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Trivedi, Disha S. ***Development, Antimicrobial Properties and Mechanical Properties of Starch-Based Biodegradable Films Incorporated with Antimicrobial-Nanoparticle System***

Abstract

Increasing plastic waste has led to a shift in the use of materials for food packaging from plastic to natural polymers. Antimicrobial agents (AM) and Nanoparticles (NP) have been used to improve certain properties of the bio-based films. In particular, two AM agents, sodium benzoate (Bz) and natamycin (Nat), were incorporated into starch to form starch-based films. Two incorporation methods were studied, directly incorporating AM into starch matrix, and incorporating AM-NP systems into starch matrix. In the latter method, AM were first loaded onto NP, layered double hydroxide (LDH), called LDH-Bz and LDH-Nat, and then incorporated into starch. Five films were prepared: starch without AM, starch with Bz, starch with Nat, starch with LDH-Bz, and starch with LDH-Nat. Mechanical properties were tested using the Tensile Strength Tester for Elongation, Tensile Strength and Elasticity. The results showed that addition of AM has significant effect on elongation at break but no significant effect on tensile strength. The elongation increased for films with 2% of LDH-Bz. Antimicrobial tests revealed that films most effective in inhibiting the molds were all Natamycin films i.e. Standard Nat 0.4% (50% salt and 50% lactose) and Starch LDH-Nat, whereas none of the films showed effectiveness against bacterial species.

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Chapter I: Introduction and Objectives

Food Packaging has many functions like containment, protection/preservation, communication and utility of a product. Different materials from different natural and synthetic sources have been used as packaging for food products for e.g. plastics (Polypropylene, Polyvinyl chloride, etc.), paper and paper-based materials (kraft paper, bleached paper, waxed paper, paperboard, etc.), metals, glass, etc. (Shin & Selke, 2014). One of the important function of packaging is protection and preservation of food product that it encases and there are different methods to ensure food is of the highest quality when it reaches the consumer. Some of these methods include adding preservatives, freezing, drying, reduction of oxygen content and water activity, irradiation, packaging, etc. Packaging can be modified in various ways to protect the food it holds by using Modified Atmosphere packaging (MAP), Active packaging, using Biodegradable and Edible Coatings, etc. (Gorris & Peppelenbos, 2007). One of the common bio-based material used for packaging is starch. Major advantages of using starch are that it is cheap, renewable and abundant in nature due to its availability from different sources. A study by Ghasemlou and colleagues in 2013 showed that starch films did not have good mechanical as well as barrier properties but could be used as packaging with incorporation of external agents like antimicrobial agents which ultimately led to improved properties (Ghasemlou et al., 2013).

On the other hand, nanoparticles are a recent addition in the food packaging industry. They can be used to provide antimicrobial properties to food packaging systems and increasing the shelf life of food products (Rhim, Park, & Ha, 2013). Some of nanomaterials used in the food sector are silver nanoparticle, titanium nitride nanoparticle, and nano-titanium dioxide, nano-zinc oxide, and nanoclay (Tager, 2014). Layered Double Hydroxide (LDH) is one such nanoparticle that has been used for food packaging. Its structure is generally expressed as:

$[M^{II}_{1-x} M^{III}_{x} (OH)_2]^{x+} (A^{n-})_{x/n} . yH_2O$, where M^{II} and M^{III} are divalent and trivalent metal ions respectively. The divalent metal ions can be Magnesium (Mg^{2+}), Zinc (Zn^{2+}), etc. and trivalent metal ions can be Aluminum (Al^{3+}), Chromium (Cr^{3+}), Iron (Fe^{3+}), etc. A recent study done by Rodica and others in 2016 showed that polymeric films developed with Polypropylene, Polyurethane foam and incorporation of LDH in both, showed improved mechanical and antimicrobial properties. Dou and et.al also studied the oxygen barrier properties of films incorporated with LDH, which showed enhanced oxygen-barrier behavior due to the addition of LDH.

One of the basic methods of food preservation includes addition of preservatives, different chemical methods, modification of packaging, etc. (Gorris & Peppelenbos, 2007). Active packaging is a packaging system developed by adding active ingredients to the packaging system and/or using actively functional polymers (Kim, Kim, Han, & Kimmel 2008). Abreu and others in 2015 studied starch films that were incorporated with montmorillonite modified with a quaternary ammonium salt C30B/starch nanocomposite and silver nanoparticles, separately. Mechanical, barrier and antimicrobial properties were enhanced after the incorporation of nanoparticles (Abreu et al., 2015). A recent study on functional food packaging films developed using Starch/Polyvinyl Alcohol/Citric Acid Ternary Blend, showed increased antimicrobial activity against E. coli and L. monocytogenes (Wu et al., 2017).

Food processing includes different steps in which the food product is handled and distributed till it reaches the consumer, for e.g. growing, harvesting, processing, packaging and distribution. Glass and metal packaging could provide a good barrier, but it is important to choose polymeric materials with good mechanical and barrier properties for plastic packaging to avoid damages during transportation and/or handling. Furthermore, selection of packaging material is also crucial as the intrinsic properties of the packaged food product as well as environmental factors like temperature could affect the mechanical and barrier properties of the packaging (Siracusa, 2012). Starch films on its own do not have a good mechanical structure and tended to be brittle and sensitive to moisture (Lloyd & Kirst, 1963). On the other hand, packaging films incorporated with antimicrobials for development of active packaging, showed better diffusion properties and release kinetics but not physical properties like mechanical and barrier properties (Bastarrachea, Dhawan, & Sablani, 2011). Therefore, several researchers tried to study nanoparticle matrices that would impart good physical properties and have controlled release of antimicrobials in the food system. A recent investigation studied the physical (mechanical and barrier) as well as antimicrobial properties of PHB (polyhydroxybutyrate) and PHBV (polyhydroxybutyrate-co-valerate) -based LDH composite films with 2% LDH (modified with sodium benzoate and gallate), where the researcher concluded that there was a sizeable improvement in most of mechanical and barrier properties along with controlled release of the antimicrobials (Degruson, 2014).

Purpose of the Study

In this study, the overall goal was to develop starch-based biodegradable films incorporated with LDH nanoparticles – antimicrobial system with improved mechanical properties and additional function, antimicrobial property.

Hypothesis of the Study

It was hypothesized at the beginning of this research that:

a. Using starch along with LDH nanoparticles that were modified with commonly used antimicrobials would show improved mechanical properties.
b. The mechanical properties would be in the order of best to worst i.e.

Starch – Modified LDH films > Starch – unmodified LDH films / Starch – antimicrobial films > Native starch films.

Limitations of the Study

The limitations of the study were:

- Using only native starch (with no modification) for the research
- The concentration studied was only 2%
- The antimicrobial tests done could have been prolonged for 2-3 months

Chapter II: Literature Review

Plastics, for many years have been the most common and widely used material for a variety of purposes. The main reason being its low cost, availability of abundant resources and easy process ability. For packaging functions, type of plastic to be used depends on the physical and chemical characteristics of the product as well as the environmental conditions in which the product is stored and/or distributed. Usually, the raw materials used for the production of plastics are petroleum products obtained from the refining process and tend to be non-biodegradable leading to pollution (Thomas et al., 2015). According to the recent statistics, 33.6 million tons of plastic was discarded in the United States in 2014, out of which only 9.5% was recycled (Columbia University, 2017). Therefore, for a few years with increase in plastic waste, scientists have been working on using biodegradable and/or biobased packaging materials.

Bio-Based Polymers

Polymers produced from renewable sources are bio-based materials. On the other hand, biodegradable polymers undergo complete deterioration when subjected to micro-organisms, carbon dioxide (aerobic process), methane (anaerobic process) or even just water. Most bio-based materials are biodegradable but the same cannot be assumed vice-versa (Babu, Oconnor, & Seeram, 2013).

Bio-based polymers can be produced using one of the following ways:

a. Using natural bio-based polymers with partial modification to meet the requirements (e.g., starch).
b. Producing bio-based monomers by fermentation/conventional chemistry followed by polymerization (e.g., polylactic acid, polybutylene succinate, and polyethylene).
c. Producing bio-based polymers directly by bacteria (e.g., polyhydroxyalkanoates).

A summary of approaches that can be taken to produce bio-based polymers can be seen in the following figure:

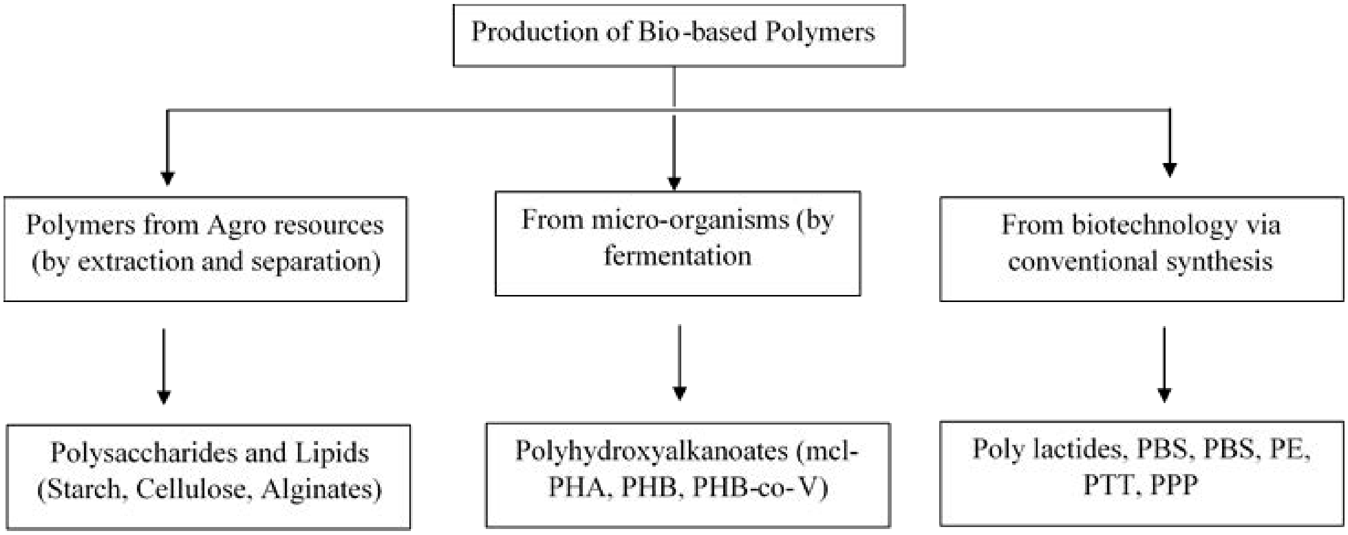

Figure 1. Most common categories of bio-based polymers produced by various processes.

Polylactic acid (PLA). Polylactic acid belongs to the family of aliphatic polyesters with lactic acid as the main functional unit. Synthesis of PLA can be done by using lactic acid in a direct polycondensation reaction or ring-opening polymerization of the lactide monomer. Different types of PLA and its copolymers can be obtained depending on the ratio and stereochemical nature of the lactide monomer (L or D). In food packaging, PLA is mainly used in food trays, tableware such as plates and cutlery, water bottles, candy wraps, cups, etc. (Babu et al., 2015)

Polyhydroxyalkanoates. Polyhydroxyalkanoates (PHAs) belong to the family of polyesters that are produced by bacterial fermentation. Polyhydroxybutyrate (PHB) is one of the simplest PHA. Most PHAs can be converted into different shapes and forms like film and sheet, fibers, laminates, and coated articles. Food packaging applications like caps and closures, disposable items such as forks, spoons, knives, tubs, trays, and hot cup lids involve the use of PHAs (Babu et al., 2013).

Starch

Considered as a common constituent of higher plants, starch is the major form in which carbohydrates are stored. Corn or maize (Zea mays L.), can be considered one of the major sources of starch. Starch is majorly composed of two polymers: amylose and amylopectin. When isolated, amylose is a linear polymer consisting of α -D-glucopyranosyl units linked by (1→ 4) bonds, whereas amylopectin is a branched polymer consisting of (1 → 4)-linked α - glucan chains joined by frequent α -(l → 6)-branch points. The amylose and amylopectin content vary depending on the source of starch, for e.g. waxy maize starch contains <1% amylose whereas normal maize may contain 70-80% amylose and 20-30% amylopectin. Chemical processes and isolation can help in the production of dry, unmodified corn starch which is a white powder. Alterations are made to the in-place milling process for corn, to obtain its different by-products like starch and ethanol (BeMiller & Whistler, 2009).

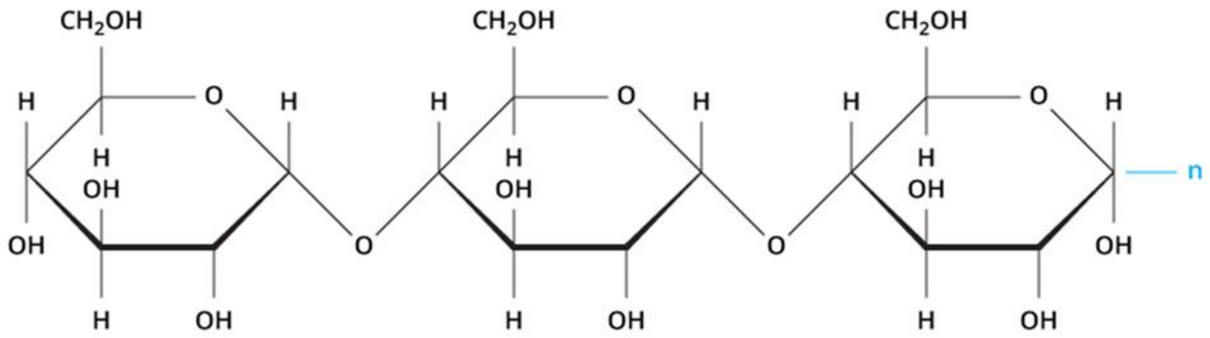

Figure 2. Simple starch molecule.

Properties of corn starch. Kernels of corn have 3 main parts, the seed coat or pericarp, the starchy endosperm, and the embryo, commonly called the germ. The endosperm consists the majority of starch, almost 90% (BeMiller & Whistler, 2009). The molecular formula for corn starch could be written as $C_{27}H_{48}O_{20}$. According to the National Center for Biotechnology

Information (NCBI), the molecular weight of corn starch is 692.661 g/mol and it also has great thickening power. Its chemical composition mainly consists of highly oxygenated carbon compounds. Starch, based on the amylose content can be either waxy maize starch (with almost 100% amylopectin) or normal corn starch (70-80% amylose).

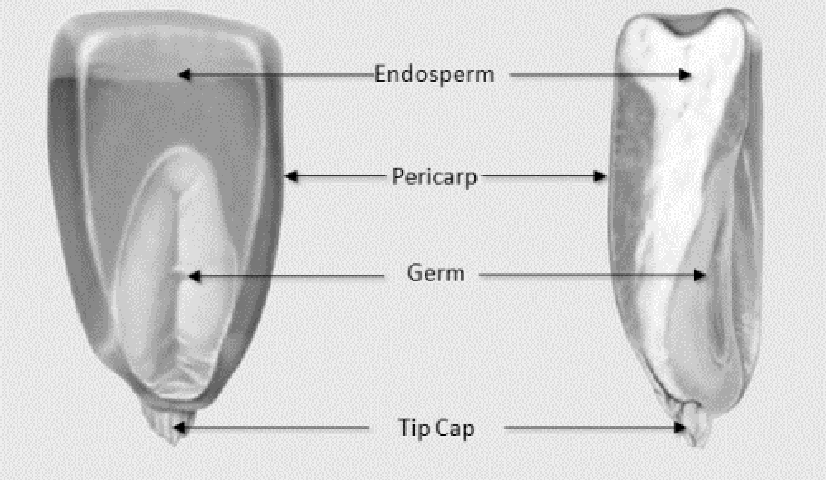

Figure 3. Corn kernel composition.

Several studies have investigated properties of starch and have concluded that starch on it own, is quite unfavorable in terms of process ability, dimensional stability and mechanical properties depending on the end product (Zou, Gu, Tan, & Zhang, 2015). Properties of starch are dependent on its water uptake capacity, granule swelling, formation of a viscoelastic paste during heating, followed by reassociation of dispersed starch chains on cooling and formation of a gel. Gelatinization i.e. process of breaking down the intermolecular bonds of starch molecules in the presence of water and heat and Retrogradation i.e. gelatinized starch molecules begin to reassociate in an ordered structure are important processes in determining the glass transition temperature of starch (T_g) (BeMiller & Whistler, 2009; Wang et al., 2015). When starch is subjected to thermal energy (heat), viscosity is increased leading to starch gaining rubbery and flexible character. This change in the polymer backbone is referred to as Glass Transition temperature (T_g). starch from different sources have varied T_g and hence, different physical and chemical properties.

Plasticizers are one of the additives used in the development of starch films as they plasticize starch which in turn enhances their film flexibility by reduction of intramolecular hydrogen bonding along polymer chains. This starch is then called Thermoplastic starch. Common examples of plasticizers include polyols (ethylene glycol, glycerol, sorbitol, etc.) and amines (formamide and urea) (Zou et al., 2013).

Production of corn starch. Usually for the production of starch, wet milling process is used. Briefly, wet milling process can be described as a process in which the feed material is steeped in water in order to soften the kernel and enhance the separation of kernel's various components. A summary of the process can be seen in the following flowchart.

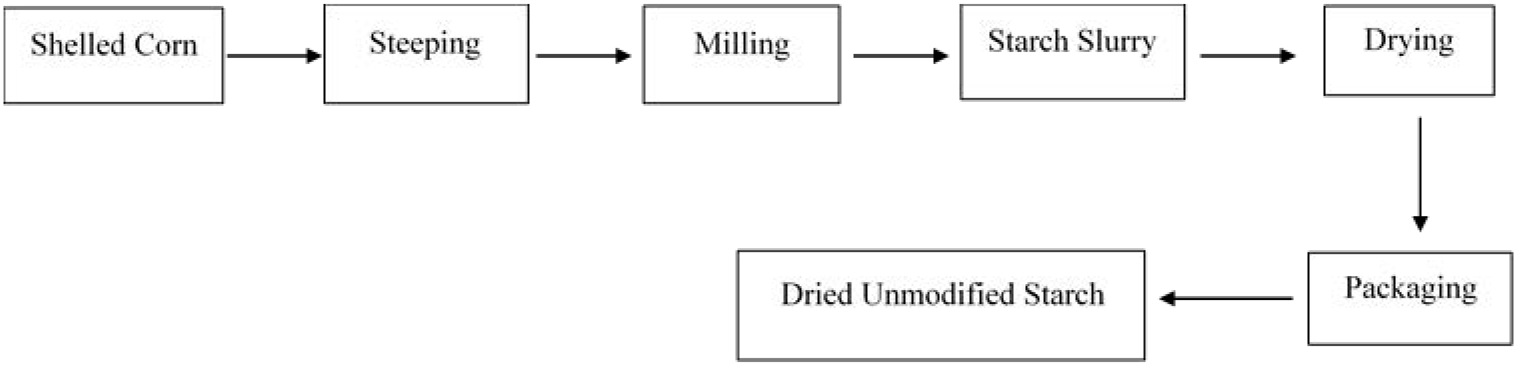

Figure 4. Production of dried unmodified starch.

Nanotechnology

The United States National Nanotechnology Initiative defines Nanotechnology as science, engineering, and technology conducted at the nanoscale, which is about 1 to 100 nanometers. Briefly, nanotechnology is the study and application of extremely small things which can be used across all different fields. One nanometer (nm) is a billionth part of a meter i.e. 10^{-9} (National Nanotechnology Initiative, n.d.). Nanotechnology has been used in the food industry to improve the shelf life of the food products and also to ensure maximum food preservation till the product reaches the consumer. Some examples include creation of functional foods (products developed to improve targeted physiological functions), use of nano-biosensors for the rapid detection of pathogens and contaminants in food and the 'electronic tongue' which is used as a tool for taste and flavor perception during product development stages (Abbas, Saleh, Mohamed, & MohdAzan, 2009). Typically, nanomaterials can be classified into 3 categories; particulates, platelets and fibers (Schmidt et al., 2002).

Nanotechnology has also been implemented for food packaging. The nano-sized dimensions of nanomaterials provide a large surface to volume ratio and surface activity. Therefore, they could be used to improve the properties of compatible polymers and develop an appropriate packaging system (Uskokovic, 2007).

Layered double hydroxide (LDH). Layered double hydroxides (LDH) is a hydrotalcite like clay material with surface layers made of mixed metal hydroxides of divalent and trivalent metals with exchangeable intercalated negatively charged species in between the two surface layers. LDH can be molecularly expressed as $[M^{II}_{1-x} M^{III}_{x} (OH)_2]^{x+} (A^{n-})_{x/n}. yH_2O$, where M^{II} and M^{III} are divalent and trivalent metal ions respectively. The divalent metal ions can be Magnesium (Mg^{2+}), Zinc (Zn^{2+}), etc. and trivalent metal ions can be Aluminum (Al^{3+}), Chromium (Cr^{3+}), Iron (Fe^{3+}), etc. (Mondal et al., 2016). The structure of LDH can be observed in the following image.

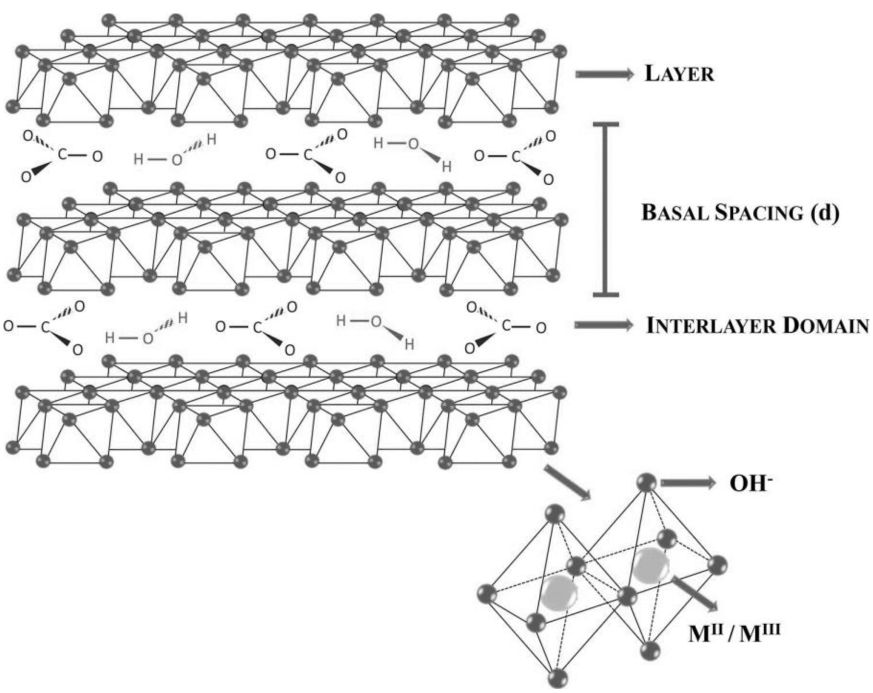

Figure 5. LDH basic structure.

Food Packaging

Food comes under the category of perishable commodities. Hence, it requires protection and preservation for it to be used for longer periods of time. Food preservation primarily involves prevention or slowing down the growth of micro-organisms like bacteria, mold, yeasts, etc. to increase the shelf life of the product. Different methods of food preservation include using different techniques alone or in combination with each other. Some of these techniques include avoiding invasion of micro-organisms by aseptic techniques, inhibiting the growth and activity of micro-organisms by freezing, refrigeration, drying, anaerobic conditions, chemicals or antibiotics, using different packaging techniques to prevent it from environmental spoilage (Rahman, 2007).

Packaging of a food product could be described as an organized system of preparing food for transport, distribution, storage, retailing, and end-use by the consumers. Major function of a food packaging system includes; containment, protection/preservation and communication.

During storage and transportation, foods are exposed to different types of damages such as shock, vibrations, environmental damages like exposure to light, water, micro-organisms, etc. Hence, a packaging system must be developed considering the characteristics of the food it encloses, which will help maintain and/or increase its shelf-life (Shin & Selke, 2014).

Over the years, researchers and food manufacturers have used different types of packaging technologies for preserving and increasing the shelf-life of the food products. Some of these technologies include aseptic packaging, modified atmosphere packaging (MAP), active and intelligent packaging, etc. (Robertson, 2013).

Antimicrobial packaging. Active packaging is a broad concept and its purpose is one or a combination of the following; remove an unwanted compound, add a desirable compound, prevent microbial growth, etc. Antimicrobial packaging is one such example of an active packaging system, where it works to reduce, inhibit or retard the growth of spoilage causing micro-organisms (Robertson, 2013). Packaging of a food product can be altered to create unfavorable environments for micro-organisms targeted to that specific product. This can be done by eliminating essential growth requirements and/or rendering contact of the incorporated antimicrobials with the target micro-organisms (Ebnesajjad, 2013). The Food and Drug Administration (FDA) has defined antimicrobial agents as “substances used to preserve food by preventing growth of microorganisms and subsequent spoilage, including fungistats, mold and rope inhibitors” (Davidson et al., 2006). Antimicrobial packaging could include antimicrobial (AM) agents in the packed food or the packaging material itself. This can be accomplished in one of the following ways:

- Incorporation of AM agents directly into polymers,
- Coating AM onto polymer surfaces,

- Use of polymers that may indirectly act as an AM agent, etc.

To act as an antimicrobial packaging, the AM agent must prolong the lag period and reduce the growth rate of the growth rate of the spoilage causing micro-organisms and in due course increase the shelf-life and maintain food safety. Additionally, the AM agents also must be present at the food surface above their minimum inhibitory concentration (MIC) to be effective against the target microbial species. Common AM agents that have been used in the food industry are alcohols, organic acids and their salts (benzoate, propionate and sorbates), fungicides, enzymes (glucose oxidase, peroxidase and lysozyme), extracts from herbs and spices, etc. (Robertson, 2013).

Sodium Benzoate. Benzoic acid can be considered as one of the oldest chemical preservatives used in the cosmetic, food and drug industries. It was also the first chemical to be approved by the FDA (Jay, 2000). The molecular formula for Benzoic acid is C_6H_5COOH and for Sodium Benzoate it is C_6H_5COONa.

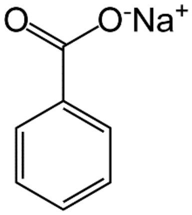

Figure 6. Molecular structure of Sodium Benzoate.

Sodium benzoate is a white, crystalline powder with molecular weight 144.1 and because of its increased solubility in water has been used more than benzoic acid for preservation purposes. Literature has shown that sodium benzoate can be used to inhibit the growth of yeasts and molds at 0.05% and 0.1% respectively, whereas for antibacterial effects, it can be used at 0.01% to 0.02%. But, several factors like the pH, temperature, genus and species of micro-organisms targeted, intrinsic characteristics of the food product, etc. are responsible for determining the MIC of the AM agent (Davidson & Junja, 1990). Researchers have investigated and concluded that sodium benzoate can be effective against bacterial species like Bacillus cereus at pH 6.3 and MIC 500 ppm and Escherichia coli at pH 5.2 – 5.6 and MIC 50 – 120 ppm. Molds were also inhibited at pH 5.1 and MIC 100 ppm for Cladosporium herbarum and pH 5.0 and MIC 2000 ppm for Penicillium citrinum (Chipley, 1993; Davidson & Junja, 1990; Russell, 1991).

Natamycin. Natamycin has been used for several years for the prevention of yeast and/or mold growth in certain foods and beverages. Selected Streptomyces strains can be used for the isolation of Natamycin by its controlled fermentation in a dextrose-based media. Chemically, Natamycin is a tetraene polyene macrolide with a molecular weight of 665.7 Daltons. The molecular formula for Natamycin is $C_{33}H_{47}NO_{13}$ and its structure can be seen in the figure below.

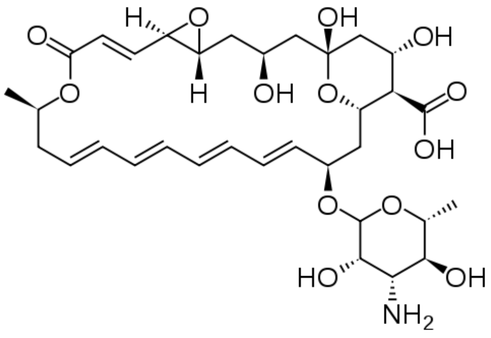

Figure 7. Molecular structure of Natamycin.

Natamycin, when isolated is a white to cream colored powder with no odor and taste. Researchers and scientists after several experiments have concluded that Natamycin is effective against nearly all yeasts and molds but is ineffective against bacteria, protozoa and viruses. MIC for Natamycin ranges from 0.5 to 6 µg/ml, and for some resistant species the concentration can go up to 25 µg/ml. Natamycin forms a complex with sterols, specifically ergosterol, which is an important constituent in the fungal cell membranes. Therefore, this mode of action of Natamycin explains its antifungal nature and also its ineffectiveness against bacteria (Robertson, 2013).

Several studies have shown the antimicrobial nature with the controlled release of AM agents like Natamycin and Sodium Benzoate. Neetoo and colleagues in 2013 studied the use of antimicrobial films and edible coatings incorporated with chemical and biological preservatives to control the growth of Listeria monocytogenes. The research concluded that cellulose-based coatings incorporated with Nisin, Potassium sorbate and Sodium benzoate showed a significant reduction in the Listeria growth after 4 weeks of refrigerated storage (Neetoo & Mahomoodally, 2014). On the other hand, studies have also shown improvement in the controlled release of the AM agents from the starch-based biodegradable films when incorporated alongside nanoparticles and/or nanocomposites. Ghasemlou and et.al in 2013 studied the physical, mechanical and barrier properties of corn starch films incorporated with plant essential oils i.e. Zataria multiflora Boiss (ZEO) or Mentha pulegium (MEO) and they concluded that there was no significant change in the tensile strength of the films after the incorporation of the plant essential oils, there was antimicrobial activity for the films with ZEO against Escherichia coli and Staphylococcus aureus (Ghasemlou et al., 2013). In a research done by Dou et.al (2013), it was investigated that cellulose acetate films incorporated with Layered Double Hydroxide (LDH) showed increased oxygen barrier properties along with the films’ durability against humidity, temperature and light irradiation.

Chapter III: Development of Starch-Based Biodegradable Films

Packaging in the food industry is a large section and comprises of various materials that can be used for the same. Plastics form a major ingredient for food packaging. Greek word ‘plastikos’ is the inspiration behind the word plastics, which means moldable. Using plastic has many advantages like, cheap, durable, inert, disposable, etc. (Plastics, n.d.). But one of the controversial disadvantages of suing plastic is the increase in waste and pollution due to use of plastics in major industrial sectors. According to Environmental Protection Agency (EPA), in 2014, about 258 million tons of Municipal Solid waste (MSW) was generated among which 136 million tons were landfilled and plastic accounted for 33.3 million tons of waste (EPA, 2014). Researchers have been taking serious steps in order to develop sustainable packaging for effective replacement of plastic. Naturally existing and renewable sources could be used as a material for biodegradable packaging (e.g. corn) (Ebnesajjad, 2013). Biodegradable and/or biobased polymers are polymers that can either be produced by biological systems (micro-organisms, plants, etc.) or chemically synthesized from biological starting materials (natural fats or oils, starch, etc.). Starch is one such polysaccharide polymer that could be used solely or in combination with other polymers for the purpose of packaging. Agricultural plants are the sources of starch where it is produced in the form of hydrophilic granules. Common sources include corn, wheat, potatoes and rice. Chemically, starch is composed of two molecules; amylose (poly-α-1, 4-D-glucopyranoside), which is linear and amylopectin (poly-α-1, 4-Dglucopyranoside and α-1, 6-D-glucopyranoside), which is branched and amorphous (Pawar and others, 2013).

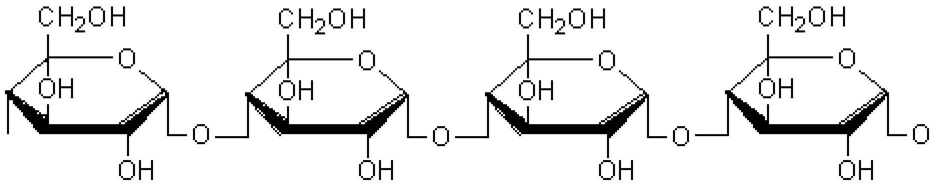

Figure 8. Amylose molecule.

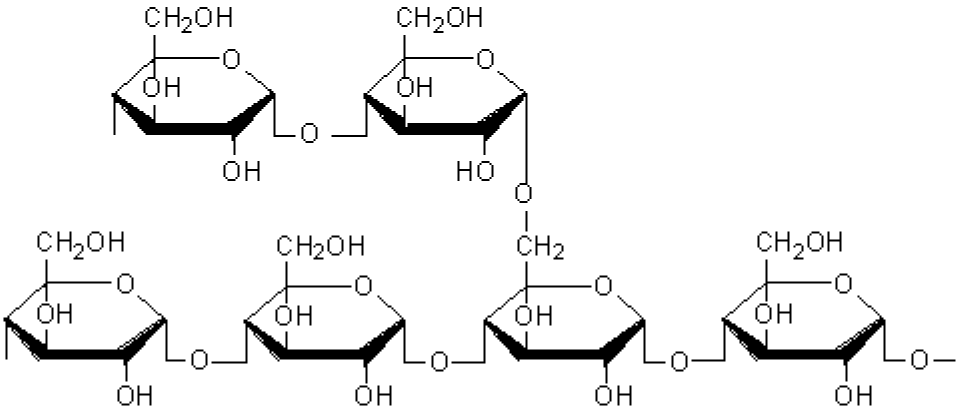

Figure 9. Amylopectin molecule.

The amount of both amylose and amylopectin depends on the source of extraction which ultimately also leads to materials with varying mechanical properties and biodegradability (Pawar & Purwar, 2013). Corn starch, when isolated from the source is a dry, soft and white powder. The starch granules when gradually heated in water suspension, will eventually begin to absorb water and lose their characteristic birefringence (optical property of a material having a refractive index that depends on the polarization and propagation direction of light) and opacity to form a gel or a paste. This process is called as gelatinization (Corn Refiners Association, 2006). Retrogradation, on the other hand is the process which occurs when gelatinized starch molecules begin to reassociate in an ordered structure (BeMiller & Whistler 2009).

Starch can also be chemically modified through processes like etherification, esterification, cross-linking, decomposition or physical treatment (Korma, 2016). Modification causes changes in basic structure as well as starch gelatinization and retrogradation properties. Cationic corn starch is positively charged, anionic corn starch is negatively charged, amphoteric corn starch has dual charges and starch acetate is commonly produced with acetic acid and acetic anhydride as starch esterification reagents. (Industrial starch products, n.d.) (Korma, 2016).

Layered Double Hydroxide (LDH) nanoparticles have recently become a subject of interest in the development of starch-based biodegradable films with enhanced physical and barrier properties. Dou et.al in 2014 concluded that LDH in combination with cellulose acetate could potentially be used in the future as flexible packaging for food and drugs (Doue et al., 2014). Following research was hypothesized on the basis of LDH nanoparticles enhancing the mechanical and release properties of Starch-based biodegradable films.

Materials and Methods

Initially, several tests were carried out to finalize the process for development of starch-based films. Following was the finalized procedure developed, which was then used throughout the research.

Preparation of starch-based films. Corn starch was used as the raw material for the preparation of films using the Solvent – Casting method. Initially, 2% starch along with 0.3g/gram of starch of Glycerol (plasticizer) were added to a 3-necked round bottom flask containing De-Ionized water. The mixture was brought to the temperature 85°C along with magnetic stirring, after the starch dissolves completely, where it was held for 20 minutes for the starch to gelatinize completely. While making control films, this process would go on for 1 more hour. Films with modified nanoparticles or antimicrobials involved additional steps to ensure their optimum dispersion in the solvent i.e. water. Probe sonicator was used for the same, where the nanoparticles or antimicrobials (2% of starch) were sonicated for 5 minutes [30 sec pulse on and 20 sec pulse off time], stirred magnetically for 10 minutes at 1100rpm and sonicated again for 5 minutes with the same settings. The dispersed nanoparticles were then mixed with the gelatinized starch for 1 hour at 85°C to allow proper inclusion of LDH into the starch. The mixture of nanoparticle and gelatinized starch were sonicated as well using the same settings (5 minutes total process time 30s pulse on and 20s pulse off). The resultant solution was casted on Teflon-coated petri-plates (60ml) and then vacuum dried for 5 minutes at 40°C to eliminate trapped air molecules. Lastly, the cast films were dried for 24-28 hours at 40°C and 55% relative humidity. After drying, all films were stored at room temperature and 55% RH after which they were tested. Natamycin used were of two types i.e. one containing 50% salt and the other 50% lactose. Initial films and tests were performed by using Natamycin with 50% salt. The films tested were Control films i.e. starch without Antimicrobial agents (AM), starch with sodium benzoate (Starch-Bz), starch with natamycin (Starch-Nat), starch with LDH-Bz and Starch with LDH-Nat.

Mechanical test. The starch films were tested for mechanical properties like Ultimate Tensile Strength, % Elongation and Elastic Modulus using 200R Resources Tensile Tester. The tests were performed following the Standard Test Method for tensile properties of thin plastic sheeting, ASTM D-882-02 (ASTM 2002). Films were cut into rectangular specimens with a width less than 1 inch and length 3-4 inches, approximately. Tensile strength (psi) was calculated by dividing the maximum load on the film before failure by the cross-sectional area of the initial specimen. Percentage elongation was defined as the percentage change in the length of the specimen to the original length between the grips. Elastic modulus (psi) was calculated as the ratio of stress to strain over the linear part of stress-strain curve. At least three replicates of each film were tested.

Statistical analysis. Statistical analysis was done using IBM SPSS v24. One-way ANOVA test and Tukey's multiple comparison test at 95% confidence level were carried out to study the differences in mechanical properties of the tested films.

Results and Discussion

Following were the results obtained after performing the mechanical tests on the starch-based films.

Table 1

Mechanical Properties of Starch-Based Films

Sample	UTS (psi)	EL (%)	EM (psi)
Starch	3370 ± 280.10^{a}	6.75 ± 1.77^{a}	84611.10 ± 70157.82^{a}
Starch-Bz	2548.33 ± 761.59^{a}	8.26 ± 1.40^{a}	31042.94 ± 25314.83^{a}
Starch-LDH-Bz	2556.67 ± 208.95^{a}	17.69 ± 2.83^{b}	14183.97 ± 14004.85^{b}
Starch-Nat	2541.33 ± 383.05^{a}	8.49 ± 2.61^{a}	29037.82 ± 15901.67^{a}
Starch-LDH-Nat	1473.33 ± 306.89^{a}	2.48 ± 0.33^{a}	61845.51 ± 6291.55^{a}

Note: values are the mean ± SD. Means in the same column followed by the same letter are not significantly different (P> 0.05)

According to the results, it can be seen that the films showed a significant difference with the addition of antimicrobial agents on the elongation at break of the films ($P < 0.05$). On the other hand, the addition of antimicrobial agents did not show any effects on the UTS ($P > 0.05$). The control films had the lowest elongation i.e. 6.75% compared to Starch-LDH-Bz films which had the highest elongation i.e. 17.69%.

Conclusion

Initially, based on the literature it was hypothesized that addition of nanoparticles would improve the mechanical properties of the starch-based films. However, statistical analysis showed that there was some improvement in the percent elongation of the films incorporated with nanoparticles, but not a significant difference in the Ultimate tensile strength (UTS) or the Elastic modulus (EM). Further studies by using different types of starch with varied nanoparticles would help in understanding the depth of mechanical properties of the starch-based films for packaging.

Chapter IV: Antimicrobial Properties of Starch-Based Biodegradable Films Incorporated with Antimicrobial-Nanoparticle System

Food can be briefly described as raw, processed or formulated material that are consumed orally by humans, animals for growth, health, satisfaction, etc. The composition of food which is comprised of lipids, fats, carbohydrates, etc. make it a rich source of nutrients not only for humans but also harmful micro-organisms. Preservation and processing ensures that the shelf life of food increases by preventing or slowing down the growth of spoilage causing micro-organisms including molds, yeasts and bacteria. The basic principles of food preservation are; prevention or delay of the growth of micro-organisms, prevention or delay of self-decomposition and prevention of damage from insects or animals. Common methods include sterilization, pasteurization, extrusion, aseptic processing, packaging, etc. (Rahman, 2007).

Food products can be contaminated by different microbial species like bacteria, molds and yeasts. It is the responsibility of the food processors and manufacturers to ensure that the food product is safe and free from any sort of contamination till and after it reached the consumer. Any illness caused after consumption of contaminated food and showing symptoms like nausea, vomiting and diarrhea, can be briefly grouped under the umbrella of food poisoning. Food related illness could either be intoxication i.e. illness caused by consumption of food that has been contaminated by the toxins of the bacteria (e.g. Clostridium botulinum, Bacillus cereus, etc.) or infection i.e. directly consuming foods containing live micro-organisms which would grow in the human intestinal tract. (Branch, 2017; University of Nebraska Lincoln, n.d.). Center for Disease Control (CDC) has estimated that each year 48 million people get sick from a foodborne illness of which 128,000 are hospitalized and 3000 die (CDC, 2017). Due to growing cases of foodborne illnesses, manufacturers and researchers have been working on different ways to improve the shelf life of food products and ways to minimize the contamination during all stages of processing. One of the recent but studied way of decreasing the ways of contamination is using Antimicrobial Active food packaging.

Antimicrobial active packaging involves addition of antimicrobial agents in the packaging itself so as to prevent contamination of the food it encases by blocking the entry of oxygen and moisture (two key components that micro-organisms require for growth) and also controlled release of antimicrobials from the packaging into the food system (Kim et al., 2008). Ehivet and others in 2011 studied the antimicrobial properties of sweet potato starch-based edible films containing Origanum (Thymus capitatus) oil and concluded that the films incorporated with the antimicrobial showed increased mechanical properties and showed inhibitory effects against S. enteritidis and E. coli O157:H7 (Ehivet, Min, Park, & Oh, 2011). In a research done in 2015, researchers investigated the properties of antimicrobial nanostructured starch films developed with Montmorillonite modified with a quaternary ammonium salt C30B/starch nanocomposite (C30B/ST-NC), silver nanoparticles/starch nanocomposite and found that the films incorporated with nanoparticles showed improved mechanical properties and antimicrobial properties against Staphylococcus aureus, Escherichia coli and Candida albicans (Abreu et al., 2015).

Materials and Methods

The antimicrobial properties of the starch-based films were tested using two methods. Primarily, the disc-diffusion test was performed to provide a baseline as to how effective the films were in inhibiting the microorganisms.

Antimicrobial properties. The starch-based films developed by incorporation of antimicrobial-nanoparticle systems or only antimicrobials were tested for the antimicrobial properties against common spoilage causing organisms i.e. generic Salmonella species, generic E. coli and molds like *Cladosporium cladospoiroid*es and *Penicillium commune*. Antimicrobial free 1-year old sharp Cheddar cheese (14% total fat, 7% cholesterol, 7g protein) for testing was bought from Cady Cheese Factory, Wilson Wisconsin. Freeze dried mold cultures were obtained from the American Type Culture Collection (ATCC).

Disc-diffusion test. Before beginning the testing of the films, the initial step involved isolating the freeze-dried cultures of *Cladosporium cladospoiroid*es and *Penicillium commune* were isolated. The freeze-dried cultures were transferred to a sterile test tube containing sterile water and then surface inoculated on Potato Dextrose Agar (PDA) plates and incubated at room temperature (RT) for 48-72 hours to allow optimum growth. After the incubation time ended, the molds were then transferred into a test tube containing sterile water to prepare suspension to test it against cheese and the starch-based films incorporated with antimicrobial-nanoparticle system.

The preliminary step involved isolating the bacterial cultures from the stock. Raw chicken breasts from the local market was used to isolate generic species of Escherichia coli. Sterile peptone water was used to absorb the surface microbial flora from the chicken breasts which was then inoculated in peptone enrichment broth and incubated at 37°C for 24 to 48 hours for it to grow optimally. The inoculum from the enrichment broth was then transferred (0.1ml) to Petri films commercially designed for the detection of coliforms, which was then incubated at 37°C for 24 to 48 hours to allow maximum growth. After sufficient isolation from the Petri films, the generic E. coli colonies were then made into a suspension and used for the Kirby-Bauer Disc-Diffusion test.

Primarily Kirby-Bauer's disc-diffusion test was done to check the qualitative nature of the films.

The Kirby-Bauer Disc-Diffusion test involved using sterile Mueller Hinton agar plates. A single hole puncher in a sterile condition was used for preparing the discs (6mm) from the starch-based biodegradable films. The bacterial or mold suspension was then surface inoculated (0.1ml) on the Mueller Hinton agar plates, after which the starch film discs were placed on the plates in a sterile condition. The experiment was carried out in triplicates to minimize any inaccuracies in the results. Results were observed after incubation at 37°C for 24-48 hours.

Challenge testing. Cheddar cheese was cut into 1" x 1" blocks in a sterile environment and placed in sterile petri plates. The mold or bacterial suspension (1 loop = 1µl) was then spread on the cut cheese in triplicates i.e. 3 for *Cladosporium cladospoiroid*es and 3 for *Penicillium commune* and 3 for *Salmonella spp.* and 3 for *E. coli* for each sample. The experimental design was 8 x 6, where 8 samples of the films i.e. Control films with no antimicrobial agents or nanoparticles, Starch-Bz 1%, Starch-LDH-Bz 2%, Starch-Nat 0.4% (50% salt), Starch-Nat 0.4% (50% lactose), Starch-LDH-Nat 1, Starch-LDH-Nat 2 and Starch-LDH-Nat 4 were tested for the antimicrobial efficiency in triplicates for both the mold cultures. For bacterial species, the experimental design was 4 x 6, where the film samples tested were Control films, Starch-Bz 1%, Starch-Bz 2% and Starch-LDH-Bz. The film samples were also cut in 1" x 1" pieces and they were used to cover the inoculated cheese blocks on both sides (surface and bottom). These petri plates were then incubated at RT (for molds) or 37°C for 24-48 (for bacteria) in a saturated chamber with 55% relative humidity (to prevent drying of films) for 48-72 hours (for molds) or an incubator (for bacteria).

Results and Discussions

The results are divided into two parts and also the categories of the microbial species used for tests i.e. bacteria or molds.

Kirby-Bauer Disc Diffusion test for molds. The Kirby-Bauer disc diffusion test showed the following results.

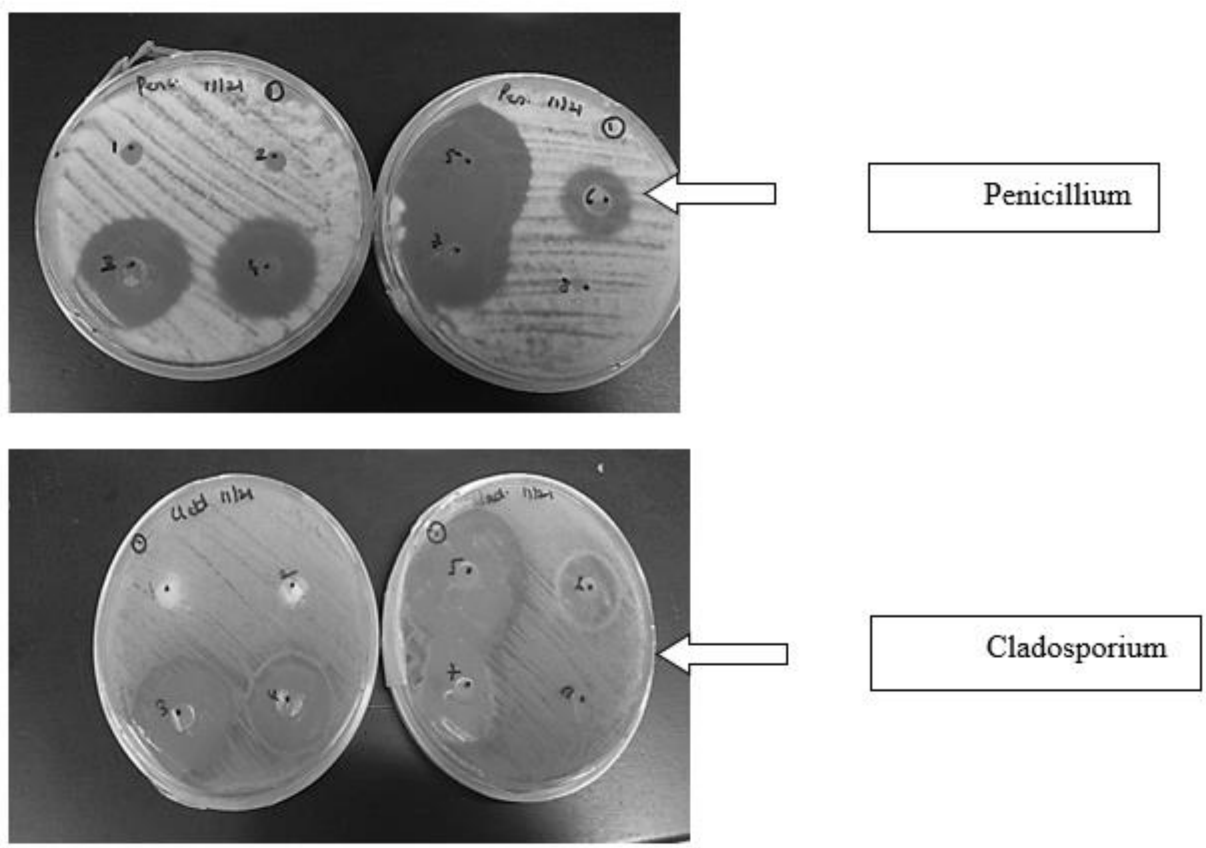

Figure 10. Disc diffusion results for molds.

Table 2

Disc Diffusion Test Results for Molds

Sample no. on the plate	Sample	Zone of inhibition in mm	
		Penicillium spp.	Cladosporium spp.
1	Std. Bz 1%	0	0
2	Starch LDH-Bz 2%	0	0
3	Std. Natamycin 0.4% (50%salt)	1.5	1.5
4	Std. Natamycin 0.4% (50%lactose)	1.4	1.4
5	Starch LDH-Natamycin 1 2%	3.6	3.5
6	Starch LDH-Natamycin 2 2%	1.5	1.5
7	Starch LDH-Natamycin 4 2%	2.7	2.5
8	Control Starch Films 2%	0	0

As it can be seen from the table, no inhibition of the molds was observed for the Standard Benzoate 1% films, Starch LDH-Bz 2% films and also the control films. But, it is also evident that the films that were most effective in inhibiting the molds were all films incorporated with Natamycin i.e. Standard Natamycin 0.4% (50% salt and 50% lactose) and Starch LDH-Natamycin 2% (1, 2 and 4).

Challenge testing for molds. The challenge tests done on the cheese blocks also showed some similar results for molds after incubation at room temperature for 48-72 hours.

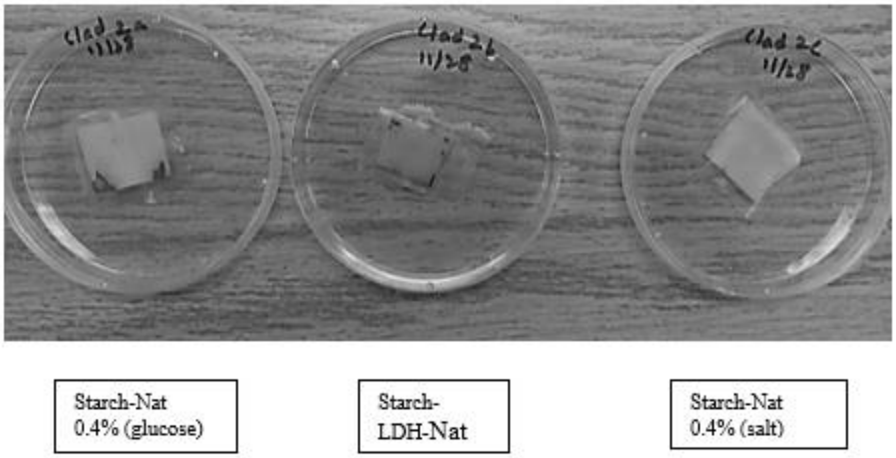

Figure 11. Results for Cladosporium spp.

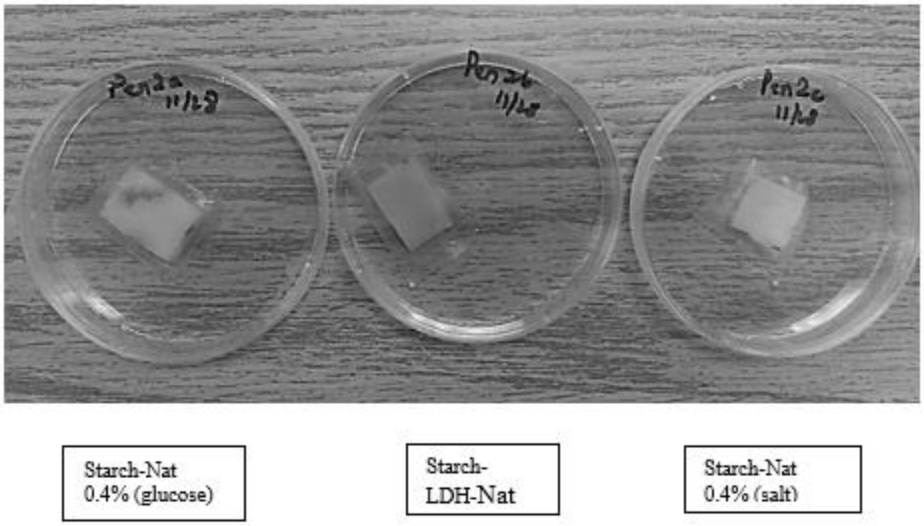

Figure 12. Results for Penicillin spp.

Sample	**Growth observed**	
	Penicillium spp.	Cladosporium spp.
Control Starch Films 2%	+	+
Starch LDH-Natamycin 1 2%	-	-
Starch LDH-Natamycin 2 2%	-	-
Starch LDH-Natamycin 4 2%	-	-
Std. Natamycin 0.4% (50%salt)	-	-
Std. Natamycin 0.4% (50%lactose)	-	-

Figure 13. Results for antimicrobial tests on cheese. Key: + = growth of molds, - = no growth of molds till 5 days.

The results for challenge studies was observed after 5 days. The control films showed mold growth i.e. no inhibition after 24-48 hours, whereas, films incorporated with Natamycin showed no growth till 5 days. After 5 days, beginning of the mold growth could be seen on the edges of the cheese blocks. Therefore, it can be said that starch-based films incorporated with Natamycin-nanoparticle system proved to be effective against the molds *Penicillium commune* and *Cladosporium cladospoiroides* at room temperature and 55% relative humidity for up to 5 days.

Kirby-Bauer disc diffusion test results for bacteria. The Kirby-Bauer disc diffusion results for bacteria were as follows.

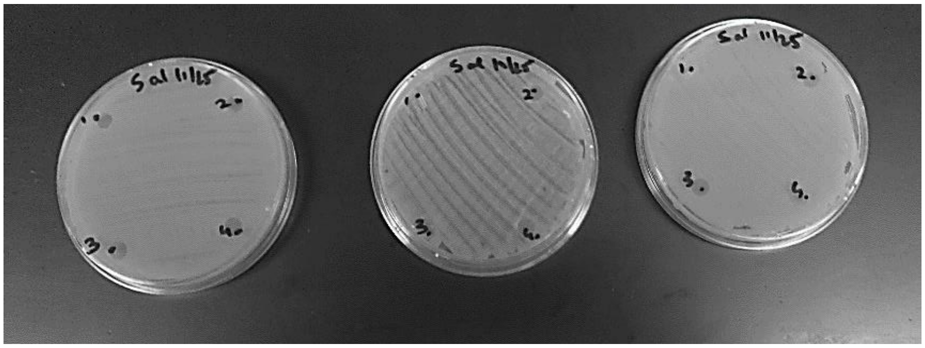

Figure 14. Kirby-Bauer disc diffusion results for Salmonella spp.

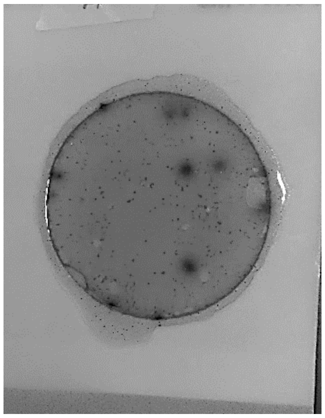

Figure 15. Petri films for E. coli spp.

The disc diffusion tests i.e. the films tested on bacterial species were:

- Control i.e. Starch 2% - sample 1
- Starch-Bz 1% - sample 2
- Starch-Bz 2% - sample 3
- Starch-LDH-Bz 2% - sample 4

The bacterial species i.e. salmonella spp. and generic E. coli were not inhibited by any of the above-mentioned films. The primary reason for this could be the ineffectiveness of sodium benzoate at pH ranges higher than 4.5, whereas the pH of Mueller Hinton agar is 7.3 ± 0.1. Therefore, the results of the disc diffusion test could not be considered reliable.

Challenge test results for bacteria. For more evidence of the effectiveness of sodium benzoate against bacterial species, antimicrobial tests on cheese blocks were done. The results obtained were as follows.

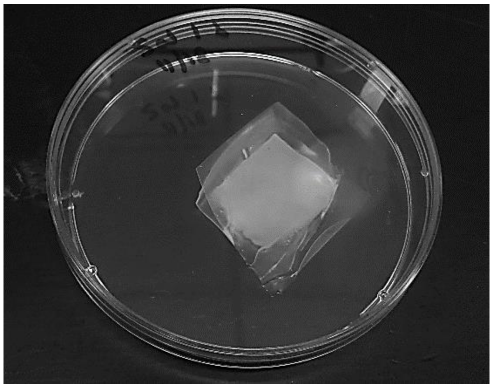

Figure 16. Challenge test results for Starch-Bz 1% for salmonella.

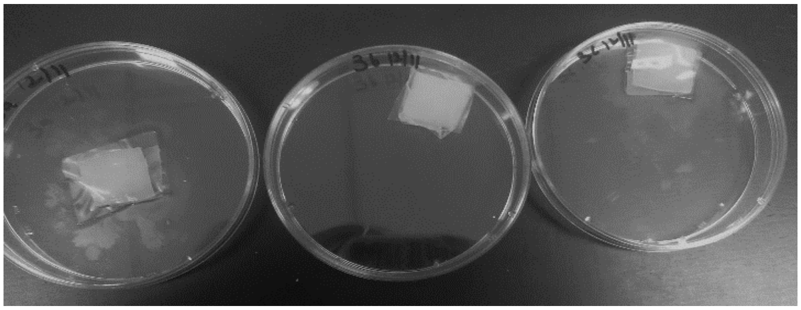

Figure 17. Challenge test results for Starch-LDH-Bz 2% for E. coli.

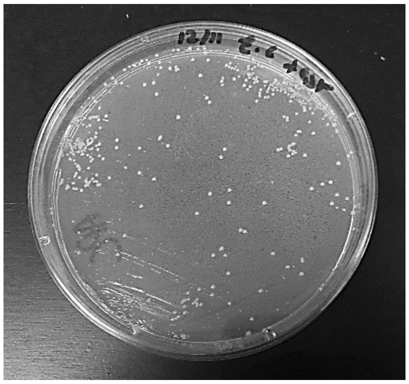

Figure 18. Isolation of bacteria from challenge testing plates.

The antimicrobial test done on cheese was also difficult to comprehend due to the cheese being creamish white and the bacteria culture being colorless. But, after the incubation, as it can be seen in figure 18, the fat was inoculated with the help of a nichrome loop on Mueller Hinton agar and incubated for 24-48 hours at 37°C. after incubation. Colonies were observed for both the bacterial species i.e. Salmonella and E. coli, indicating that they were not inhibited by the starch-based films incorporated with sodium benzoate at 2%, 1% and LDH-Bz 2% complex.

Chapter V: Summary and Future Research

According to the proposed hypothesis, the Starch based films incorporated with Benzoate (Starch-Bz) and nanoparticle antimicrobial complex (Starch-LDH-Bz) should have inhibited the growth of the bacterial species because of benzoate being the commonly used antimicrobial agent in the food industry. But, the results show that it was not as effective as hypothesized. There could be many interpretations for this result; the benzoate could not form a complete complex with the nanoparticle resulting in its inefficient release from the starch-based films. On the other hand, the type of starch i.e. native corn starch used for the study, could have been a factor in deciding the antimicrobial property of the finished product i.e. Starch-LDH-Bz. Future research could concentrate on using varied types of starch, different antimicrobials and/or using another type of nanoparticle.

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