THE ROLE OF POTASSIUM SORBATE IN MULTI-LAYER CHITOSAN-BASED ON ASPERGILLUS NIGER

A

THESIS

Presented to the Department of Materials Science and Engineering

African University of Science and Technology, Abuja

In partial Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

By

Joseph Tarnue Barbu

Abuja, Nigeria

December 2014

THE ROLE OF POTASSIUM SORBATE IN MULTI-LAYERED CHITOSAN-BASED FILMS ON ASPERGILLUS NIGER

By

Joseph Tarnue Barbu

A THESIS APPROVED BY THE MATERIALS SCIENCE AND ENGINEERING DEPARTMENT

RECOMMENDED: .....

Supervisor, Professor Wole Soboyejo

APPROVED: .....

Head, Department of Materials Science and Engineering

.....

Chief Academic Officer

11 Dec., 2014

Date

ABSTRACT

This paper presents the use of multilayered chitosan films incorporated with antimicrobial agent, potassium sorbate to enhance effective food packaging. In this study, potassium sorbate was incorporated into multilayer chitosan films for antimicrobial food packaging applications. The effectiveness of the films was tested through measurement of release kinetics and antimicrobial activity on a selected microorganism, *Aspergillus niger*. All the films prepared with potassium sorbate showed growth inhibition on *Aspergillus niger*. FTIR was used to characterize the presents of amino group's presents in the chitosan film, whiles SEM and Celestron microscope were used to examine the morphologies of the films. UV-Vis was used to analyze the amount of potassium sorbate release over time. The most significant characteristics of these films were their asymmetric porous structure. The results were then discussed for potential applications of potassium sorbate incorporated into a multilayer chitosan-films for controlled release of antimicrobial agents for food packaging.

ACKNOWLEDGEMENTS

I am sincerely grateful to the almighty God for giving me the opportunity to study Materials Science and Engineering Master at the African University of Science and Technology. I am also thankful to him for the health, shelter, strength and wisdom He granted me doing my study. Many thanks goes to my sponsor, DTCA, for the scholarship which allow me to study at AUST. Not forgetting about the government of Liberia for their support towards my successful stay at AUST.

My profound gratitude goes to Prof. Winston Oluwole Soboyejo, my supervisor, a treasure image of a supervisor, lecturer and father, who has proven to be very caring, loving, understanding and whose life leaves impact and legacy for us all to follow. My sincere thanks goes to Dr. Shola Odusanya for all his support towards this research. I also acknowledge Mrs. Ann Seliskar and her daughter, Mrs. Stephanie Seliskar, for their support towards my successful educational endeavors.

I am forever grateful to Danyuo Yiporo, a PhD candidate at AUST, who assisted me a lot during my project. I acknowledge the SHESTCO administration for allowing me to use their laboratory for my experimental work. Many thanks goes to Mrs. Onodugo Chinweoma Dympla, staff member at SHECTO who worked with me tirelessly in achieving my results. I also acknowledge Mrs. Stella Dozie-Nwachukwu and all the IT students most especially Murhyel Nathaniel, for their supports towards my experimental work.

My sincere thanks and appreciation goes to AUST staff, AUST Academics, AUST Accounts Department, all the PhD candidates of AUST, my outstanding Liberian buddies (Moses K. Flomo, Aloysius Kotee, Bill Landlord Gontor, Frankine Bundoo, Yei Danlette Suah, Emmanuel Gbarnjah, Nuwoe Kellen and Edwin Barquoi II) and finally to the class of 2014 (materials

science and engineering) for your contributions towards my successful stay at AUST and the success of this research work. I pray that God will reward you all graciously.

DEDICATION

I dedicate this work to Jesus Christ for His provision of the scholarship which allowed me to study at AUST. I recognize His guidance and strength that I enjoyed during my master program. I also remember my Sponsors (DCTA), my dear mother (Korto Barbu), my wife to be (Winnie J. Daniel), my daughter; (Josephine K. Barbu), my son; (Joseph Stephen Barbu Jr.), my brothers, my sisters, Stephanie Seliskar and Ann Seliskar for their prayers, love, support, and guidance through my study.

Table of Contents

List of Figures

List of Tables

CHAPTER ONE: BACKGROUNG AND INTRODUCTION

1.0 BACKGROUNG AND INTRODUCTION

1.1 Background

In Africa, about one third of the harvest is lost between the farm and the market [1]. This is due to lack of adequate food preservation methods. Also, currently, close to one third of Africa population lives in chronic hunger [2]. There is, therefore, a need for the development of new methods of food packaging that use locally available materials for food preservation. This research used natural polymer (chitosan) for food preservation. The swelling and releasing kinetics of potassium sorbate and inherent antimicrobial agent of chitosan are studied using experimental procedures.

Antimicrobial packaging is a promising and rapidly emerging technology for food preservation [3]. It involves the incorporation of antimicrobial agents into food packaging materials [4]. This prolongs the shelf lives of packaged food, due to the reductions in the colony of microorganisms. Active packaging systems that have been used so far include: oxygen scavenging systems, which are applied to most food classes [5]; carbon dioxide production systems which are applied to most foods affected by moulds and water vapor removal systems that are applied to dried and meld-sensitive foods; ethylene removal systems that are applicable to horticultural produce and ethanol release systems that are applied to baked foods, and antimicrobial activity systems that are applied to most food classes.

Chitosan has great applications due to its biodegradability, biocompatibility, antimicrobial activity, nontoxicity and versatile chemical and physical properties [6]. It can also be formed into films, gels, sponges, beads or nanoparticles [7]. Chitosan also has high antimicrobial activity

against a variety of pathogenic and spoilage microorganisms. These include: fungi, gram positive and gram negative bacteria. However, there are relatively few studies that have explored the potential role of chitosan as a packaging material in which the controlled release of antimicrobial agents is used to extend the shelf lives of packaged foods.

1.2 PROBLEM STAMEMENT

In traditional food packaging applications, antimicrobial agents are directly mixed with food or applied to the package to control microbial growth and extend shelf-life. Direct addition of the antimicrobial agents into the food has the following two major disadvantages: (i) the concentration decreases due to its diffusion into the interior parts of the food product, thereby reducing the shelf life; (ii) neutralization of the added agent occurs and leads to the utilization of excessive amounts of chemical additives in food materials, which changes the taste of the food. Antimicrobial packaging offers an alternative approach that can be used to overcome the above limitations of conventional food packaging.

1.3 OBJECTIVES OF THE STUDY

The objective of this work is to produce chitosan powder and films for food packaging. To study the absorption and release of the antimicrobial agent (potassium sorbate) using experimental methods. Carry out UV-spectroscopy analysis to analyze the concentration of potassium sorbate that is release. Study the zone of inhibition of the films by applying the films on Aspergillus niger in a PDA media. Characterized the films with Celestron microscope and SEM to observe the surface morphologies. Discuss the implications of the results.

1.4 SCOPE OF THE STUDY

This thesis is organized into five chapters. Following the background and introduction (chapter 1), the literature review is presented in chapter 2. This is followed by the experimental methods in section 3. The results and discussion are then presented in section 4 before summarizing the salient conclusions from this work in section 5.

1.5 REFERENCES

[1] E. Royte, “One-Third of Food Is Lost or Wasted : What Can Be Done,” *Natl. Geogr. USA*, pp. 1–16, 2014.

[2] T. Folarnmi, “Food insecurity and malnutrition in Africa: Current trends, causes and consequences,” *Consult. Africa Intell. Your Africa Partn. Super. reseach Anal.*, no. September 2012, pp. 1–10, 2014.

[3] P. SUPPAKUL, J. MILTZ, K. SONNEVELD, and S. W. BIGGER, “Active Packaging Technologies with an Emphasis on Antimicrobial Packaging and its Applications,” *JFS Concise Rev. Hypotheses Food Sci. Act. Packag. Technol. with an Emphas. Antimicrob. Packag. its Appl.*, vol. 68, no. 2, 2003.

[4] D. S. Yildirim, “Active Packaging Antimicrobial Films for Food Packaging,” *Zurich Univ. Appl. Sci. Inst. für Leb. und Getränkeinnovation Campus Reidbach Postfach Wädenswil*, pp. 1–22, 2011.

[5] T. F. Group, “Active and Intelligent Packaging,” *Free. US Ind. Study with Forecast. 2015 2020*, vol. 8, no. 2772, p. 302, 2011.

[6] F. N. Hafdani and N. Sadeghinia, “A Review on Application of Chitosan as a Natural Antimicrobial,” *World Acad. Sci. Eng. Technol.*, vol. 50, pp. 225–229, 2011.

[7] P. K. Dutta, S. Tripathi, G. K. Mehrotra, and J. Dutta, “Perspectives for chitosan based antimicrobial films in food applications,” *Food Chem.*, vol. 114, no. 4, pp. 1173–1182, Jun. 2009.

CHAPTER TWO: LITERATURE REVIEW

2.1 Introduction to Active Packaging

Active packaging involves the controlled release of antimicrobial agents embedded within the package [8]. Such controlled release can help to increase the shelf lives of the package components [4]. Current research on active packaging also includes: the development of active packing for the scavenging of carbon dioxide, ethylene, oxygen and water vapor in the atmosphere [9]. This can be achieved by the controlled release of natural antimicrobials, aromatic substances, antioxidants etc. Such controlled release can be used to increase product shelf life by adjusting the control release to the specific food requirements [5].

Active packaging is very important to food because it leads to increase in the shelf life of food [10]. It causes the food to look natural, safe and keep the nutritional and sensorial qualities of the food for a very long time. It leads to the use of limited quantity of additives but increases the effect when the release is controlled. It causes optimum conservation of products and keeps the food safe throughout the distribution cycle [6].

Active packaging has been studied for more than 40 years [11]. Ever since passive packaging embraced oxygen and water vapor barriers for the protection of food and beverage products during distribution [12]. Active packaging has been used globally to preserve food from farm to consumers [13]. It has also been shown to protect food degradation due to microbe, oxygen, water vapor and light. Hence, the type of packaging determines the shelf life of a food. 'Active' packaging can also be designed to react to events that occur within a package [4].

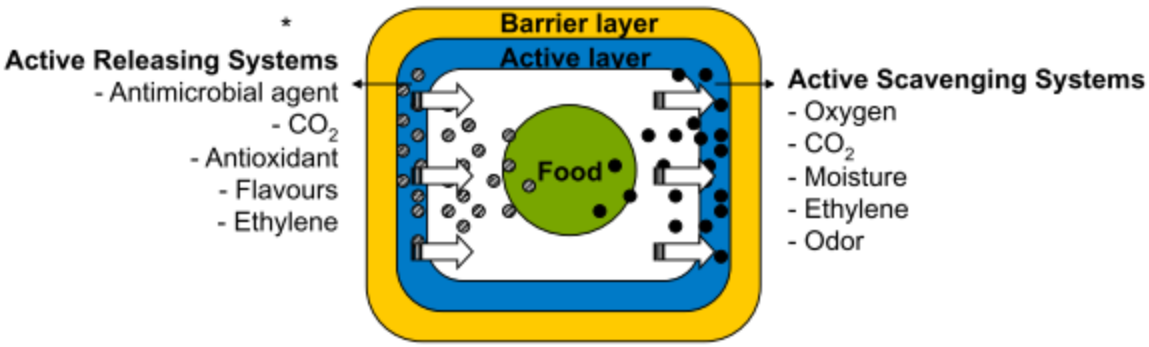


Figure 2.1: **Various Conditions that can Cause Food Spoilage**

(Adopted from: Yildirim, Dr. Selcuk; Active Packaging Antimicrobial Films for Food Packaging)

2.2 What can occur inside a Food Package*?*

Fresh plant and animal foods contain microbes [14]. They are still active biological systems, and the environments within the package change as gases and moisture are produced during metabolic processes. The type of packaging used will also influence the environment around the food [15]. This is because some plastics have poor barrier properties to gases and moisture. Whereas, as water is produced, the humidity of the headspace of the package builds up. This enhances the growth of spoilage microorganisms and damages the fruit and vegetable tissue [7].

Ethylene is produced by many food plant produce as part of their normal metabolic cycle. This simple organic gas triggers ripening and aging of fruits and vegetables. This explains why fruits, such as bananas and pear, ripen quickly when kept with ripe or damaged fruits in a single container. It also causes broccoli to turn yellow, even when kept in the refrigerator [4].

Extensive trials have revealed that each fresh food has its own optimal gas composition and humidity level for maximizing its shelf life. Active packaging offers promise in this area. However, it is difficult (with conventional packaging) to optimize the composition of the headspace in a package.

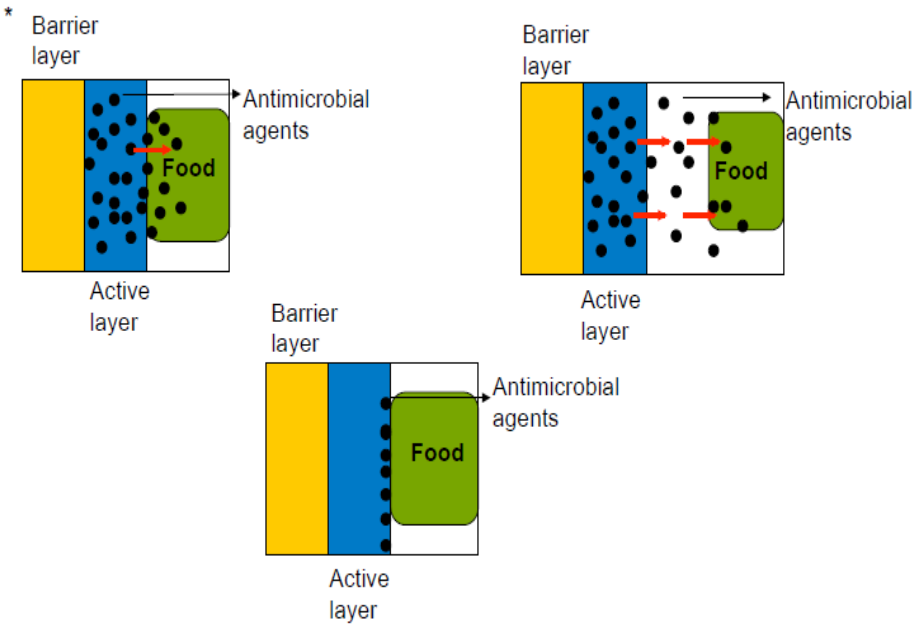


Figure 2.2: **Various Antimicrobial and Food Interaction Mechanisms**

(Adopted from: Yildirim, Dr. Selcuk; Active Packaging Antimicrobial Films for Food Packaging)

The environment surrounding food also influences the shelf life of processed foods. For some processed foods, reducing the oxygen level is beneficial, since it can slow down the discoloration of cured meats and powdered milk. It can also prevent rancidity in nuts and other high fat foods. On the other hand, high carbon dioxide and low oxygen levels can pose a problem in fresh products, leading to anaerobic metabolism and rapid rotting of the food. However, in fresh and processed meats, cheeses and baked goods, carbon dioxide may have a beneficial antimicrobial effect [4].

2.3 Active Packaging Systems

Active packaging generally contains a packaging material that interacts with the internal gas environment to extend the shelf life of a food. This new technology continuously modifies the gas environment. It can also could interact with the surface of the food by removing gases or adding gases to the headspace inside a package. Current innovations for the control of specific

gases within a package involve the use of chemical scavengers to absorb a gas or alternatively other chemicals that may release a specific gas as required [16].

Antimicrobials	**Polymer/carrier**	**Main target microorganisms**
Organic acids / anhydrides: Propionic, benzoic, sorbic, acetic, lactic, malic	Edible films, EVA, LLDPE	Molds
Inorganic gases: Sulfur dioxide, chlorine dioxide	Various polyolefins	Molds, Bacteria, Yeasts
Metal: Silver	Various polyolefins	Bacteria
Fungicide: Benomyl, imazalil	LDPE	Molds
Bacteriocins: Nisin, pediocins, lacticin	Edible films, cellulose, LDPE	Gram-positive bacteria
Enzymes: Lysozyme, glucose oxidase	Cellulose acetate, PS, Edible films	Gram-positive bacteria
Chelating agents: EDTA	Edible films	Gram-negative bacteria
Spices: Cinnamic, caffeic, p-coumaic acids Horscradish (allylisothiocyanate)	Nylon/PE, cellulose	Molds, yeast, bacteria
Essential oils (plant extracts): Grapefruit seed extract, hinokitiol, bamboo powder, Rheum palmatum, coptis chinesis extracts	LDPE, cellulose	Molds, yeast and bacteria
Parabens: Propylparaben, ethylparaben	Clay-coated, cellulose, LDPE	Mold
Miscellaneous: Haxemethylenetetramine	LDPE	Yeasts, anaerobes and aerobes

Abbreviations: EVA (ethylene vinyl acetate); LLDPE (linear low density polyethylene); LDPE (low density polyethylene); PS (Polystyrene); PE (polyethylene)

Table 2.1**: Shows Antimicrobials, Polymer/carriers and Main Target Microorganisms**

(Adopted: Appendini and Hotchkiss 2002)

Active packaging is a substitutive method to the existing preservation methods. It can be used to control undesirable micro-organisms in foods. Direct addition of antimicrobial agents results in some loss of their activities, as they diffuse into the food matrix. Hence, the use of packaging films (containing antimicrobial agents) is more efficient than the direct addition of such agents onto the food. Surface consequently, the controlled migration of the active compound (from the packaging material into the food) enables not the initial inhibition of undesirable microorganisms (present in food), as well as residual activity over time. This includes residual activity during transport, storage and of food distribution [17].

Packaging System	**Application**
Oxygen scavenging	Most food classes
Carbon dioxide production	Most food affected by moulds
Water vapour removal	Dried and meld-sensitive foods
Ethylene removal	Horticultural produce
Ethanol release	Baked foods (where permitted)

Table 2.2**: Packaging Systems and Their Applications**
(Adopted: Appendini and Hotchkiss 2002)

2.3.1 Ethylene Scavenging

Ethylene is a colorless flammable gas of unsaturated hydrocarbon (C_2H_4), incorporated into the packaging film. It causes the ripening of fruits or vegetables. The reaction is irreversible and only small quantities of the scavenger (a chemical agent that is added to a chemical mixture to counteract the effects of impurities) is required to remove ethylene at the concentrations at which

it is produced. A feature of the ethylene scavenging system is its pink color, which can be used as an indicator of the extent of reaction and shows when the scavenger is used upon [13]

2.3.2 Oxygen Scavenging

The presence of oxygen in food packages speed up the spoilage of many foods. Oxygen can cause food to lose its colour, flavour or nutrients. It can also lead to microbial growth. Several different systems are being investigated for prevention of oxygen building for preservation of different foods. One of the most promising applications of oxygen scavenging systems in food packages is the control of mould growth. Most moulds require oxygen to grow. Mould growth frequently limits the shelf lives of packaged baked goods, such as cakes and crumpets. Research has also shown that mould growth (on some baked products) can be stopped for at least 30 days with active packaging methods [18],[19]. Significant improvements in the shelf lives of packaged cheese has also been obtained [4].

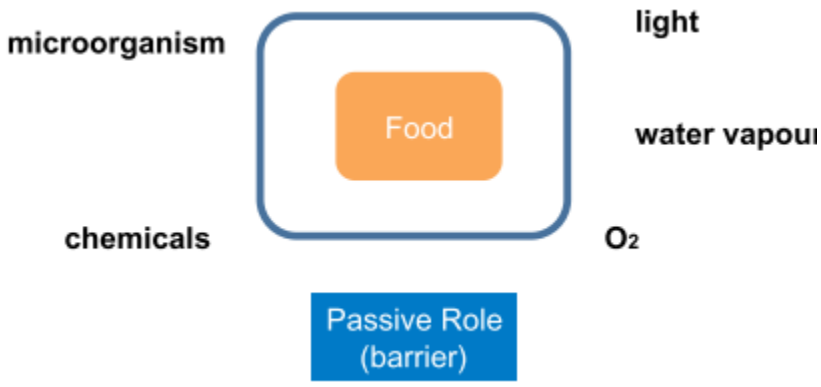


Figure 2.3: **Preservation of Food from the Major Destructive Agents**
(Adopted from: Yildirim, Dr. Selcuk; Active Packaging Antimicrobial Films for Food Packaging)

Similarly, another promising application in the food industry is the use of active packaging to delay the oxidation of food and, therefore, rancidity development in vegetable oils. Again the use

of discrete sachets containing oxygen absorbents has already found commercial applications. In this example, the scavenging material is mostly finely divided iron oxide. These sachets have been used in some countries to protect the colour of packaged cured meats from oxygen in the headspace. They have also been used to slow down staling and meld growth in baked products, e.g. pizza crusts [20].

The method of inserting a sachet into the package is effective, but also meets with resistance among food packers [21],[22]. The active ingredients in most systems consist of a non-toxic brown/black powder or aggregate that is usually unappealing if the sachet breaks. Another significant approach would be the use of a transparent packaging plastic as the scavenging medium [18].

The key factor that reduces the shelf life of food is the presence of oxygen, which causes oxidation. Oxidation leads to changes in odor, color, and flavor, thereby damaging the nutrients and causing growth of insect, molds and aerobic bacteria [23]. It is, therefore, important to remove oxygen from the food. This method is achieved by the oxidation of 1 or more of the following substances: enzymes (such as glucose, oxidase and ethanol oxidase), iron power, ascorbic acid, rice extract, immobilized yeast on a solid substrate photo-sensitive dyes, or unsaturated fatty acids [24], which are normal in a sachet. Vacuum packaging has also been widely used to eliminate oxygen in the package prior to sealing, but not oxygen contained in packaging materials [25].

Meanwhile, modified atmosphere packaging (MAP) is often used as a substitute to the reduction of the O_2 inside food packaging. Moreover, for many foods, the levels of residual oxygen that can be achieved by regular (MAP) technologies are too high for maintaining the desired quality and for achieving the desired shelf lives [26]. The use of oxygen scavenging packaging materials

means that oxygen dissolved in the food, or present initially in the headspace, can potentially be reduced to levels much lower than those achievable by modified atmosphere packaging [27],[28].

2.3.3 Humidity Control

Sweating is a significant problem in many kinds of packaged fruit and vegetables. It is of particular concern in cartooning of fresh flowers, for which there is important export trade [29]. Unless the relative humidity around flowers is maintained at about 98 percent, water will be lost from the branches. Such high humidity levels portray there is a very big threat of condensation occurring during transport, as the temperature of the flowers could fluctuate by several degrees. When one part of the package becomes cooler than another, water is likely to condense in the cooler areas [30].

If water is kept away from the produce, there will be little harm to the fruit. However, when the condensation wets the produce, nutrients leak into the water, encouraging rapid mould growth.

When the condensation is controlled inside the packages, the food remains dry without drying out the product itself. Therefore, sensitive products, such as flowers and table grapes, are protected from contact with water. This helps to reduce mould growth [4].

2.3.4. Carbon Dioxide Release

Maximum carbon dioxide levels are acceptable in some food packages because they inhibit surface growth of microorganisms. Fresh fish, meat, poultry, cheeses and strawberries are foods that can benefit from packaging in a high carbon dioxide atmospheres.

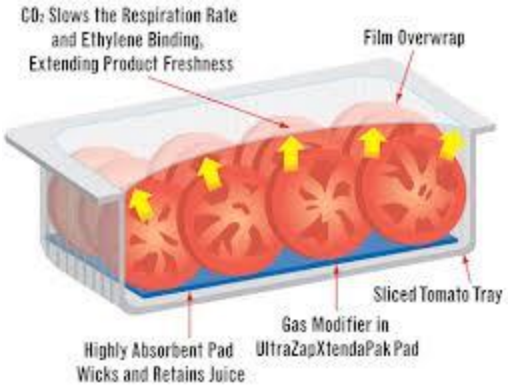


Figure 2.4: **Typical Antimicrobial Releasing and Scavenging Systems**
(Adopted from: Wikipedia 2014)

Meanwhile, with the introduction of modified atmosphere packaging, there is a need to get varying concentrations of carbon dioxide to suit specific food requirements. Since carbon dioxide is more permeable through plastic films than is oxygen, carbon dioxide will need to be actively produced in some applications to maintain the desired atmosphere in the package [33].

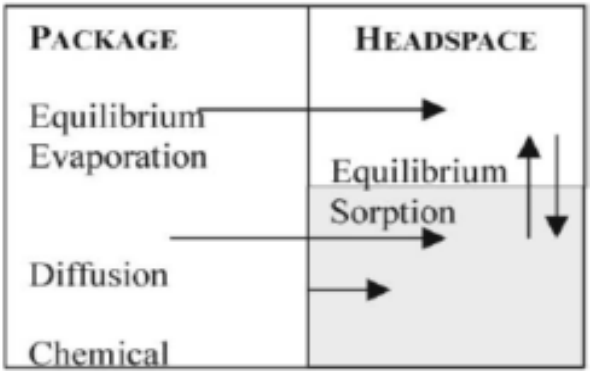


Figure 2.5: **Package/ Headspace/food System Relative Behavior of Active Substances**
(Adopted from: Han 2000)

So far, the problems associated with diffusion of gases, especially carbon dioxide, through the package, have not been resolved and this remains an important research topic [10].

2.3.5 Natural Antimicrobial Agents

Natural antimicrobial agents occur naturally in nature, or are isolated from microbial plants or animal sources in nature. Natural antimicrobial agents produced by microorganisms include bacteriocins, such as nisin or pediocin, antibiotics such as natamycin, organic acids, such as sorbic and benzoic acids and enzymes, such as lysozyme. Those produced from plant antimicrobial agents include: extracts of spices, such as thyme, oregano etc. Chitosan is an example of natural antimicrobial polymer obtained by deacetylation of chitin. It can be obtained commercially from shrimp and crab shell.

Nisin is one of the bacteriocins that is mostly employed as a food preservative. It has a very high antibacterial activity with very little toxicity to humans. It is most often used in food preservation against the growth of bacteria in meat, dairy and canned food. The bacteriocin nisin, was discovered in England by Rogers and Whittier in 1928. It is produced from Lactococcus lactis strains of bacteria. Nisin belongs to a group of bacteriocins known as "lantibiotics" (class-I), which are peptides. They are smallish in size and their source is the Gram positive bacteria of different genre [34].

2.4. Release of Microbial Inhibitors

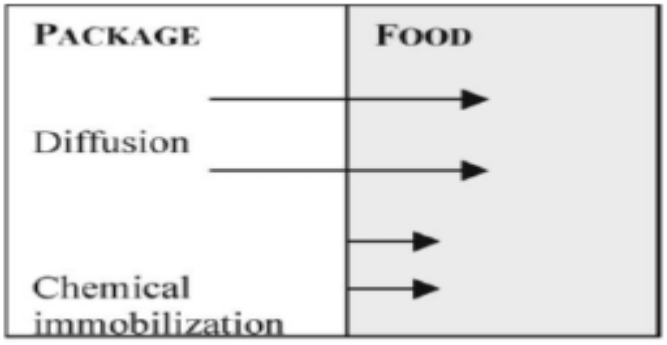


Figure 2.6: **Package/Food System and Relative Behavior of Active Substances**

(Adopted from: Han 2000)

2.4.1 Ethanol

The antimicrobial activity of ethanol or common alcohol is well known and is used in medical and pharmaceutical applications. It has been proven to increase the shelf lives of bread and other baked products, when sprayed onto the surfaces of the product, prior to packaging.

As outstanding technique for generating ethanol vapour is through the use of an ethanol releasing system that is enclosed in a small sachet. This has been included in a food package, based on a recent development in Japan. This method is achieved by food grade of ethanol that is absorbed onto a fine inert powder enclosed in a sachet. This permeable to water vapour. Meanwhile, moisture is absorbed from the food by the inert powder and ethanol vapour is released and permeates the sachet into the food package headspace. This system is approved and used in Japan to extend the free-free shelf life of various cakes[3].

2.4.2 Sulfur Dioxide

Sulfur dioxide is mainly used to control mould growth in some fruits. Serious loss of table grapes can occur unless precautions are taken against mould growth. It is necessary to refrigerate grapes in combination with fumigation using low levels of sulfur dioxide. Fumigation can be conducted in the fruit cool stores, as well as in the cartons. Carton fumigation consists of a combination of quick release and slow release systems, which emit small amounts of sulfur dioxide.

When the temperature of the packed grapes rises due to inadequate temperature control, the slow release system fails releasing all of its sulfur dioxide quickly. This can lead to illegal residues in the grapes and unsightly bleaching of the fruit. Considerable amount of work is done to develop systems, which gradually release sulfur dioxide and are less sensitive to high temperature and

moisture than those presently used. These systems have the potential for application on the packaging of fresh grapes and processed foods permitted to contain sulfur dioxide. These include: dried tree fruits and wine [9], [35].

Active packaging system	Mechanisms	Food applications
Oxygen scavengers	1. Iron based 2. Metal/acid 3. Metal (e.g. platinum) catalyst 4. Ascorbate/metallic salts	Bread, cakes, cooked rice, biscuits, pizza, pasta, cheese, cured meat and fish, coffee, snack foods, dried foods and beverages
Carbon dioxide scavenger/emitters	1. Iron oxide/calcium hydroxide 2. Ferrous carbonate/metal halide 3. Calcium oxide/active charcoal 4. Asorbate/sodium bicarbonate	Coffee, fresh meats and fish, nuts and other snacks food products and sponge cakes
Ethylene scavengers	1. Potassium permanganate 2. Activated carbon 3. Activated clays/zeolites	Fruit, vegetables and other horticultural products
Preservative releasers	1. Organic acids 2. Silver zeolite 3. Spice and herb extracts 4. BHA/BHT antioxidant 5. Vitamin E antioxidant 6. Volatile chlorine dioxide/sulphur dioxide	Cereals, meats, fish, bread, cheese, snack food, fruit and vegetables
Ethanol emitters	1. Alcohol spray 2. 2. Encapsulated	Pizza crusts, cakes, bread, biscuits, fish and bakery products
Moisture absorbers	1. PVA blanket 2. Activated clays and minerals 3. Silica gel	Fish, meats, poultry, snack foods, cereals, dried foods, sandwiched, fruits and vegetables
Flavor/odour absorbers	1. Cellulose triacetate acetylated paper 2. Acetylated paper 3. Citric acid 4. Ferrous salt/ascorbate 5. Activated carbon/clays/zeolites	
Temperature control packaging	1. Non-woven plastics 2. Double walled containers 3. Hydrofluorocarbon gas 4. Lime/water 5. Ammonium nitrate/water	Ready meals, meats, fish, poultry and beverages

Table 2.3: **Selected Examples of Active Packaging Systems**

Adopted from: Active packaging (Brian P.F. Day)

2.5 Other Developments

The methods presented above are only some of the commercial and non-commercial applications of active packaging. This technology is the subject of research in many countries and rapid developments are expected. The other systems that are used for active packaging which include:

- Sachets with iron powder and calcium hydroxide, which scavenge both oxygen and carbon dioxide. These sachets are used to extend the shelf lives of ground coffee.
- Film containing microbial inhibitors other than those noted above. Other inhibitors being investigated include metal ions and salts of propionic acid.
- Specially fabricated films to absorb flavors and odors or, conversely, to release them into the package [3].

Class	Examples	References
Acid Anhydride	Benzoic anhydride	Weng and Hotchkiss (1993), Huang and others (1997), Dobias and others (2000)
	Sorbic anhydride	Weng and Chen (1997)
Alcohol	Ethanol	Luck and Jager (1997)
Amine	Hexamethylenetetramine (HMT)	Luck and Jager (1997), Devlieghere and others (2000b)
Ammonium Compound	Silicon quaternary ammonium salt	
Antibiotic	Natamycin	Luck and Jager (1997)
Antimicrobial Peptide	Attacin	Dillon (1994)
	cecropin	Dillon (1994)
	Defensin	Dillon (1994)
	Magainin	Abler and others (1995)
Antioxidant Phenolic[1]	Butylated hydroxyanisole (BHA)	Hotchkiss (1997)
	Butylated hydroxytoluene (BHT)	Hotchkiss (1997)
	Tertiary butylhdroquinone (TBHQ)	Hotchkiss (1997)

Bacteriocin	Bavaricin	Nettles and Barefoot (1993)
	Brevicin	Nettles and Barefoot (1993)
	Carnocin	Nettles and Barefoot (1993)
	Lacticin	Nettles and Barefoot (1993), An and others (2000), Scannell and others (2000)
	Mesenterocin Nisin	Nettles and Barefoot (1993)
	Nisin	Luck and Jager (1997), An and others (2000), Natrajan and Sheldon (2000a, b), Scannell and others (2000)
	Pediocin	Barnby-Smith (1992), Nettles and Barefoot (1993)
	Sakacin	Nettles and Barefoot (1993)
	Subtilin	Barnby-Smith (1992)
Chelator	Citrate	Hotchkiss (1997)
	Conalbumin	Conner (1993)
	EDTA	Luck and Jager (1997), Rodrigues and Han (2000)
	Lactoferrin	Conner (1993)
	Polyphosphate	Shelef and Sieter (1993)
Enzyme	Chitinase	Fuglsang and others (1995)
	Ethanol oxidase	Fuglsang and others (1995)
	β −Glucanase	Fuglsang and others (1995)
	Glucose oxidase	Fuglsang and others (1995)
	Lactoperoxidase	Conner (1993), Fuglsang and others (1995)
	Lysozyme	Conner (1993), Fuglsang and others (1995), Appendini and Hotchkiss (1997), Luck and Jager (1997), Rodrigues and Han (2000)
	Myeloperoxidase	Fugisang and others (1995)
Fatty acid	Lauric acid	Ouattara and others (1997; 2000b)
	Palmitoletic acid	Ouattara and others (1997)
Fatty Acid Ester	Monolaurin (lauricidin ®)	Luck and Jager (1997)
Fugicide	Benomyl	Halek and Garg (1989)

	Imazalil	Hale and others (1986), Weng and Hotchkiss (1992)
	Sulfur dioxide	Thomas and others (1995), Christie and others (1997), Luck and Jager (1997), Opperman and others (1999)
Inorganic acid	Phosphoric acid	Hotchkiss (1997)
Metal	Copper	Ishitani (1995)
	Copper silver	Ishitani (1995), Luck and Jager (1997), An and others (1998), Chung and others (1998)
Miscellaneous	Reuterin	Helander and others (1997)
Natural Phenol	Catechin	Walker (1994)
	p-Cresol	Hotchkiss (1997)
	Hydroquinones	Hotchkiss (1997)
Oligosaccharide	Chitooligosaccharide	Cho and others (2000), Hong and others (2000)
Organic Acid	Acetic acid	Doores (1993), Ouattara and others (2000a, b), Luck and Jager (1997)
	Benzoic acid	Luck and Jager (1997), Weng and others (1997), Chen and others (1999), Weng and others (1999)
	Citric acid	
	Lactic acid	Doores (1993), Luck and Jager (1997)
	Malic acid	Doores (1993)
	Propionic acid	Doores (1993), Ouattara and others (2000a, b), Luck and Jager (1997)
	Sorbic acid	Luck and Jager (1997), Weng and others (1999)
	Succinic acid	Doores (1993)
	Tartaric acid	Doores (1993)
Organic Acid Salt	Potassium sorbate	Chen and others (1996), Han and Floros (1997, 1999), Devlieghere and others (2000a)
	Sodium benzoate	Chen and others (1996)
Paraben	Ethyl paraben	Davidson (1993), Luck and Jager (1997), Dobias and others (2000)
	Methyl paraben	Davidson (1993), Luck and Jager

		(1997)
	Propyl paraben	Davidson (1993), Luck and Jager (1997), Dobias and others (2000)
Plant-Volatile Component	Allyl isothiocyanate (AIT)	Isshiki and others (1992), Luck and Jager (1997), Brody and others (2001)
	Carvacrol	Ouattara and others (1997), Scora and Scora (1998)
	Cineole	Lis-Balchin and others (1998), Scora and Scora (1998)
	Cinnamaldehyde	Ouattara and others (1997; 2000b)
	Citral	Lis-Balchin and others (1998), Scora and Scora (1998)
	p –Cymene	Scora and Scora (1998)
	Estragole (methyl chavicol)	Scora and Scora (1998), Suppakul and others (2002)
	Eugenol	Ouattara (1997), Scora and Scora (1998)
	Geraniol	Scora and Scora (1998)
	Hinokitiol (β -thujaplicin)	Fallik and Grinberg (1992), Brody and others (2001)
	Linalool	Lis-Balchin and others (1998), Scora and Scora (1998), Suppakul and others (2002)
	Terpineol	Scora and Scora (1998)
	Thymol	Ouattara and others (1997), Scora and Scora (1998)
Polysaccharide	Chitosan	Sudarshan and others (1992), Begin and Calsteren (1999), Hong and others (2000)
	Konjac glucomannan	Xiao and others (2000)

- [1]Although generally used as Antioxidants, they have shown also Antimicrobial activity (Hotchkiss, 1997).

Table 2.4: **Examples of Antimicrobial Agents for Potential use in Food Packaging Materials**

2.6 Chitosan Film

Chitosan is a natural, biodegradable and environmentally friendly amino-polysaccharide polymer.

It is a deacetylated and acetylated derivatives that is obtain from crustaceans. Its powder appears

white to light-red in color. It is prepared by the treatment of crustaceans shells with alkali sodium hydroxide. It is soluble in organic acid and insoluble in water. Chitosan is the third most abundant polysaccharide in the world. It has antibacterial, anti-fungal and anti-viral properties, which account for it bio-applications. Including it bio-applications, chitosan is also used in nutritional applications, skin and hair care, environmental and agriculture applications, food packaging [36].

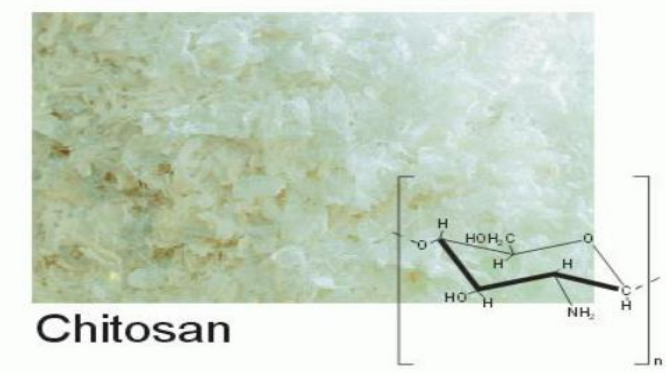


Figure 2.7: **Chitosan**
(Adopted from: Wikipedia 2014)

2.6.1 Structure of Chitosan

Chitosan is a linear polysaccharide with random monomers chains of beta (1-4) linked of D-glucosamine and N-acetyl-D-glucosamine. It is a deacetylated derivative of chitin, which is commercially derived from shells of crabs, shrimp, and lobster. The -NH_2 signifies the amino group, which is located on the C-2 of the D-glucosamine repeating unit, it protonation results in the solubilization of chitin which lead to the formation chitosan [8].

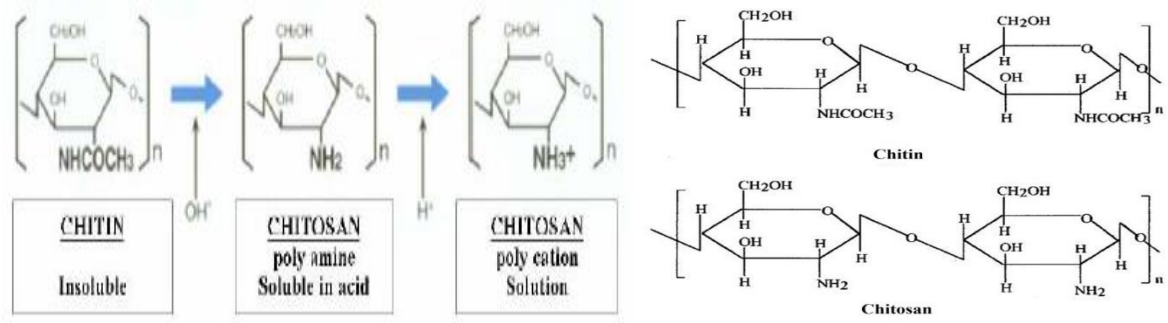
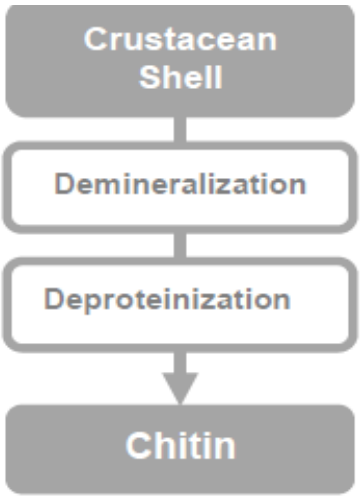


Figure2.8: **Structure of Chitosan**

(Adopted from: Wikipedia 2014)

2.6.2 Manufacturing of Chitosan



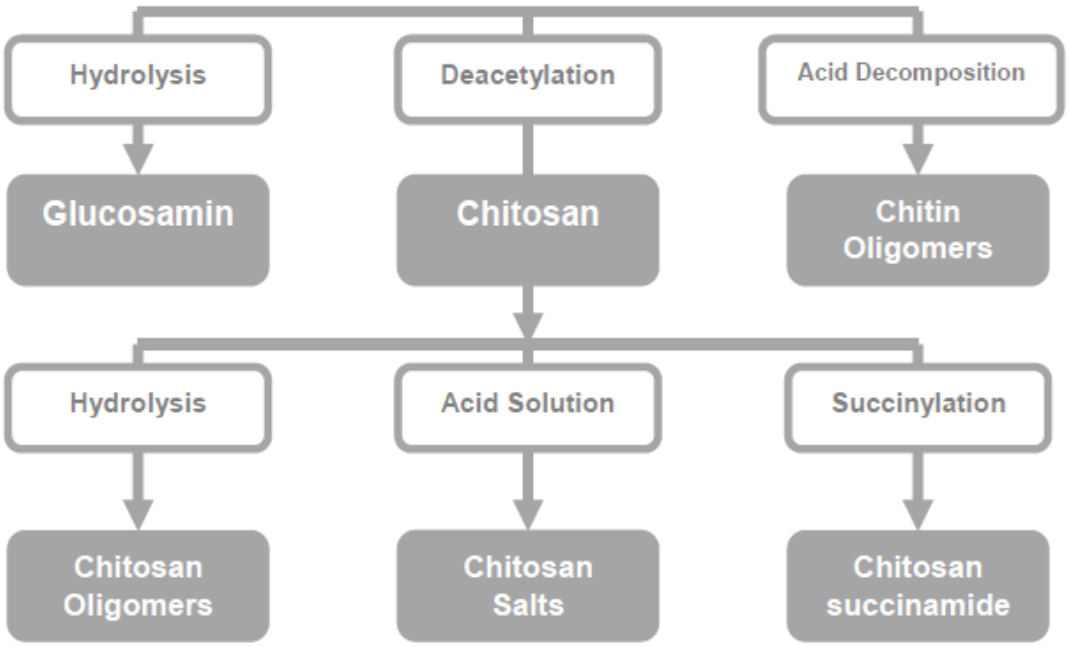


Figure 2.9: **Manufacturing Flow Chart**

(Adopted from: CHITOSAN PRODUCTION LINE OFFER by: ensymm project consulting of life Science)

Commercially, chitosan is derived from the shells of shrimp and other sea crustaceans (lobster, crab fish, crab, etc.) by deacetylation of chitin the structural element in the exoskeleton of crustaceans and the cell walls of fungi. The degree of deacetylation (%DD) can be determined by NMR spectroscopy, and the %DD in commercial chitosan ranges from 60 to 100%. The molecular weight of commercially produced chitosan is between 3800 and 20,000 Daltons. A simple method for the synthesis of chitosan is the deacetylation of chitin using sodium hydroxide in excess as a reagent and water as a solvent. This reaction pathway, when allowed to go to completion (complete deacetylation), yields up to 98% product of chitosan [37].

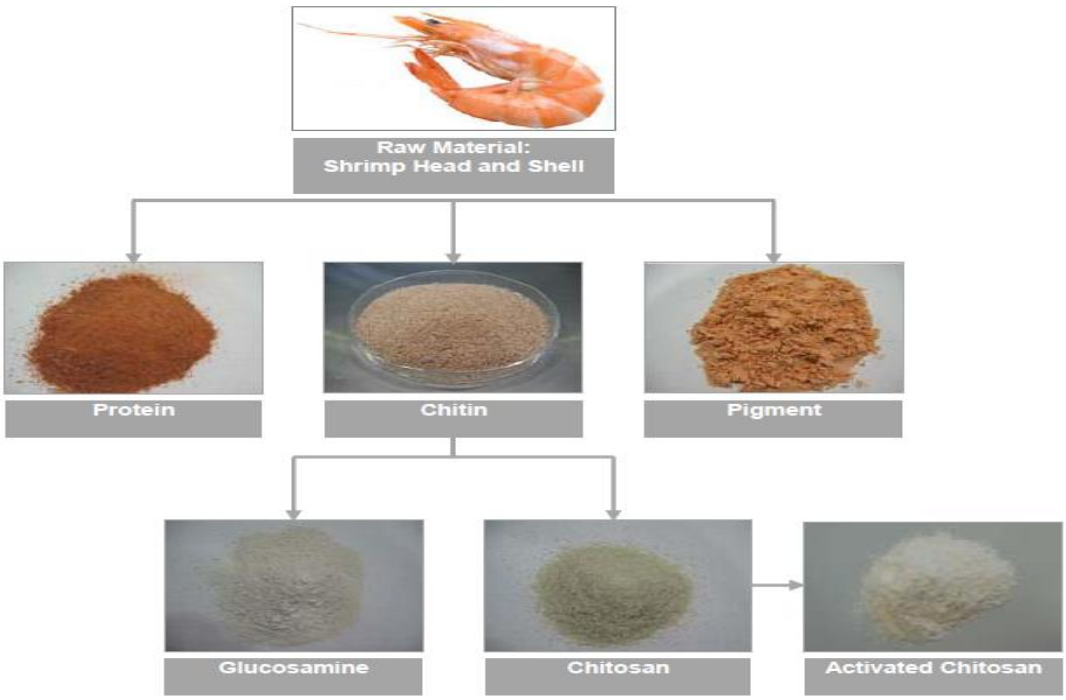


Figure 2.10: **Manufacturing Process**

(Adopted from: Wikipedia 2014)

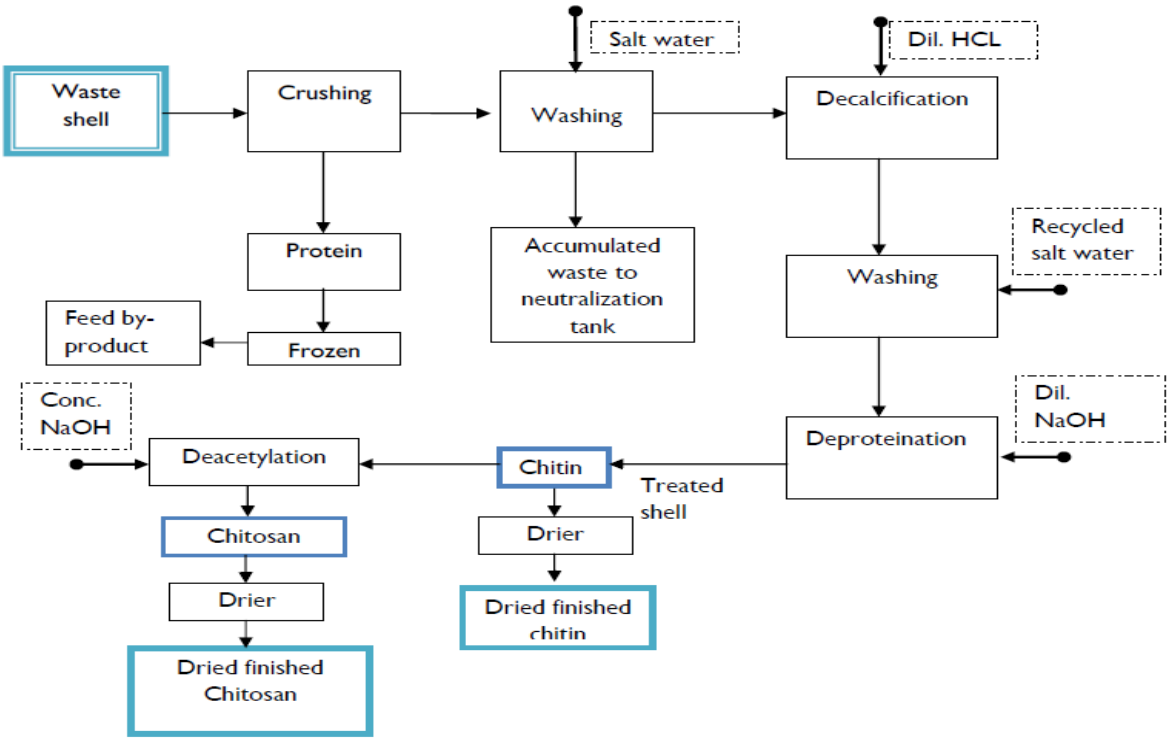


Figure 2.11: **Manufacturing Procedures**

Adopted from: Manufacturing of Chitin and Chitosan (KITCO the Consultants)

2.6.3 Chitosan Properties

Chitosan has numerous of properties that make it a highly interesting area in several fields. It is biodegradable and biocompatible (based on its antigenic behavior). It also exhibits antithrombotic (prevents blood clots) and homeostatic (maintain internal equilibrium) properties [38],[39]. Chitosan also exhibits it has a remarkable healing activity [40]. It is a cholesterol-lowering product, as all polysaccharides, it is an excellent moisturizing agent due to its water retention capacity [41]. Chitosan is also able to inhibit the development of a number of parasites and germs (among others E. coli, Pseudomonas, Candida albicans). It also has immunological and anti-tumor properties [42]. Chitosan is also an outstanding chelating agent (especially for heavy metals). It is active against yeast and moulds, and it favours plant germination and growth [37] [38].

2.6.4 Applications of Chitosan

Based on the numerous properties of chitosan it has many applications. In agriculture and horticulture, chitosan is used primarily for plant defense and yield increase [43], [44] & [45].This is based on how the glucosamine polymer influences the biochemistry and molecular biology of the plant cells [46].

Environmentally, chitosan is used in water process engineering as a part of a filtration process [47]. It causes fine sedimentary particles to bind together. The bound particles can then be removed during sand filtration. It is an important additive in the filtration process that removes phosphorus, heavy minerals, and oils from the water. Chitosan has been used to precipitate caseins from bovine milk and cheese [37][48][38].

Based on the excellent moisturizing and antimicrobial characteristics of chitosan [49], it is used in the production skin lotions [50]. In biomedicine chitosan is used in wound healing by applying it directly to wounds. From research done [51], it was shown that chitosan causes normal tissue regeneration and blood coagulation. It is also used in contact lenses sutures, obesity treatment, high cholesterol treatment, and the treatment of Cohn's disease. It is also used to treat complications that kidney failure patients on dialysis often face, including high cholesterol, "tired blood" (anemia), loss of strength and appetite, and insomnia. Some people also apply chitosan directly to their gums to treat inflammation.

In pharmaceutical manufacturing, chitosan is used as filler in tablets; as a carrier in controlled-release drugs; to improve the way certain drugs dissolve; and to mask bitter tastes in solutions taken by mouth. In addition, chitosan is used in food preservations. It is used in creating active packages that have the ability to prolong the shelf life of food [48][52].

2.7 Antimicrobial Activities of Potassium Sorbate

Potassium sorbate is a naturally occurring polyunsaturated fat. It was first discovered in the mountain ash tree in 1850 by the French [53]. It is now produced synthetically by the bonding of potassium salt with sorbic acid to form a fatty acid salt. Potassium sorbate appears in a powder form at room temperature. It forms sorbic acid when dissolved. It can be dissolved in water, ethanol, propylene glycol, acetone, chloroform, corn oil and ester, but not in benzene. It is used in wine, food and body care lotions to prevent fungi, bacteria, and mould from damaging the content. It has been approved as a preservative by the Food and Drug Administration and the Center for Science in the Public Interest. As a preservative, it is used to prolong the shelf lives of packaged foods [3][10].

2.7.1 Structure of Potassium Sorbate

Potassium sorbate is the potassium salt of sorbic acid with the formula $CH_3CH=CH-CH=CH-CO_2K$. It is a white salt that has a molar mass of 150.22 g/mol, density of 1.363 g/cm^3, melting point of 270 ^{0}C and water solubility of 58.2g/100ml (100 ^{0}C). It is a colorless, odorless and tasteless chemical. It leaves no taste in your mouth when you eat food that contains it. It can be used in place of sulfur dioxide, which usually leaves its taste in your mouth.

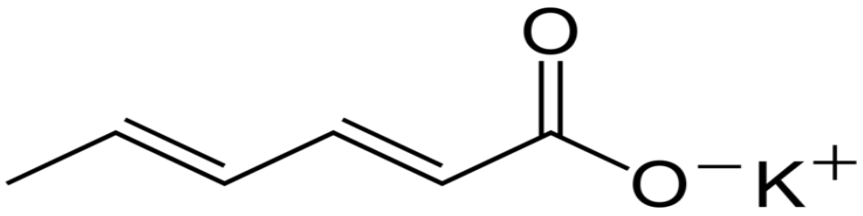


Figure 2.12: **Molecular Structure of Potassium Sorbate**
(Adopted from: Wikipedia 2014)

2.7.2 Uses of Potassium Sorbate

Based on the antimicrobial activities of potassium sorbate, it is used to prevent the growth of mould, bacteria and fungi in cheese, dried meats, baked goods, jellies, syrups, etc. It can also be added to dietary supplements, inhibiting microbes and increasing the shelf-lives of food products. Potassium sorbate is used by wine-makers during fermentation process. It helps the flavor of the yeast by consuming the sugar. It can also be used in beauty products (hair and skin lotions) [10].

2.7.3 Manufacturing of Potassium Sorbate

In the 1850s, potassium sorbate was manufactured from a mountain ash tree. However, potassium sorbate is widely produced by the chemical combination of potassium hydroxide and sorbic acid. This results in the formation of water and potassium hydroxide. After the reaction, the water is removed or the solution is used, depending the application [10].

2.7.4 Side Effects

When potassium is used in beauty products, it may cause itching, burning of the skin or eye and red swelling on the hand. Based on scientific studies, it has been shown that potassium sorbate can cause long time effects on those that consume food containing excessive potassium sorbate. These may include diarrhea and/or nausea. Also, those that are allergic to potassium are at great risk of developing negative reactions to potassium sorbate. In extreme cases, it may lead to renal or kidney problems, which may cause serious damage overtime.

2.8 Aspergillus niger

Aspergillus niger is a member of the genus Aspergillus. Aspergillus includes a set of fungi that are generally considered asexual. Geographically, they are widely distributed, and have been observed in a broad range of habitats. A. niger is commonly found as a saprophyte growing on stored grain, dead leaves, compost piles, and other decaying vegetation. The spores are widespread, and are often associated with organic materials and soil.

2.8.1 Significance of Aspergillus niger to Human

Aspergillus niger is use in the food industry for the production of many enzymes such as amylase, amyloglucosidase, cellulases, lactase, invertase, pectinases, and acid proteases (Bennett, 1985a; Ward, 1989). Additionally, the annual production of citric acid from *A. niger* or *Candida* yeast through fermentation, is now approximately 350,000 tons. Citric acid fermentation using *A. niger* is carried out commercially in both surface culture and in submerged processes (Berry et al., 1977; Kubicek and Rohr, 1986; Ward, 1989). Meanwhile, *A. niger* is also used to test the efficacy of preservative treatments (Jong and Gantt, 1987). There is also interest in using this fungus to perform certain enzymatic reactions that are very difficult to accomplish by strictly chemical means, such as specific additions to steroids and other complex rings (Jong and Gantt, 1987).

2.8.2 Human Health Hazard of Aspergillus *Niger*

The growth of the *Aspergillus* in human tissue or within air ways, such as bronchus or pulmonary cavity, is termed *aspergillosis* (Bennett, 1979a). Human exposure to *Aspergillus* must be nearly universal but disease is rare. The physiological condition of the exposed individual thus appears to be of paramount importance. Patients exhibiting aspergillosis are generally immunocompromised, and thus susceptible to otherwise common and usually harmless microorganisms. Factors that may lead to immunosuppression include an underlying debilitating disease (e.g., chronic granulomatous diseases of childhood), chemotherapy, and the use of supraphysiological doses of adrenal corticosteroids (Bennett, 1980).

Pulmonary aspergillosis is the most common clinical manifestation of aspergillosis. The most common symptoms of pulmonary aspergillosis are a chronic productive cough and hemoptysis (coughing up blood). *Aspergillus* can colonize ectatic bronchi, cysts, or cavities in the lung. The fungus rarely invades the wall of the cavity, cyst, or bronchus in such patients" (Bennett, 1979a). Although *Aspergillus niger* is regarded as an opportunistic pathogen (Padhye, 1982; Walsh and Pizzo, 1988), an earlier report shows that it can cause otomycosis in healthy, uncompromised persons who have no underlying disease (Austwick, 1965). Otomycosis is the name given to the growth of *Aspergillus*, often *A. niger*, on ceruman and desquamated debris in the external auditory canal.

2.8.3 Treatment Methods of Aspergillus Niger

Simple colonization of A. niger is treatable, but if the simple colonization becomes chronic or invades neighboring tissues, the infection becomes more difficult to treat (McGinnis, 1980). Surgical excision has been used successfully to treat invasive aspergillosis of the paranasal sinus

as well as non-invasive sinus colonization. Intravenous amphotericin B has resulted in arrest or cure of invasive aspergillosis when immunosuppression is not severe (Bennett, 1980).

2.9 References

[8] C. M. P. Yoshida, C. Eduardo, N. Bastos, and T. T. Franco, “Modeling of potassium sorbate diffusion through chitosan films,” *LWT - FOOD Sci. Technol.*, vol. 43, pp. 584–589, 2010.

[9] R. S. Cruz, G. P. Camilloto, and A. Clarissa, “Oxygen Scavengers : An Approach on Food Preservation,” *Food Tecnhology Dep. Fed. Univ. Viçosa, Viçosa, MG, Brazil*, 2012.

[10] U. Metin, “PREPARATION OF CONTROLLED RELEASE ANTIMICROBIAL FOOD PACKAGING MATERIALS,” *Izmir Inst. Technol. Thesis*, pp. 1–85, 2009.

[11] J. Vermeiren, L., Devlieghere, F., Van Beest, M., De Kruijf, N., & Debevere, “Developments in the active packaging of foods. Trends in food science & technology,” *Trends food Sci. Technol.*, vol. 10, no. 3, p. 1999, 1999.

[12] S. Silvestre, C., Duraccio, D., & Cimmino, “Food packaging based on polymer nanomaterials.,” *Prog. Polym. Sci.*, vol. 36, no. 12, p. 2011, 2011.

[13] Y. Zhou, G. H., Xu, X. L., & Liu, “Preservation technologies for fresh meat–A review.,” *Meat Sci.*, vol. 86, no. 1, p. 2010, 2010.

[14] D. A. Jay, J. M., Loessner, M. J., & Golden, “Modern food microbiology.” 2005.

[15] S. K. (2014). DASH, “MODIFIED ATMOSPHERE PACKAGING OF FOOD. Polymers for Packaging Applications,” p. 337, 2014.

[16] C. Fuciños, P. Fuciños, M. Míguez, I. Katime, L. M. Pastrana, and M. L. Rúa, “Temperature- and pH-sensitive nanohydrogels of poly(N-Isopropylacrylamide) for food

packaging applications: modelling the swelling-collapse behaviour.," *PLoS One*, vol. 9, no. 2, p. e87190, Jan. 2014.

[17] J. Dutta, S. Tripathi, and P. K. Dutta, *Progress in antimicrobial activities of chitin, chitosan and its oligosaccharides: a systematic study needs for food applications*, vol. 18, no. 1. 2012, pp. 3–34.

[18] G. Petersen, K., Væggemose Nielsen, P., Bertelsen, G., Lawther, M., Olsen, M. B., Nilsson, N. H., & Mortensen, "Potential of biobased materials for food packaging.," *Trends Food Sci. Technol.*, vol. 10, no. 2, p. 1999, 1999.

[19] W. M. (1989). Labuza, T. P., & Breene, "APPLICATIONS OF 'ACTIVE PACKAGING' FOR IMPROVEMENT OF SHELF-LIFE AND NUTRITIONAL QUALITY OF FRESH AND EXTENDED SHELF-LIFE FOODS," *J. Food Process. Preserv.*, vol. 13, no. 1, pp. 1–69, 1989.

[20] P. T. C/Albert 1, "ACTIVE PACKAGING TO EXTEND THE PRODUCT SHELF LIFE," *ITENE*, vol. 1, pp. 1–2, 2000.

[21] L. R. Brody, A. L., Strupinsky, E. P., & Kline, "Active packaging for food applications," *CRC Press*, p. 2001, 2001.

[22] M. (Eds. . Lang, T., & Heasman, "Food wars: the global battle for mouths, minds and markets," *Earthscan*, p. 2004, 2004.

[23] C. Film, I. Powder, A. Acid, and T. Dioxide, "Oxygen-Scavenger Oxygen Scavenging Packaging Mean Oxygen Scavenging Packaging Aid Copolymer * Copolymer *

substances Activation † heat during extrusion # self-activated (humidity) h□: Titanium Dioxide *," pp. 2–3, 2012.

[24] N. SundaraBaalaji, "Invited Lecture. In Book of Abstracts." p. 47, 2001.

[25] J. Vermeiren, L., Devlieghere, F., Van Beest, M., De Kruijf, N., & Debevere, "Developments in the active packaging of foods.," *Trends food Sci. Technol.*, vol. 10, no. 3, p. 1999, 1999.

[26] M. Miltz, J., & Perry, "Evaluation of the performance of iron-based oxygen scavengers, with comments on their optimal applications.," *Packag. Technol. Sci.*, vol. 18, no. 1, p. 2005, 2005.

[27] A. C. Cruz, R. S., Camilloto, G. P., & dos Santos Pires, "Oxygen Scavengers: An Approach on Food Preservation.," p. 2012, 2012.

[28] M. L. Rooney, "Active packaging in polymer films. In Active food packaging," *Springer US*, pp. 74– 110, 1995.

[29] L. com Inc, "Food Poisoning and Waterborne Illness: How to Prevent 1.8 Million Deaths Every Year," *Product. Publ.*, p. 2012, 2012.

[30] G. A. Ayala-Zavala, J. F., Del-Toro-Sánchez, L., Alvarez-Parrilla, E., & González-Aguilar, "High Relative Humidity In-Package of Fresh-Cut Fruits and Vegetables: Advantage or Disadvantage Considering Microbiological Problems and Antimicrobial Delivering Systems?," *J. Food Sci.*, vol. 73, no. 4, pp. R41–R47, 2008.

[31] Y. Pranoto, S. K. Rakshit, and V. M. Salokhe, “Enhancing antimicrobial activity of chitosan films by incorporating garlic oil, potassium sorbate and nisin,” *LWT - Food Sci. Technol.*, vol. 38, no. 8, pp. 859–865, Dec. 2005.

[32] “ACTIVE PACKAGING.”

[33] I. Active, W. Can, and H. Inside, “30) ACTIVE PACKAGING , INTELLIGENT PACKAGING,” *Goolge B.*, pp. 1–5.

[34] A. Lucera, C. Costa, A. Conte, and M. a Del Nobile, “Food applications of natural antimicrobial compounds.,” *Front. Microbiol.*, vol. 3, p. 287, Jan. 2012.

[35] R. S. Cruz, G. P. Camilloto, and A. Clarissa, “Oxygen Scavengers : An Approach on Food Preservation,” 2012.

[36] K. Juntarapun and C. & Satirapipathkul, “ANTIMICROBIAL ACTIVITY OF CHITOSAN AND TANNIC ACID ON COTTON FABRIOUS MATERIALS,” *RMUTP Int. Conf. Bangkok Thail.*, pp. 1–6, 2012.

[37] M. Rinaudo, “Chitin and chitosan : Properties and applications,” *Sci. Direct*, vol. 31, pp. 603–632, 2006.

[38] P. K. Dutta, J. Dutta, and V. S. Tripathi, “Chitin and chitosan : Chemistry , properties and applications,” *J. Sci. Ind. Res. (India).*, vol. 63, pp. 20–31, 2004.

[39] V. S. (2004). Dutta, P. K., Dutta, J., & Tripathi, “Chitin and chitosan: Chemistry, properties and applications,” *J. Sci. Ind. Res.*, vol. 63, no. 1, pp. 20–31, 2004.

[40] V. (2000). Tsigos, I., Martinou, A., Kafetzopoulos, D., & Bouriotis, “Chitin deacetylases: new, versatile tools in biotechnology. Trends in biotechnology,” *Trends Biotechnol.*, vol. 18, no. 7, p. 2000, 2000.

[41] N. (2010). Jayakumar, R., Prabaharan, M., Nair, S. V., Tokura, S., Tamura, H., & Selvamurugan, “Novel carboxymethyl derivatives of chitin and chitosan materials and their biomedical applications.,” *Prog. Mater. Sci.*, vol. 55, no. 7, pp. 675–709, 2010.

[42] S. P. Dube, D., Gupta, M., & Vyas, “Nanocarriers for Drug Targeting to Macrophages: Emerging Options for a Therapeutic Need,” *Proc. Natl. Acad. Sci. India Sect. B Biol. Sci.*, vol. 82, no. 1, p. 2012, 2012.

[43] M. Á. Falcón-Rodríguez, A. B., Costales, D., Cabrera, J. C., & Martínez-Téllez, “Chitosan physico–chemical properties modulate defense responses and resistance in tobacco plants against the oomycete and Phytophthora nicotianae,” *Pestic. Biochem. Physiol.*, vol. 100, no. 3, pp. 221–228, 2011.

[44] L. A. Hadwiger, “Multiple effects of chitosan on plant systems,” *Solid Sci. or hype. Plant Sci.*, pp. 42–49, 2013.

[45] R. N. Srinivasa, P. C., & Tharanathan, “Chitin/chitosan—Safe, ecofriendly packaging materials with multiple potential uses.,” *Food Rev. Int.*, vol. 23, no. 1, pp. 53–72, 2007.

[46] D. Ocloo, F. C., Adu-Gyamfi, A., Quarcoo, E. A., Serfor-Armah, Y., & Asare, “PRELIMINARY STUDIES ON ANTIFUNGAL PROPERTIES OF RADIATION PROCESSED CHITOSAN FROM CRAB SHELLS.,” *Rep. 2nd RCM on" Dev. radiation-Process. Prod. Nat. Polym. Appl. Agric. Heal. Ind. Environ.*, p. 101, 2009.

[47] B. Krajewska, “Membrane-based processes performed with use of chitin/chitosan materials,” *Sep. Purif. Technol.*, vol. 41, no. 3, pp. 305–312, 2005.

[48] R. Nicu, E. Bobu, and J. Desbrieres, “CHITOSAN AS CATIONIC POLYELECTROLYTE IN WET-END PAPERMAKING SYSTEMS,” *Cellul. Chem. Technol.*, pp. 105–111, 2011.

[49] C. N. Cutter, “Opportunities for bio-based packaging technologies to improve the quality and safety of fresh and further processed muscle foods,” *Meat Sci.*, vol. 74, no. 1, pp. 131–142, 2006.

[50] G. Sebag, H., & Vanlerberghe, “U.S. Patent No. 3,953,608 . Washington, DC: U.S. Patent and Trademark Office.,” *Washington, DC U.S. Pat. Trademark Off.*, no. 3\, 1976.

[51] W. Material, “Report of the 2nd RCM on ‘ Development of radiation-processed products of natural polymers for application in agriculture , healthcare , industry and environment ’ Reims , France,” pp. 1–234, 2010.

[52] D. Jianglian and Z. Shaoying, “Application of Chitosan Based Coating in Fruit and Vegetable Preservation : A Review,” *FOOD Process. Technol.*, vol. 4, no. 5, pp. 5–8, 2013.

[53] J. M. Quicho, “Efficacy of ultraviolet light in combination with chemical preservatives for the reduction of Escherichia coli in apple cider,” *Dr. Diss. Virginia Polytech. Inst. State Univ.*, p. 2005, 2005.

CHAPTER THREE: MATERIALS AND METHODS

3.1 Materials

Chitosan was produced locally from shrimp shells. A Pack of 10 flint glass petri dish (10 mm diameter), potassium sorbate (KS), Whatmann No.3 filter paper, Tween 20, Sodium hydroxide (NaOH) used for deproteination, hydrochloric acid (used for deactylation), beakers, a pack of hand gloves, environmental vacuum oven (Model 19), autoclave, test tubes, were all purchased from Sigma Aldrich Co. (St. Louis, MO, USA). Meanwhile, acetic acid was purchased from Mallinckrodt, Argentina. While the distilled water used was locally produced.

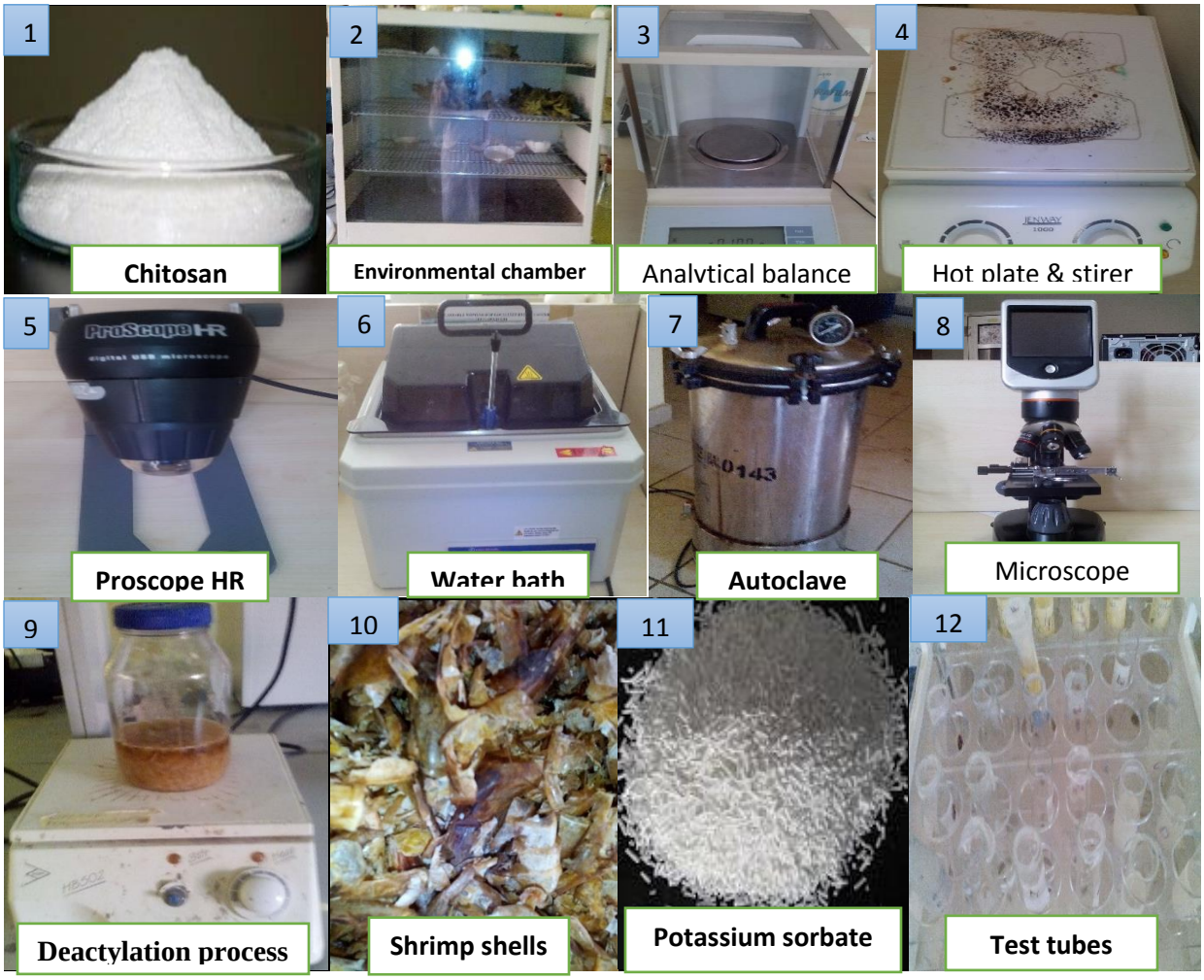


Figure 3. 1: **Some Chemicals, Apparatus and Instruments Used in this Research**

3.1.1 Production of Chitosan Powder

Different procedures have been used to obtain high quality chitin by removing protein, inorganic material (mainly CaCO3) and pigments and lipid [54]. Demineralization is usually done by treating the shrimp shells with HCl, while deproteination is done by the treatment of shrimp with NaOH. However, other researchers have used other methods [37][38]. However, in most cases, chitin producing industries conduct deproteination after demineralization [54].

The shrimps were purchased from Lagos, Nigeria and transported to Abuja, Nigeria by air. They were stored at SHESTCO Environmental Biotechnology Laboratory prior to research. Upon commencement of the research work, the shells were removed from the shrimps. They were washed with fresh water several times to remove residual materials from the shells. The shrimp shells were then dried in a Memmert oven (Sigma, St Louis, MO, USA) at 45^0C.

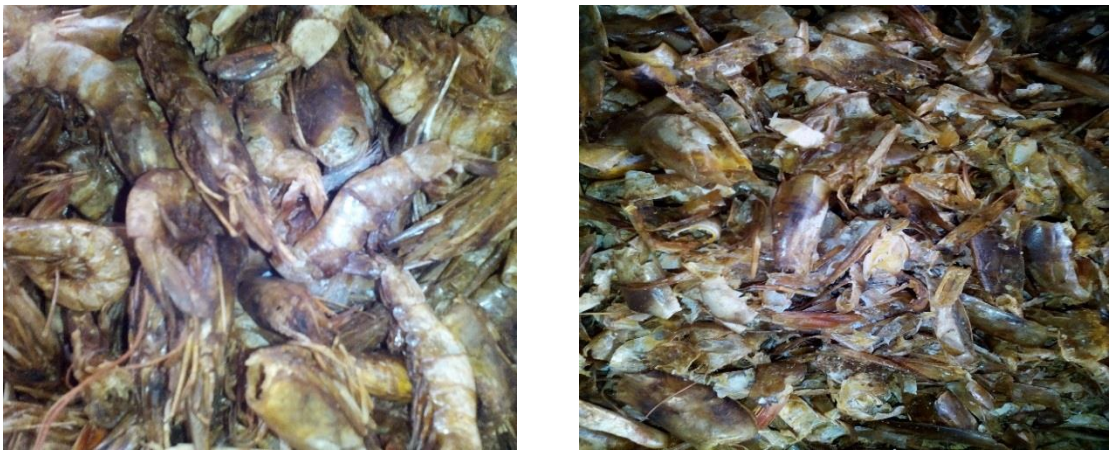


Figure 3. 2: **Shrimps (Left) and Shrimp Shells (Right)**

Upon drying, the shells were ground using a Binatone blender (Model BLG-401, made in China). Chitin extraction was achieved using alkali acid (6N HCl) treatment. The shrimp was then treated with 1% NaOH (w/w) form chitosan.

Figure 3. 3: **Washing of Shrimp Shells**

3.1.2 Experimental Methods

The shrimps were stripped of their shells and washed several times using fresh water. This was done to remove debris. The shells were then dried at a temperature of 45^0C using Memmert oven (Model 500, Rombai Instruments, Mumbai - 400102, Maharashtra, India). They were then sieved. Deproteination was carried out by dissolving 2g of the powdered shells into 20 ml of 1% NaOH at 60^0C for 24 hours. Thereafter, the samples were washed several times with distilled water and filtered using Whatmann paper No. 3.

Two grams of the deproteinized shrimp shells powder was weighed into a clean Bama bottle while 30 ml of 6N HCl was added on the 2g of deproteinized shrimp shell powder for 24 hours under serious agitation produced by a magnetic stirrer at room temperature. This was done to remove the minerals. Subsequently, the sample was washed with sterilized distilled water and filtered using Whatmann No. 3 filter paper.

Figure 3. 4: **Deproteination of the Crashed Shrimp Shells (Left) and Chitin formed after deproteination (Right)**

The residue on the filter paper was scrapped off into another clean Bama bottle using spatulas. The sample was then dissolved into 97 ml of 40% NaOH that was dispensed into a Bama bottle containing the sample. This was heated at 80^0C for 24 hours. This process is a called the deacetylation process. After 24 hours, the sample was filtered and washed by the addition of distilled water to residue on the Whatmann No. 3 filter paper. This was continued until the coloring was removed from the sample. The crude chitosan was then scrapped off from the Whatmann No.3 paper into a falcon tube. It was subsequently dried overnight in a Memmert oven (Model 500, Rombai Instruments, Mumbai - 400102, Maharashtra, India) at 100 ^{0}C.

Figure 3. 5: **Deacetylation process**

The multilayer chitosan films were prepared by dissolving 1 g of shrimp chitosan in 100 ml of 1% acetic acid solution. Potassium sorbate on the other hand was dissolved separately in water (0.5% w/w) and the solution was pour drop wise into the acetic acid (1% and 4%) solution

containing the chitosan while stirring. To enhance the stirring, the solution was placed on a magnetic stirrer. The stirring of the mixture continued until all the potassium sorbate solution was added and dissolved into the acetic acid containing the chitosan.

Once the homogeneous mixture was obtained, the solution was casted in petri dishes and place in a Precision vacuum oven (Model 19) to remove bubbles. Immediately after that, the films forming solution was placed into an environmental chamber (Siemens, Simatic OP7, Massa Martana, Italy) and dried for 2h at 50°C relative humidity. Then the films were peeled out from the Petri dishes. Another film was formed with the same concentration of acetic acid and chitosan solutions but without potassium sorbate to serve as the control.

Figure 3. 6: **Chitosan powder produced (Left) and Potassium Sorbate (Right)**

3.2.1 Procedures for the Formation of the multilayer chitosan film

In the multilayer film, the potassium sorbate concentration in the film forming solution was kept constant while the acetic acid (1% to 4%) and chitosan (0.5g to 1g) solution concentrations were changed in the multilayer films. The first layer was casted in the petri dish and the vacuum oven was used to degas. After 5 minutes, the second layer was casted upon the first layer. Similarly, the third layer was casted after degasing for 6 minutes. Thereafter, the films were dried for 2h at 50°C, relative humidity in environmental chamber (Siemens, Simatic OP7, Massa Martana, and

Italy). Upon removing them from the chamber, the films were analysis using proscope HR (Sigma Aldrich Co. (St. Louis, MO, USA) and celestron microscope (Model 44345, RoHS, and made in China).

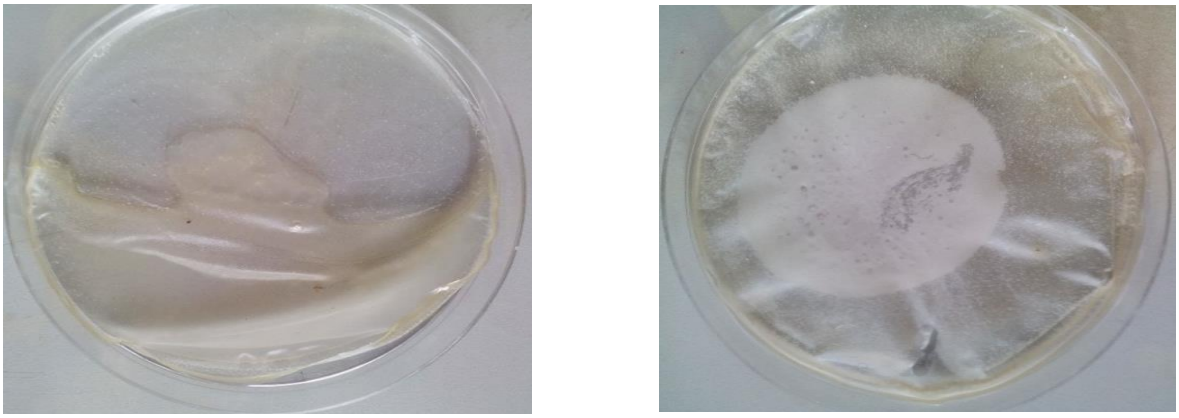


Figure 3. 7: **Multilayer Chitosan Film without Potassium Sorbate (Left) and Multilayer Chitosan Film with Potassium Sorbate (Right)**

3.3. Swelling Ratios and Diffusion Measurement

3.3.1 Swelling Ratios of Chitosan Multilayer Films

The swelling ratios of multilayer chitosan films were determined by immersing the dried multilayer chitosan films into 40 mL of distilled water at 25^0C and 37^0C. The swelling and release behavior of the antimicrobial agent (potassium sorbate and inherent) were then studied. The average swelling ratios (SR_A) were obtained by soaking the multilayer chitosan films in distilled water at temperatures between 25 ˚C and 37˚C. The average swelling ratios (SR_A) of the films were computed using the equation:

$$SR_A = (M_o - M_t)/M_o \quad (5)$$

Where M_t is the mass of the multilayer chitosan films at time t and M_o is the mass of the dried multilayer chitosan films at time, t = 0. The equilibrium swelling ratio (SR_{eq}) was obtained from the following expression [55]:

$$SR_{eq} = (M_{eq} - M_o)/M_o \quad (6)$$

Where M_{eq} is the mass of the film at equilibrium (after 648,000 seconds of soaking) and M_o is the initial mass of the film. The fluid uptakes by the film were obtained from [56]:

$$M_{rel} = M_t/M_s \quad (7)$$

Where M_s is the swollen mass at any given temperature and M_t is the mass of the hydrogel at time, t. The equilibrium volume was obtained from:

$$V_{eq} = \frac{\left(\frac{\pi D^2}{4}\right)}{\left(\frac{\pi D_\circ^2}{4}\right)} = \frac{D^2}{D_\circ^2} \quad (8)$$

Where D_o and D, are the diameters of the films before and after equilibrium swelling.

Release Exponents (n) Associated with Sample Geometry			**Drug Release Mechanism**	**Rate (dM_t/dt) as a function of time**
Slab	**Cylinder**	**Sphere**		
0.5	0.45	0.43	Controlled Diffusion (Fickian Diffusion)	t^{-n}
0.5 < n < 1.0	0.45 < n < 0.89	0.43 < n < 0.85	Anomalous Transport (Non Fickian Transport)	t^{n-1}
1.0	0.89	0.85	Controlled Swelling (Case II-Transport)	Zero-order (time-dependent) release
n > 1	n > 0.89	n > 0.85	Super-Case-II transport	t^{n-1}

Table 3. 1: **Summary of Exponents Associated with Diffusion Mechanisms in Antimicrobial Agents Eluting from Polymeric Films (Siepmann and Siepmann 2008, Peppas, 1985).**

The figures below show the different classes of non-Fickian sorption.

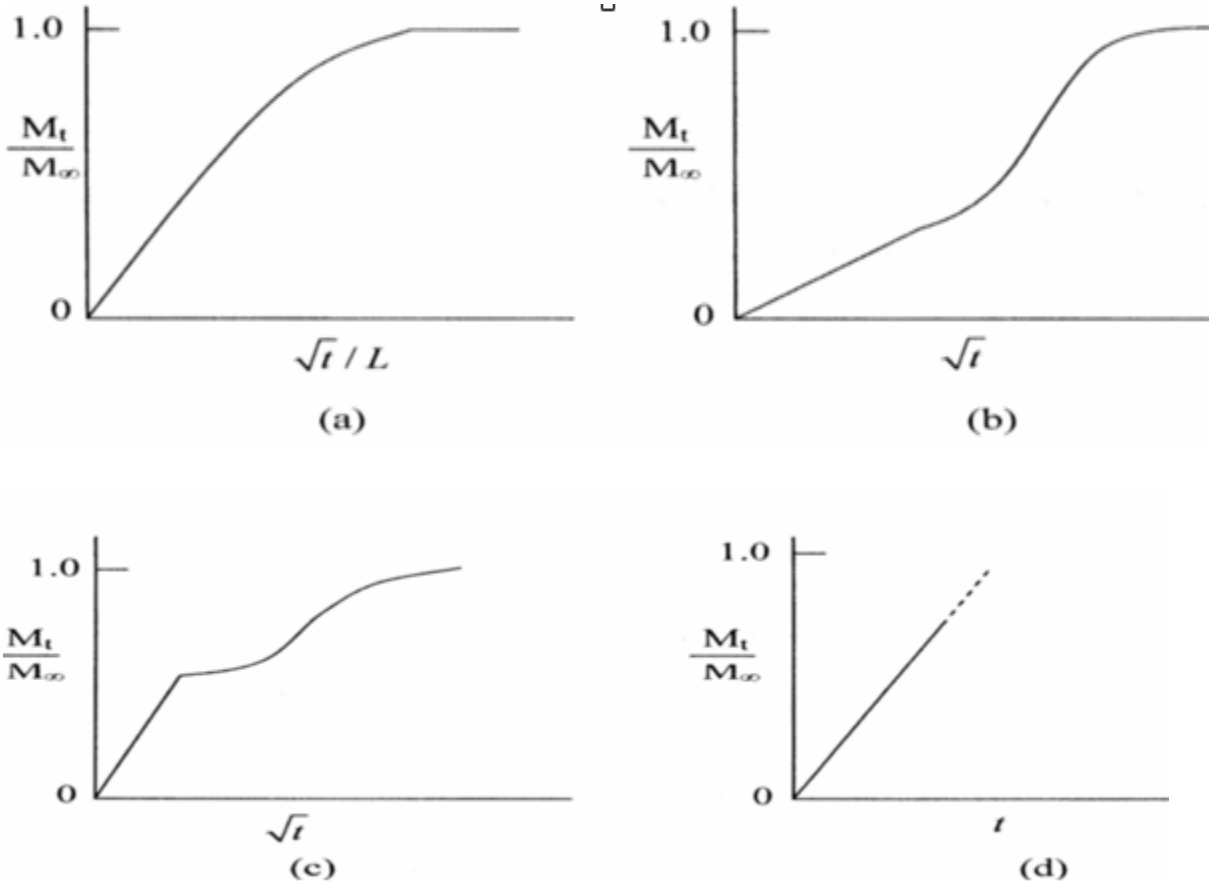


Figure 3. 8: **Different Classes of non-Fickian Sorption: (a) Classical; (b) Sigmoidal; (c) Two-step; and (d) Case II (Kee et al., 2005).**

3.5.2 Diffusion Mechanisms

The fluid release exponent, n, and the diffusion constant, k, were determined from the equation (9) [57]:

$$\frac{m_t}{m_i} = 4\left(\frac{Dt}{\pi\delta^2}\right)^n = kt^n \qquad (9a)$$

$$m_t = m_o - m_f \quad (9b)$$

where $\frac{m_t}{m_i}$ is the fraction of fluid or antimicrobial agent release at time, t, δ is the thickness of the film, D is the diffusivity, k is the geometric constant of the release system, m_i is the absolute cumulative amount of antimicrobial agent released at time, t, m_t is the amount of antimicrobial agent /fluid remaining in the film at time, t during antimicrobial agent elution, δ is the thickness of the film. From equation (9b), m_o is the mass of the swollen film at equilibrium state prior to antimicrobial agent release and m_f is the final mass of the film after antimicrobial agent elution. The fractional release of antimicrobial agent from the polymers is exponentially related to the release time. The release exponent, n, corresponds to the mechanism of antimicrobial agent release. For a cylindrical sample, n = 0.45 corresponds to Fickian diffusion, and the release rate is then dependent on $t^{-0.5}$. When n is between 0.45 and 0.85, non-Fickian diffusional release occurs, while n = 0.89 is a case-II transport [58][59]. The constants k and n were obtained from the linear plot of equation (9a). This gives:

$$ln\left({}^{m_t}/_{m_i}\right) = \log k + n \log t \quad (10)$$

Where k and n are obtained, respectively, from the intercepts and slopes of the plots of $\ln(m_t/m_i)$ versus ln (t). The diffusion coefficient, D, was obtained from:

$$D = \frac{k\pi\delta^2}{4} \quad (11)$$

Where δ the thickness of the film, and k is a geometric factor of the gel.

The swelling ratios were achieved by weighing the sample before swelling and after every one hour until the values of the sample were observed to not increase after several hours. The below tables identify the values achieved.

RESULTS FOR SWELLING OF THE CHITOSAN FILMS				
	At 25^{0}C		At 37^{0}C	
	Sample (A) no KS	**Sample (B) with KS**	**Sample (A) no KS**	**Sample (B) with KS**
Diameter of film before swelling (m)	0.0103	0.0103	0.0103	0.0103
Thickness of film before swelling (m)	0.002	0.002	0.002	0.002
Weight of film before swelling (g)	0.004	0.009	0.004	0.002
Time (sec.)	**Mass 1 (g)**	**Mass 2 (g)**	**Mass 1 (g)**	**Mass 2 (g)**
54000	0.034	0.048	0.041	0.022
108000	0.035	0.055	0.044	0.03
162000	0.043	0.059	0.046	0.031
216000	0.044	0.06	0.048	0.035
270000	0.044	0.08	0.049	0.037
324000	0.046	0.082	0.05	0.04
378000	0.047	0.085	0.051	0.041
432000	0.048	0.088	0.052	0.043
486000	0.049	0.088	0.069	0.046
540000	0.049	0.118	0.072	0.06
594000	0.049	0.118	0.072	0.06
648000	0.049	0.118	0.072	0.06

Table 3. 2: **Results Obtained from Swelling of Chitosan Films**

Similarly, the release ability of the chitosan films were tested by measuring the de-swelling of the films. The below values were the values achieved from the de-swelling of the samples at two different temperatures.

RESULTS FOR DE-SWELLING OF THE CHITOSAN FILMS				
	At 25^{0}C		At 37^{0}C	
	Sample (A) no KS	**Sample (B) with KS**	**Sample (A) no KS**	**Sample (B) with KS**
Diameter of film before swelling (m)	0.0104	0.0104	0.0104	0.0104
Thickness of film before swelling (m)	0.0004	0.0004	0.0004	0.0004
Weight of film before swelling (g)	0.033	0.057	0.074	0.026
Time (sec.)	**Mass 1 (g)**	**Mass 2 (g)**	**Mass 1 (g)**	**Mass 2 (g)**
54000	0.03	0.05	0.56	0.021
108000	0.023	0.028	0.029	0.06
162000	0.019	0.012	0.016	0.06

216000	0.011	0.006	0.008	0.023
270000	0.008	0.006	0.005	0.015
324000	0.006	0.005	0.004	0.002
378000	0.006	0.005	0.004	0.002
432000	0.004	0.005	0.004	0.002
486000	0.004	0.005	0.004	0.002
540000	0.004	0.005	0.004	0.002
594000	0.004	0.005	0.004	0.002
648000	0.004	0.005	0.004	0.002

Table 3. 3: **Results Obtained from Swelling of Chitosan Films**

3.4 Morphological Characterization of Films

Morphology of the films was examined by celestron microscope (Model 44345, made in China) and scanning electron microscopy (SEM) on a Philips XL-30SFG model. SEM and EDX analysis was used to make elemental analysis of the samples using same SEM device. The SEM samples were coated with gold palladium using a Magnetron Sputter Coating Instrument.

3.5 Culturing of Microbes to Test the Antimicrobial Activity of the Film

3.5.1 Preparation of Potatoes Dextrose Agar (PDA) and Culture Media

To prepare the PDA solution, we used an analytical weighing balance and weighed 90g of chopped Irish potatoes placed it in 200ml of distilled water. The mixture was boiled in a water bath for 1 hour at 100°C. Meanwhile, the normal saline solution was formed by dissolving 0.9g of sodium chloride in 200ml of distilled water and thereafter, solution was subjected to shaking to ensure complete dissolution of the salt in water.

The source of nutrient for the microbes in PDA media are the potatoes broth and sucrose. In so doing, 6g sucrose of which was well dispensed in 300ml of distilled water using the standard of 20g of sucrose in 1liter of distilled water. Also, 4.5g of agar was dispensed in 300ml distilled water using a standard of 15g of agar in 1liter distilled water. The sucrose solution, agar solution

and 0.6g of chloramphenicol (to inhibit the growth of bacteria) were dispensed in potatoes broth that had been made up to 300mL and this combined solution is called potatoes dextrose agar (PDA).

The saline solution, PDA solution, bijou bottles and the petri dishes were all autoclaved at 121°C for 15minutes. After autoclaving for 15minutes, they were all allow to cool for 15minutes. Then they were transferred to the biosafety cabinet that has been sterilized and the PDA solution was pour in each of the Petri dishes and bijou bottle and allowed to jell. Upon jelling, portion of the soil sample (normal saline) was place in each of the Petri dishes and were gently spread out.

3.5.2 Preparation of the soil sample

Soil samples which was used to isolate the fungi were collected from the land fill refuse bin at Galadimawa, Abuja Nigeria. From the both soil samples, two grams of each of the soil were measured and place in a bijou bottles label A and B. Additionally, six pieces of bijou bottles for soil samples A and B were prepared.

For soil sample A and B, 2g of the soil from the refuse dump site were poured into the bijou bottles with labels ‘sample A, 24/10/2014’ and ‘sample B, 24/10/2014’ respectively. Then 9ml of normal saline solution were poured into each of the twelve bijou bottles so that it would be used for cell subculture.

3.5.3 Culturing of the Microbes

A stock solution was prepared using 2g of soil dispensed in 8ml normal saline solution. The bijou bottles were labeled 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}. Using syringe 1ml of the stock solution was added to the bottle labeled 10^{-1} which contains 9ml of the normal Saline solution to make it up to 10mL solution. Then 1ml of the content of the 10^{-1} bijou bottle was taken and then transferred to

10^{-2} bijou bottle, another 1ml of the stock was transferred to 10^{-3} and finally 1ml was transferred to 10^{-4}. Then two bottles the one label 10^{-4} and 10^{-4} were used to collect the sample to be transferred to the Petri dishes for culturing. Little quantity of both were place in separate Petri dishes for culturing. The growth of microbes were observed after 72 hours. Then the spores were subculture into another Petri dishes containing jell PDA solution by the used of wire lobe. This action was repeated several time in order to grow the different types of spores that were forming on the PDA media.

After several day of Sub-culturing the different types of spores that where found growing on the PDA media, they were view using light microscope. Aspergillus niger was identify from the results of the samples and was subculture for the zone of inhibition test.

3.5.4 Preparation of Test Organism

Aspergillus niger colonies were transferred form PDA slant and dissolved in 5ml sterile water. The culture was then incubated at 25°C for 2 hours and 100µl portions of it were dispersed onto PDA agar plates. The agar plates were incubated at 25°C for 5 days to develop Aspergillus niger spores. At the end of this period, the grown Aspergillus niger spores were harvested by sterile twee n-80 solution (0.05% w/ v) and added to the PDA slants. These slants were then further incubated at 25°C for 7 days and once more harvested by using sterile tween-80 solution.

3.5.5 Determining the Number of Aspergillus niger Spores

The 50 ml solution containing Aspergillus niger spores was centrifuged and the supernatant was discarded. The precipitated pellet was dissolved in sterile peptone water (0.1% w/v) and then the number of spores was determined. In order to adjust the spore number, 100 µl of sterile peptone water containing precipitated pellet was taken and diluted with 1 ml distilled water and the

number of spores were counted under microscope by using a Thoma Counting Chamber. After necessary dilutions the spore number was adjusted to $24x10^6$ spores/ml.

3.5.6 Zone of Inhibition Test

The antimicrobial activity was determined by the classical zone of inhibition test. For this purpose 100 µl of the Aspergillus niger culture was transferred and spread onto PDA agar plates. Then, 1.3 cm diameter discs of films was obtained aseptically with surgical blade and was place with the wire loop at the two extreme of each Petri dish for the sample with potassium sorbate. Meanwhile, the samples with potassium sorbate were placed in the middle of the petri dishes. The Petri dishes were incubated at 25°C during 7 days and monitored for growth of Aspergillus niger and zone formation. The diameter of the zones was measured with a caliper after days.

3.6 FTIR (Fourier Transform Infrared) spectroscopy analysis.

The deacetylation step in the production of chitosan is very essential and if it is not done properly, chitosan cannot be achieved. To provide if the powder achieved was chitosan, FT-IR analysis was done on the powder. Unlike many researchers who prepared their samples with potassium bromide (KBr) before analyzing with FT-IR, the sample used for this FT-IR analysis was not prepared with KBr. The produced powder after deacetylation was analysis at Nigerian Turkish Nile University; Abuja, Nigeria using the IR Affinity – 1 FT-IR spectrometer without the combination of any other chemical.

This was done because, FTIR offers quantitative and qualitative analysis for organic and inorganic samples. The Fourier Transform Infrared Spectroscopy (FTIR) used, identified the chemical bonds in the chitosan powder by producing an infrared absorption spectrum. The spectra produce a profile of the chitosan. FTIR is an effective analytical instrument for detecting

functional groups and characterizing covalent bonding information. Below is a chat showing the standard absorption frequencies of the functional groups.

Characteristic IR Absorption Frequencies of Organic Functional Groups			
Functional Group	**Type of Vibration**	**Characteristic Absorptions (cm-1)**	**Intensity**
Alcohol			
O-H	(stretch, H-bonded)	3200-3600	strong, broad
O-H	(stretch, free)	3500-3700	strong, sharp
C-O	(stretch)	1050-1150	Strong
Alkane			
C-H	stretch	2850-3000	Strong
-C-H	bending	1350-1480	Variable
Alkene			
=C-H	stretch	3010-3100	Medium
=C-H	bending	675-1000	Strong
C=C	stretch	1620-1680	Variable
Alkyl Halide			
C-F	stretch	1000-1400	Strong
C-Cl	stretch	600-800	Strong
C-Br	stretch	500-600	Strong
C-I	stretch	500	Strong
Alkyne			
C-H	stretch	3300	strong, sharp
-C≡C-	stretch	2100-2260	variable, not present in symmetrical alkynes
Amine			
N-H	stretch	3300-3500	medium (primary amines have two bands; secondary have one band, often very weak)
C-N	stretch	1080-1360	medium-weak
N-H	bending	1600	Medium
Aromatic			
C-H	stretch	3000-3100	Medium

C=C	stretch	1400-1600	medium-weak, multiple bands
Analysis of C-H out-of-plane bending can often distinguish substitution patterns			
Carbonyl	Detailed Information on Carbonyl IR		
C=O	stretch	1670-1820	strong
(conjugation moves absorptions to lower wave numbers)			
Ether			
C-O	stretch	1000-1300 (1070-1150)	strong
Nitrile			
CN	stretch	2210-2260	medium
Nitro			
N-O	stretch	1515-1560 & 1345-1385	strong, two bands

IR Absorption Frequencies of Functional Groups Containing a Carbonyl (C=O)

Functional Group	Type of Vibration	Characteristic Absorptions (cm^{-1})	Intensity
Carbonyl			
C=O	Stretch	1670-1820	strong
(conjugation moves absorptions to lower wave numbers)			
Acid			
C=O	Stretch	1700-1725	strong
O-H	Stretch	2500-3300	strong, very broad
C-O	Stretch	1210-1320	strong
Aldehyde			
C=O	Stretch	1740-1720	strong
=C-H	Stretch	2820-2850 & 2720-2750	medium, two peaks
Amide			
C=O	Stretch	1640-1690	strong
N-H	Stretch	3100-3500	Un-substituted have two bands
N-H	Bending	1550-1640	
Anhydride			
C=O	Stretch	1800-1830 & 1740-1775	two bands
Ester			

C=O	Stretch	1735-1750	strong
C-O	Stretch	1000-1300	two bands or more
Ketone			
Acyclic	Stretch	1705-1725	strong
Cyclic	Stretch	3-membered - 1850 4-membered - 1780 5-membered - 1745 6-membered - 1715 7-membered – 1705	strong
α,β-unsaturated	Stretch	1665-1685	strong
aryl ketone	Stretch	1680-1700	strong

Table 3. 4: **Characteristic IR Absorption Frequencies of Organic Functional Groups**

(Silverstein, R.M.; Bassler, G.C.; and Morrill, T.C. Spectrometric Identification of Organic Compounds. 4th Ed. New York: John Wiley and Sons, 1981. QD272.S6 S55)

3.7 UV-VIS Spectrometer Measurements

An ultra-violet-visible (UV-VIS) spectrometer (CECIL 7500 Series, Buck Scientific Inc., East Norwalk, USA) was used to obtain the absorbance and wavelengths of potassium sorbate diffused from the multilayer chitosan film at 37˚C. The absorbance of potassium sorbate was obtained at wavelengths of 250. The UV analysis was done by dissolved a cylindrical shape chitosan film, which contained potassium sorbate, in a 10 ml distilled water. Five milliliters of the distilled water was removed from the sample daily and kept in a cylindrical bottle. After several days, the samples were taken to SHESTCO laboratory for UV analysis. A quartz cell was filled half way with distilled water and its absorbance was obtained (controlled sample). Subsequently, each of the samples were loaded alone with the distilled water and the absorbance were recorded. This was done at 37˚C.

From Beer-Lambert law, the concentration, absorbance, A, and molar absorptivity, ε, were related to UV-Visible adsorption spectrograph via (Shane et al., 2006):

$$\varepsilon = \frac{A}{LC} \quad (1)$$

Where A is the absorbance of the potassium sorbate, L is the path length of the quartz cell used (1 cm), and C is the concentration of the potassium sorbate.

3.8 References

[37] M. Rinaudo, "Chitin and chitosan : Properties and applications," *Sci. Direct*, vol. 31, pp. 603–632, 2006.

[38] P. K. Dutta, J. Dutta, and V. S. Tripathi, "Chitin and chitosan : Chemistry , properties and applications," *J. Sci. Ind. Res. (India).*, vol. 63, pp. 20–31, 2004.

[54] N. Van Toan, "Production of Chitin and Chitosan from Partially Autolyzed Shrimp Shell Materials," *Open Biomater. J.*, vol. 1, pp. 21–24, Oct. 2009.

[55] O. O. and Y. Dogu, "Swelling – Deswelling Kinetics of Poly(Nisopropylacrylamide) Hydrogels Formed in PEG Solutions," *Wiley Period.*, 2005.

[56] K. Nam, K., Watanabe, J., & Ishihara, "Modeling of swelling and drug release behavior of spontaneously forming hydrogels composed of phospholipid polymers," *Int. J. Pharm.*, vol. 275, no. 1, pp. 259–269, 2004.

[57] A. N. Peppas, "Analysis of Fickian and Non-Fickian Drug Release from polymers," *Pharm. HEL.*, vol. 60, no. 4, p. 1985, 1985.

[58] I. R.W. Baker, H.K. Lonsdale, "Tanquary A.C. and Lacey R.E. Controlled Release of Biologically active Agents.," *Plenum Publ. New York*, pp. pp15 – 71, 1974.

[59] A. A. Lamberti, G., Galdi, I., & Barba, "Controlled release from hydrogel-based solid matrices. A model accounting for water up-take, swelling and erosion," *Int. J. Pharm.*, vol. 407, no. 1, pp. 78–86, 2011.

[60] S. A. (2009). Gemili, S., Yemenicioğlu, A., & Altınkaya, “Development of cellulose acetate based antimicrobial food packaging materials for controlled release of lysozyme,” *J. Food Eng.*, vol. 90, no. 4, pp. 453–462, 2009.

CHAPTER FOUR: RESULTS AND DISCUSSION

4.1 Results

4.1 Chitosan Powder analysis

In this work, the chitosan powder was produced from shrimp shells which was used for the production of multilayer films. The chitosan powder was produced by extracting the shells from the shrimps. Chitin extraction which was achieved by using alkali acid (6N HCl) to treat shrimp shells powder. The chitin achieved was then treated with 1% NaOH (w/w) so as to form the chitosan. The chitosan powder was characterized using (IR affinity-1) FT-IR spectrometer.

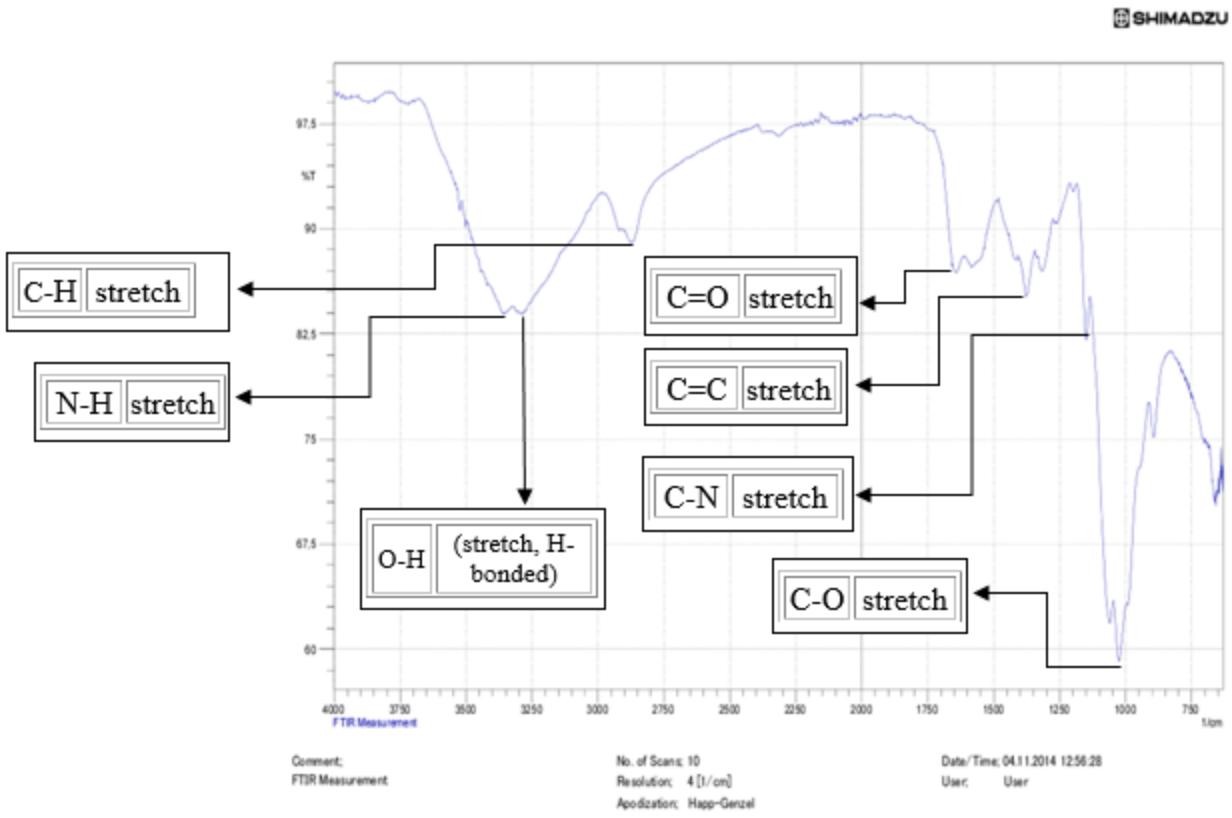


Figure 4. 1: **FT-IR Spectroscopy Result for Produced Chitosan**

The FTIR spectra were recorded in the middle infrared (4000 cm^{-1} to 400 cm^{-1}) with a resolution of 4 [1/ cm] in the absorbance mode for 10 scans at room temperature. The spectra was measured using a deuterated triglycerinesulphate detector (DTGS) with a specific detectivity of 1×10^{9} $cmHz^{1/2}$ w^{-1}. From the spectra observed in the above figure, it is shown that there are various peaks present. Each of the peaks represent a specific functional group. Those of significant importance to this research are highlighted in the figure 4.1. The amine peak can be seen in the figure at the peak of 3435 (N- H). The presence of amine (most especially NH_2) is significant in proving that the powder is chitosan. It also reveals that the deacetylation of chitin to chitosan was done properly. The below table shows the various peaks present in the FT-IR result and their functional groups.

Approximated Peak	Normal Peak range	Functional group
3435	(3100-3500)	N-H Stretch
3300	(2500-3300)	O-H Stretch, H-bounded
1687	(1640-1690)	C=O Stretch
1437	(1400-1600)	C=C Stretch
1031	(1000-1300)	C-O Stretch

Table 4. 1: **Peaks Present in the FT-IR Result and their Functional Groups**

In addition to the FT-IR analysis to prove that the powder achieved was chitosan, the chitosan powder was dissolved in acetic acid. After few seconds, the powder was able to dissolve 90% and also was able to jell. These two in addition to the FT-IR result proved that the power produced was chitosan. Additionally, these results proved that it is easy to produce chitosan on a large scale in Africa for films production and other applications.

Figure 4. 2 **Chitosan powder (Left) and Chitosan film (Right)**

4.2 Films Morphology

The morphological features of the films prepared by casting method changed based on varying the processing conditions. Among these conditions, the composition of the initial casting solution was found to play a significant role on the structure of the films [60][61]. The initial casted films appeared to be denser as compared to the porous middle layers. From the physical analysis carried out, the films appeared very smooth and flexible. The microscopic analysis revealed that the sample without potassium sorbate had lot of smaller sizes of pores with the average diameter of 4.4 μm. While the sample with potassium sorbate had larger sizes of pores with average diameter of 10.5 μm but fewer in number. The difference in the porous sizes is due to the chemical interaction between potassium sorbate and chitosan which led to the increase in the bond angles.

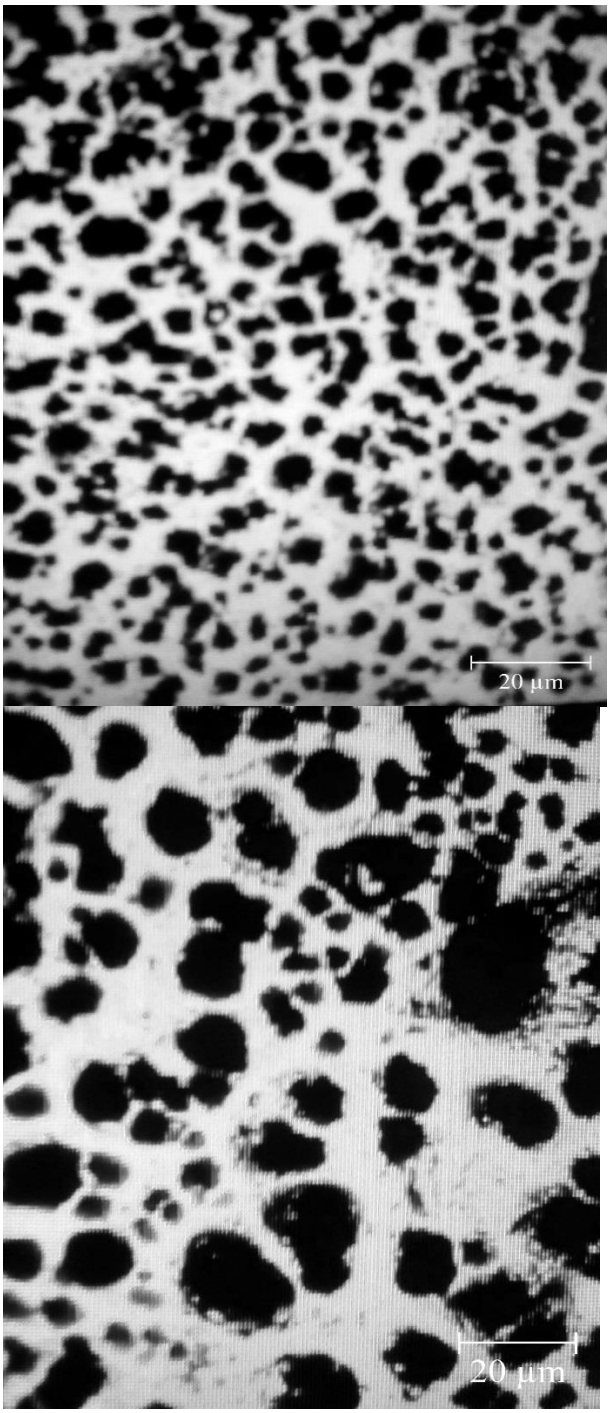


Figure 4. 3 **Microstructure of Chitosan Film without Potassium Sorbate (Left) and Microstructure of Chitosan Film with Potassium Sorbate (Right) at Magnification of 16000x**

In addition to the microscopic analysis, scanning electron microscope (SEM) was also used to analyze the multilayer chitosan films. The samples used for characterization were label A1, A2

and A3 for the films without potassium sorbate and B1, B2, B3, C1, C2, C3 and C4 for films with potassium sorbate. The films prepared in this study are not only porous but they also have asymmetric structures with a dense skin layer at the top and a porous layer in the bulk. This asymmetric structure leads to more crystal formation with larger sizes on the porous in the middle of the films.

Although higher drying temperature favors increased crystallization rates, the decrease in the porosity and pore size of the films becomes a more dominant factor in controlling the crystal formation. Thus, the number of crystals formed in the membrane decreased with increased drying temperature as shown by SEM pictures.

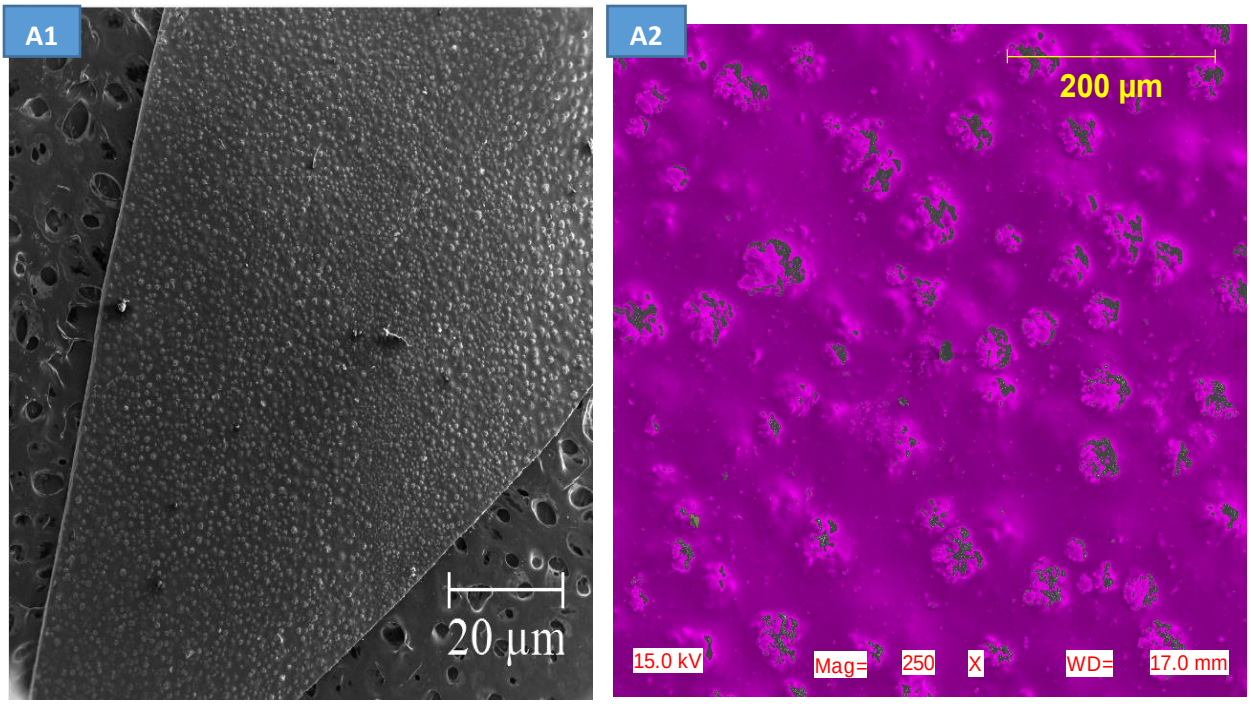


Figure 4. 4: **SEM Microstructure of Chitosan Film without Potassium Sorbate at Magnification of 100x (Left) and SEM Microstructure of Chitosan Film without Potassium sorbate at Magnification of 250x (Right)**

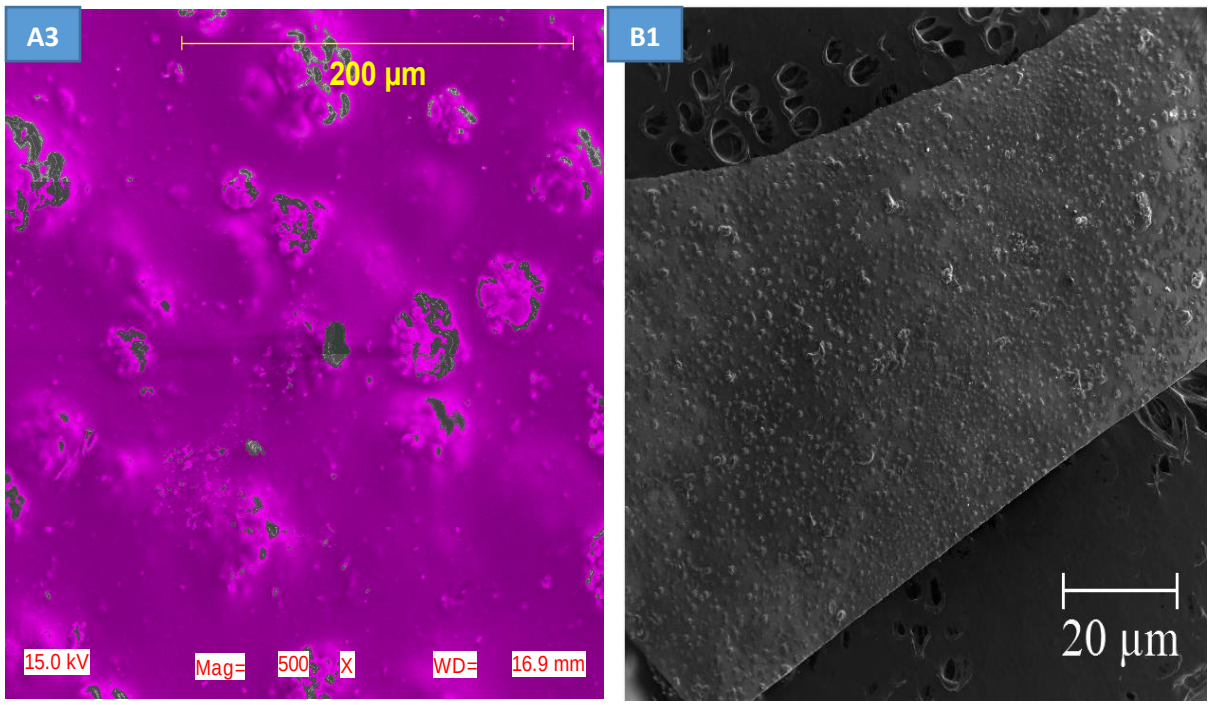


Figure 4. 5: **SEM Microstructure of Chitosan Film without Potassium Sorbate at Magnification of 500x (Left) and SEM Microstructure of Chitosan Film with Potassium Sorbate at Magnification of 100x (Right)**

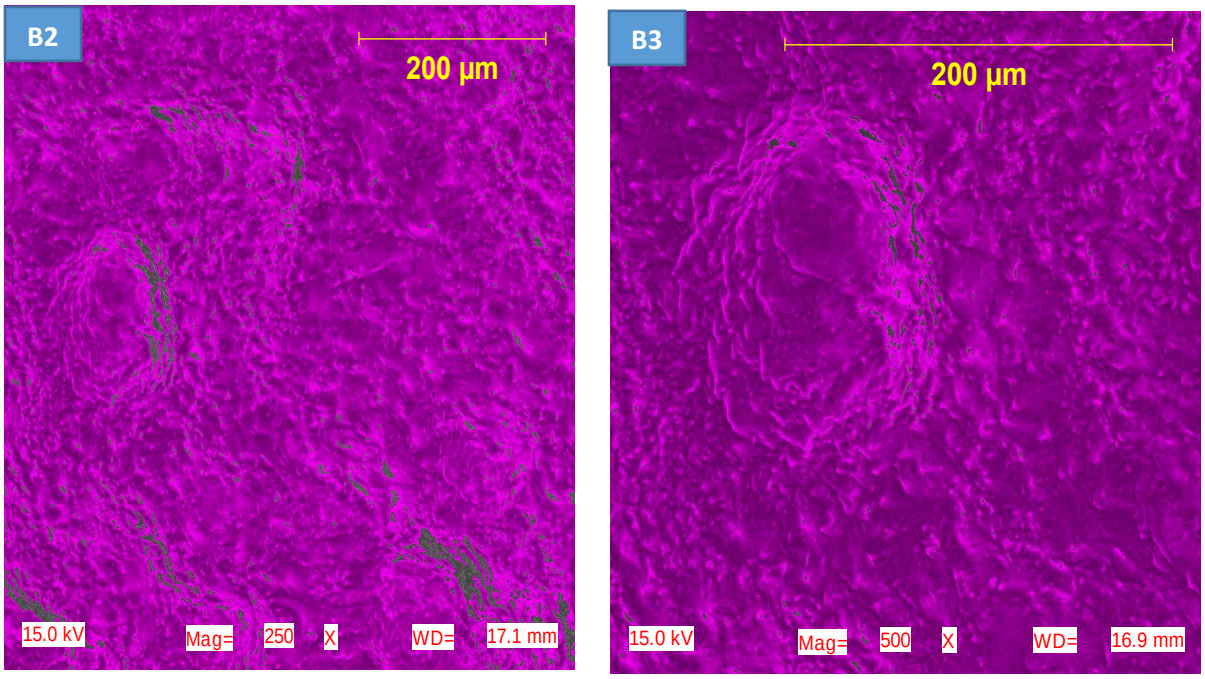


Figure 4. 6: **SEM Microstructure of Chitosan Film with Potassium Sorbate at Magnification of 250x (Left) and SEM Microstructure of Chitosan Film with Potassium Sorbate at Magnification of 500x (Right)**

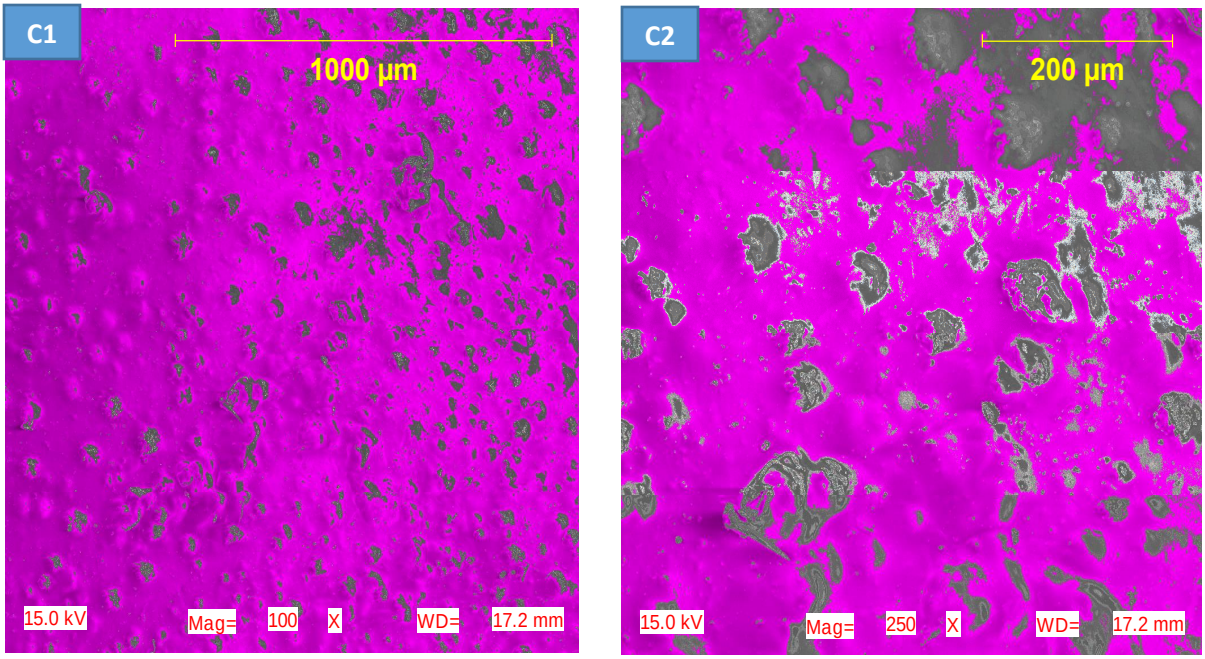


Figure 4. 7: **SEM Microstructure of Chitosan Film with Potassium Sorbate at Magnification of 100x (Left) and SEM Microstructure of Chitosan Film with Potassium Sorbate at Magnification of 250x (Right)**

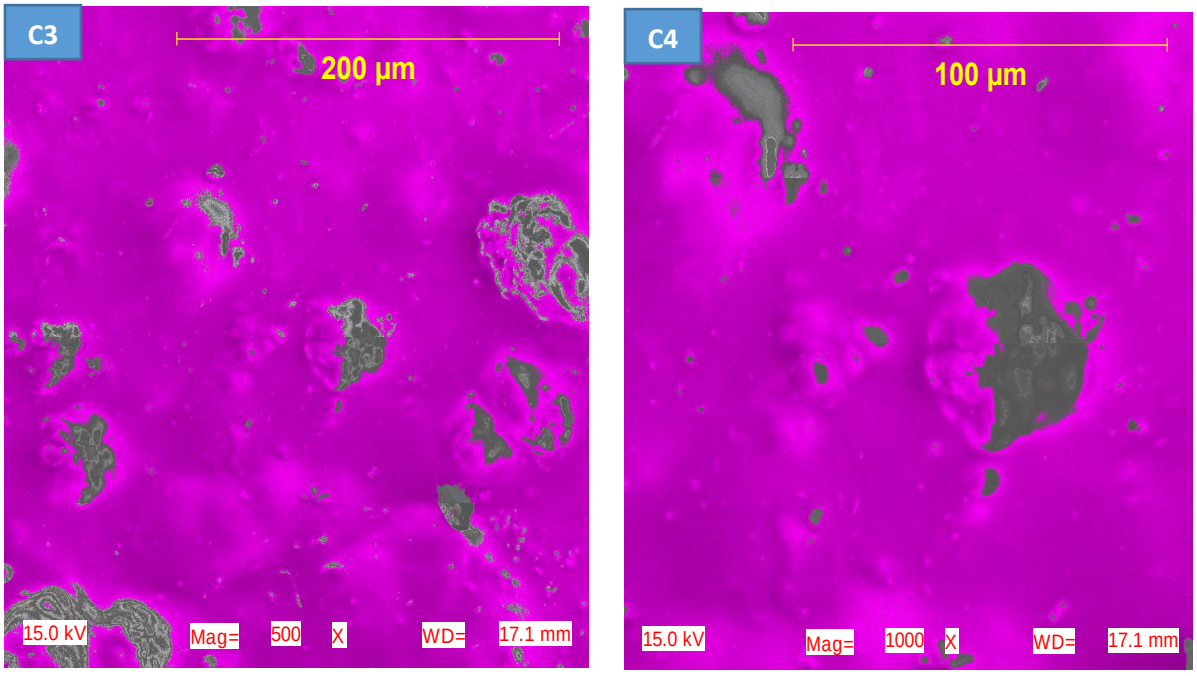


Figure 4. 8 **SEM Microstructure of Chitosan Film with Potassium Sorbate at Magnification of 500x (Left) and SEM Microstructure of Chitosan Film with Potassium Sorbate at Magnification of 1000x (Right)**

From these samples, it is realized that the films are not only porous but they also have asymmetric structures with a dense skin layer at the top and a porous layer in the bulk. Figure (A1) and (B1) reveal the presence of the dense layers from the porous layers. Meanwhile, the surface morphologies show the presence of some dendritic structures. These came about after the phase separation occurred in the environmental chamber, further drying in the vacuum oven led to the penetration of potassium sorbate into the pores and an increase in its concentration in the pores. This increases supersaturation and induces crystal growth in the pores. Similar phenomena were observed by Caussy (2006) in stones and porous sedimentary rocks which are exposed to sea-salts [62][63].

4.3 Swelling and De-swelling Analysis

In line with the previous observations, three different casting compositions were selected as shown in Table 4.2. The difference in the casting composition was done to enhance the release from the films. The pores in these simples ranging from 4.4 µm to 10.5 µm which were measured using Gwyddion software. The measured porous size reveals that the films had excellent swelling and diffusion ability. To further prove this, the swelling and de-swelling abilities of the films were study by dissolving the films in a distilled water and weighing them after every one hour. The below charts and plots reveal the values achieved at two different temperatures, 25^0C and 37^0C.

Weight of Four Components Used in forming the film			
Polymer (Chitosan)	**Solvent (Acetic acid)**	**Non-Solvent (Distilled water)**	**Antimicrobial Agent (Potassium sorbate)**
1g	1%	24.5 ml	0.5g
2g	4%	48ml	2g

Table 4. 2: **The Compositions of Polymer, Solvent, non-solvent and Antimicrobial Agent in the Film Forming Solutions**

RESULTS FOR SWELLING OF THE CHITOSAN FILMS At 25^{0}C	
Sample (A) no KS	
Diameter of film before swelling (m)	0.0103
Thickness of film before swelling (m)	0.002
Weight of film before swelling (g)	0.004

Time (t)	**Ln t (s)**	**Sample (A) no KS**	**Average swelling ratio**	**Equilibrium swelling**	**Water Retention**	**Initial & final weight difference**	**Release Fraction**	**ln (M_o/M_t)**
		Mass (g)	Sra =(M_t-M_0)/M_0	Sreq=(M_{eq}-M_0)/m_0	**Mrel**	**Mass (g)**	M_o/M_t	
54000	**10.8967**	0.034	0	0.441176	1	0.015	2.266667	0.81831
108000	**11.5899**	0.035	0.029412		1.029412		2.333333	0.847298
162000	**11.9954**	0.043	0.264706		1.264706		2.866667	1.05315
216000	**12.283**	0.044	0.294118		1.294118		2.933333	1.076139
270000	**12.5062**	0.044	0.294118		1.294118		2.933333	1.076139
324000	**12.6885**	0.046	0.352941		1.352941		3.066667	1.120591
378000	**12.8426**	0.047	0.382353		1.382353		3.133333	1.142097
432000	**12.9762**	0.048	0.411765		1.411765		3.2	1.163151
486000	**13.094**	0.049	0.441176		1.441176		3.266667	1.18377
540000	**13.1993**	0.049	0.441176		1.441176		3.266667	1.18377
594000	**13.2946**	0.049	0.441176		1.441176		3.266667	1.18377
648000	**13.3816**	0.049	0.441176		1.441176		3.266667	1.18377

Table 4. 3: **Results for the Swelling of the Multilayer Chitosan Film at 25^{0}C (Sample A)**

From the values achieved after twelve hours of swelling and weighing, we were able to calculate the following parameters: the average swelling ratio, equilibrium swelling, water retention, initial

& final weight difference and release fraction. From the valves achieved after the calculations of the average swelling ratio, water retention and release fraction, we released that between 486000 to 684000 seconds there was no change in the results. This was because, at these time interval, the film had reached its peak of swelling and there was no Swelling occurring. Meanwhile, from the calculations, we were able to plot the natural log of release ratio against the natural log of the time so as to observe what was going on.

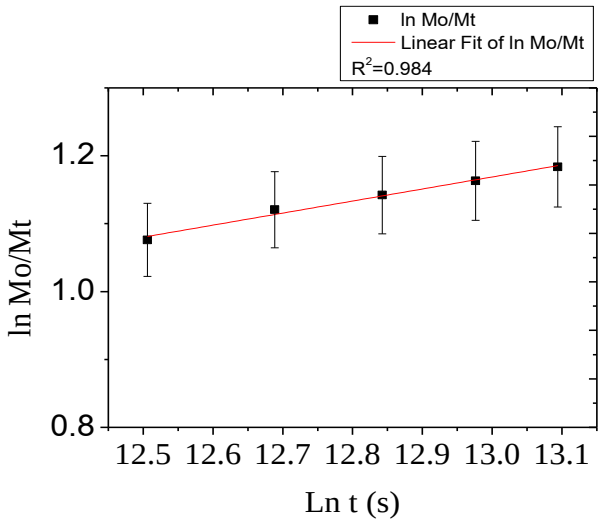


Figure 4. 9: **Plot Showing the Swelling of the Multilayer Chitosan Film without Potassium Sorbate at 25^0C (Sample A)**

From the plot, it is shown that the chitosan film without potassium sorbate was able to swell at the temperature of 25^0C. It reveals that multilayer chitosan films have the potential of swelling at low temperature.

RESULTS FOR SWELLING OF THE CHITOSAN FILMS At 25^0C Sample (B) with KS	
Diameter of film before swelling (m)	0.0103

Thickness of film before swelling (m)						0.002		
Weight of film before swelling (g)						0.009		
Time (t)	**Ln t (s)**	**Sample (A) no KS**	**Average swelling ratio**	**Equilibrium swelling**	**Water Retention**	**Initial & final weight difference**	**Release Fraction**	**ln (M_o/M_t)**
		Mass (g)	Sra =(M_t-M_0)/M_0	Sreq=(M_{eq}-M_0)/m_0	**Mrel**	**Mass (g)**	M_o/M_t	
54000	**10.8967**	0.048	0	1.458333	1	0.07	0.685714	-0.37729
108000	**11.5899**	0.055	0.145833		1.145833		0.785714	-0.24116
162000	**11.9954**	0.059	0.229167		1.229167		0.842857	-0.17096
216000	**12.283**	0.06	0.25		1.25		0.857143	-0.15415
270000	**12.5062**	0.08	0.666667		1.666667		1.142857	0.133531
324000	**12.6885**	0.082	0.708333		1.708333		1.171429	0.158224
378000	**12.8426**	0.085	0.770833		1.770833		1.214286	0.194156
432000	**12.9762**	0.088	0.833333		1.833333		1.257143	0.228842
486000	**13.094**	0.088	0.833333		1.833333		1.257143	0.228842
540000	**13.1993**	0.118	1.458333		2.458333		1.685714	0.522189
594000	**13.2946**	0.118	1.458333		2.458333		1.685714	0.522189
648000	**13.3816**	0.118	1.458333		2.458333		1.685714	0.522189

Table 4. 4: **Results for the Swelling of the Multilayer Chitosan Film at 25^0C (Sample B)**

From the valves achieved after the calculations of the average swelling ratio, water retention and release fraction, we released that between 540000 to 684000 seconds there was no change in the results. This was because, at these time interval, the film had reached its peak of swelling and there was no Swelling occurring. Therefore, comparing the results achieved form the films with potassium sorbate to that of the without potassium sorbate, we realized that the values achieved from the films with potassium sorbate where much higher as compare to the films without

potassium sorbate. This was due to the bond angles between potassium sorbate and chitosan which lead to the increase in the porous sizes.

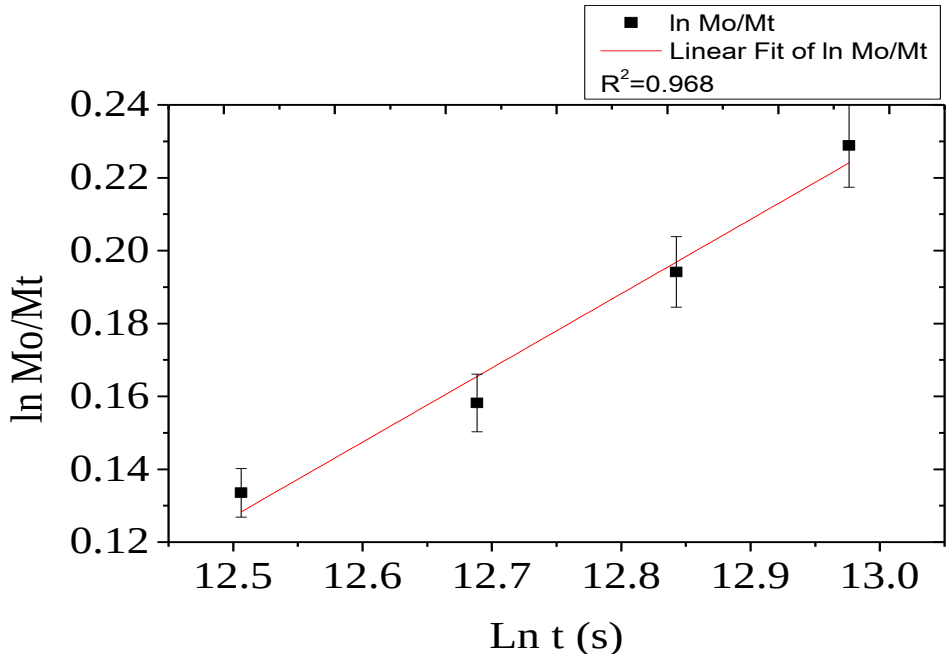


Figure 4. 10: **Plot Showing the Swelling of the Multilayer Chitosan Film with Potassium Sorbate at 25^{0}C (Sample B)**

From this plot, it is shown that the chitosan film produced with incorporated potassium sorbate was able to swell at the temperature of 25^{0}C. This reveals that chitosan films with other chemicals or natural antimicrobial agents have the potential of swelling at low temperature.

RESULTS FOR SWELLING OF THE CHITOSAN FILMS At 37^{0}C **Sample (A) with no KS**								
Diameter of film before swelling (m)					0.0103			
Thickness of film before swelling (m)					0.002			
Weight of film before swelling (g)					0.004			
Time (t)	**Ln t (s)**	**Sample (A) no KS**	**Average swelling ratio**	**Equilibrium swelling**	**Water Retention**	**Initial & final weight difference**	**Release Fraction**	**ln (M_o/M_t)**

		Mass (g)	Sra=(M_t-M_0)/M_0	Sreq=(M_{eq}-M_0)/m_0	Mrel	Mass (g)	M_o/M_t	
54000	**10.8967**	0.041	0	0.756098	1	0.031	1.322581	0.279585
108000	**11.5899**	0.044	0.073171		1.073171		1.419355	0.350202
162000	**11.9954**	0.046	0.121951		1.121951		1.483871	0.394654
216000	**12.283**	0.048	0.170732		1.170732		1.548387	0.437214
270000	**12.5062**	0.049	0.195122		1.195122		1.580645	0.457833
324000	**12.6885**	0.05	0.219512		1.219512		1.612903	0.478036
378000	**12.8426**	0.051	0.243902		1.243902		1.645161	0.497838
432000	**12.9762**	0.052	0.268293		1.268293		1.677419	0.517257
486000	**13.094**	0.069	0.682927		1.682927		2.225806	0.800119
540000	**13.1993**	0.072	0.756098		1.756098		2.322581	0.842679
594000	**13.2946**	0.072	0.756098		1.756098		2.322581	0.842679
648000	**13.3816**	0.072	0.756098		1.756098		2.322581	0.842679

Table 4. 5: **Results for the Swelling of the Multilayer Chitosan Film at 37^0C (Sample A)**

From the valves achieved after the calculations of the average swelling ratio, water retention and release fraction, we released that between 540000 to 684000 seconds there was no change in the results. This was because, at these time interval, the film had reached its peak of swelling and there was no swelling occurring. Meanwhile, comparing the results achieved at 37^oC to that 25^oC for films without potassium sorbate, we realized that at elevated temperature, the swelling increased. This reveals that at higher temperature, chitosan film without potassium sorbate can swelling faster as compare to those at low temperature.

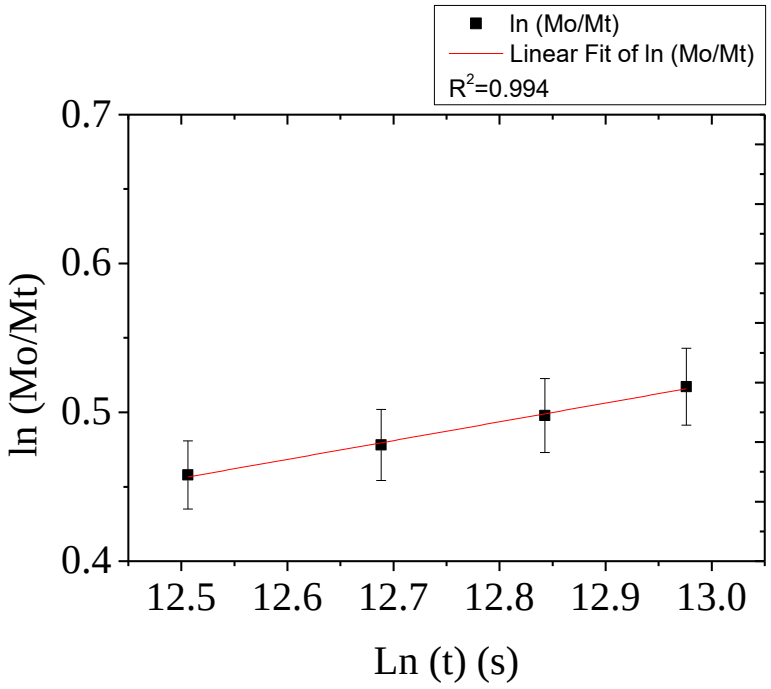


Figure 4. 11: **Plot Showing the Swelling of the Multilayer Chitosan Film at 37^{0}C (Sample A)**

From this plot, it is shown that the chitosan film produced at elevated temperature was still able to swell significantly. This also reveals that at higher temperature, the chitosan films without added antimicrobial agents have the ability of swell.

RESULTS FOR SWELLING OF THE CHITOSAN FILMS At 37^{0}C Sample (B) with KS								
Diameter of film before swelling (m)					0.0103			
Thickness of film before swelling (m)					0.002			
Weight of film before swelling (g)					0.004			
Time (t)	**Ln t (s)**	**Sample (A) no KS**	**Average swelling ratio**	**Equilibrium swelling**	**Water Retention**	**Initial & final weight difference**	**Release Fraction**	Ln (M_o/M_t)
		Mass (g)	Sra=(M_t-M_0)/M_0	Sreq=(M_{eq}-M_0)/m_0	**Mrel**	**Mass (g)**	M_o/M_t	
54000	**10.8967**	0.022	0	1.727273	1	0.038	1.727273	0.546544

108000	**11.5899**	0.03	0.363636		1.363636		1.266667	0.236389
162000	**11.9954**	0.031	0.409091		1.409091		1.225806	0.203599
216000	**12.283**	0.035	0.590909		1.590909		1.085714	0.082238
270000	**12.5062**	0.037	0.681818		1.681818		1.027027	0.026668
324000	**12.6885**	0.04	0.818182		1.818182		0.95	-0.05129
378000	**12.8426**	0.041	0.863636		1.863636		0.926829	-0.07599
432000	**12.9762**	0.043	0.954545		1.954545		0.883721	-0.12361
486000	**13.094**	0.046	1.090909		2.090909		0.826087	-0.19106
540000	**13.1993**	0.06	1.727273		2.727273		0.633333	-0.45676
594000	**13.2946**	0.06	1.727273		2.727273		0.633333	-0.45676
648000	**13.3816**	0.06	1.727273		2.727273		0.633333	-0.45676

Table 4. 6: **Results for the Swelling of the Multilayer Chitosan Film at 37°C (Sample B)**

Based on the data acquired, it is shown that at elevated temperature, the chitosan film with potassium sorbate was able to swell despite the presence of potassium sorbate. The swelling was enhanced by the increased in temperature as compare to the sample at 25°C.

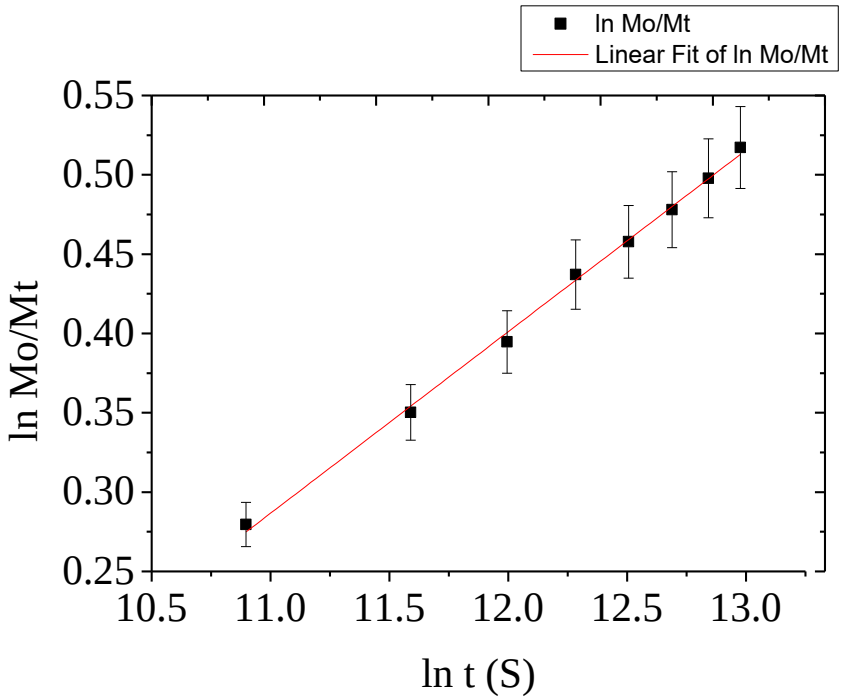


Figure 4. 12: **Plot Showing the Swelling of the Multilayer Chitosan Film at 37^0C (Sample B)**

From the plot, it is shown that the chitosan film produced at elevated temperature was absorb significant amount of distilled. This also reveals that at higher temperature, the chitosan films have the ability of swelling.

Between the tenth and twelfth hours of weighing, it was observed that the values were constant. No changes were observed doing these reading. This reveal that the film had reach it pick of swelling and could swell no more. Therefore, the samples were weighed and kept in an environment of 25^0 C and 37^0 C respectively. This was done to study the de-swelling ability of the films by weighing after every hour. Below are the various value achieved and some calculated values for them.

RESULTS FOR DE-SWELLING OF THE CHITOSAN FILMS At 25^0C		
	Sample (A) no KS	**Sample (B) with KS**

Diameter of film before swelling (m)				0.0104		0.0104	
Thickness of film before swelling (m)				0.0004		0.0004	
Weight of film before swelling (g)				0.033		0.057	
Diffusion coefficient (Ds)				$7.0x10^{-9}m^2/s$		$3.76x10^{-12}m^2/s$	
Slope (n)				0.775		0.509	
Time (t)	**lnT/s**	**Sample (A) no KS[Mass(g)]**	**(M_t/M_o)**	**ln(M_t/M_o)**	**Sample (B) with KS KS[Mass(g)]**	**(M_t/M_o)**	**Ln (M_t/M_o)**
54000	**10.8967**	0.03	10	2.302585	0.05	1	0
108000	**11.5899**	0.023	13.04348	2.568288	0.028	1.785714	0.579818
162000	**11.9954**	0.019	15.78947	2.759343	0.012	4.166667	1.427116
216000	**12.283**	0.011	27.27273	3.305887	0.006	8.333333	2.120264
270000	**12.5062**	0.008	37.5	3.624341	0.006	8.333333	2.120264
324000	**12.6885**	0.006	50	3.912023	0.005	10	2.302585
378000	**12.8426**	0.006	50	3.912023	0.005	10	2.302585
432000	**12.9762**	0.004	75	4.317488	0.005	10	2.302585
486000	**13.094**	0.004	75	4.317488	0.005	10	2.302585
540000	**13.1993**	0.004	75	4.317488	0.005	10	2.302585
594000	**13.2946**	0.004	75	4.317488	0.005	10	2.302585
648000	**13.3816**	0.004	75	4.317488	0.005	10	2.302585

Table 4. 7: **Result for De-swelling of the Chitosan Film at 25^0C for Both Samples**

From the valves achieved after the calculations of the average swelling ratio, water retention and release fraction, we released that between 432000 to 684000 seconds there was no change in the results. This was because, at these time interval, the film had finally dried up and there was no water left with each of the porous. This result shows that multilayer chitosan films have the ability of retaining chemical and release it after long time.

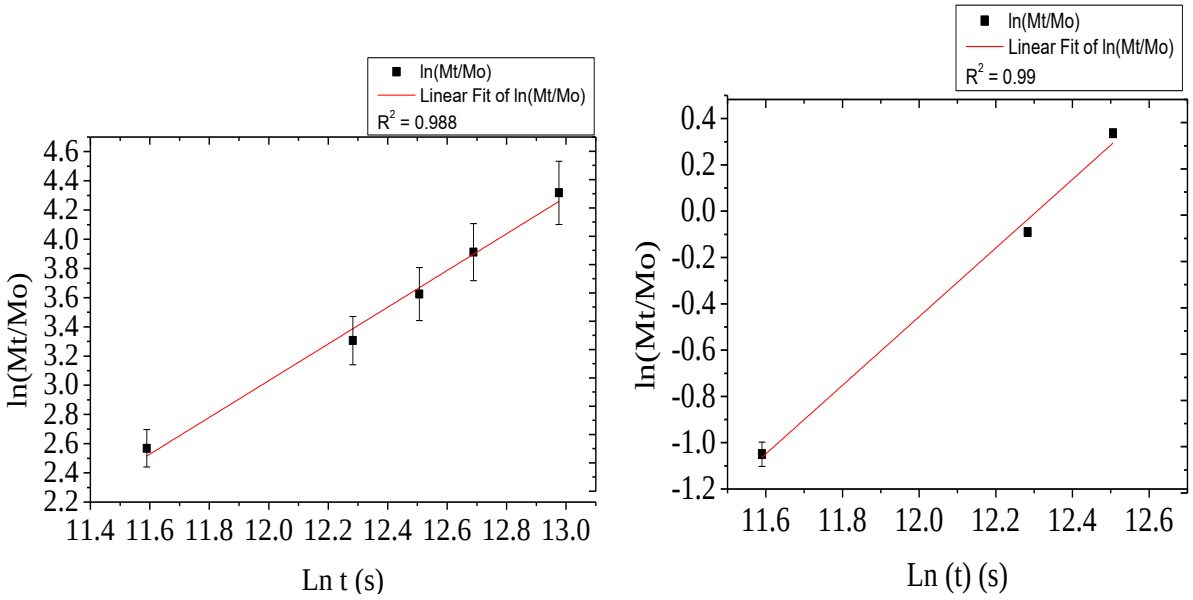


Figure 4. 13: **De-swelling of Sample (A) at 25^{0}C without Potassium Sorbate (Left) and De-swelling of Sample (B) at 25^{0}C with Potassium Sorbate (Right)**

Even though the films were thin and small in diameter, they were observed, on the average, to release in a control minor. They released until the eighth hour were no release were observed on the average.

RESULTS FOR DE-SWELLING OF THE CHITOSAN FILMS At 37^{0}C							
				Sample (A) no KS		**Sample (B) with KS**	
Diameter of film before swelling (m)				0.0104		0.0104	
Thickness of film before swelling (m)				0.0004		0.0004	
Weight of film before swelling (g)				0.074		0.026	
Diffusion Coefficient (Ds)				$7.287x10^{-12}m^2/s$		$3.7637x10^{-9}m^2/s$	
Slope (n)				0.873		0.549	
Time (t)	**Ln t (s)**	**Sample (A) no KS Mass(g)**	**(M_t/M_o)**	**ln(M_t/M_o)**	**Sample (B) with KS[Mass(g)]**	**(M_t/M_o)**	**ln(M_t/M_o)**
54000	**10.8967**	0.56	1	0	0.021	1	0
108000	**11.5899**	0.029	19.31034	2.960641	0.06	0.35	-1.04982
162000	**11.9954**	0.016	35	3.555348	0.06	0.35	-1.04982
216000	**12.283**	0.008	70	4.248495	0.023	0.913043	-0.09097

270000	**12.5062**	0.005	112	4.718499	0.015	1.4	0.336472
324000	**12.6885**	0.004	140	4.941642	0.002	10.5	2.351375
378000	**12.8426**	0.004	140	4.941642	0.002	10.5	2.351375
432000	**12.9762**	0.004	140	4.941642	0.002	10.5	2.351375
486000	**13.094**	0.004	140	4.941642	0.002	10.5	2.351375
540000	**13.1993**	0.004	140	4.941642	0.002	10.5	2.351375
594000	**13.2946**	0.004	140	4.941642	0.002	10.5	2.351375
648000	**13.3816**	0.004	140	4.941642	0.002	10.5	2.351375

Table 4. : **Results for De-swelling of Multilayer Chitosan Films at 37^{0}C**

From the valves achieved after the calculations of the average swelling ratio, water retention and release fraction, we released that between 324000 to 684000 seconds there was no change in the results. This reveals that at these time intervals, the films had finally dried up and there was no water left in each of the porous. Comparing the results achieved at 37^{o}C to that achieved at 25^{o}C, we released that at elevated temperature, the film's release a little faster as compared to the lower temperature. This is because the bond between chitosan and potassium sorbate stretched at increased temperatures, and result in the water that did not formed a strong bond with the chitosan and potassium sorbate to be release easily. This result shows that multilayer chitosan films have the ability of retaining chemical and release it over some essential time.

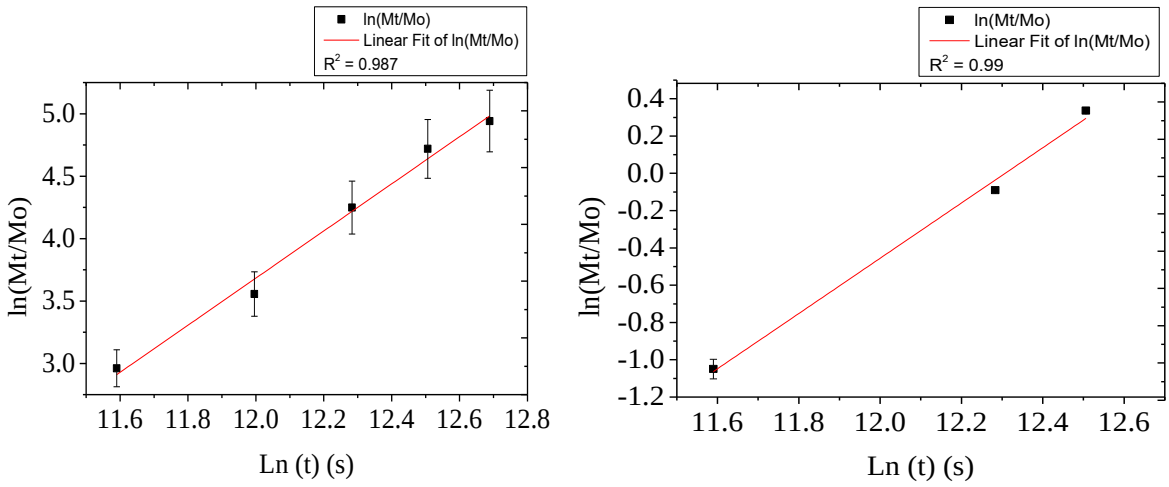


Figure 4. 14: **De-swelling of Sample (A) at 37^{0}C without Potassium Sorbate (Left) and De-swelling of Sample (B) at 37^{0}C with Potassium Sorbate (Right)**

Diffusivity or diffusion coefficient has been identified in this work as the major packaging parameter that directly influences the release rate of potassium sorbate from the multilayer chitosan films. Diffusivity is a material property and a polymer parameter. When the target release

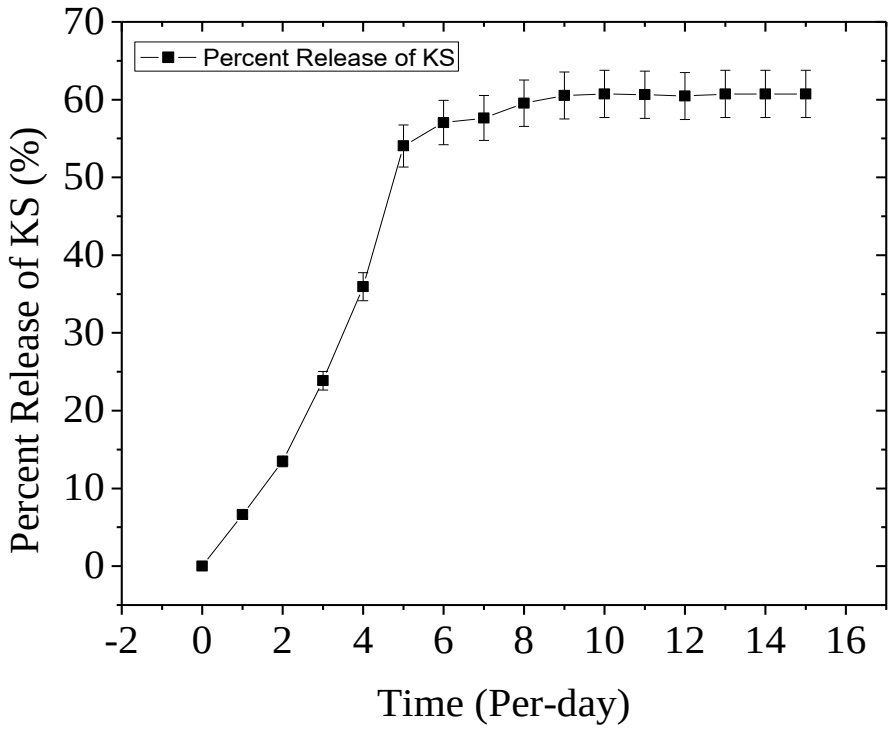
rate is quantified based on diffusivity, it can easily be translated to the type of packaging material used in the manufacturing of the films. The diffusivity was measured in this work so as to compare the release rate of potassium sorbate from the chitosan films. Also in this work, the diffusivity was calculated to be $7.0x10^{-9}m^2/s$ for the film without potassium sorbate at 25°C and $3.76x10^{-12}m^2/s$ for the films with potassium sorbate at 25°C. Similar calculation was done with the chitosan films at the temperature of 37°C. The films without potassium sorbate had the diffusivity of $7.287x10^{-12}m^2/s$ while the films with potassium sorbate had the diffusivity of $3.7637x10^{-9}m^2/s$.

From these results, we realized that the diffusivity reduced from 25°C to 37°C which was due to the fast the bond between the chitosan films were not stretched. Meanwhile, for the films with potassium sorbate, we increased in the diffusivity from 25°C to 37°C. This is because at elevated temperature, the bond between chitosan and potassium sorbate are stretched. This can results in increased diffusivity of the films.

4.4 UV-spectroscopic analysis

In addition to the swelling and de-swelling test of the films, UV- spectroscopy analysis was carried out on the samples. The UV-spectroscopic test was done to reveal the amount of potassium sorbate that released from the films. To obtain a standard curve for potassium sorbate,

2% w/w of potassium sorbate was dissolved in distilled water and placed in quartz cell and distilled water used as the reference. Similarly, the highest peak was observed at 252.0nm with the absorbance of 2.992A. Meanwhile, the films used for the analyses were casted in cylindrical shape of diameter of 13,000 µm. Then, they were place in a 10 ml distilled water. After every 12 hours, 5 ml of the distilled water were removed for the sample and kept. This was done for ten days. The samples collected were pull into the quartz cell one after the other and was place in the UV - spectrometer to measure the peak.



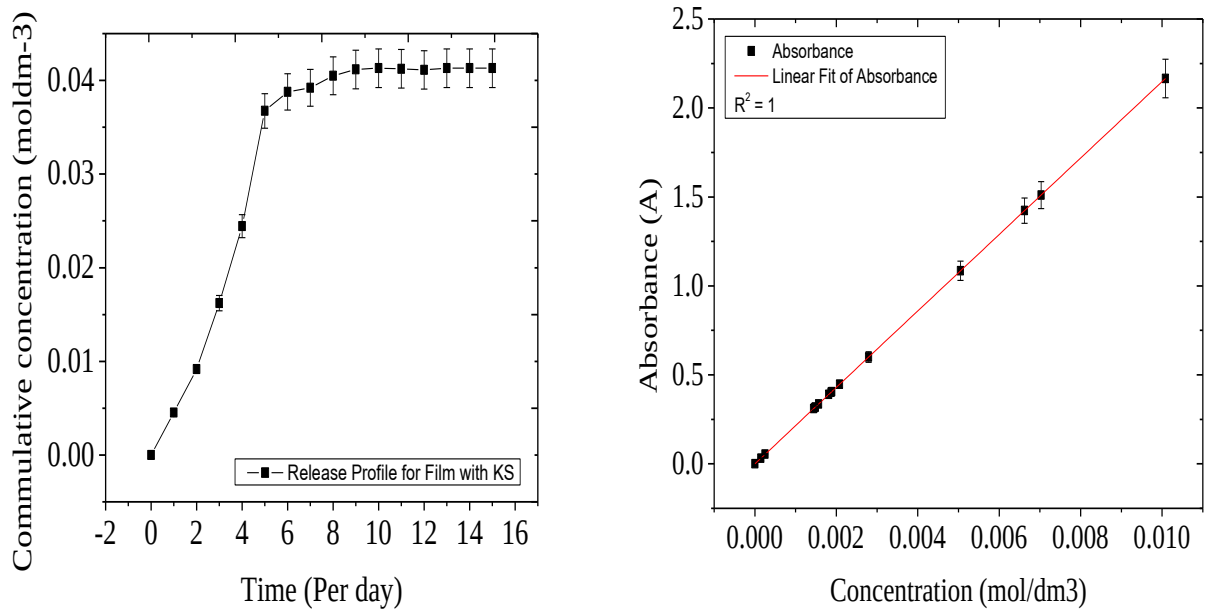


Figure 4. 15: **Percent Release Profile for Multilayer Chitosan Film with Potassium Sorbate (Top), Release Profile for Multilayer Chitosan Film with Potassium Sorbate (Left) and Standard Curve for Potassium Sorbate Release from Multilayer Chitosan Films (Right)**

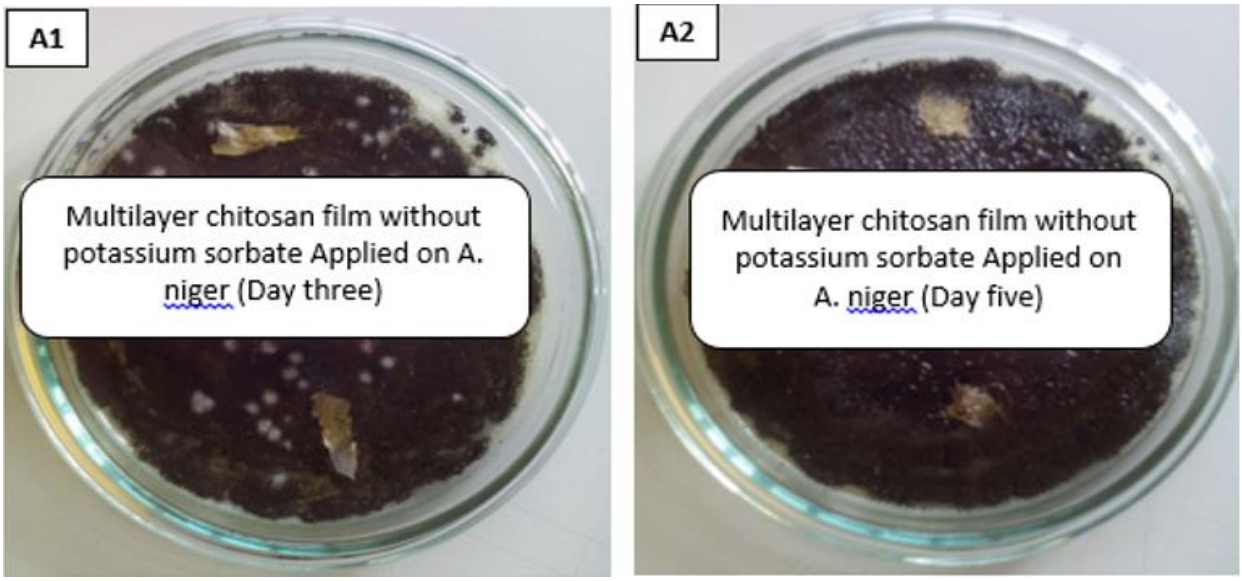
The highest peak of each sample was recalled and was then used to plot figures 4.15. It is observed in figures 4.15 (Right) that the concentrations of the potassium sorbate increases with increasing absorbance. This signifies that as the day pass by, the overall release of potassium increase. Similarly, the release profile (figure 4.15 (Left) clear shows how the potassium sorbate is been released from the film.

From the percentage release profile of the multilayer chitosan film with potassium sorbate at the top, we released that for the fifteen days period of examination, about 61% of the potassium sorbate has been released. Using the formula below, we were able to release the amount of potassium sorbate that was released.

$\frac{61}{100}$ x (concentration equivalent to the peak absorbance) $= \frac{61}{100}$ x $0.0123 = 0.0075$ moldm^{-3} of PS has been released over the time frame.

4.5 Zone inhibition of potassium sorbate against Aspergillum Niger

The chitosan films produced (with and without potassium sorbate) were applied on Aspergillus niger so as to observe it inhibitory effects on this micro-organism. Some section chitosan films without potassium sorbate were applied on aspergillum niger in a PDA media. It was observed that it did not inhibit the growth of the fungi. This was due to the limited ability of the film without any antimicrobial agents to inhibit the growth of microbes. Also, there were over growth of the fungi which might had contributed to the inability of the films without potassium sorbate to inhibit the growth of the fungi. Figure 4.16 below clearly show the results that were achieved after day three, five, seven and nine.



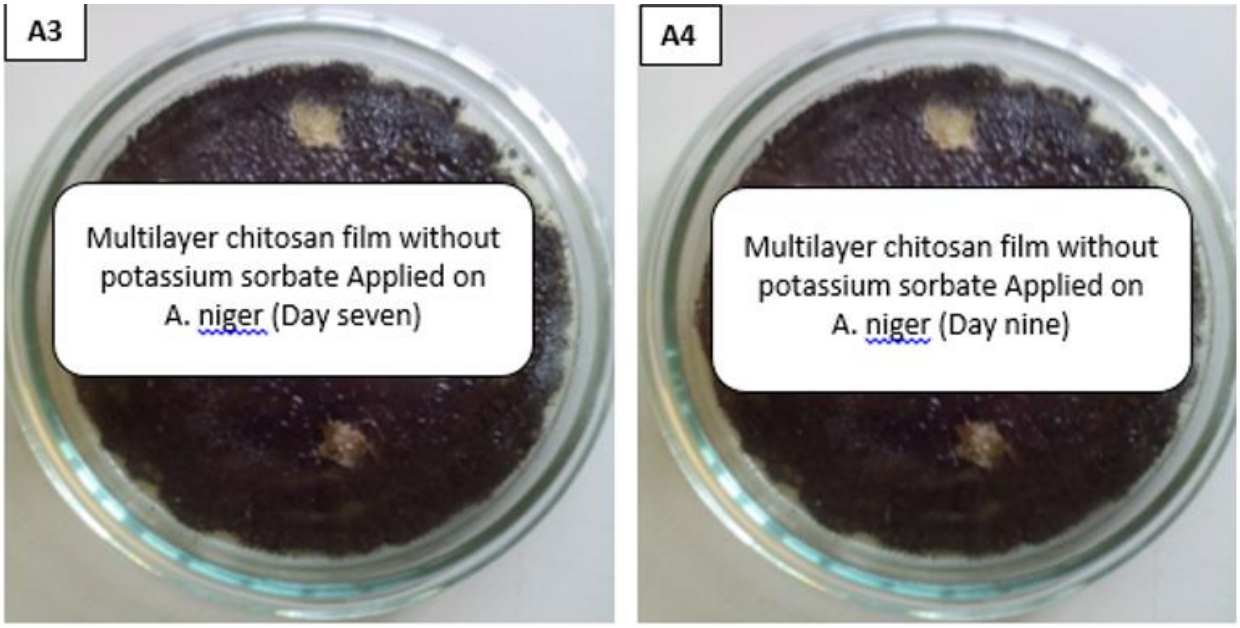


Figure 4. 16: **Multilayer Chitosan Films without Potassium Sorbate Applied on Aspergillus niger for Third Day (A1), Fifth Day (A2), Seventh Day (A3) and Ninth Day (A4)**

Meanwhile, using another films with the same diameter and high concentration of potassium sorbate (20 wt %), we were able to observed zone of inhibition. To test the antimicrobial efficiency, *aspergillum niger* was selected as the test microorganism since it is an important food-borne fungus that can be isolated from soil and various foods such as bread, nuts, meat, and dairy products etc. It can cause the deterioration of foods. The number of spores of aspergillum niger used in the tests was adjusted to $24x10^6$ spores/ml. The zone of inhibition method was used to measure the antimicrobial activity of the films. The dense surfaces of the square shape samples of the film were placed onto PDA agar and the clear zone formed around sample which is shown in the below in figures 4.27.

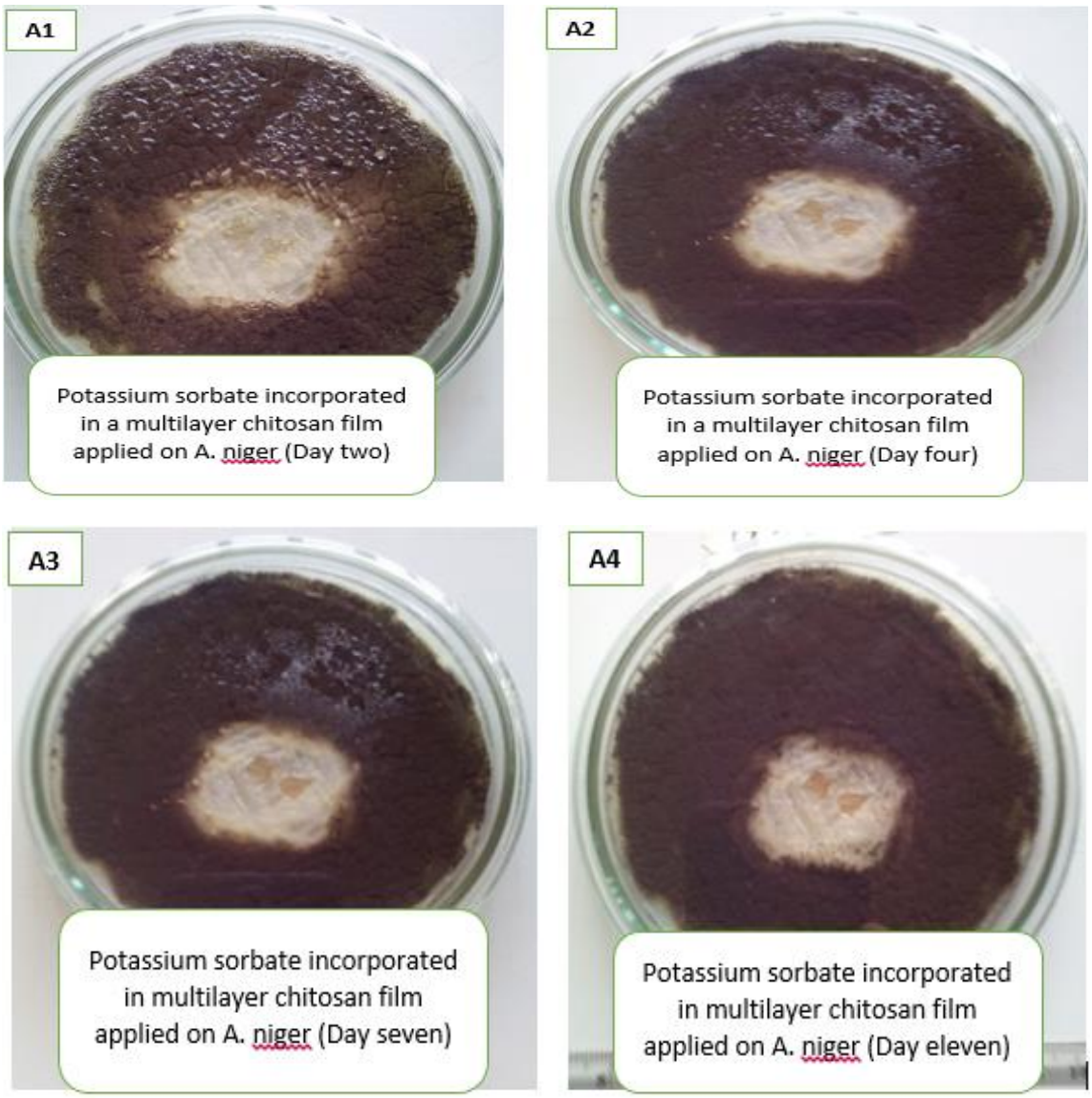


Figure 4. 17: **Multilayer Chitosan Films with Potassium Sorbate Applied on Aspergillus niger for Day Two (A1), 0Day Four (A2), and Day seven (A3)**

The clear zone of inhibition indicates the diffusion of potassium sorbate from the multilayer chitosan films into the agar medium and subsequent inhibited the growth of *Aspergillus niger* in the medium. The antimicrobial efficiency of different samples were quantified by measuring the diameter of the zones with a digital caliper. The areas of the inhibition zones were calculated based on the diameter measurement on second (1.8 cm), fourth (1.11 cm) and seventh days (0.8

cm). Doing an inspection on the ninth day, we realized that the zone was closing up. This shows that multilayer layer chitosan when applied on a fungi, *potassium sorbate*, has the ability of inhibiting its growth with in seventh days. But, this is based on several facts; if the fungi is already in the stage of overgrowth or if it is applied directly on the fungi with similar conditions as those in the PDA media in the autoclaved petri dishes. If the conditions are not the same, the growth time maybe extend above the seven days period.

The reduction in antimicrobial activity of preservatives such as potassium sorbate with heat treatment was reported by Han and Floros (1998). It was found that potassium sorbate loses its antimicrobial activity exponentially with temperature and linearly with heating time (Han 1996). Even though the films prepared in this study are dried at 50°C for a period of 24 hours, they have still shown antimicrobial activity on Aspergillus niger.

4.6 References

[60] S. A. (2009). Gemili, S., Yemenicioğlu, A., & Altınkaya, "Development of cellulose acetate based antimicrobial food packaging materials for controlled release of lysozyme," *J. Food Eng.*, vol. 90, no. 4, pp. 453–462, 2009.

[61] S. A. (2010). Gemili, S., Yemenicioğlu, A., & Altınkaya, "Development of antioxidant food packaging materials with controlled release properties.," *J. Food Eng.*, vol. 96, no. 3, pp. 325– 332, 2010.

[62] H. Berkowitz, B., Cortis, A., Dentz, M., & Scher, "Modeling non-Fickian transport in geological formations as a continuous time random walk.," *Rev. Geophys.*, vol. 44, no. 2, 2006.

[63] B. Cortis, A., & Berkowitz, "Anomalous transport in 'classical' soil and sand columns," vol. 68, no. 5, pp. 1539–1548, 2004.

[64] N. A. Ritger, P. L., & Peppas, "A simple equation for description of solute release II. Fickian and anomalous release from swellable devices.," *J. Control. releas*, vol. 5, no. 1, pp. 37–42, 1987.

[65] N. A. Sinclair, G. W., & Peppas, "Analysis of non-Fickian transport in polymers using simplified exponential expressions.," *J. Memb. Sci.*, vol. 17, no. 3, pp. 329–331, 1984.

CHAPTER FIVE: CONCLUSION AND RECOMMENDATION FOR FUTURE WORK

5.1 Conclusion

In this work, chitosan powder was produced for local shrimp shells. The chitosan produced shows some significant qualities of jelling and dissolving. This was proved by the presence of the amine and hydroxide group in the FT-IR results. The diffusion and swelling kinetics of chitosan films studied at temperatures of 25˚C and 37 ˚C shows that the films have good diffusion and swelling abilities. This further proved that the chitosan films have the potential of preserving food for a long time. The swelling capacities of the multilayer chitosan films were found to decrease with increasing temperature. The release rates were governed by the earlier time approximation, while the diffusion of the antimicrobial agent was strongly influenced by temperature. Non-Fickian diffusion dominated the results obtained from the power law equation (Sung and Yoo, 2000**)**. Furthermore, since chitosan is biocompatible (Approved by American Food and Drug Association), it is recommended as a packaging material for controlled release. (Oni et al., 2011). The porous structures of the chitosan films also enhance the controlled release of antimicrobial agent.

5.2 Recommendation

The results of release and antimicrobial tests suggest that the films prepared in this research may be used as significant controlled release food packaging materials. However, further studies are needed to test the effectiveness of these films on selected food systems on which A. niger grow. To the best of our knowledge, this is the first study which has shown the application of chitosan films containing potassium sorbate on Aspergillus niger.

5.3 References

[1] E. Royte, “One-Third of Food Is Lost or Wasted : What Can Be Done,” *Natl. Geogr. USA*, pp. 1–16, 2014.

[2] T. Folarnmi, “Food insecurity and malnutrition in Africa: Current trends, causes and consequences,” *Consult. Africa Intell. Your Africa Partn. Super. reseach Anal.*, no. September 2012, pp. 1–10, 2015.

[3] P. SUPPAKUL, J. MILTZ, K. SONNEVELD, and S. W. BIGGER, “Active Packaging Technologies with an Emphasis on Antimicrobial Packaging and its Applications,” *JFS Concise Rev. Hypotheses Food Sci. Act. Packag. Technol. with an Emphas. Antimicrob. Packag. its Appl.*, vol. 68, no. 2, 2003.

[4] D. S. Yildirim, “Active Packaging Antimicrobial Films for Food Packaging,” *Zurich Univ. Appl. Sci. Inst. für Leb. und Getränkeinnovation Campus Reidbach Postfach Wädenswil*, pp. 1–22, 2011.

[5] T. F. Group, “Active and Intelligent Packaging,” *Free. US Ind. Study with Forecast. 2015 2020*, vol. 8, no. 2772, p. 302, 2011.

[6] F. N. Hafdani and N. Sadeghinia, “A Review on Application of Chitosan as a Natural Antimicrobial,” *World Acad. Sci. Eng. Technol.*, vol. 50, pp. 225–229, 2011.

[7] P. K. Dutta, S. Tripathi, G. K. Mehrotra, and J. Dutta, “Perspectives for chitosan based antimicrobial films in food applications,” *Food Chem.*, vol. 114, no. 4, pp. 1173–1182, Jun. 2009.

[8] C. M. P. Yoshida, C. Eduardo, N. Bastos, and T. T. Franco, “Modeling of potassium sorbate diffusion through chitosan films,” *LWT - FOOD Sci. Technol.*, vol. 43, pp. 584–589, 2010.

[9] R. S. Cruz, G. P. Camilloto, and A. Clarissa, “Oxygen Scavengers : An Approach on Food Preservation,” *Food Tecnhology Dep. Fed. Univ. Viçosa, Viçosa, MG, Brazil*, 2012.

[10] U. Metin, “PREPARATION OF CONTROLLED RELEASE ANTIMICROBIAL FOOD PACKAGING MATERIALS,” *Izmir Inst. Technol. Thesis*, pp. 1–85, 2009.

[11] J. Vermeiren, L., Devlieghere, F., Van Beest, M., De Kruijf, N., & Debevere, “Developments in the active packaging of foods. Trends in food science & technology,” *Trends food Sci. Technol.*, vol. 10, no. 3, p. 1999, 1999.

[12] S. Silvestre, C., Duraccio, D., & Cimmino, “Food packaging based on polymer nanomaterials.,” *Prog. Polym. Sci.*, vol. 36, no. 12, p. 2011, 2011.

[13] Y. Zhou, G. H., Xu, X. L., & Liu, “Preservation technologies for fresh meat–A review.,” *Meat Sci.*, vol. 86, no. 1, p. 2010, 2010.

[14] D. A. Jay, J. M., Loessner, M. J., & Golden, “Modern food microbiology.” 2005.

[15] S. K. (2014). DASH, “MODIFIED ATMOSPHERE PACKAGING OF FOOD. Polymers for Packaging Applications,” p. 337, 2014.

[16] C. Fuciños, P. Fuciños, M. Míguez, I. Katime, L. M. Pastrana, and M. L. Rúa, “Temperature- and pH-sensitive nanohydrogels of poly(N-Isopropylacrylamide) for food packaging applications: modelling the swelling-collapse behaviour.,” *PLoS One*, vol. 9, no. 2, p. e87190, Jan. 2014.

[17] J. Dutta, S. Tripathi, and P. K. Dutta, *Progress in antimicrobial activities of chitin, chitosan and its oligosaccharides: a systematic study needs for food applications*, vol. 18, no. 1. 2012, pp. 3–34.

[18] G. Petersen, K., Væggemose Nielsen, P., Bertelsen, G., Lawther, M., Olsen, M. B., Nilsson, N. H., & Mortensen, “Potential of biobased materials for food packaging.,” *Trends Food Sci. Technol.*, vol. 10, no. 2, p. 1999, 1999.

[19] W. M. (1989). Labuza, T. P., & Breene, “APPLICATIONS OF ‘ACTIVE PACKAGING’ FOR IMPROVEMENT OF SHELF-LIFE AND NUTRITIONAL QUALITY OF FRESH AND EXTENDED SHELF-LIFE FOODS,” *J. Food Process. Preserv.*, vol. 13, no. 1, pp. 1–69, 1989.

[20] P. T. C/Albert 1, “ACTIVE PACKAGING TO EXTEND THE PRODUCT SHELF LIFE,” *ITENE*, vol. 1, pp. 1–2, 2000.

[21] L. R. Brody, A. L., Strupinsky, E. P., & Kline, “Active packaging for food applications,” *CRC Press*, p. 2001, 2001.

[22] M. (Eds. . Lang, T., & Heasman, “Food wars: the global battle for mouths, minds and markets,” *Earthscan*, p. 2004, 2004.

[23] C. Film, I. Powder, A. Acid, and T. Dioxide, “Oxygen-Scavenger Oxygen Scavenging Packaging Mean Oxygen Scavenging Packaging Aid Copolymer * Copolymer * substances Activation † heat during extrusion # self-activated (humidity) h : Titanium Dioxide *,” pp. 2–3, 2012.

[24] N. SundaraBaalaji, “Invited Lecture. In Book of Abstracts.” p. 47, 2001.

[25] J. Vermeiren, L., Devlieghere, F., Van Beest, M., De Kruijf, N., & Debevere, “Developments in the active packaging of foods.,” *Trends food Sci. Technol.*, vol. 10, no. 3, p. 1999, 1999.

[26] M. Miltz, J., & Perry, “Evaluation of the performance of iron-based oxygen scavengers, with comments on their optimal applications.,” *Packag. Technol. Sci.*, vol. 18, no. 1, p. 2005, 2005.

[27] A. C. Cruz, R. S., Camilloto, G. P., & dos Santos Pires, “Oxygen Scavengers: An Approach on Food Preservation.,” p. 2012, 2012.

[28] M. L. Rooney, “Active packaging in polymer films. In Active food packaging,” *Springer US*, pp. 74– 110, 1995.

[29] L. com Inc, “Food Poisoning and Waterborne Illness: How to Prevent 1.8 Million Deaths Every Year,” *Product. Publ.*, p. 2012, 2012.

[30] G. A. Ayala-Zavala, J. F., Del-Toro-Sánchez, L., Alvarez-Parrilla, E., & González-Aguilar, “High Relative Humidity In-Package of Fresh-Cut Fruits and Vegetables: Advantage or Disadvantage Considering Microbiological Problems and Antimicrobial Delivering Systems?,” *J. Food Sci.*, vol. 73, no. 4, pp. R41–R47, 2008.

[31] Y. Pranoto, S. K. Rakshit, and V. M. Salokhe, “Enhancing antimicrobial activity of chitosan films by incorporating garlic oil, potassium sorbate and nisin,” *LWT - Food Sci. Technol.*, vol. 38, no. 8, pp. 859–865, Dec. 2005.

[32] “ACTIVE PACKAGING.”

[33] I. Active, W. Can, and H. Inside, “30) ACTIVE PACKAGING , INTELLIGENT PACKAGING,” *Goolge B.*, pp. 1–5.

[34] A. Lucera, C. Costa, A. Conte, and M. a Del Nobile, “Food applications of natural antimicrobial compounds.,” *Front. Microbiol.*, vol. 3, p. 287, Jan. 2012.

[35] R. S. Cruz, G. P. Camilloto, and A. Clarissa, “Oxygen Scavengers : An Approach on Food Preservation,” 2012.

[36] K. Juntarapun and C. & Satirapipathkul, “ANTIMICROBIAL ACTIVITY OF CHITOSAN AND TANNIC ACID ON COTTON FABRIOUS MATERIALS,” *RMUTP Int. Conf. Bangkok Thail.*, pp. 1–6, 2012.

[37] M. Rinaudo, “Chitin and chitosan : Properties and applications,” *Sci. Direct*, vol. 31, pp. 603–632, 2006.

[38] P. K. Dutta, J. Dutta, and V. S. Tripathi, “Chitin and chitosan : Chemistry , properties and applications,” *J. Sci. Ind. Res. (India).*, vol. 63, pp. 20–31, 2004.

[39] V. S. (2004). Dutta, P. K., Dutta, J., & Tripathi, “Chitin and chitosan: Chemistry, properties and applications,” *J. Sci. Ind. Res.*, vol. 63, no. 1, pp. 20–31, 2004.

[40] V. (2000). Tsigos, I., Martinou, A., Kafetzopoulos, D., & Bouriotis, “Chitin deacetylases: new, versatile tools in biotechnology. Trends in biotechnology,” *Trends Biotechnol.*, vol. 18, no. 7, p. 2000, 2000.

[41] N. (2010). Jayakumar, R., Prabaharan, M., Nair, S. V., Tokura, S., Tamura, H., & Selvamurugan, “Novel carboxymethyl derivatives of chitin and chitosan materials and their biomedical applications.,” *Prog. Mater. Sci.*, vol. 55, no. 7, pp. 675–709, 2010.

[42] S. P. Dube, D., Gupta, M., & Vyas, “Nanocarriers for Drug Targeting to Macrophages: Emerging Options for a Therapeutic Need,” *Proc. Natl. Acad. Sci. India Sect. B Biol. Sci.*, vol. 82, no. 1, p. 2012, 2012.

[43] M. Á. Falcón-Rodríguez, A. B., Costales, D., Cabrera, J. C., & Martínez-Téllez, "Chitosan physico–chemical properties modulate defense responses and resistance in tobacco plants against the oomycete and Phytophthora nicotianae," *Pestic. Biochem. Physiol.*, vol. 100, no. 3, pp. 221–228, 2011.

[44] L. A. Hadwiger, "Multiple effects of chitosan on plant systems," *Solid Sci. or hype. Plant Sci.*, pp. 42–49, 2013.

[45] R. N. Srinivasa, P. C., & Tharanathan, "Chitin/chitosan—Safe, ecofriendly packaging materials with multiple potential uses.," *Food Rev. Int.*, vol. 23, no. 1, pp. 53–72, 2007.

[46] D. Ocloo, F. C., Adu-Gyamfi, A., Quarcoo, E. A., Serfor-Armah, Y., & Asare, "PRELIMINARY STUDIES ON ANTIFUNGAL PROPERTIES OF RADIATION PROCESSED CHITOSAN FROM CRAB SHELLS.," *Rep. 2nd RCM on" Dev. radiation-Process. Prod. Nat. Polym. Appl. Agric. Heal. Ind. Environ.*, p. 101, 2009.

[47] B. Krajewska, "Membrane-based processes performed with use of chitin/chitosan materials," *Sep. Purif. Technol.*, vol. 41, no. 3, pp. 305–312, 2005.

[48] R. Nicu, E. Bobu, and J. Desbrieres, "CHITOSAN AS CATIONIC POLYELECTROLYTE IN WET-END PAPERMAKING SYSTEMS," *Cellul. Chem. Technol.*, pp. 105–111, 2011.

[49] C. N. Cutter, "Opportunities for bio-based packaging technologies to improve the quality and safety of fresh and further processed muscle foods," *Meat Sci.*, vol. 74, no. 1, pp. 131–142, 2006.

[50] G. Sebag, H., & Vanlerberghe, "U.S. Patent No. 3,953,608 . Washington, DC: U.S. Patent and Trademark Office.," *Washington, DC U.S. Pat. Trademark Off.*, no. 3\, 1976.

[51] W. Material, "Report of the 2nd RCM on ' Development of radiation-processed products of natural polymers for application in agriculture , healthcare , industry and environment ' Reims , France," pp. 1–234, 2010.

[52] D. Jianglian and Z. Shaoying, "Application of Chitosan Based Coating in Fruit and Vegetable Preservation : A Review," *FOOD Process. Technol.*, vol. 4, no. 5, pp. 5–8, 2013.

[53] J. M. Quicho, "Efficacy of ultraviolet light in combination with chemical preservatives for the reduction of Escherichia coli in apple cider," *Dr. Diss. Virginia Polytech. Inst. State Univ.*, p. 2005, 2005.

[54] N. Van Toan, "Production of Chitin and Chitosan from Partially Autolyzed Shrimp Shell Materials," *Open Biomater. J.*, vol. 1, pp. 21–24, Oct. 2009.

[55] O. O. and Y. Dogu, "Swelling – Deswelling Kinetics of Poly(Nisopropylacrylamide) Hydrogels Formed in PEG Solutions," *Wiley Period.*, 2005.

[56] K. Nam, K., Watanabe, J., & Ishihara, "Modeling of swelling and drug release behavior of spontaneously forming hydrogels composed of phospholipid polymers," *Int. J. Pharm.*, vol. 275, no. 1, pp. 259–269, 2004.

[57] A. N. Peppas, "Analysis of Fickian and Non-Fickian Drug Release from polymers," *Pharm. HEL.*, vol. 60, no. 4, p. 1985, 1985.

[58] I. R.W. Baker, H.K. Lonsdale, "Tanquary A.C. and Lacey R.E. Controlled Release of Biologically active Agents.," *Plenum Publ. New York*, pp. pp15 – 71, 1974.

[59] A. A. Lamberti, G., Galdi, I., & Barba, "Controlled release from hydrogel-based solid matrices. A model accounting for water up-take, swelling and erosion," *Int. J. Pharm.*, vol. 407, no. 1, pp. 78–86, 2011.

[60] S. A. (2009). Gemili, S., Yemenicioğlu, A., & Altınkaya, "Development of cellulose acetate based antimicrobial food packaging materials for controlled release of lysozyme," *J. Food Eng.*, vol. 90, no. 4, pp. 453–462, 2009.

[61] S. A. (2010). Gemili, S., Yemenicioğlu, A., & Altınkaya, "Development of antioxidant food packaging materials with controlled release properties.," *J. Food Eng.*, vol. 96, no. 3, pp. 325– 332, 2010.

[62] H. Berkowitz, B., Cortis, A., Dentz, M., & Scher, "Modeling non-Fickian transport in geological formations as a continuous time random walk.," *Rev. Geophys.*, vol. 44, no. 2, 2006.

[63] B. Cortis, A., & Berkowitz, "Anomalous transport in 'classical' soil and sand columns," vol. 68, no. 5, pp. 1539–1548, 2004.

[64] N. A. Ritger, P. L., & Peppas, "A simple equation for description of solute release II. Fickian and anomalous release from swellable devices.," *J. Control. releas*, vol. 5, no. 1, pp. 37–42, 1987.

[65] N. A. Sinclair, G. W., & Peppas, "Analysis of non-Fickian transport in polymers using simplified exponential expressions.," *J. Memb. Sci.*, vol. 17, no. 3, pp. 329–331, 1984.