	8.6	8.4	2.32 ±0.68

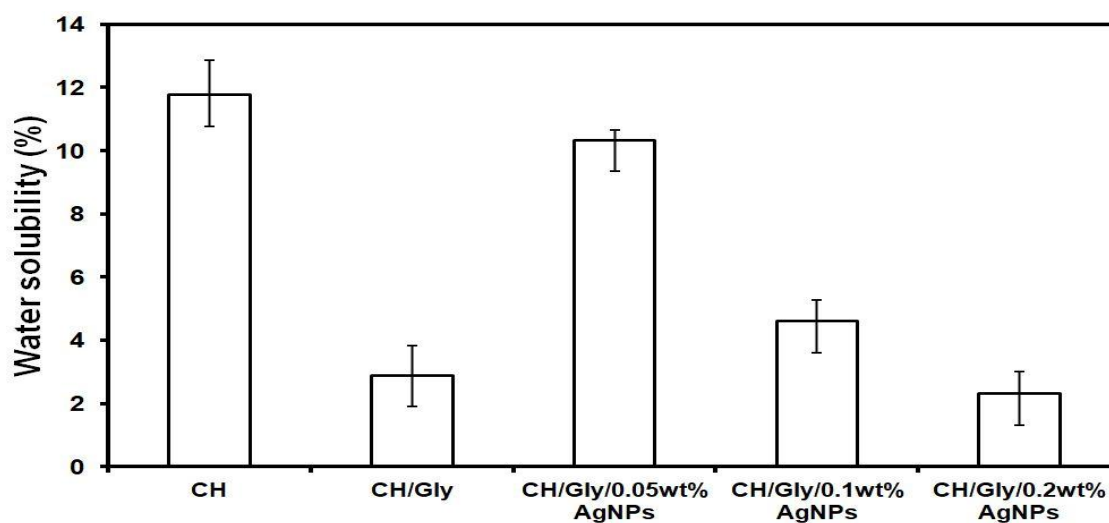


Figure 19: Water solubility of five different CH films

4.9 Swelling Study

The water absorption of films depends on its matrix and composition. Swelling reached the equilibrium gradually within 6 hours, regardless of film composition. However, the films with acidic content show higher swelling values. This is due to the intrinsic property of CH biopolymer to absorb water at lower pH. With the increase of the empty spaces for water penetration due to higher free volume for the diffusion of water molecules, there is increase in the swelling of the films. The decrease in the swelling values is due to increase in bonds, which reduced free volume and chain mobility, thus, hindering the water entry in the network. A constant increase in different CH films were observed till weight becomes constant. The increase in weight represents the swelling of the films. Each film has different capacity of absorbing water. Table 7 shows that this study was done in duplicates and average of the two values was taken. Figure 20 represents the graph showing percentage swelling of different CH films. The maximum swelling was observed in the CH/Gly film due to the hydrophilic nature of the Gly. The Gly interacts through hydrogen bonds with CH and with water molecules, leading to higher water contents absorption. And the least swelling was observed in CH/Gly/0.2wt% AgNPs film. This lowering in the swelling capacity is attributed due to binding of AgNPs with oxygen and nitrogen atoms of hydroxyl and amine groups present in CH chains. The film swelling kinetics was observed and is shown in figure 21.

Table 7: Swelling percentage of five different CH films.

Type of chitosan film	Swelling study 1 (%)	Swelling study 2 (%)	Average (%)
CH	85.71 ±3.89	83.12 ±4.83	84.42 ±4.29
CH/Gly	90.74 ±3.13	91.79 ±4.78	91.27 ±4.78
CH/Gly/0.05wt% AgNPs	56.25 ±2.78	58.11 ±3.38	57.18 ±3.26
CH/Gly/0.1wt% AgNPs	35.29 ±2.12	33.25 ±2.91	34.27 ±2.36
CH/Gly/0.2wt% AgNPs	26.31 ±2.78	27.25 ±2.78	26.78 ±2.16

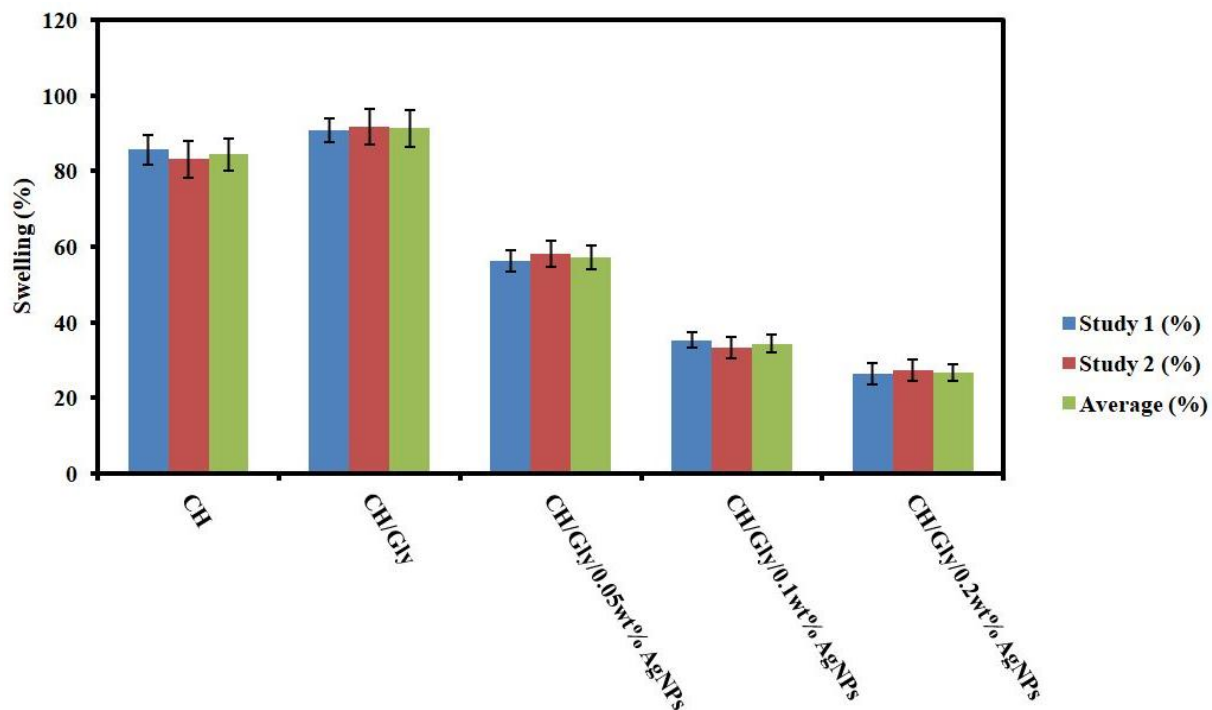


Figure 20: Represents swelling study of different films

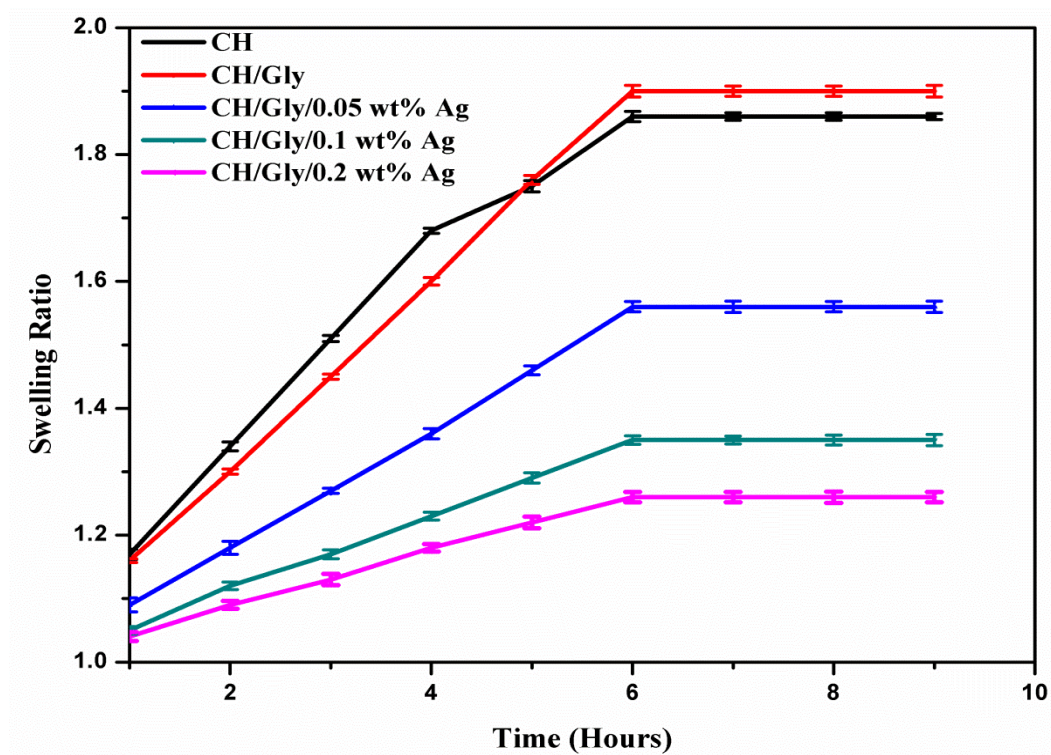


Figure 21: Swelling kinetics of different CH films at room temperature

4.10 Moisture Content

The size 20 mm × 20 mm of each of the five film samples were weighed and dried at 105 ±0.5 °C. The moisture content was previously observed by weighing the films at 25 °C to check the sensitivity of the five different CH films. The CH films maintain the moisture levels and physical integrity in order to avoid the loss of functional properties during its application. The table 8 shows the moisture content level which increases with the increase in Ag concentration in the films. This was observed due to decreased weight change of the higher Ag concentration embedded films. The moisture content ranges from 12.24% to 20.93%. The average moisture content of all the five different CH film is 16.28%. The lower moisture content depicts the strong bonds (hydroxyl and amine group) between one molecule of CH to another in the film matrix. The plasticizer addition increased moisture content of films, due to its hydrophilic character. It has been observed that with increase in nano-silver concentration, the moisture content decreases. This is probably due to the addition of the hydrophobic metallic AgNPs, which reduced the hydrophilicity of polymer.

Table 8: Percentage moisture content of the various CH films

Type of CH film	Average M_w of films before conditioning at 105° C (mg)	Average M_d of films after conditioning at 105°C (mg)	Average difference in films $M_w- M_d$ (mg)	Moisture content (%)
CH	9.8	8.6	1.2	12.24 ±0.18
CH/Gly	3.4	2.9	0.5	14.70 ±0.12
CH/Gly /0.05wt% AgNPs	4.3	3.4	0.9	20.93 ±0.2
CH/Gly /0.1wt% AgNPs	5.3	4.3	1	18.86 ±0.178
CH/Gly /0.2wt% AgNPs	7.5	6.4	1.1	14.66 ±0.19

4.11 Water Vapor Permeability (WVP) of Films

Water permeability refers to penetration of water molecules from one side of the film to the other side through diffusion at a given temperature. The WVP of a film is related to its chemical structure, morphology and temperature of environment. The polysaccharide films are hydrophilic in nature; hence have poor water barrier property. Table 9 shows WVP of the CH/Gly film is 32.50 g.mm/m².day.KPa, which is higher than all other films. This represents that in pure CH film, Gly has positive impact due to their reinforced molecular interactions and its hydrophilic character. Gly interacts through hydrogen bonds with CH and with water molecules, leading to increase in water vapor permeation through the film. Also, glycerol decreases the intermolecular interactions which lead to interchain spacing. The water holding capacity of a film can be increased with addition of Gly. WVP is not related to the thickness of the films. The addition of AgNPs shows decrease in WVP of the films is shown in figure 22.

Table 9:- Calculated result of WVP values of different CH films

Type of CH films	Initial weight of film (mg)	Weight of film after vacuum (mg)	Weight of film after providing moisture (mg)	Water Vapor Permeability (g.mm/m ² .day .KPa)
CH	11.9	6.6	10.5	29.10 ±1.89
CH/Gly	8.1	7.2	10.5	32.50 ±1.45
CH/Gly /0.05wt% AgNPs	7.4	6.4	7.0	7.9 ±0.78
CH/Gly /0.1wt% AgNPs	11.3	10.3	10.8	4.6 ±0.34
CH/Gly /0.2wt% AgNPs	17.5	14.9	15.17	2.55 ±0.05

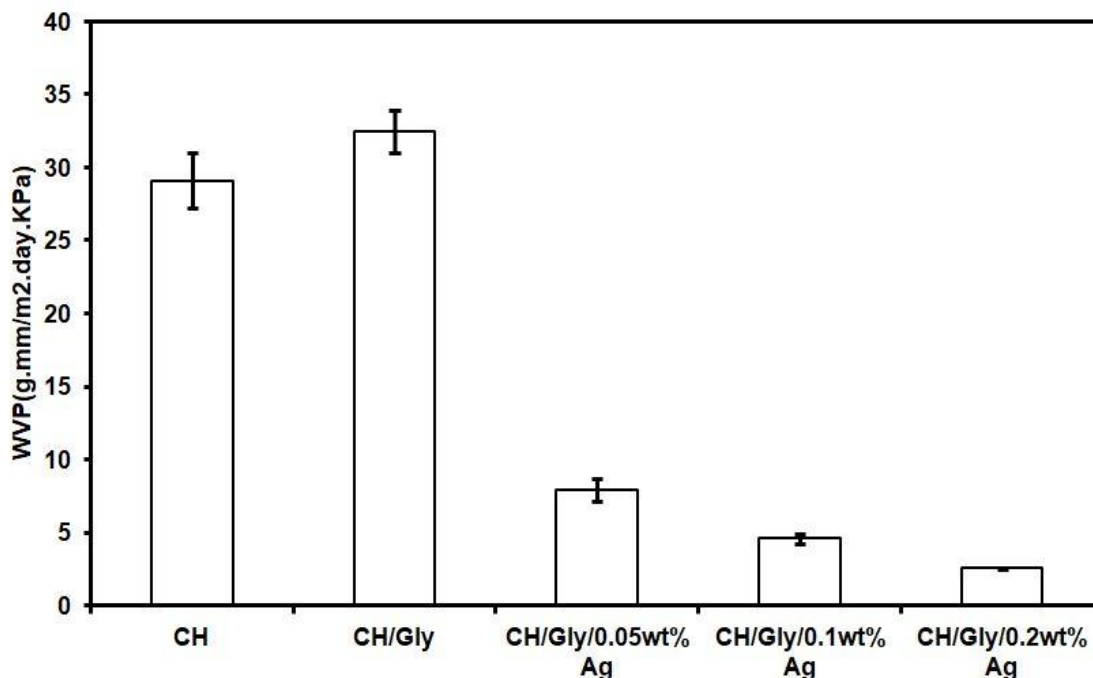


Figure 22: Water-vapor permeability of various types of CH films

This is due to formation of compact network structure by the dispersed AgNPs in the polymer matrix which does not allow water molecules to easily pass through it. This forces water vapor to travel through tortuous path through the polymer matrix dispersed with particles, thereby which increase the effective path length for diffusion. Also, the nano-silver particles have hydrophobic nature, which reduced the hydrophilicity of polymer. The mean WVP for three Ag embedded CH films is 5.02 g.mm/m².day.KPa at 25 °C with 75% RH.

4.12 Antioxidant Activity of Films

The antioxidant activity of the films was characterized by DPPH free radical scavenging assay. The DPPH radical has been widely used to test the ability of compounds to act as free radical scavengers and thus to evaluate the antioxidant activity. This assay is based on the ability of DPPH, a stable free radical, to be quenched and thereby decolorize in the presence of antioxidants resulting in a reduction in absorbance values. DPPH is a stable free radical that is dissolved in methanol and its purple color shows a characteristic

absorption at 517 nm. Antioxidant molecules scavenge the free radical by hydrogen donation and the color from the DPPH assay solution becomes light yellow resulting in a decrease in absorbance. Table 10 shows different DPPH free radical scavenging activity of the five different CH films.

Table 10: Absorbance and DPPH activity of various CH film solutions

Type of film	Absorbance at 517nm	DPPH activity (%)
Positive control	0.014	80.82 $\pm$ 2.99
Negative control	0.073	-
CH	0.049	30.13 $\pm$ 2.02
CH/Gly	0.039	44.9 $\pm$ 2.45
CH/Gly /0.05wt% AgNPs	0.035	50 $\pm$ 2.34
CH/Gly /0.1wt% AgNPs	0.033	52 $\pm$ 2.67
CH/Gly /0.2wt% AgNPs	0.006	108.88 $\pm$ 3.89

The activity is observed due to interaction between biomolecules of CH or Gly and AgNPs. The scavenging activity enhanced with increase in the nano-silver concentration in the films is shown in figure 23. AgNPs synthesized showed significant positive correlation of antioxidant potential. This is because of proper solubility of the AgNPs in the DPPH solution. In the films containing 0.2wt% Ag, the antioxidant activity increased in more than 70% in relation to the pure CH solution sample. The higher antioxidant activity of the films will protect the fruits and vegetables from the oxidative damages or free radical production.

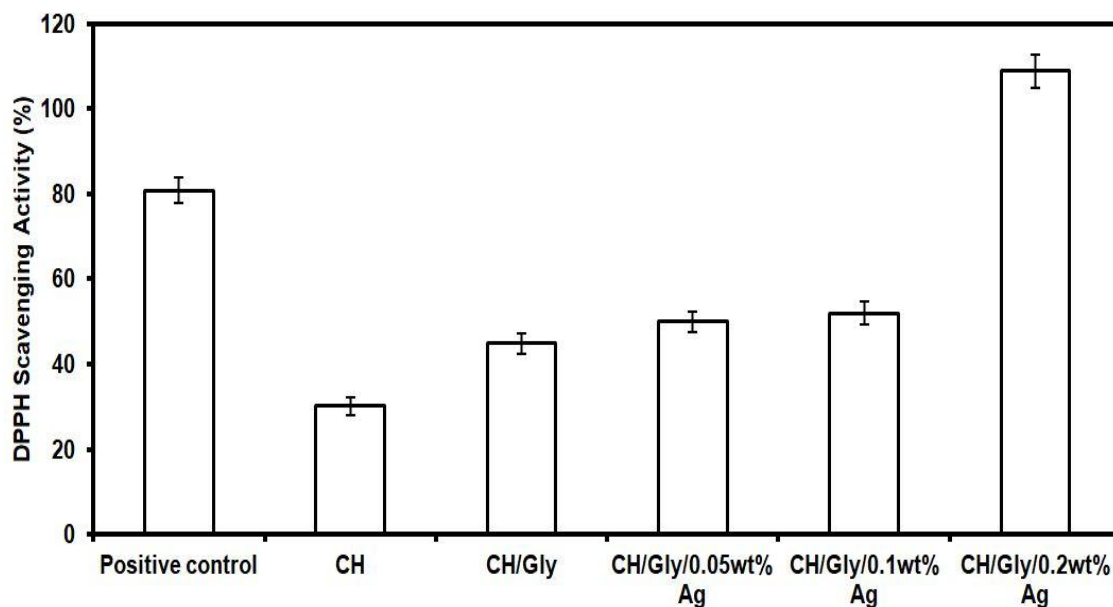


Figure 23: Antioxidant activity of different CH blended film solution.

4.13 Antibacterial Activity of the Films

The antibacterial activity of CH/Gly/Ag nanocomposite film was evaluated towards the Gram positive bacteria, *Enterobacter aerogenes* and *Staphylococcus aureus* and Gram-negative bacteria, *E.coli*. In figure 24 (A, C, E); 1,2,3,4 represents CH/Gly film, CH/Gly/0.05wt% AgNPs, CH/Gly/0.1wt% AgNPs and CH/Gly/0.2wt% AgNPs, respectively. With the increase in the concentration of the AgNPs, the antibacterial property of the film increased and bacterial growth was completely inhibited. This is due to contact inhibition of bacteria with films. This can be evidenced by the zero count of colony formation on the films shown in figure 24 (A, C, E) at number 4. Based on figure, it can be concluded that synthesized AgNPs had significant antibacterial action on both the Gram classes of bacteria. The differential sensitivity of Gram-negative and Gram-positive bacteria towards AgNPs possibly depends upon their cell surface characteristics and their interaction with the charged AgNPs. Besides, the cell wall of Gram-negative bacteria consists of an outer membrane composed of lipids, proteins and lipopolysaccharides which act as barrier and provide effective protection against

antibacterial agents which is absent in Gram-positive bacteria (Maneerung *et al.*, 2008; Xu *et al.*, 2006).

The antimicrobial activity of Ag^+ is also related to its ability to modify the DNA replication mechanisms as well as to cause abnormalities in the size, cytoplasmic contents, cell membrane, and outer cell layers of sensitive cells. The antimicrobial activity of different types of CH films varied distinctively depending on films and microorganisms tested. The lower amount of Ag embedded film possesses lower antimicrobial activity than the blend film of higher concentrations. The AgNPs do not leach out from the film to show the antimicrobial activity.

Also, the figures 24 (B, D, F) clearly showed the contact inhibition of bacterial growth under the film. The areas under the control film as well as bionanocomposite films have no bacterial growth. This signifies that CH alone can show bacteriostatic activity. CH attacks on the external surface of bacteria; reason for antimicrobial property is the positively charged amino group on the CH surface which binds with the negatively charged surface of microbial cell membranes. This leads to agglutination which causes outflow of proteinaceous and intracellular constituents of the microbial cells.

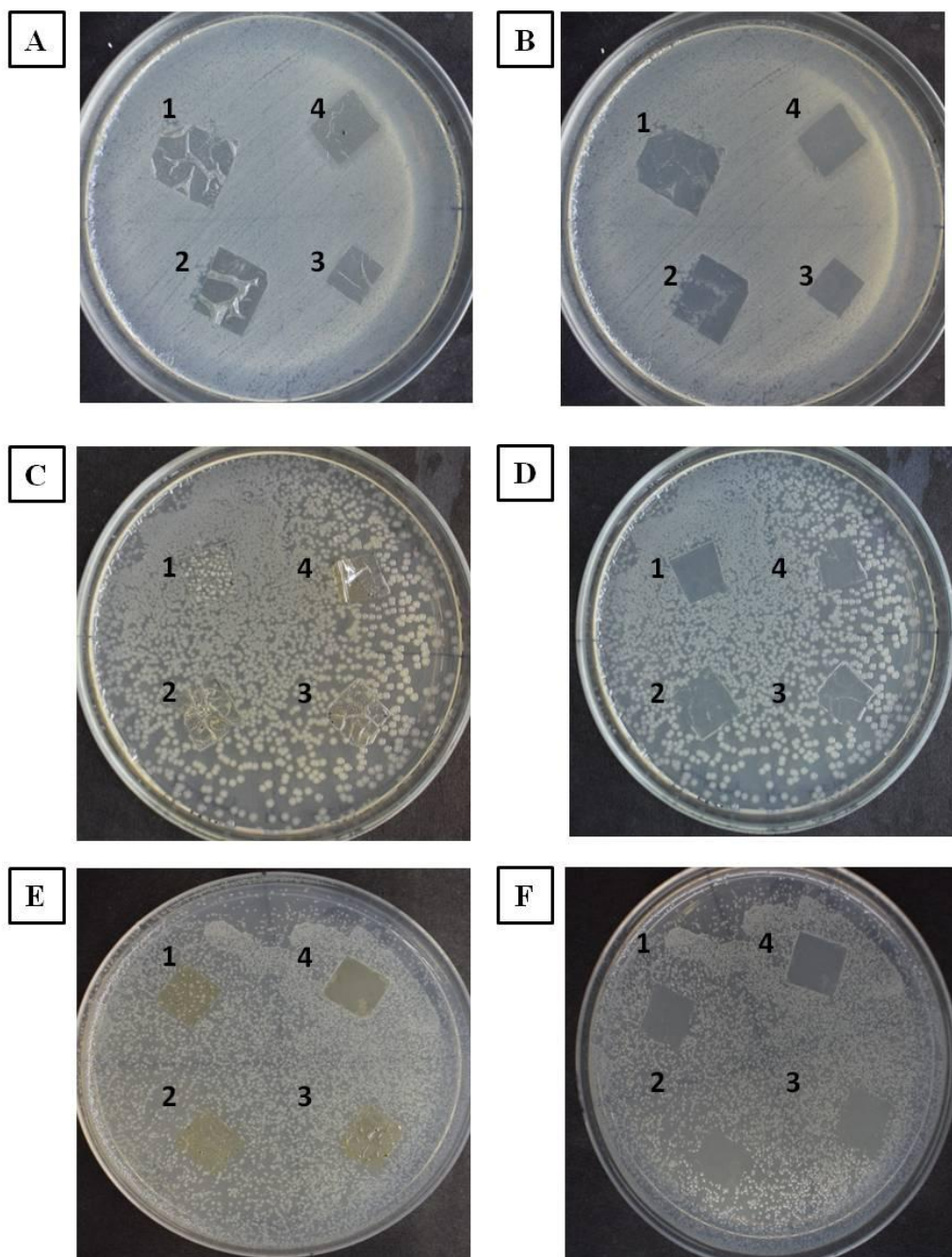


Figure 24: Results for antibacterial assay by contact inhibition against, *E.coli* (A) with film (B) without film; *Enterobacter aerogenes* (C) with film (D) without film; *Staphylococcus aureus* (E) with film (F) without film

4.14 Fruit Packaging Studies

The preservation studies were conducted by subjecting cherries wrapped in different types of films under storage conditions at 4 °C. The films were tied with the thread properly and softly. The samples were checked every day. The different CH films showed better properties and increased shelf-life of the cherry for more than two weeks. It is proved to be a better choice with no foul smell. The difference in cherries wrapped in different films can be easily observed. With the passage of time the darkening of the cherry color was observed at 4 °C. In the figure 25, it can be observed that the texture of the packed cherries remained same, fresh and red till ten days. In nano-silver packed cherries, there is slow wrinkling of cherry skin. The wrinkling of cherry is a natural process due to loss of water due to catabolism in the fresh fruits which is an attribution to the process by respiration and other senescence related metabolic processes during storage.

The first change was observed in the surface appearance of the unwrapped cherry on seventh day. There was slight wrinkling of the fruit surface without change in the color of the cherry. There was no change observed in the fruit till eight days of being wrapped around the CH/bionano-composite film. Durability test of unpackaged cherry was very less of 11 days with skin drying and also because of occurrence of fungal growth in the control unpackaged cherry. The wrapped films remained same even after the deterioration of the fruit skin texture. It can be concluded that the bionanocomposite films contributes in the shelf life of the fruit significantly. Also, increased shelf-life has been observed in cherry packaged with highest AgNPs concentration than other which have small amount of AgNPs in film. The bionanocomposite/CH/CH-Gly film wrapped around cherries is known to show lesser water loss percentage. The single layer of packaging maintains the heat escape and moisture from film surface. The complete shrinkage of all the cherries was marked at seventeenth day. AgNPs are known to have antimicrobial effect over different packaging CH films; hence these films can be the potential candidate in the field of food preservation.

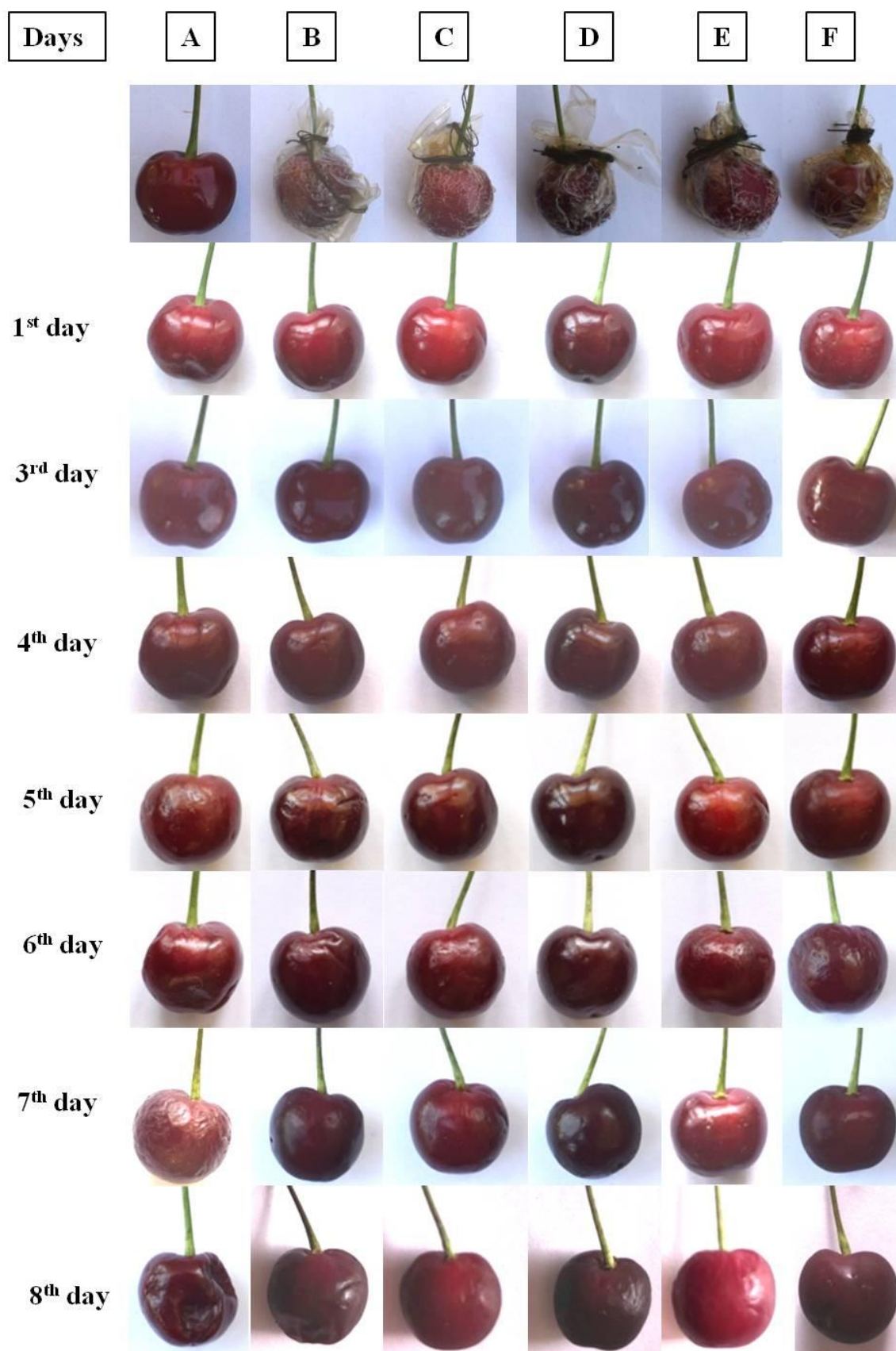




Figure 25: Changes in the physical appearance of fruit (cherry) over time when subjected to packaging. A) Unpacked control cherry, B) Packed with CH film, C) Packed with CH/Gly film, D) Packed with CH/Gly/0.05wt%AgNPs, E) Packed with CH/Gly/0.1wt%AgNPs, F) Packed with CH/Gly/0.2wt% AgNPs

CONCLUSIONS

The main aim of this study was to obtain bionano-composite films consisting of CH, Gly and AgNPs. The biodegradable CH, Gly and nano-silver films were made by homogenous dispersion of particle. The addition of AgNPs into the CH matrix increased translucency and darker appearance of the composite films. The CH film prepared from nano-silver solution had greater thickness than that prepared from only CH solution. FTIR analysis indicated good compatibility between the polymers. The nano-composite films prepared have shown improved antioxidant and antibacterial activity. As regard to the film properties, the higher the AgNPs content, the WVP decreases. These films have shown excellent barrier properties to UV Visible light, which could prevent food from oxidation. Addition of AgNPs into the CH films decreased the moisture content, swelling percentage, water solubility, transparency but increased opacity, thickness. These films showed strong bactericidal activity against gram negative food borne bacteria (*E.coli*), and gram positive bacteria (*Enterobacter aerogenes* and *Staphylococcus aureus*). Interestingly, with increase in nano-silver concentration in film, the bactericidal activity also enhanced. These active films when wrapped around cherries, improved shelf-life of the fruit. Owing to their versatility, non-toxicity and biodegradability, these films can be commercially used for preservation of fruits at low temperature (4 °C) with secured food quality. These antimicrobial films can be proved as novel method to store food products such as fruits and vegetables with proper quality maintenance and extended shelf-life.

Currently nanotechnology and nanomaterials are investigated for the formation of microcarriers. The formation of core template was visualized using SEM. The synthesis of nano-silver particles through L-B-L assembly was an innovative method. The AgNPs were made by reduction with TSC. The stability of formed AgNPs was found to be high for extend period of time. The effective size of each type of particle prepared in the process of AgNPs synthesis was estimated by DLS.

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