CONTROLLED RELEASE OF FUNGICIDAL ANTIMICROBIALS ON PACKAGED FOODS

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ABSTRACT

This paper presents the effect of polymer concentration on the release of fungicidal antimicrobial (AM) agent to prolonging the shelf life of packaged food. This project aimed to produce and test AM films with varying polymer ratio to control the release of fungicidal AM agent. The effects of potassium sorbate (PS) released on peanut and fresh breads was tested for nineteen days to determine the effect of AM agent to inhibit the growth of *Aspergillus niger* (*A. niger*). The microbe was cultured on a potato dextrose agar (PDA) medium to obtain the activity of AM film. AM film contains mixtures of CA: acetone in 15% w/w, 13% w/w and 10% w/w ratios. Each ratio was mixed with 8 ml PS (from stock solution containing 20 g PS in 80 ml distilled water). PS was used as the AM agent during film formation. The film was formed in a petri dish at room temperature and the mixture was degassed in a vacuum oven. The *A. niger* was cultured from the soil and the AM activities of the films gave a clear zone indicated around the film for seven days. PS incorporated into CA based film has the capacity to inhibit the growth of *A. niger* in peanuts and fresh bread. The release kinetics of PS from the films was obtained by studying the de-swelling properties of PS loaded film at room temperature (24˚C) and at 37˚C. The diffusion coefficients of PS released through the film network were obtained to be between 1.97×10^{-8} m^2s^{-1} – 5.29×10^{-7} m^2s^{-1} at 24˚C and 1.3×10^{-10} m^2s^{-1} – 1.80×10^{-8} m^2s^{-1} at 37˚C. The fractional release exponent n-values for the film ranges from 0.16 – 0.54 at 24˚C and 0.5 - 1.1 at 37˚C. The geometrical constant (k) ranges from 0.0063 – 0.19 at 24˚C and 0.000041 – 0.0057 at 37˚C. The average swelling ratio of the films also ranges from 1.026 - 1.56 at 24˚C and 0.71 - 1.51 at 37˚C. SEM images were obtained to observe the morphology of the films and UV-Vis spectrophotometer was also used to study the amounts of PS released from the films.

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DEDICATION

To my late parents Sunday and Marion Aina

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CHAPTER ONE

1.0 Introduction

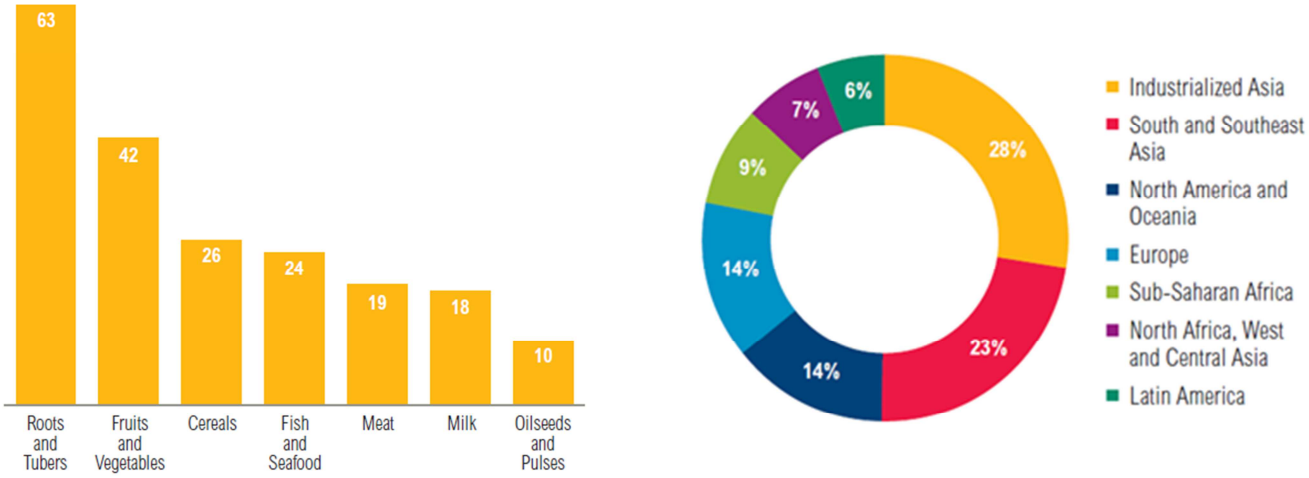
Controlled release packaging (CRP) is a form of packaging technology that is currently used in the food packaging industry. CRP controls the rate at which an active agent such as antimicrobials, antioxidants are been delivered to the packaged food to improve upon the quality and safety of the food. CRP extends the shelf life of foods (Kit. L. Yam and Xuntao Zhu. 2014). The active agent tends to create an enabling environment for the packaged food item ().

Antimicrobial agents is defined as “substances used to preserve food by preventing the growth of microorganisms and subsequent spoilage, including fungi, mold, yeast and rope inhibitors”(FDA, 2011). The current work explores the application of a type of fungicidal antimicrobial, incorporating it into a form of mono-layered film of different polymer ratiofor food packaging. Studies provided a controlled release of the fungicidal antimicrobial agent into the food in order to prolong the shelf life of food packaged in plastic containers.

1.1 Food degradation

To ensure that food produced from the farm gets to the target consumers, there is need to reduce or prevent the rate of its degradation. Looking at the statistics given by the United Nations Food and Agriculture Organization 2011, it has been estimated that 32 percent of all food produced based on weight in the world were lost/wasted in 2011. This lost amounts to 24% in calories of food lost globally (FAO 2011). It is also important to note that 56% of the total food lost/wasted occurs in developed countries like China and Japan while 44% occurs in developing countries such as Nigeria, Congo among others (FAO 2009). Based on the stages of food value chain, 24% of global food lost/wasted occurs at production, 24% during handling and storage and 35% at consumption, these three amounts to 80

percent of global food lost/wasted. This is shown in the figure 1 a-b. This statistics calls for concerns to reduce food wasted by improving on existing methods of food preservation such as the use of antimicrobial agents on both fresh and packaged food, in a way that helps to minimize losses of both fresh and processed food. Values displayed are of waste as a percentage of food supply, defined here are the sums of the "Food" and "Processing" (FAO Food Balance Sheet 2009).



(a) (b)

Figure 1.1: (a) Share of Commodity Lost or Wasted, 2009 (Percent of kcal) and (b) Share of Global Food Loss and Waste by Region, 2009 (100% = 1.5 quadrillion kca). (Source: WRI analysis based on FAO. 2011)

1.2 Fungicidal and antimicrobial food packaging

AM food packaging is a method of food packaging that can kill or prevents the growth of pathogenic micro-organisms in food by the use of AM agents thereby preventing spoilage and contamination of packaged food. If the AM agent is targeted at fungi, molds and yeasts it is called "fungicidal antimicrobial agent". AM food packaging also allows a controlled release of active agents into the food surface during storage and distribution of the packaged food item. AM food packaging can take one of these forms (Balasubramaniams, 2012):

- Addition of sachets or pads containing volatile antimicrobial agents into packages.
- Incorporation of antimicrobial agents directly into polymers.
- Coating or adsorbing antimicrobials to polymer surfaces.
- Immobilization of antimicrobials by covalent linkage.
- Use of polymers that are inherently antimicrobial.

Table 1.2.1 Showing antimicrobials incorporated directly into polymers used for food packaging

Fungicidal Antimicrobials	Polymer /Packaging	Main Target Microorganisms	Reference
Organic acid/anhydride: Propionic, benzoic, sorbic, acetic, lactic and malic	Edible films, EVA, LLDPE	Molds	Guilbert (1998), Baron & Summer (1993), Torres & Karel (1985), Devlieghere, et al., (2000), Weng and Hotchkiss, 1993.
Spices: cinnamic, caffeic, *p*-coumaic acid	Nylon/PE, cellulose	Molds and yeast	Hashino, Iijima, Hayashi & Shibata (1998), Anon (1995), Nielson & Rios (2000)
Essential oils (plant extracts): grapefruit seed extract, hinokitiol, bamboo poeder, rheum palmatum, coptis chinesis extracts.	LDPE, Cellulose	Molds and yeast	Lee, Hwang & Cho (1998), Imakura, Yamada & Fukazawa (1992), Oki (1998), Chung, Cho & Lee (1998), Hong et al. (2000).

^{EVA}(ethylene vinyl acetate), ^{LLDPE}(linear low density polyethylene), ^{LDPE}(low density polyethylene), ^{PS}(polystyrene) and ^{PE}(polyethylene).

1.3 Unresolved issues in antimicrobial (AM) food packaging

Some of the issues that needed to be addressed in controlled release of AM food packaging include the following:

- The need to extend the study of controlled release of antimicrobial food packaging to testing its efficacy on selected food items.
- Applying design and mathematical models to optimize antimicrobial food package.

- Achieving further shelve life extension through the design of model packaging to further control the release of AM agents to packaged food stuffs.
- Design smart package that can notify the store keeper of the fact that the packaged food will soon expire.
- Controlling the release of AM agent into packaged food so that the amount released over a given time frame can be ascertained.
- Forming polymeric pellets that have antimicrobial agent incorporated into it.

1.4 Scope of work

Potassium sorbate (PS) would be incorporated into cellulose acetate (CA) (a polymer) to form three different mono-layers of AM film with different composition of the polymer to controlling the release of the fungicidal AM agent to the food. The film formed would be degassed in a vacuum oven and then dried at room temperature (24°C). The mono layered film formed will be placed in a medium to test the rate of release of the antimicrobial agent over a giving time frame. The absorbance will also be tested using the UV Spectrophotometer. The release of the PS will be tested on *Aspergillus niger* (Fungi). The PS release and the method of diffusion to the packaged food will be determined by swelling and de-swelling method, the life of a selected type of food will also be ascertained. Scanning Electron Microscope (SEM) would be used to examine the surface morphology of the films produced. The schematic of a model to predict target release of an antimicrobial agent to food is as shown below

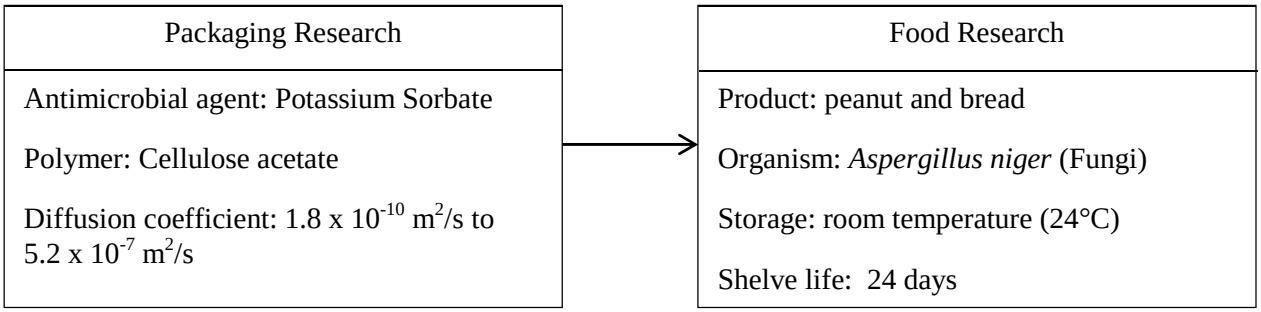


Figure 1.4.1: The application of antimicrobial agent to packaged food (Balasubramaniams, 2012)

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Balasubramaniams, A., et al., *Predicted Target Release Profile of Antimicrobials from Controlled Release Packaging*. A Dissertation, 2012. 1: p. 5

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CHAPTER TWO

2.0 Antimicrobial food packaging

Antimicrobial food packaging (AFP) consists of packaging system(s) adopted to control the growth of harmful microbes in food products. In order to ensure that fresh and processed food reach desired consumers with little or no degradation, the food must be packaged in an environmental condition that limits the tendency of degradation by fungi attack during the period between packaging and consumption by the consumer. To curtail the damage to the packaged food by microbes a system is been adopted known as antimicrobial food packaging system. In this technology, the antimicrobial agent is introduced into the packaged food to inhibit the growth of harmful microorganism in the packaging (Lück and Jager 1997). The usual method for food preservation is to prevent the packaged food from having contact with oxygen and moisture, thereby preventing the growth of the harmful microbes.

Several studies have been carried out in the area of antimicrobial food packaging. Seyhum Gemili 2009 studied the effect of incorporating lysozyme into cellulose acetate (CA). The maximum release of the antimicrobial agent into the packaged food was obtained with 5% CA and 1.5% lysozyme and asymmetric CA film has good tendency to achieve a controlled release of the antimicrobial agent from the packaging system. The release of antimicrobial agents can be accomplished either by direct contact of the food with the package or by diffusion of the packing layer to the surface of food (Coma, 2008; Conte, Buonocore, Bevilacqua Sinigaglia and Del Nobile 2006; Gemili, Yemenicioglu, and Altinkaya, 2009). To address this challenge, the use of “Smart packaging” was introduced which allows for the addition of different kinds of active agents to the polymer, hence aiding food preservation (Han 2000).

2.1 Statistics on methods of food packaging

Earlier on in the food industries, there was little packaging of individual units. Liquid and solid food products were made available to consumers in bulk containers (Driscoll and Paterson 1999). In the mid-18th century, the Dutch preserved roasted beef and salmon by placing hot food into tin-plated iron cans, covering with hot fat and quickly soldering on the lid (Robertson 2006). The first half of the 20th century saw the development of cellophane, aluminum foil and aluminum foil-laminated paperboard. After the Second World War, the use of polyethylene (PE), polyvinyl chloride (PVC) and polyvinylidene chloride (PVDC) were developed. In the second half of the 20th century, ethylene vinyl alcohol (EVOH) copolymer, polypropylene (PP) and polyethylene terephthalate (PET) for beverage packaging were developed.

Modern methods of preserving and packaging protects food ensures that less than 2% of all food produced in developed countries is wasted, while up to 30 to 50% is lost/wasted in developing countries (Driscoll and Paterson 1999).

2.2 Classical antimicrobial food packaging systems

Classical antimicrobial food packaging system refers to the method of packaging food used in the olden days (Metin U. 2009). In ancient times, the Roman and Egyptians used silver vessel for water and other food storage. In the late eighties, organic acids together with surfactants were incorporated into sucking pads and when these pads were used on trays for packaging meats and poultry they have the capacity to absorb meat exudates which prevents microbial growth (Hansen et al. 1989).

Studies have shown that polymer networks can allow the transport of active molecules (Papadokostaki et al. 1997). The use of heat and gamma irradiation can produce cross-linking between protein molecules and this brings about a better physical and functional properties of edible films. Some of the agents that have been proposed and tested for antimicrobial packaging includes potassium sorbate, bacteriocine, garlic oil, silver and copper ions etc (Han and Floros 1997). Some of the food packaging materials are polymers like low density polyethylene, (LDPE), cellulose acetate, chitosan etc.(Huang *et al.*,1997, Weng *et al.*,1997, Han and Floros, 1997), ceramics (glass e.g. borosilicate glass, sodium calcium glass, etc) and metals (aluminum, tin etc).

Incorporating 1.0% w/w Potassium Sorbate in low density polyethylene film (0.4 mm thick) lowered the growth rate of yeast and prolonged the lag period before mold growth became evident (Han and Floros, 1997). Imazalil concentration of 2000 mg/kg incorporated in 5.1 μm thick LDPE film delayed the growth of A. Toxicarius on a potato dextrose agar, but when the concentration of the Imazalil in the LDPE was reduced to 1000 mg/kg it could inhibit the growth of *penicillium sp.* in cheese.

A suitable method for fabricating polyethylene-co-methacrylic acid films was studied and it was discovered that with thickness ranging from 0.008mm to 0.010mm while incorporating sorbic or benzoic acids as the antimicrobial agent into the film had the capacity to inhibit the growth of fungi (Wen et al. 1999).

2.3 Types of antimicrobial agents

Antimicrobial agents can be divided into two categories; natural antimicrobial agents and chemical antimicrobial agents. These divisions are on the basis of their source (Appendini and Hotchkiss, 2005).

2.3.1 Natural antimicrobial agents

Natural antimicrobial agents derive their origin from plants, animals and microorganisms examples of natural antimicrobial agents are; enzymes derived from microorganisms (Volesky and Luong, 1982), other are nisin which could be derived from bacteria (Rogers and Whittier, 1928), lysozyme, etc.

Nisin is one of the bacteriocin that is mostly employed as a food preservative, it possess a very high antibacterial activity with very little toxicity for humans. It is most often used in food preservation against the growth of bacteria in dairy, meat and canned food. The bacteriocin nisin (or a group N inhibitory substance), was discovered in England by Rogers and Whittier in 1928, and is produced by certain strains of *Lactococcus lactis.* Nisin belong to a group of bacteriocins known as "lantibiotics" (class-I). Lantibiotics are peptides that are smallish in size and their source is the Gram positive bacteria of different genera (Breukink E. et al., 1999, Carr, F. J. et al., 2002, Ross R.P et al. 2002).

Lysozyme is a type of enzyme called hydrolytic enzyme which originates from cells that has been purified or from secretions and tissues of essentially all living organisms and viruses. Several studies has shown that Lysozyme possess antimicrobial activities towards bacteria, fungi, protozoan and viruses (Seyhum and Gemili 2009); moreover, it is essentially known for its antibacterial activity and has found lots of application in food preservation. Lysozyme is currently used as a preservative in many foods, such as cheese, fish, meat, fruit, vegetables and wine (Daniela et al., 2010).

2.3.2 Chemical antimicrobial agents

Antimicrobials used in prolonging the shelf life of food are classified as "preservatives." Chemical preservatives are chemical that which when added to food can prevent or retard deterioration (FDA, 2000)

The whole microbial spectrum, mode of action, and the efficacy of chemical compounds are generally reliant on the chemical and physical properties of the antimicrobial agent. Top on this list is the polarity of the chemical compound, water solubility is also essential (Robach, 1980). Examples of chemical antimicrobial agents include; Sorbic acid, sorbates, benzoic acid, benzoates, sulfur dioxide etc.

Sorbic acid can prevent yeasts and molds from growing on food, as well as against many bacteria (Sofos, 2000). Sorbic acid has found lots of application in various foods, such as dairy products, bakery items, fruit and vegetable products, edible fat emulsion products, certain meat and fish products, and sugar and confectionery items (Sofos, 1989).

Sulfur dioxide has aided fruit storage for example dipping mandarin orange and storing it in a bad of polyethylene showed that the fruit maintained its quality for over a year (Fishman and Karalidze 1984). Using only 0.2% of sulfur dioxide and no storage in a bag showed that the fruit only lasted for 60 days to the mold growth in mandarin oranges (Yakobashvili and Georgadze, 1984).

2.4 Fungicidal antimicrobial agents

Fungicidal antimicrobial agents are antimicrobial agents used to preserve food by preventing the growth of fungi and subsequent spoilage of the food thus extending the shelf life of the food (Han 2000). Examples of fungicidal antimicrobial agents are benzoic acid, benzoates, sorbic acid, sorbates, natamycin, sulfites etc.

2.4.1 Sorbic acid

The properties of sorbic acid as fungicidal antimicrobial agent were first discovered in Germany and United states, by E. Miller in the late 1930s and Gooding in the late 1940s (Luck. 1976; Sofos and Busta, 1981). Study has shown that the conjugated double bond in the sorbic acid (e.g. 2,4-hexadienoic acid; CH_3-CH=CH-CH=CH-COOH) having the molecular weight of 112.13 $gmol^{-1}$. It plays an important role in making sorbic acid to possess fungicidal antimicrobial properties (Wedzicha and Brook. 1989; Sofos. 1989). Sorbic acid can inhibit the growth of fungi (e.g. molds, yeasts) and bacteria (Sofos, 2000).

2.4.2 Antimicrobial activity of potassium sorbate

Potassium sorbate is a salt that derived its origin from sorbic acid having a chemical formula of CH_3CH=CH-CH=CH-CO_2K. It is soluble in water to release sorbic acid, and it has found lots of application in the food industry because it could prevent or act as a hurdle to the growth of microbes in foods. The reactive nature of the carboxyl group and the conjugated double bonds of the sorbic acid make potassium sorbate an efficient antimicrobial agent (Sofos, 2000). Figure 2.4.2. below shows the structure of potassium sorbate.

The efficiency of potassium sorbate is highly dependent on pH and it increases with decrease in the pH of the substrate as it gets closer to the dissociation constant (pK_a=4.76) (Cowles, 1941; Hoffman et al., 1944; Rahn and Conn, 1944; Luck, 1976, 1980; Sofos and Busta, 1981; Cerruti et al., 1990). Potassium sorbate are effective at pH value of 6.5 and more effective at even lower pH values than 6.5 (Bell et al., 1959, Lück, 1976; Sofos et al., 1979a; Sofos and Busta, 1980. 1981). It is important to note

that the concentration of sorbates in foods lie within the range of 0.02% to 0.30% (Davidson, A. Branen, and J. Sofos, Editors. 2005).

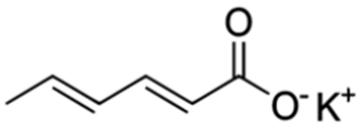

Figure 2.4.2.1 Structure of Potassium

Potassium sorbate has the capacity to inhibit the following microorganisms; Fungi (e.g. *Penicillium Commune*), Mold (e.g. *Alternaria,Cephalosporium, Helminthosporium,Penicillium*), Yeast (e.g. *Brettanomyces, Hansenula, Sporobolomyces*), Bacteria (e.g. *Acetobacter, Clostridium, Vibrio* (Davidson, et al., 2005). A good example of the areas of application of Potassium sorbate is seen in Cheese where 1000 μg/mL of potassium sorbate was able to inhibit the growth of *Penicillium verrucosum var. cyclopium*, also $\leq 6\%$ of potassium sorbate was able to inhibit the growth of *Penicillium roqueforti; Mucor miehi.*

The antimicrobial activity of sorbates depends on the composition of the sorbates, the method of processing, environmental factors like, concentration, pH, water activity, a_w, temperature, gas atmosphere, packaging, microbial flora and inoculum size all of these factors could enhance or reduce the antimicrobial potency of sorbates (Sofos and Busta, 1981; Sofos, 1989; Steels et al., 2000, Sofos, 1989, 1992).

Sorbates can alter the structure, integrity and functions of microbial cell membrane, it can also prevent the transport and metabolic activity (Sofos, 1989). Sorbates can also alter *in vitro* activity of several enzyme e.g. sulfhydryl-containing enzymes (Kouassi and Shelef, 1995a,b) thereby preventing

transport functions, cell metabolism, growth and replication (Davidson, et al., 2005). Sorbates also get in the way of substrate and electron transport mechanisms thereby causing cell starvation (Deak and Novak, 1970; Sheu and Freese,1972, 1973; Sheu et al., 1972, 1975; Freese and Levin, 1978). Sorbates can also prevent synthesis or lessening of Adenosine Tri-Phosphate (ATP), and restrain the metabolic energy used by the amino acid transport system (Sofos, 1989).

2.5 Controlled release food packaging systems

Controlled release food packaging (CRFP) is a new technology that involves keeping food in a container (made of polymer, metal or glass) which acts as a transport system to enable the delivery of active compounds (antimicrobials) at a desired rate so as to retard the reaction kinetics required to cause food deterioration. This prolongs the shelve life of the food and maintains food quality during extended storage (Park, and Marth, 1972).

CRFP consists of a barrier layer, an active layer that houses the active compounds (e.g. antimicrobial) CRFP ensures that the active compound released from the active layer to the food is controlled at time, t. This is achieved by slowing down the reaction kinetics that could result in the deterioration of the packaged food. The graph in figure 2.5.1 also depicts the rate of diffusion of the active compound from the active package with respect to time and the deterioration index of the food with respect to time, thereby prolonging the shelf life of the food (Balasubramaniams, 2012)

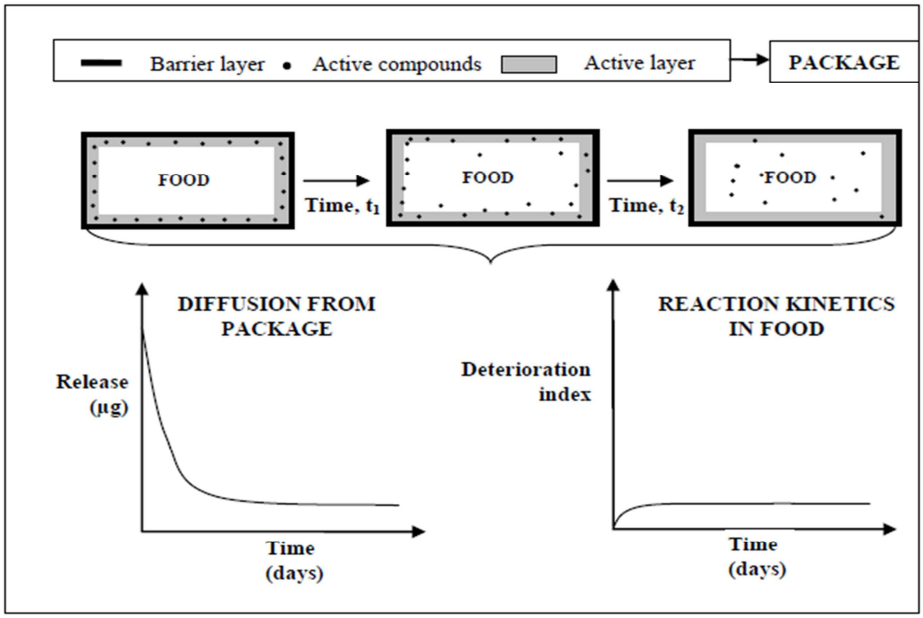

Figure 2.5.1 Mechanism of controlled release food packaging (CRFP) system

2.5.1 Fluid release mechanisms

The Mechanism of fluid release (containing antimicrobial agent) from the film/polymer involves diffusion of the fluid within the polymer matrix. This is then transport of the fluid through the partitioning at the polymer/food interface and the diffusion or dissolution of the fluid into food. This is shown in figure 5 below (Lee, D. et al., 2008).

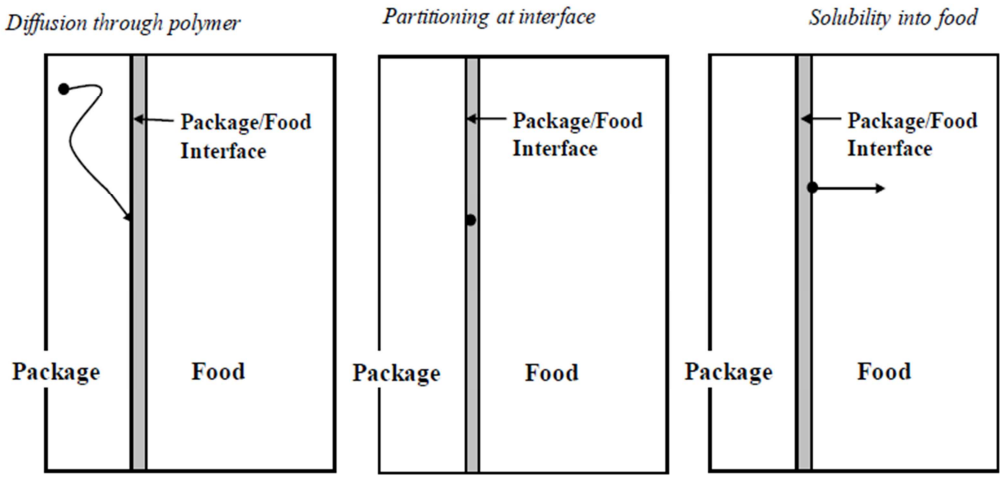

Figure 2.5.1.1 Showing fluid release mechanism from polymer to the food

2.5.2 Smart packaging

Smart Packaging involves various types of packaging designs that integrates mechanical, chemical, electrical and electronic forces, or combination of these, within the packaging. Active packaging with or without communication and Radio Frequecy Identification (RFID) enabled packaging are also other types of packaging that uses electronic smart packaging technique (Joseph, 2008). The major aim of smart packaging is to improve food quality and safety, enhance or stabilize food composition and nutrition, extend shelf-life product stability of product or build confidence. The table The table below shows application of smart packaging in food preservation.

Table 2.5.2.1 Summary of the smart packaging systems described for each group of food products according to their spoilage mechanism.

Food Product	Factors causing spoilage and/or consumer rejection	Smart packaging systems highlighted	Smart substances incorporated into the package wall	Other smart systems useful
Bread and bakery products	• Mould growth • Fat rancidity	• O_2 scavenging • Films • Ethanol emitters	• Iron, unsaturated fatty acids, natural antioxidants • Ethanol entrapped into cyclodextrins	• Antimicrobial films, aldehyde scavengers
Fruits and vegetables	• Accelerated ripening • Mould growth	• Ethylene scavengers • CO_2 controllers	• Zeolites, clay, Japanese oya • Sodium bicarbonate/Zeolites	• Humidity absorbers, antimicrobial films
Dairy products	• Lactose/cholesterol content	• Enzymatically active films	• Enzymes lactase and cholesterol reductase	• Antimicrobial films

2.6 Antimicrobial test method

The principle of all antimicrobial testing is to assess the degree of efficiency inhibiting or inactivity a selected range of organisms under specified conditions. The method of testing the efficacy of food antimicrobials are explained below.

2.6.1 In vitro methods

In vitro methods include any test in which the compound is not applied to a product under use condition. Hence this method can provide prior information to obtain the potential usefulness of the test compound in food**.** *In vitro* methods include endpoint and descriptive analyses.

2.6.1.1 End point method (agar diffusion)

The agar diffusion method which is a type of endpoint method, the antimicrobial compound is added to an agar plate on a paper disk or in a well (Acar and Goldstein, 1986; Piddock, 1990; NCCLS, 1999, 2002). The whey compound diffuses through the agar, leads to a concentration gradient that is inversely proportional to the distance from the disk or well. The degree of inhibition is indicated by a zone of no growth around the disk or well and this relies on the rate of diffusion of the compound and the cell (Barry, 1986).

Agar diffusion at most time gives qualitative results and the inhibition zone which is related to the susceptibility of the test microorganism. When the Inhibition zone is >30 to 35 mm in diameter, the microorganism is said to be susceptible while it is intermediate when the zone is 20 to 30 mm and it

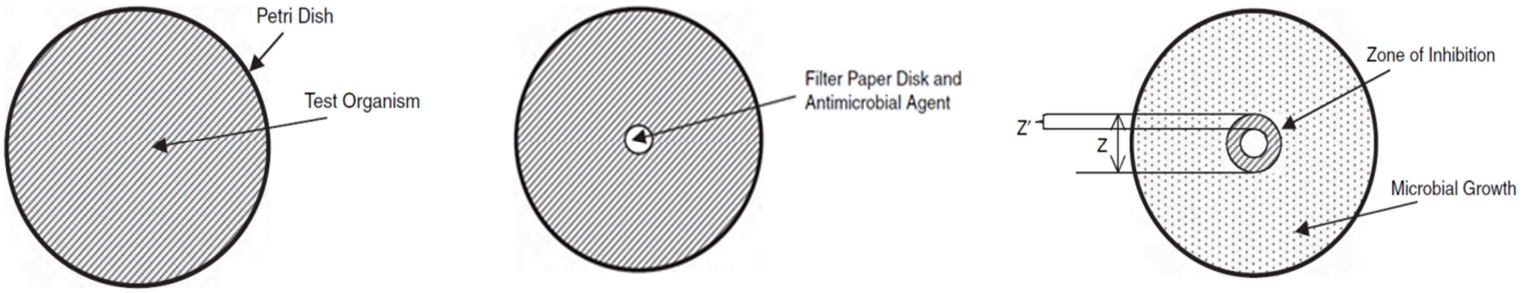

Figure 2.6.1.1 Determination of the "zone of inhibition" by the filter paper disk (agar) diffusion method (Acar and Goldstein, 1986; Piddock, 1990; NCCLS, 1999, 2002).

2.6.1.2 Dilution method

Agar and broth dilution assays are mostly used, to test activity of an antimcrobial agent to the microorganism under test. Dilution method is flexible used to determine the effectiveness of a novel antimicrobial agent. In the broth agar and broth dilution, the minimum inhibitory concentration (MIC) of the antimicrobial agent can be determined after a particular incubation period.

2.6.1.3 Descriptive methods

The descriptive method has found great application in antimicrobial food tests, and it involves the use of inhibition curve with the colony count procedure. In clinical microbiology, these inhibition curves are called "time-kill curves" (Schoenknecht et al., 1985; NCCLS, 2002). It uses the nonselective broth medium (10 to 100 mL) which is added to a single concentration of antimicrobial. The end point method can be used to determine the concentration of the antimicrobial required to totally restrain the growth of microorganism. The microorganism under test is diluted to achieve a final cell concentration of approximately log5.7 CFU/ml in the test medium (NCCLS, 2002). A lower number e.g. log 3.4 to 4 CFU/mL can be used to get the effect of the compound on the lag phase. The NCCLS (2002)

recommends against inoculum levels >log 6 CFU/mL to avoid selection of resistant mutants. A control that does not contain any antimicrobial agent should be made available as a control. At the optimum temperature under which the test is been carried out the medium is incubated for up to 48 hours. Sampling is done at regular time intervals for instance, 0, 2, 4, 8, 12, 24, and 48 hours, using spread or pour plate method the number of practicable microorganisms is determined. The type of test could give stationary-phase growth level suppression, lag-phase increase and decrease in the growth rate during log phase and lethality (Davidson and Parish, 1989). Hence endpoint method helps to determine the approximate effective concentration and the descriptive method evaluates the effect of a compound on growth over time.

Turbidity on the other hand is used to determine the number of cells. In antimicrobial food packaging, the turbidity assay test helps to determine antimicrobial effectiveness over time. The instrument for measuring turbidity is the spectrophotometer. Spectrophotometers generally require log (6.0 to 7.0) CFU/mL for detection (Brock et al., 1984; Piddock, 1990).

2.6.1.4 Combined antimicrobial test

Combined antimicrobial test involves agar diffusion, agar or broth dilution, or inhibition (time - kill) curves. Agar diffusion is a simple qualitative method that most time demands subjective interpretation of inhibiting zone shapes to determine the efficacy of combined antimicrobials (Barry, 1976). Combined antimicrobial test provides a quantity of microbial growth that has been inhibited but not the biocidal activity. Dilution method on the other hand provides quantitative data and most often conducted in conjunction with other combined concentration of two antimicrobials arranged in a “checker board”

array. The checker board is used to assess antimicrobial combinations *in vitro,* because of the following reasons.

- Its rationale is easy to understand
- The mathematical model is simple
- It can readily be performed in the laboratory using micro dilution systems
- It is the most frequently used technique in that suggested the benefit of synergistic interactions of antibiotics in clinical treatments (Eliopoulos and Moellering, 1991). The pattern formed by multiple dilutions of the two antimicrobials being tested in concentration equal to, above, and below their minimum inhibitory concentration (MIC) is termed checkerboard.

2.6.2 Previous studies on antimicrobial test methods

The methods of carrying out antimicrobial test have been reviewed and the recommendations suggested that more uniformity and standardization should be instituted in testing food antimicrobials (Davidson and Parish, 1989). One of the first methods used to examine the activity of disinfectants was developed by Robert Koch in 1881 (Block, 1983; Crémieux and Fleurette, 1983). He immersed silk thread in culture fluid containing *Bacillus anthracis Ispores* and exposed the dried threads to disinfectant for varying periods. He discovered that mercuric chloride was highly effective against the spores but carbolic acid (phenol) and ethanol had no significant antimicrobial effects. Koch was likely the first to distinguish between bacteriostasis and lethality (Block, 1983).

Assay methods involving the diffusion of compounds in agar from wells or papers disks were formulated (Piddock, 1990). The methods for determining the minimum inhibitory concentration (MIC) using broth or agar dilution were also developed.

Crushed garlic has been found to be effective against some microbes like *Mycobacterium* species, *Escherichia coli, Serratia marcescens,* and *Bacillus subtilus* (Walton et al. 1936). The test procedures of Walton and others showed that they placed crushed garlic on the lid of a petri dish and the bottom of the petri dish that contained the nutrient medium was inverted over the top. The galic vapours were allowed to penetrate into the agar for varying periods. After exposure, the medium that contained the microorganism under test was incubated to find out the rate of inhibition. The antimicrobial effectiveness was related to time of exposure of the medium to the garlic vapours. This method has formed a basis for most antimicrobial tests either directly or with little modifications (Walton et al. 1936).

The effect of lactoferrin (LF), lactoferrin hydrolysate (LFH), and lactoperoxidase systems (LPOS) were in direct contact and incorporated into edible whey protein isolates (WPI) films, were effective at inhibiting the growth of *P. Commune* (Min and Krochta, 2005). They used turbidity, disc diameter, surface spreading and film surface inoculation tests to analyse the antimicrobial effect.

In the turbidity test, the variations in the absorbance of 1% peptone water and potato dextrose broth (PDB) having varying concentration of LF, LFH, and LPOS after inoculation of *P. Commune* were investigated after 2 days with LF greater than 0.5 mg/mL and after 5 days with LF greater than 10 mg/mL. The MIC of LF was found to be 10 mg/mL to inhibit *P. Commune* from 3.6×10^3 spores/mL in 1% peptone water (50 μL of 3.6×10^5 spores/mL in 5 mL 1%(w/w) peptone water). The result shows that LF was not able to inhibit the growth of *P. Commune* in PDB medium because of the saturation of LF by divalent cations and ferric ions. It is also important to note that *P. Commune* was also resistant to LFH.

A new edible composite film, hydroxypropyl methylcellulose (HPMC)-lipid was formulated with low toxic chemicals like organic acid chemicals, parabens salt and minerals salts which had antifungal properties (Silvia et al., 2008). Using the disc diameter test they were able to assess the

antimicrobial efficiencies of some selected antimicrobial films against *Penicillium digitatum* (PD) and *Penicillium italicum* (PI) using PDA and dichloran rose-bengal chloramphenicol agar (DRBC) as a medium for the disc diameter test.

2.6.3 Swelling and de-swelling ratios

Polymer films are generally sensitive to water and environmental humidity because of the hygroscopic nature of polymers and many hydrophilic groups in the molecular chains (Kun, et al, 2008). The knowledge of swelling and de-swelling in polymer films will aid in the following applications in biomolecular electronics and sensors, drug delivery systems, wound dressings, adsorbtion of chemical materials, antimicrobial food release etc (Alan, 2002 and Pepas, et al, 1999). Hence swelling is the rate at which a solute absorbs a solvent with time while de-swelling is the rate at which a solute releases the solvent it has absorbed with time. The swelling behaviour can be calculated using the formula

$$\frac{m_t - m_0}{m_0} \text{x} 100\% \quad (1)$$

Where m_0 and m_t are weights of samples before swelling and after swelling time t, respectively. The average swelling ratios (SR_A) given by:

$$SR_A = (M_t - M_o)/M_o \quad (2)$$

The equilibrium swelling ratio SR_{eq} can be obtained from the following expression:

$$SR_{eq} = (M_{eq} - M_o)/M_o \quad (3)$$

where M_{eq} is the mass of the film at equilibrium (after 6 hours of soaking) and M_o is the initial mass of the film before soaking. The fluid uptake by the film can be obtained using the formula:

$$M_{rel} = M_t/M_s \quad (4)$$

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

$$V_{eq} = \frac{\left(\frac{\pi D^2}{4}\right)}{\left(\frac{\pi D_\circ^2}{4}\right)} = \frac{D^2}{D_\circ^2} \quad (5)$$

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

2.6.4 Fluid release from antimicrobial film

An equation based on practical experiment was proposed by Peppas and co-workers for drug release which assumes time-dependent power law function:

$$\frac{m_t}{m_i} = 4\left(\frac{D}{\pi\delta^2}\right)t^n = kt^n \quad (6)$$

$$m_t = m_o - m_f \quad (7)$$

where $\frac{m_t}{m_i}$ is the fluid/drug release fraction, k is the geometric constant of the release system, n is the fluid/drug release exponent, depicting the release mechanism, m_i is the absolute cumulative amount of drug released at time, t, m_t is the amount of drug/fluid remaining in the hydrogel/film at time, t during drug elution, δ is the thickness of the gel/film. From equation (7), m_o is the mass of the swollen hydrogel/film at equilibrium state prior to drug release, m_f is the final mass of the hydrogel/film after drug elution, and D is the diffusion coefficient.

If the release within the polymer network is diffusion controlled then Equation (6a) is used. The constants k and n can be obtained from the linear form of equation (6a). Hence:

$$ln\left({}^{m_t}/_{m_i}\right) = lnk + n\ln t \quad (7)$$

Where k, represents the intercept and, n, the slope of the plot, $\ln(m_t/\mathrm{m_i})$ versus ln(t). The intercepts on the $\ln(m_t/\mathrm{m_i})$ is equal to ln(k). The diffusion coefficients, D_s, were obtained from:

$$D_s = \frac{k\pi\delta^2}{4} \quad (8)$$

Where k, π and δ are the geometric constant of the release system, the mathematical constant reflecting the ration of a circle circumference to its diameter and the thickness of the gel, respectively.

2.6.5 Beer Lamberts law

Beer-Lambert Law is the linear relationship between absorbance and concentration of an absorbing species. The general Beer-Lambert law is usually written as**:**

$$A = \epsilon lc \quad (9)$$

Where A is absorbance (no units, since $A = \log_{10} P_0 / P$)

ϵ, is the molar absorbtivity constant with units of L mol^{-1} cm^{-1}

l, is the path length, the distance of solution that the light has to travel through (1cm)

C, is the concentration of the solution, expressed in mol L^{-1} or $moldm^{-3}$**.**

Hence, using Beer Lambert's law, the Concentration and the molar absorptivity for different types of antimicrobial film can be calculated.

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CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

100 g Potassium sorbate ($C_6H_7KO_2$) with relative molecular mass of 150.22gmol^{-2} (99.9%) was obtained from Qualikems Laboratory Reagent the mass was 100 g, New Delhi, India. While Chloramphenicol (purity of ≥97%) by Assay with model number 220551 was purchased from Merk, New Jersey, USA. Acetone with model number MFCD00008765 was obtained from sigma Aldrich, Europe. Cellulose acetate with molecular weight of 29 000 was obtained from Fluka, USA. 50 g of sucrose ($C_{12}H_{22}O_{11}$) with molecular weight of 342.29 was obtained from Kermel. Agar was obtained from Fisher Scientific, New Jersey, USA. SEM by ASPEX corporation (ASPEX 3020 PSEM2 South San Francisco, United States of America), other materials includes. Hand gloves from Supermax select Malaysia. Axiom Face Mask, Jiangsu, China.

3.2 Methods

3.2.1 Preparation of monolayer films

Potassium sorbate (Ps) and cellulose acetate (CA) were dissolved in water acetone, respectively. The Ps/water solution was added to the CA/acetone solution using a syringe in a drop wise fashion under stirring condition to obtain a homogenous mixture the solution was degassed in a vacuum oven for 30 min at 25℃ to eliminate bubbles. The sample was cast into molds to form the antimicrobial films. The concentration of PS in the film was kept constant at 2%(w/w) throughout the experiment, while the concentration of cellulose acetate used was varied (10%, 13% and 15%, by weight). Three monolayered

films were formed using glass petri dish as the mold. The codes of the films and other conditions used in the film preparation are summarized in the table 3.1.

Table 3.2.1.1 Types of films and their Codes Obtained at 24˚C

Film code	Number of layers	Film thickness (mm)	Composition of CA (%w/w)
M_{15}^{+}	1	2	15
M_{13}^{+}	1	2	13
M_{10}^{+}	1	2	10
M_{15}^{-}	1	2	15
M_{13}^{-}	1	2	13
M_{10}^{-}	1	2	10

M_{15}^{+}, M_{13}^{+} and M_{10}^{+}means a monolayer antimicrobial films with 10%, 13% and 15%, by weight of cellulose acetate in acetone and potassium sorbate

3.2.2 Release tests

Release test was carried out on the three monolayers film formed. The monolayers which contain different concentration of cellulose acetate were placed in a 10 cm glass petri dish containing 100 ml of distilled water with pH of 7 at a temperature of 25°C and the content of the petri dish is stirred using magnetic stirrer at a speed of 240 rpm (revolution per minute) with a 2 cm long Teflon coated rod.

Samples of PS-based films were place in 10 ml distilled water to measure the release of the potassium sorbate (PS) and 6 ml of the distilled water was taken at 24 h intervals, while renewing the 6 ml distilled water each time. After diluting with 1 mL distilled water, the absorbance's of the samples were measured at 250 nm using UV-Vis spectrophotometer (Biochrom Libra S22 UV/Visible Spectrophotometers, by Biochrome Ltd, Corston, UK).

3.2.3 Characterization of films

The surface morphology of the films were examined using scanning electron microscope ((ASPEX 3020 PSEM2 South San Francisco, United States of America) Spectrophotometer was also used to determine the peak absorbance of potassium sorbate in a solution containing antimicrobial film dissolved in distilled water over a period of ten days at a wavelength of 250 nm.

3.2.4 Preparation of potato dextrose agar (PDA)

90 g of chopped potato was weighed using an analytical balance (OHAUS, model number E00640, Bradford, England) and 200 ml of distilled water was added to the potato infusion that was sliced, since 300 g of the chopped potato requires 1liter of distilled water. The mixture was boiled for 1 hour at 100°C. Soil samples were collected from the land fill refuse bin at Galadimawa, Abuja Nigeria, so as to isolate the fungi from the soil sample; 2 g of the soil was measured for use. Two different soil samples were used labeled samples A and B. The experiment required six pieces of bijou bottles for soil samples A and six for soil sample B.

0.9 g of sodium chloride was dissolved in 200 ml of distilled water and the solution was subjected to shaking to ensure complete dissolution of the salt in water. This forms the diluent saline

solution. 300 ml of distilled water was added to the saline solution to make the broth. For soil sample A and B, 2 g of the soil from the refuse dump site was poured into the bijou bottles with labels sample A and B, respectively. Then 9 ml of the saline solution was poured into each of the twelve bijou bottles which were used for cell subculture.

The source of nutrient for the microbes (fungi) are the potato broth and sucrose, 6 g, which was dispensed in 300 ml of distilled water using the standard of 20 g of sucrose in 1liter of distilled water (Anonymous, 2014), 4.5 g of agar was dispensed in 300 ml distilled water using a standard of 15g of agar in 1liter distilled water (Anonymous, 2014). The sucrose solution, agar solution and 0.6 g of chloramphenicol were dispensed in potato broth that has been made up to 300 ml and this combined solution is called potato dextrose agar (PDA).

The saline solution, PDA in a conical flask and the petri dishes were all autoclaved at 121°C for 15minutes. To prepare a 70 ml of absolute ethanol (100%) was made up with 30 ml of distilled (70%w/w ethanol) which was used to kill the microorganisms in the biosafety cabinet. The biosafety cabinet was then turned on for 5 min to allow UV light to disinfect the hood. The content of the autoclaved is transferred to the biosafety cabinet, sterilized with the 70% w/w ethanol. In the biosafety cabinet is a Bunsen burner which is used to kill any microbe around the petri dish and the bijou bottles. A stock solution was prepared using 2 g of soil dispensed in 8 ml normal saline solution. The bijou bottles labeled 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}. Using syringe 1 ml of the stock solution was added to the bottle labeled 10^{-1} which contains 9 ml of the normal Saline solution to make it up to 10 ml solution. Then 1ml of the content from the 10^{-1} bijou bottle was taken and then transferred to 10^{-2} bijou bottle, by serial dilution; the process was repeated for sample B until all the bijou bottles were made up to 10 ml.

3.2.5 Preparation of test organism

Colonies of the *Aspergillus niger* (*A. niger)* prepared as stated earlier were transferred from the potato dextrose agar (PDA) slant at 25°C for 30 min from the bijou bottle to the petri dish and 80 µl section of the *A. niger* colonies were dispersed into six different glass petri dishes. After three days the grown *Aspergillus niger* spores were harvested by sterile tween-20 solution (0.05% w/v) and then added to the PDA in the slanting bijou bottles. The slants were incubated at room temperature (25°C) for 7 days and the harvest was again done again using sterile tween-20 solution.

3.3 Determining the A. niger spores

Spore suspension was prepared. Haemocytometer (Bright-Line Hemacytometer, Z359629, Gillingham Dorset, United Kingdom) was cleaning with a non linting tissue, similar tissue was used to dry the surface of the heamocytometer and the coverslip. Pipet was used to mix and then draw up 9 µl of the cell suspension into the counting chambers of the heamocytometer. Care was taken to avoid injecting bubbles into the chambers. The spores were counted in each of the four 0.1 mm^3 corner squares. The spores touching the top or left and or bottom or right borders were not counted. To determine the spore count, the total spores counted in the four corner squares were calculated using the equation:

$$\text{Spores } (ml^{-1}) = (n) \times 10^6 \qquad (3.1)$$

where: n = the average cell count per

square of the four corner squares counted.

3.3.1 Test for zone of inhibition

The antimicrobial activity exhibited by the antimicrobial agent was checked using the classical zone of inhibition test (Braid, J.J. et. al., 2002). The 80 µl of the *Aspergillus niger* culture was transferred and spread using a sterile cotton bud on the PDA in the petri dish. Then 5 mm diameter antimicrobial films of the three different CA contents were placed on a glass petri dish. The petri dishes were kept at room temperature, 25°C for seven days. The growth of the *Aspergillus niger* was monitored and the diameter of the zone was measured with a caliper on the 3rd, 5th and 7th days.

3.4 Industrial application of antimicrobial film

15 g of potassium sorbate was mixed into high density polyethylene pellets used in First Drops Nigerian Limited for producing water bottles; the mixture was mixed thoroughly and poured into the hopper after heating the pellets the heated polymer was very viscous with lots of defects on it as shown below in figure.

Figure 3.4.1 showing viscous linear high density polyethylene (HDPE) with antimicrobial agent incorporated into it

3.5 Swelling and de-swelling kinetics

Swelling and de-swelling test were carried out on six different antimicrobial film samples (M_{15}^{+},M_{13}^{+}, M_{10}^{+}, M_{15}^{-}, M_{13}^{-}, M_{10}^{-}). Where M_{15}^{+}, M_{13}^{+}, M_{10}^{+} represents antimicrobial films with 15%, 13%, 10% w/w cellulose acetate in acetone dissolved in 8 ml solution (containing 2% w/w potassium sorbate in distilled water). M_{15}^{-}, M_{13}^{-} and M_{10}^{-}, represents polymer film containing 15%, 13%, and 10% w/w cellulose acetate in acetone without any antimicrobial agent added to it. The swelling ratios were obtained from:

$$SR = (M_t - M_o)/M_o \quad (3.2)$$

The masses of the films were determined at regular intervals of 30 min for six hours. The de-swelling kinetics was also carried out for the same time frame, by placing the swollen films on a filter paper to determine the release of fluid. This was obtained using the de-swelling equation;

$$DR = (M_0 - M_t)/M_o \quad (3.4)$$

REFERENCE

Braid, J. J. and Wale, M. C. J. (2002). The antibacterial activity of triclosan-impregnated storage boxes against *Staphylococcus aureus,Escherichia coli, pseudomonas aeruginosa, Bacillus cereus and Shewanella putrefaciens* in conditions simulating domestic use. Journal of Antimicrobial Chemoteraphpy 49, 87 – 94

CHAPTER FOUR

4.0 Results and discussion

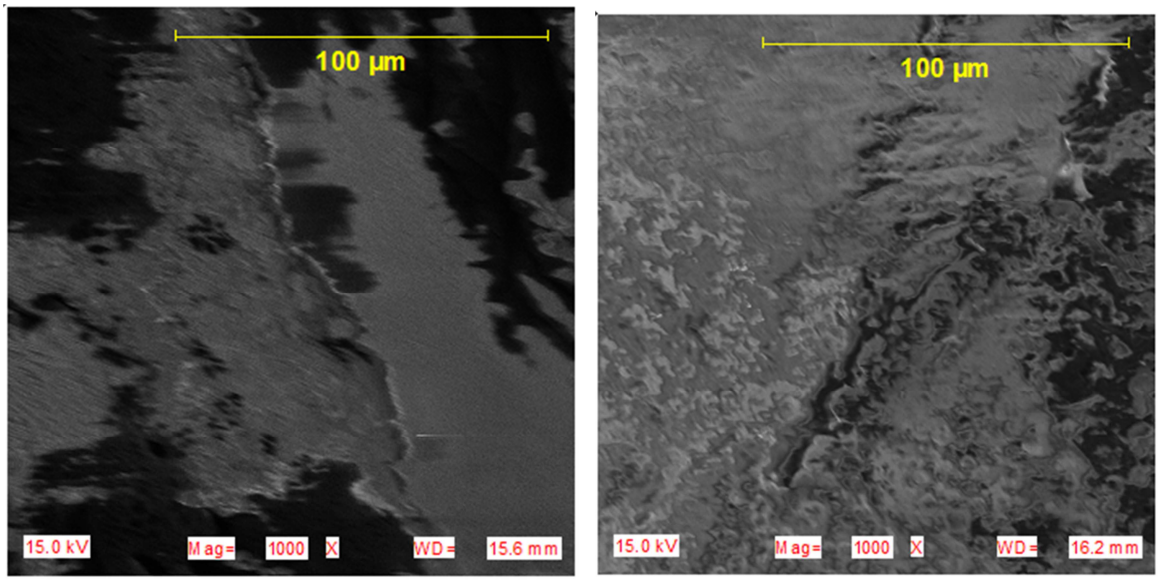
4.1 Introduction

In this study, the antimicrobial agent (AM) used was potassium sorbate (PS) incorporated into cellulose acetate (CA) where distilled water and acetone were used as the dissolution solvents, respectively. Six film samples were formed with three containing AM agents, while three others had no AM agent in it to inhibit the growth of *A. niger* on the surface morphology, the effect of the AM agent in inhibiting the growth of *A. niger* on selected food (peanut and bread), the possibility of mixing potassium sorbate with the master batch used in producing plastic food containers and the release kinetics of the antimicrobial agent from the film samples were studied.

4.2 Film morphology analyses

The results of the scanning electron microscopy analyses are shown (Figs. 4.1a-c). Microstructural analysis shows that increasing the concentration of CA in the film from 10 wt%, 13wt% and 15 wt% leads to the formation of dense films with less porosity as the amount of polymer in the films increases. As the antimicrobial film dries, the crystalline nature of the PS tends to manifest itself more (Fig. 4.1d). As the antimicrobial film dries further, the PS concentration in the pores increases which leads to increase in the crystal growth in the pores as a result of supersaturation. The result of drying the antimicrobial film at a higher temperature of 100˚C leads to further pores. Hence the amount of crystals formed in a film depends on the porosity of the films.

(a) (b)



(c) (d)

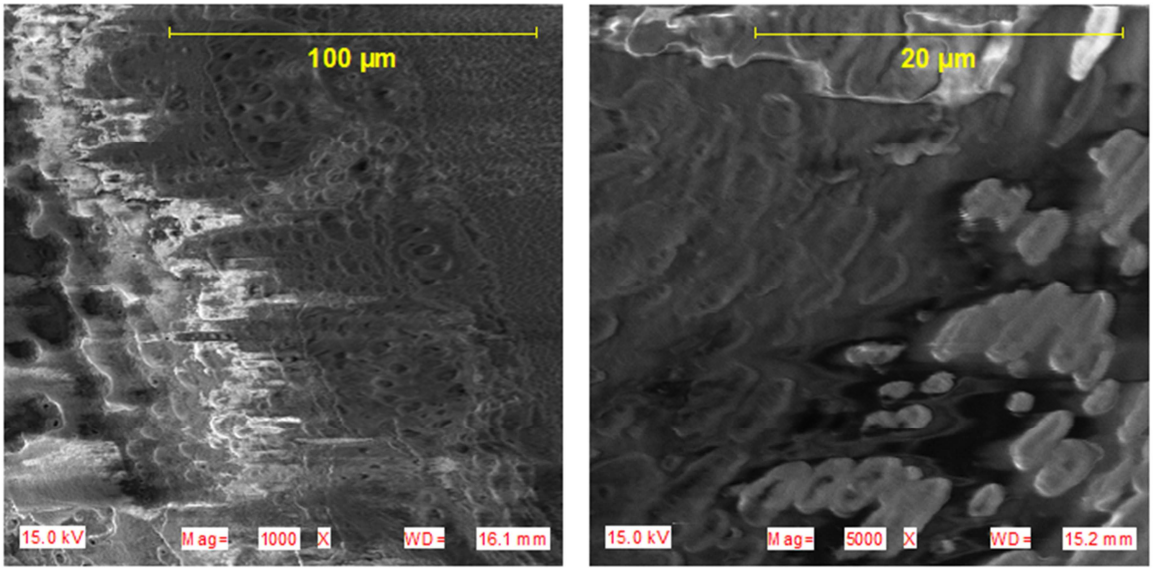


Figure 1 Figure 4.2.a-c: SEM of the cross-section of single layer CA film with 1300 µm thickness dried at 24°C: (a) CA content in the initial casting solution is 10wt%; (b) CA content in the initial casting solution is 13wt%; (c) the CA content in the initia

4.3 Swelling and de-swelling

The result of the swelling analysis shows that both at room temperature 24°C and at an elevated temperature of 37°C the film M_{13}^{+} swells the most and film swells the least of the three antimicrobial films, as shown in figure 4.3.1 (a) and (b). Hence antimicrobial film M_{13}^{+} has the highest water retention capability. For films without antimicrobial agent in them, M_{15}^{-} turns out to be the film with the highest swelling ratio and film M_{10}^{+} the least both at room temperature and at 37˚C, which implies that film M_{15}^{+} has the highest water holding capability of the three films.

The de-swelling curve on the other hand Figure 4.3.2(a - d) shows that for antimicrobial films at room temperature the de-swelling ratio of the films were close, but at 37˚C the rate of de-swelling/ water loss was more pronounced in the film with the code, M_{10}^{-} . Also for films without antimicrobial agent, film M_{10}^{-} demonstrates the highest de-swelling ratio meaning it was the fastest at losing its moisture content.

For films without antimicrobial agents, it turns out that film M_{10}^{-} had the lowest de-swelling ratio both at room temperature and at 37˚C as seen in figure 4.3.2 (c) and (d). The film with the code M_{15}^{-} had the lowest water holding capacity of the three films hence having .

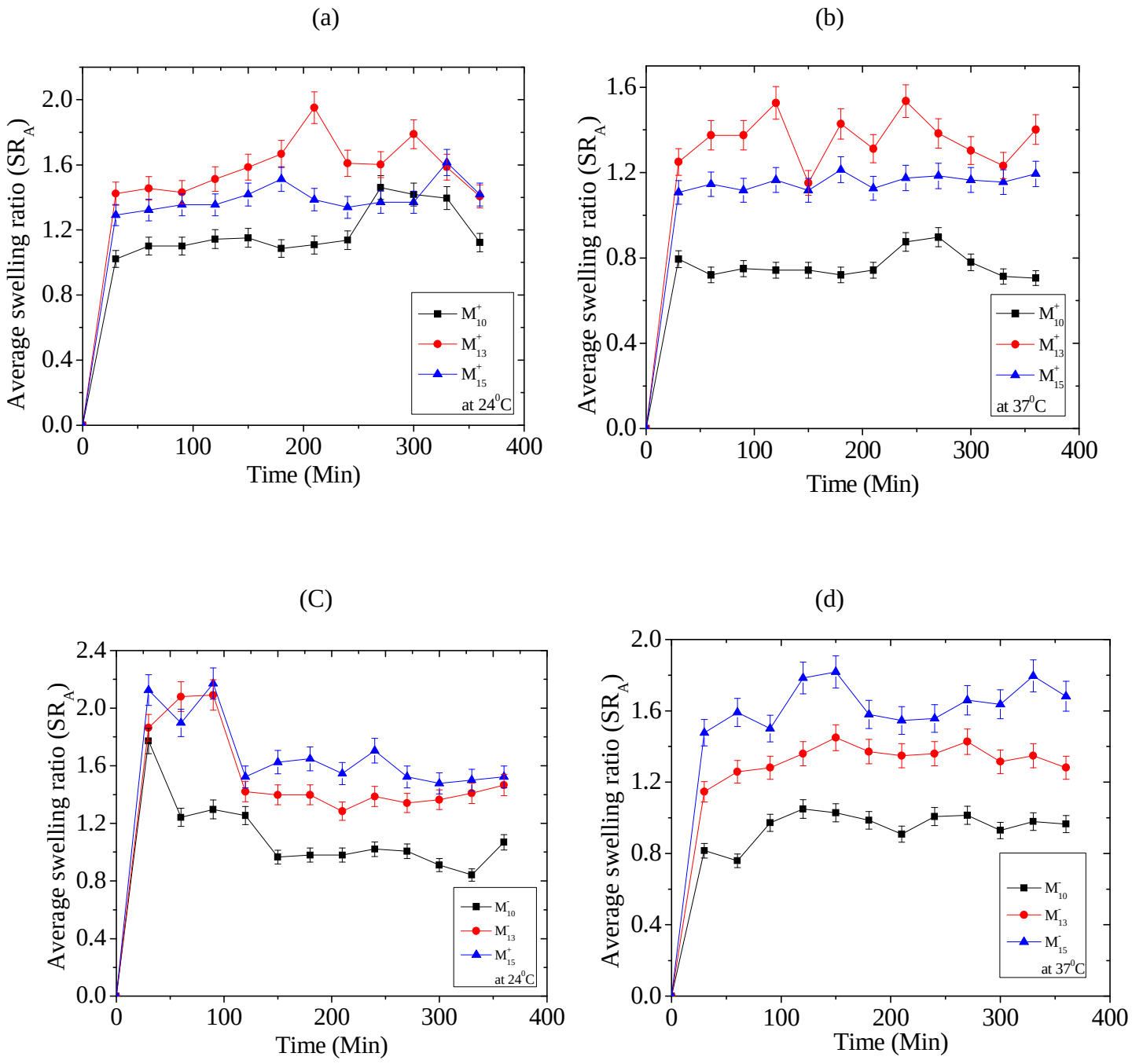


Figure 4.3.1 Shows graphs of antimicrobial films swollen (a) at room temperature 24˚C (b) at 37˚C. While (c) Films swollen at 24˚C (d) Films swollen at 37˚C. (c) and (d) has no antimicrobial agent.

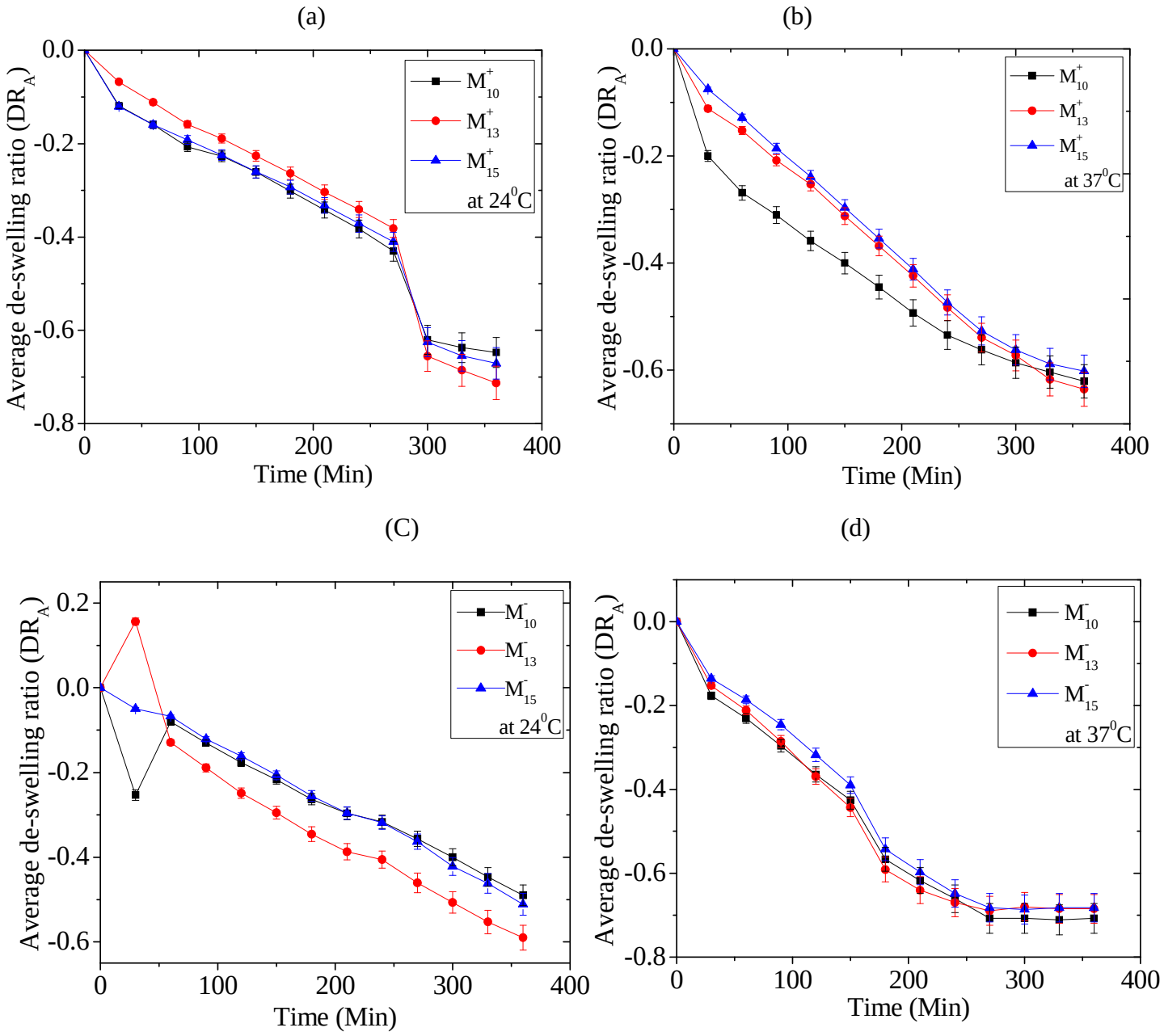


Figure 4.3.2 Shows graphs of antimicrobial films de-swelling graph at room temperature 24˚C for all the film samples.

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4.4 Determination of the film pore size

The film pore size was determined using the software called Gwyddion version 2.34. The results are as shown below.

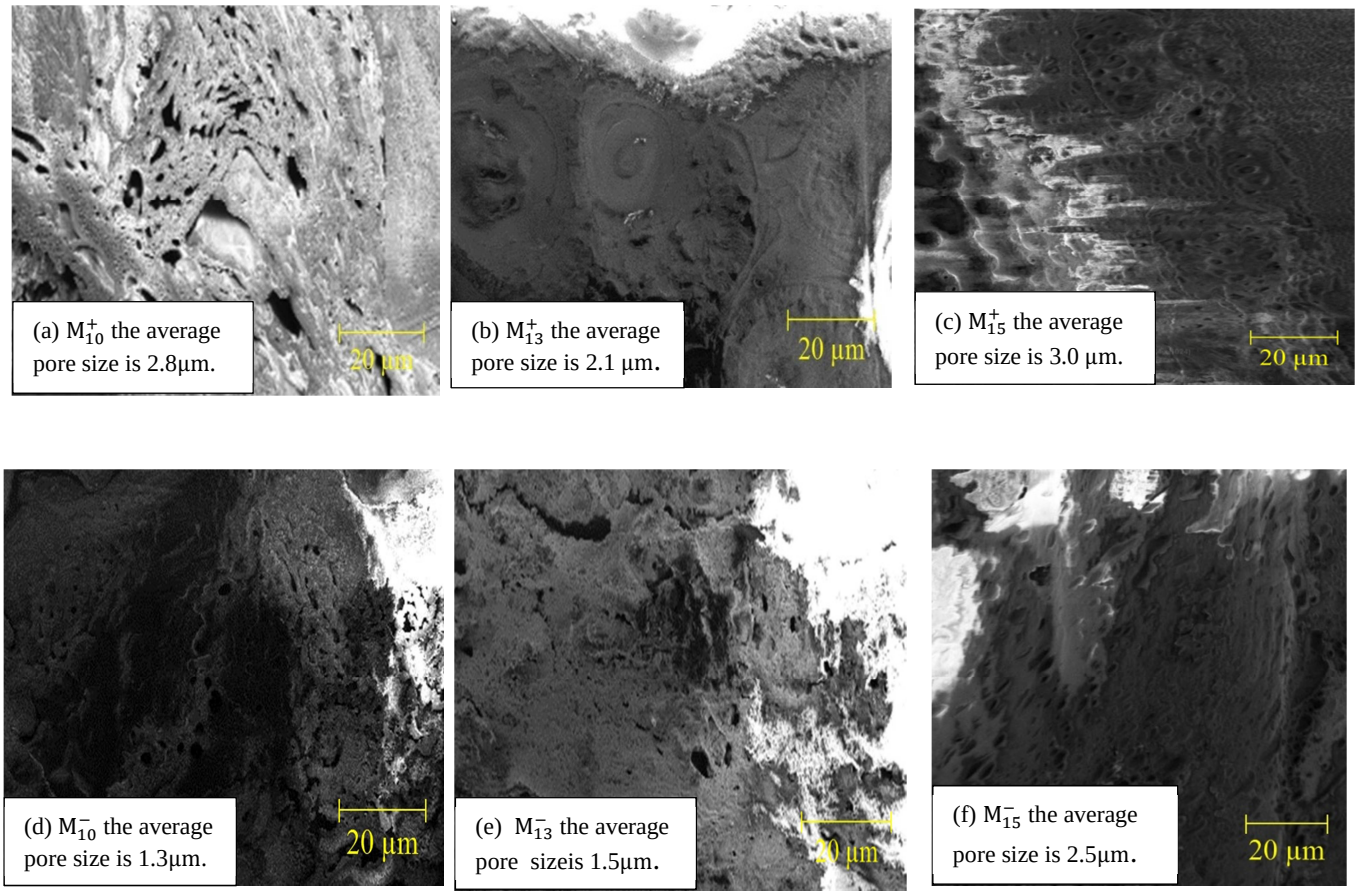


Figure 4.4.1 a - f, showing the microstructural analysis of the films to obtain the pore sizes.

The result shows that the pore sizes increases with increasing ratio of CA in the film containing antimicrobial agent and also for films without antimicrobial agent, the pore size also follows the same trend. The bar chart below shows the pictorial variation in pore sizes of the different films

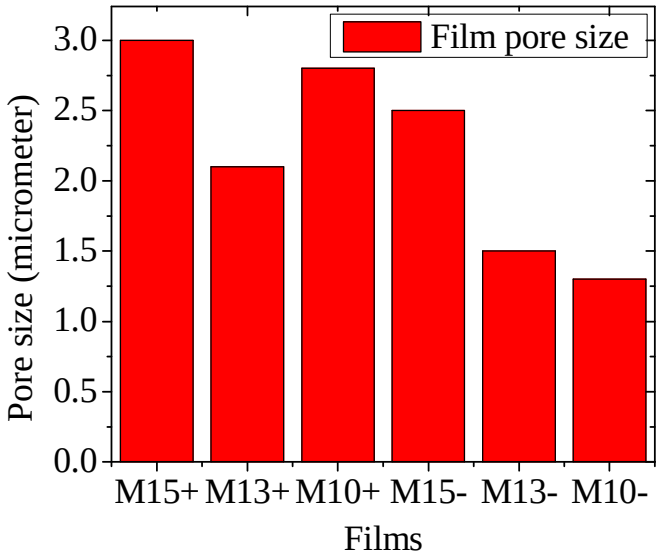


Fig.4.4.2 Showing the pore sizes of the Films produced

4.2 Swelling and de-swelling test results

The swelling and the de-swelling test was carried out on all the six different antimicrobial film samples ($M_{15}^{+}, M_{13}^{+}, M_{10}^{+}, M_{15}^{-}, M_{13}^{-}, M_{10}^{-}$). Where M_{15}^{+}, M_{13}^{+}, M_{10}^{+} represents antimicrobial films with 15%, 13%, 10% w/w cellulose acetate in acetone dissolved in 8ml solution (containing 2% w/w potassium sorbate in distilled water) and M_{15}^{-}, M_{13}^{-} and M_{10}^{-}, represents polymer film containing 15%, 13%, and 10% w/w cellulose acetate in acetone without any antimicrobial agent added to it. The swelling analyses were carried out on all the six samples and the films weight was measured at intervals of 30 minutes for six hours. The de-swelling analyses too were carried out for the same time frame, by placing the swollen film on a filter paper to see how the film's release its moisture content over time. The results are as shown in the table below.

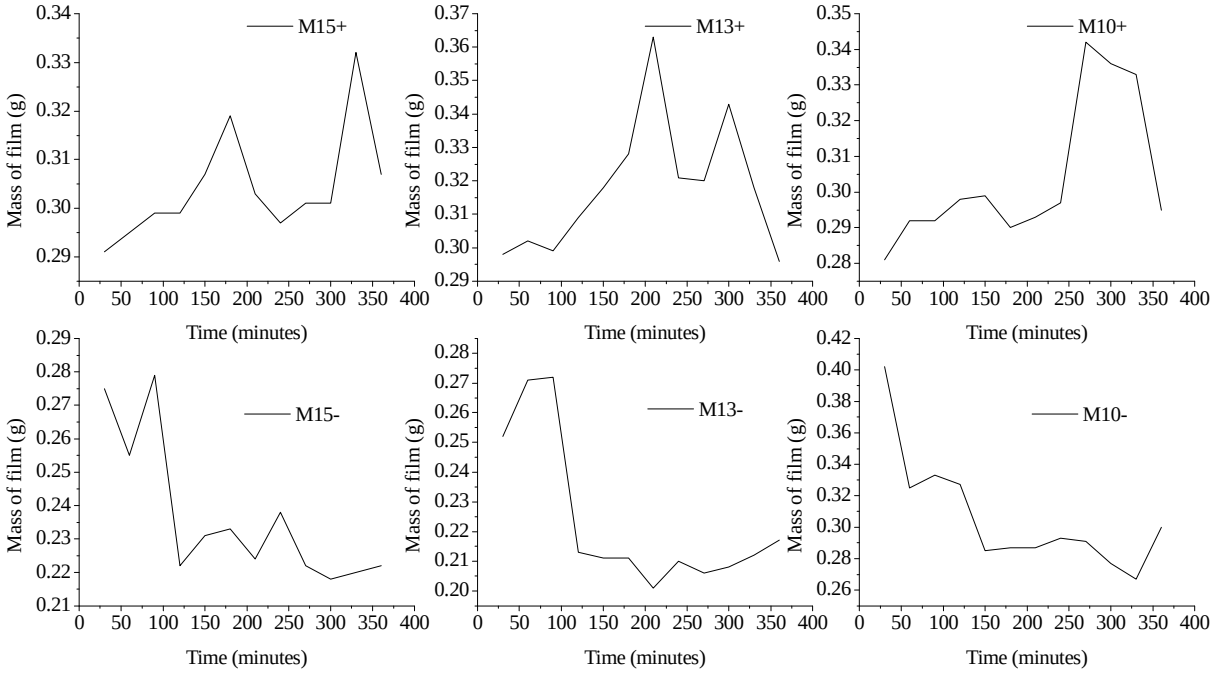


Figure. 4.2.1 Film swelling analyses at room temperature, 24˚C

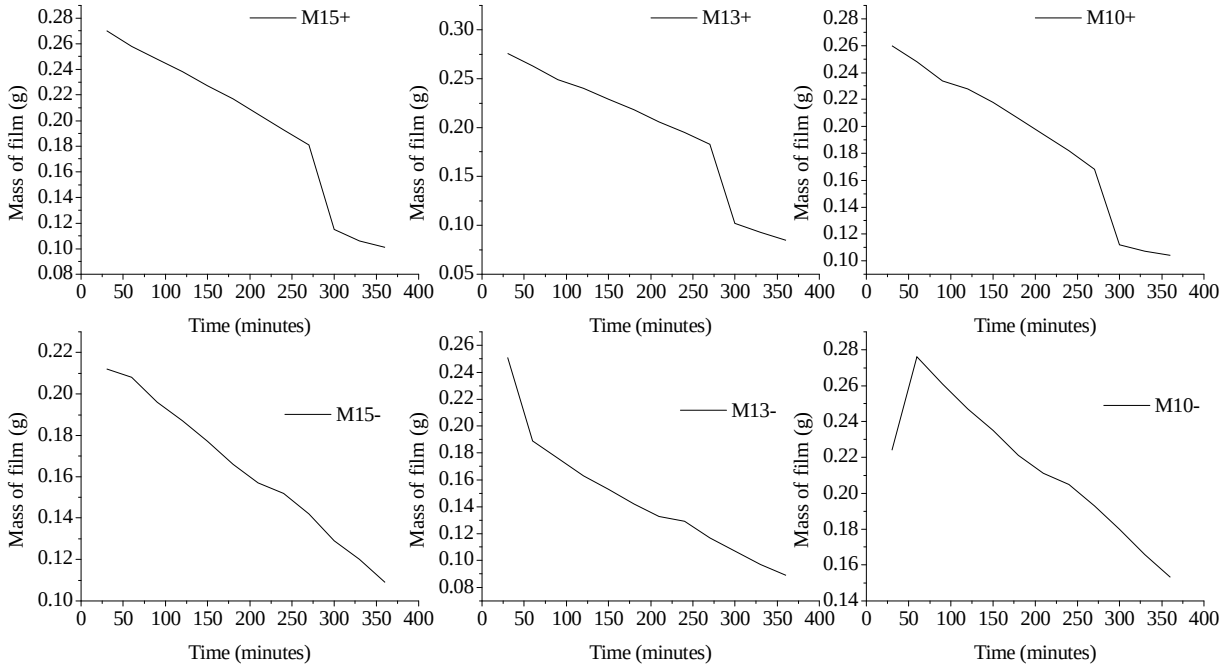


Figure. 4.2.2 Film de-swelling analyses at room temperature 24˚C

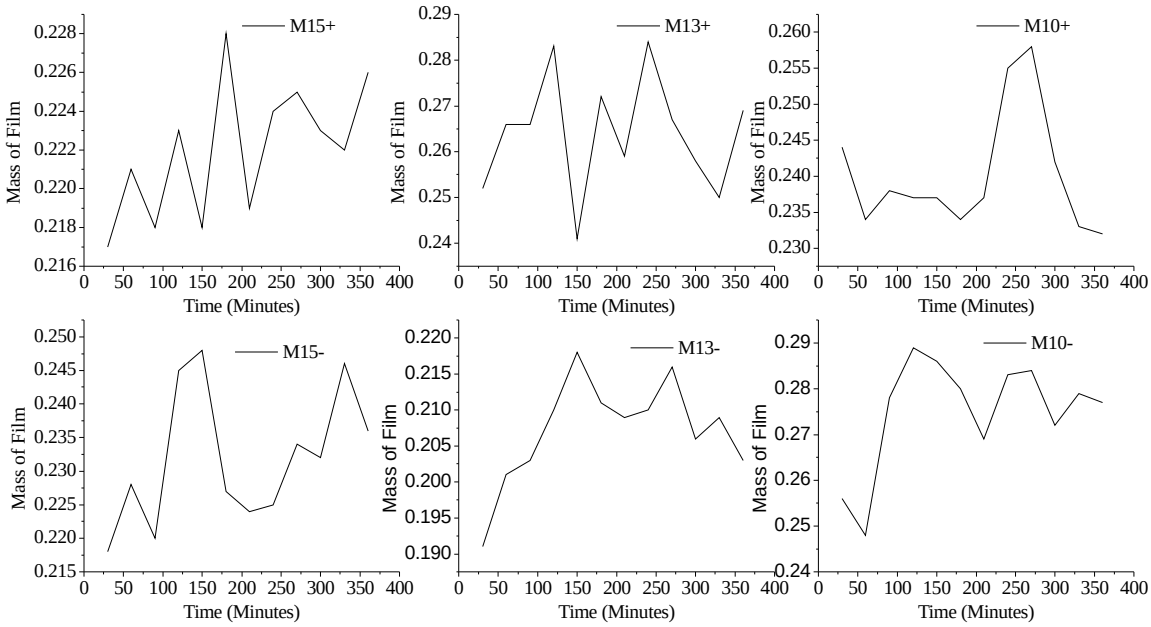


Figure. 4.2.3 Film swelling analyses at 37˚C

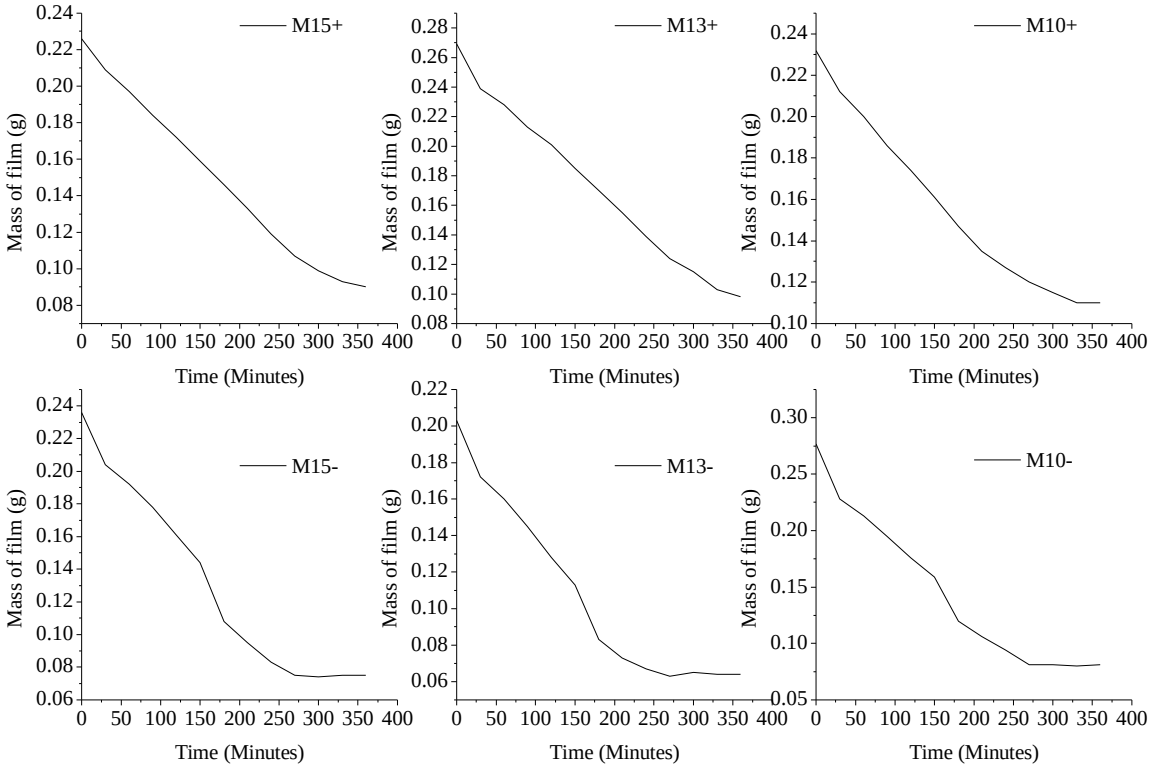


Figure.4.2.4 Film de-swelling analyses at room temperature, 24˚C

From the Data obtained from Figure 4.2.1 (a) and (b), the jagged edges of the curve ascends with tine which indicates that the antimicrobial film at room temperature has swollen from: 0.127 g to 0.332 g for film M_{15}^{+}, 0.123 g to 0.363 g for film $M_{13}^{+}$and 0.139 g to 0.342 g for film M_{10}^{+}. Films M_{15}^{-}, M_{13}^{-} and M_{10}^{-} without antimicrobial agent only swelled for the first 90 minutes and no further increase in mass was noticed again. This shows that antimicrobial films have a better swelling capability at room temperature 24˚C than films without antimicrobial agent.

From figure 4.2.2, it shows that both antimicrobial films and the films without antimicrobial agent all have a very high rate of losing their water content when exposed at room temperature. This is shown by the steep curve of the figures.

Figure 4.2.3 showed that all the films had fair swelling capability at 37˚C indicated by the jagged edges of the curves, for antimicrobial films, film M_{13}^{+}swelled the most while M_{15}^{+} swelled the least. The rate of swelling of the films without antimicrobial agent showed that film M_{10}^{-} swelled the most and film M_{13}^{-}swelled the least. Figure 4.2.4 showed that the antimicrobial films and the films without antimicrobials followed similar trend.

4.3 Swelling kinetics

The data obtained from the weight loss experiment was used to calculate the diffusion coefficient, fluid release exponent, fluid release fraction, geometric constant, average and equilibrium swelling ratios of the films are as shown statistically below. Figure 4.3.1 show that at room temperature, the geometrical constant (k) for antimicrobial films is highest for film M_{15}^{+} and lowest for filmM_{15}^{+} at room temperature while at 37˚C, film M_{10}^{+} had the highest geometric constant and film M_{13}^{+} had the least value of geometrical constant.

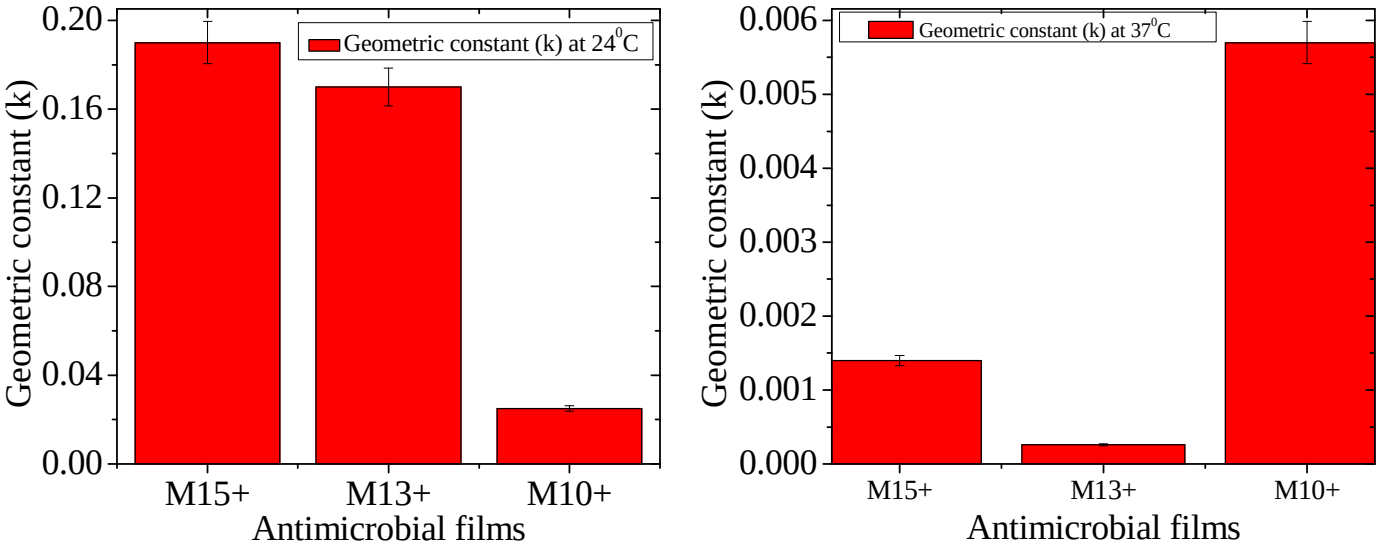


Figure 4.3.1 Bar chart showing geometrical constant (k) for antimicrobial films at (a) room temperature 24˚C and (b) at 37˚C

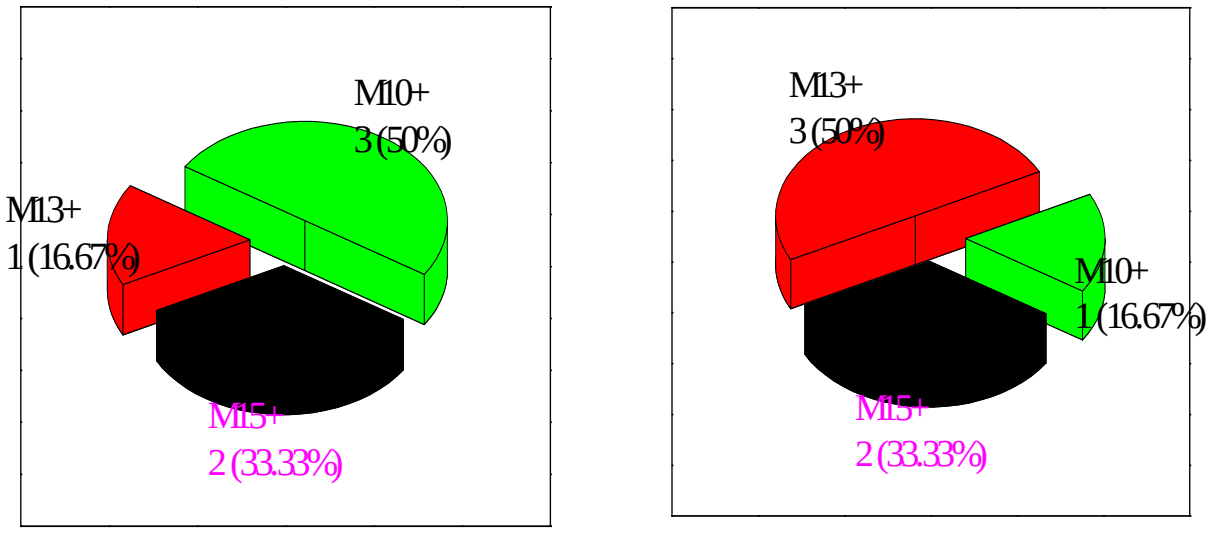
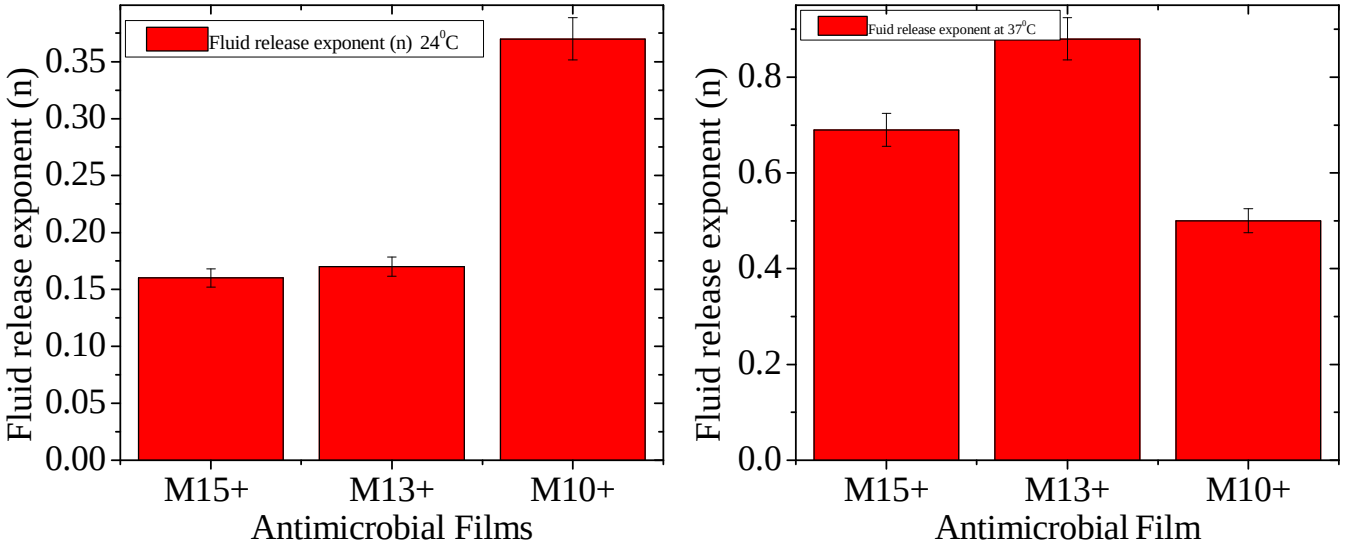
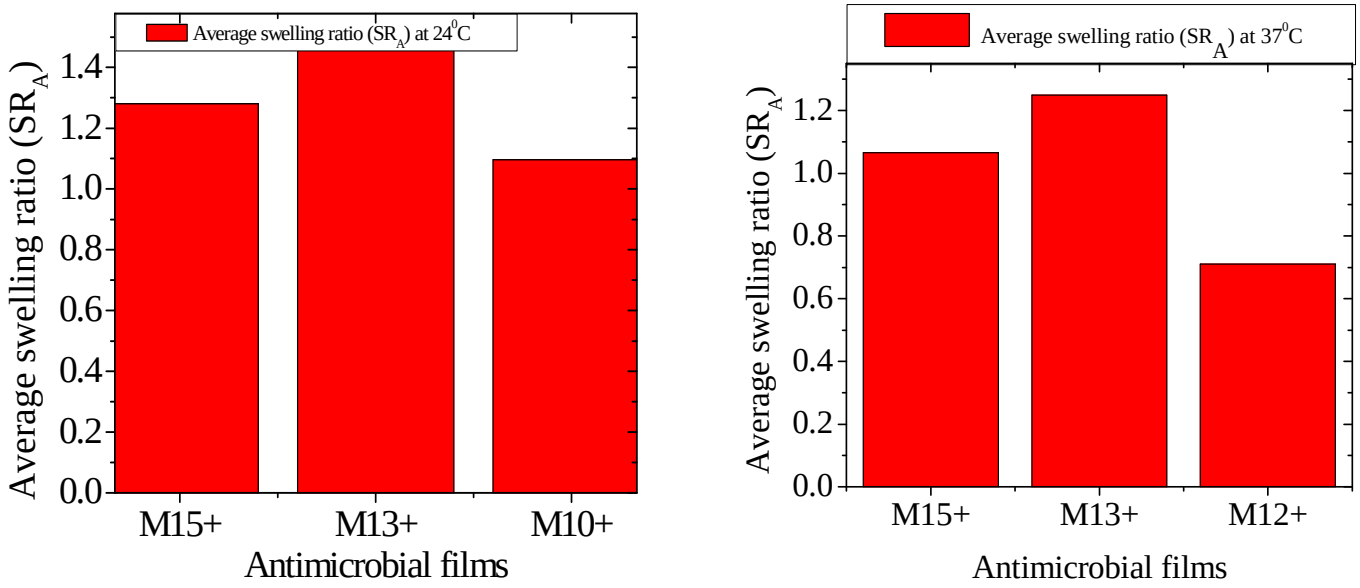


Figure. 4.3.2 Pie chart showing the diffusion coefficient (D) of the films at (a) 24˚C and (b) 37˚C



(a) (b)

Figure 4.3.3 Bar chart showing the fluid release exponent for each of the films at (a) 24˚C and (b) 37˚C



(a) (b)

Figure 4.3.4 Bar chart showing the average swelling ratio of films at (a) 24˚C (b) 37˚C

At room temperature, the rate of diffusion of fluid through film M_{10}^{+} was the highest and lowest in film M_{13}^{+}. At 37˚C on the other hand, the rate of diffusion of fluid out of the film $M_{13}^{+}$was most pronounced and least pronounced in film M_{10}^{+}.

The fluid release exponents also validates the values of diffusion coefficients as it can be seen in figure 4.3.3, with film M_{10}^{+} having the highest fluid release rate at room temperature and at 37˚C film M_{13}^{+} had the highest release rate.

The values of the average swelling ratios shows that temperature has no much effect on it, this is because from the bar chart, there was no much significant change in the shape of the bar chart both at room temperature and at 37˚C. As in figure 4.3.4 (a) and (b).the results are summarized in the table below.

Table 4.2.1 The values of the swelling and de-swelling ratios and the k, n and D values of the antimicrobial films at room temperature 24˚C.

Film Samples	Film thickness (δ) in mm	Geometric constant (k)	Fluid release exponent (n)	Diffusion coefficient,D, ($m^2 s^{-1}$)	Average swelling ratio (SR_A)	Equilibrium swelling ratio (SR_{eq})
M_{15}^{+}	2.2	0.19	0.16	7.33×10^{-7}	1.28	1.42
M_{13}^{+}	2.0	0.17	0.17	5.29×10^{-7}	1.46	1.41
M_{10}^{+}	2.3	0.025	0.37	7.87×10^{-8}	1.096	1.12
M_{15}^{-}	2.2	0.037	0.33	1.16×10^{-7}	1.56	1.52
M_{13}^{-}	2.0	0.0063	0.54	1.97×10^{-8}	1.42	1.47
M_{10}^{-}	2	0.078	0.24	2.46×10^{-7}	1.026	1.07

Table 4.2.2 The values of the swelling and de-swelling ratios and the k, n and D values of the antimicrobial films at 37˚C.

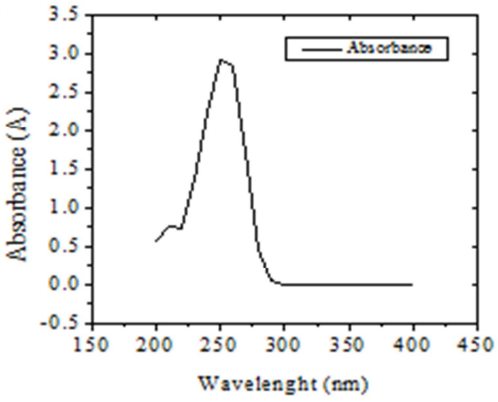
Film Samples	Film thickness (δ) in mm	Geometric constant (k)	Fluid release exponent (n)	Diffusion coefficient,D, ($m^2 s^{-1}$)	Average swelling ratio (SR_A)	Equilibrium swelling ratio (SR_{eq})
M_{15}^{+}	2	0.0014	0.69	4.52×10^{-10}	1.066	1.19
M_{13}^{+}	2	0.00026	0.88	8.12×10^{-10}	1.25	1.40
M_{10}^{+}	2	0.0057	0.50	1.80×10^{-8}	0.71	0.71
M_{15}^{-}	2	0.00068	0.82	2.15×10^{-9}	1.51	1.68
M_{13}^{-}	2	0.0025	0.68	7.85×10^{-9}	1.23	1.28
M_{10}^{-}	2	0.000041	1.10	1.30×10^{-10}	0.8778	0.96

From the table above at room temperature the method of fluid release is not by diffusion because the values of 'n' is not equal to 0.45, so for samples M_{15}^{+}, M_{13}^{+}, M_{10}^{+}, M_{15}^{-}, M_{13}^{-} and M_{10}^{-} exhibits anomalous mode of transport. For samples $M_{15}^{+}, M_{13}^{+}, M_{10}^{+}$, M_{15}^{-}, and M_{13}^{-} with the fluid release exponent ranging from $0.45 < n < 0.89$ is said to be an anomalous mode of fluid transport. The last sample with the release exponent been greater than 0.89, exhibits a super-case II transport (Peppas 1985). So the mode of diffusion is not by ficks law and the reason could be because of the inhomogeneity in the mixture used in forming the film samples, non-uniformity in the film thickness and manual method of laying the films.

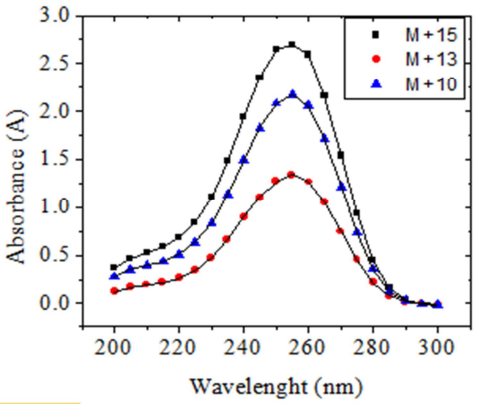
4.4 UV Spectrophotometer result

The peak absorbance of PS 2.992A, was obtained at 252.0 nm wavelength. Similarly the peak absorbance of PS released from CA film for three other films-samples were 2.644, 1.271 and 2.084 for

films M_{15}^{+}, M_{13}^{+} and M_{10}^{+}, respectively at day one as shown below. Similar plots were obtained for the other days. Standard curves of PS released on daily bases were obtained by a plot of absorbance versus concentration were obtained. The results show that, PS was released on a daily basis for a period of ten da



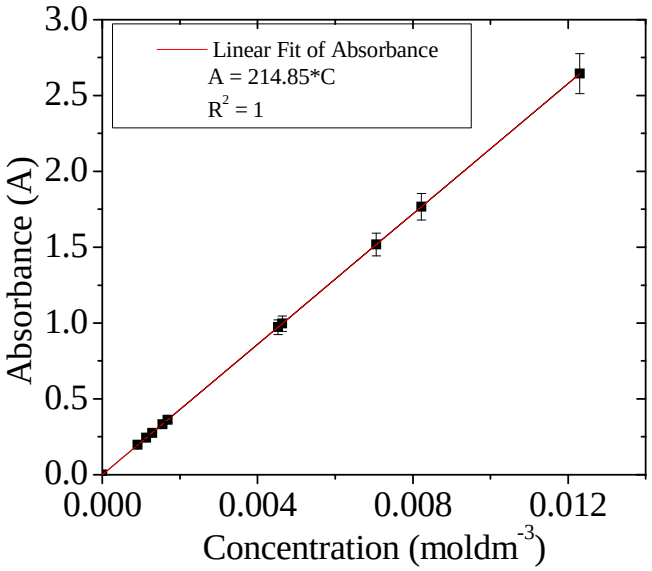
(a)



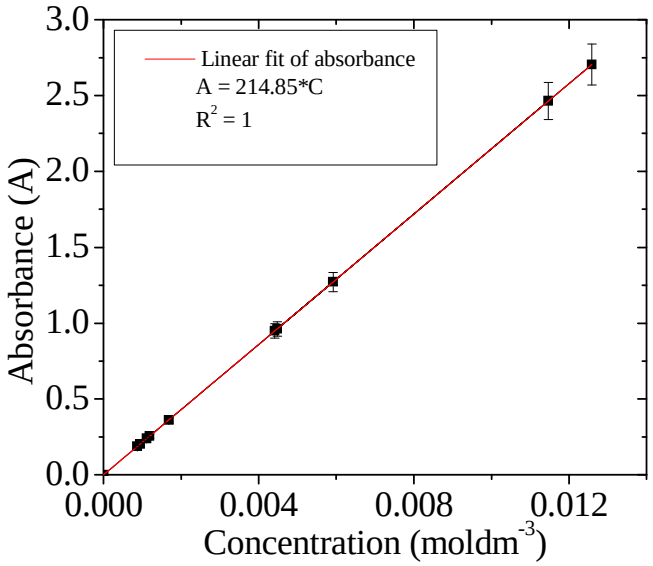
(b)

Figure 4.4.1 shows (a) the peak value of potassium sorbate absorbance in UV spectrophotometer (b) shows the peak absorbance of potassium sorbate in the three film samples.

To obtain a standard curve that shows that PS is been released on a daily basis for a period of ten days when the antimicrobial film was placed in distilled water it turns out that the presence of potassium sorbate was noticed on the samples that were picked on daily bases and the standard curve is as shown below for the three film samples.



(a)



(b)

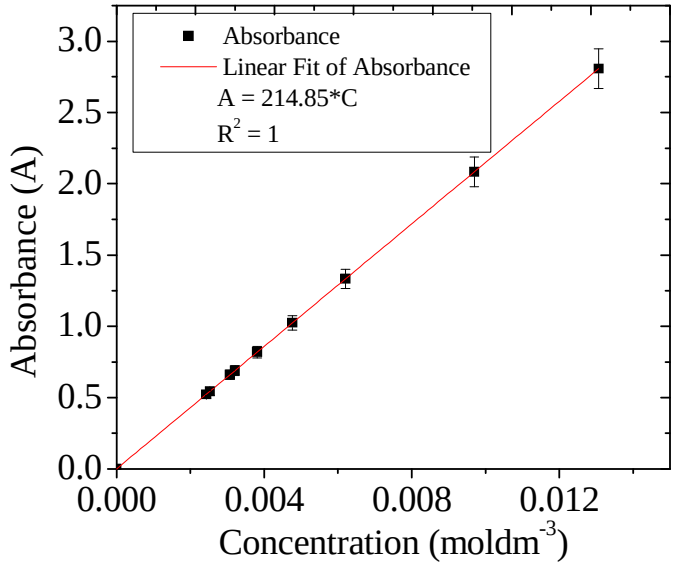
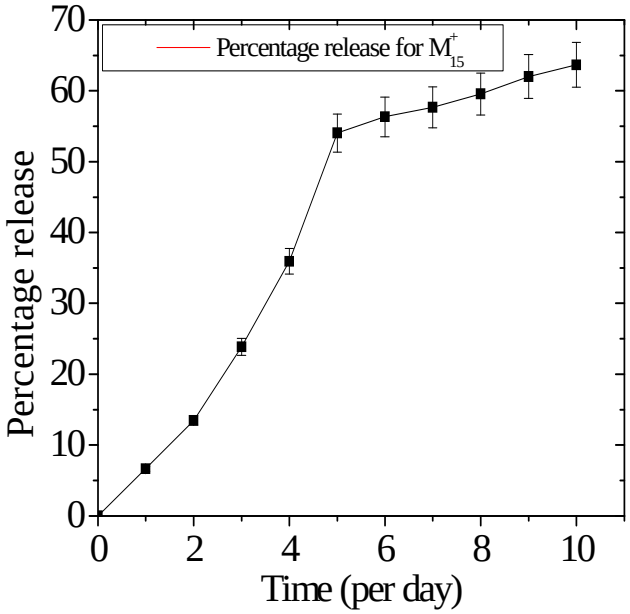
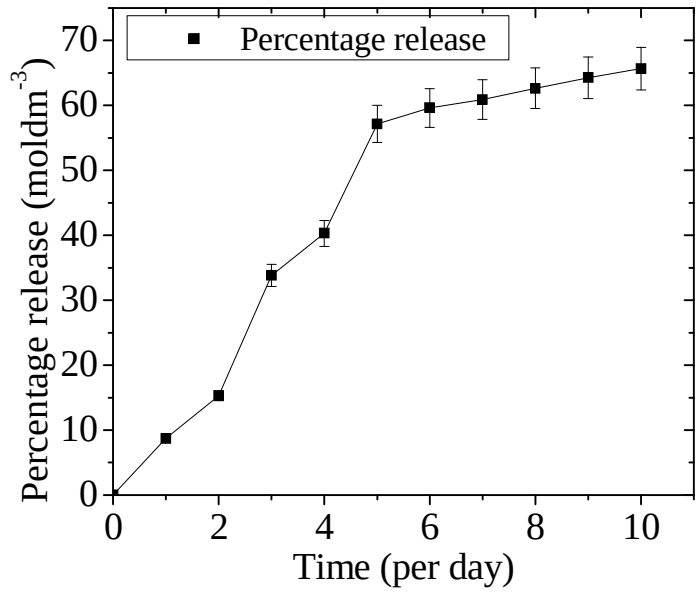


Figure 4.4.2 Showing standard curve for films (a) M_{15}^{+} (b) M_{13}^{+} and (c) M_{10}^{+}

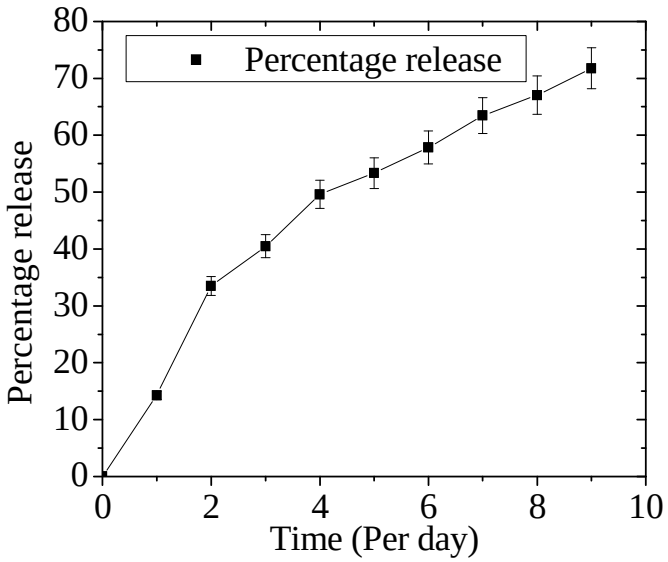
Figure 4.4.2 shows that there is a linear relationship between absorbance and the concentration using linear fit plot the value of the concentration can be obtained at any value of the peak absorbance.



(a)



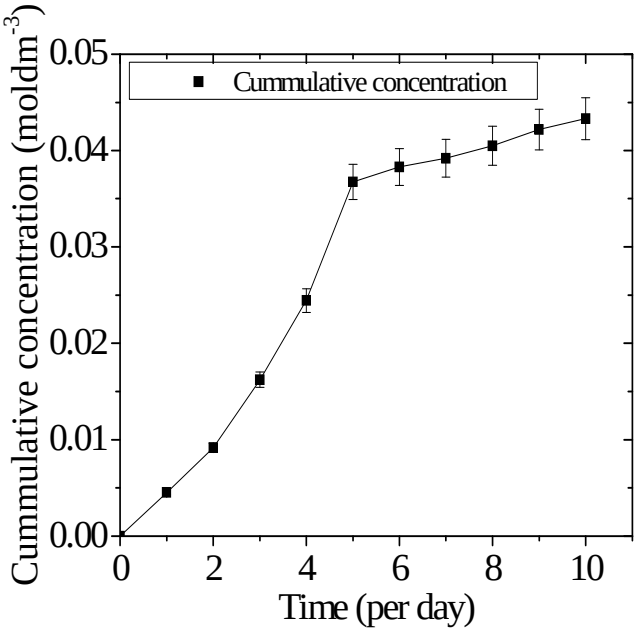
(b)



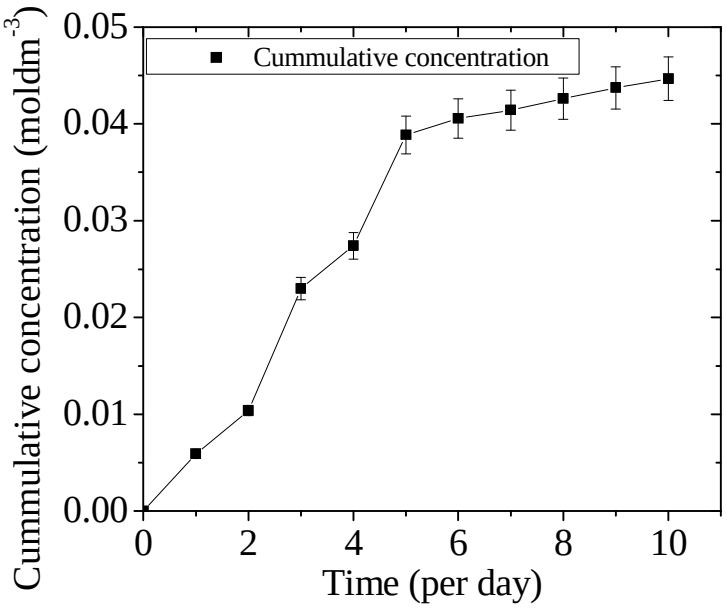
(c)

Figure 4.4.3 Cummulative release curve of film

(a) M_{15}^{+} (b) M_{13}^{+} and (c) M_{10}^{+}



(a)



(b)

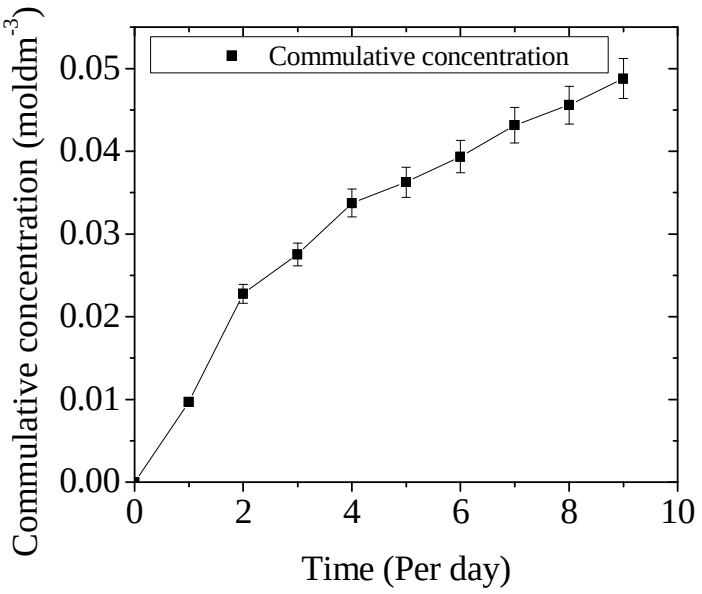


Figure 4.4.4 shows the commulative release with time of films (a) M_{15}^{+} (b) M_{13}^{+} and (C) M_{10}^{+}

From the graphs above the potassium sorbate is been released for the ten days period of examination. Cumulatively, about 64%, 66% and 72% of PS was been released over a period of ten day for samples; M_{15}^{+}, M_{13}^{+} *and* M_{10}^{+}, respectively. The concentration for each percentage can directly be related to the absorbance using:

$C = \frac{64}{100}$ x (A); $C = \frac{66}{100}$ x (A) and $C = \frac{72}{100}$ x (A) for samples M_{15}^{+}, M_{13}^{+} *and* M_{10}^{+}, respectively. where A is the equivalent peak absorbance of the concentration release over the time frame. These percentage release corresponds to the concentrations 0.00787 moldm^{-3} of PS, 0.00831moldm^{-3} of PS and 0.00941moldm^{-3} respectively.

4.5 Determination of A. niger spores

To determine the spore count, the total spores counted in the four corner squares were calculated using the equation:

Spores (ml^{-1}) = (n) x 10^6 (3.1)

where: n = the average cell count per square of the four corner squares counted.

It turns out that in this experiment

n = 30 cells/ml = (n) x 10^4 OR

spores /ml = 30 x 10,000 = 3 x 10^5 spores /ml**.**

4.5 Microstructure of the Aspergillus niger

The microstructure of the fungi, *Aspergillus niger* was observed using a celestron microscope after staining the fungi with methyl blue on a petri dish. This is shown in figure 4.5.1 below.

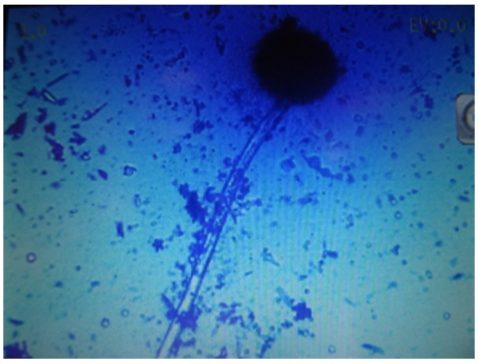
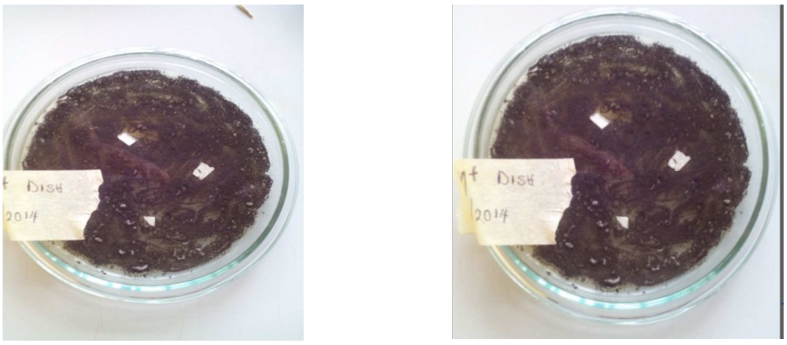


Figure 4.5.1 showing the Microstructure of Aspergillus niger. Magnification 1650 X

The image showed that *A. niger* is a filamentous fungus which belongs to the Fungi kingdom and the *Aspergillus* genus.

4.6 Antimicrobial test

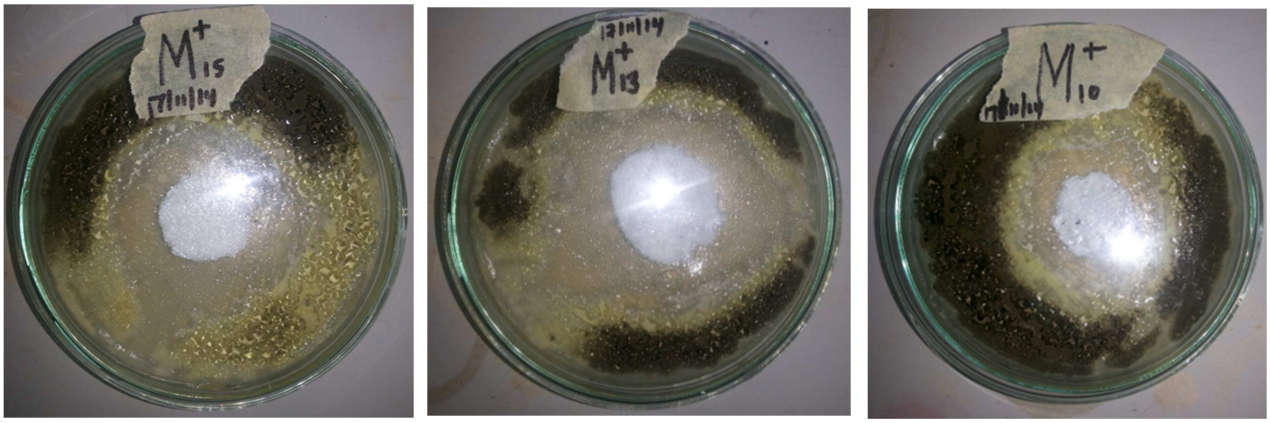
The effect of the antimicrobial agent in restraining the growth of a named food fungi, *A. niger*, was investigated. The antimicrobial agent containing 15%, 13% and 10% CA with each film containing



(a) (b)

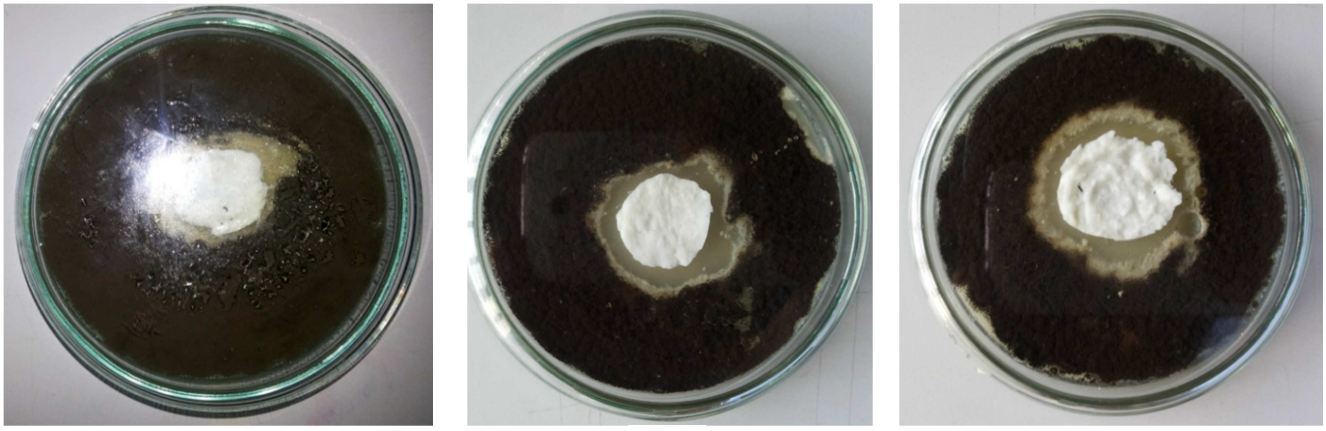
Figure 4.6.1 Antimicrobial activity of mono layer CA films on A. niger. The films in (a) and (b) are antimicrobial films containing 15%, 13% and 10% w/w of CA with 8ml of antimicrobial fluid

8ml of antimicrobial agent (2 – 20 g PS) dissolved in distilled water. The results are as shown below in figure 4.6.1. It turns out that after seven days there was no visible zone of inhibition with antimicrobial film containing 2g wt% of CA. Further increase in the concentration of PS to 20g wt% gave a visible clear zone around the antimicrobial film as shown below. Figure 4.6.2



(a) (b) (c)

Figure 3Figure 4.6.2 Antimicrobial activity of mono layer CA films on A. niger. At room temperature of 24°C with (a) M_{15}^{+} with 15 wt% CA (b) M_{13}^{+} with 13 wt% CA (c) M_{10}^{+} with 15 wt% CA, all in 8 ml PS (Containing 20g w/w PS in distilled water) after three days



(a) (b) (c)

Figure 4.6.3 Antimicrobial activity of mono layer CA films on Aspergillus niger. At room temperature of 24°C with (a) M_{15}^{+} with 15 wt% CA (b) M_{13}^{+} with 13 wt% CA (c) M_{10}^{+} with 15 wt% CA, all in 8 ml PS (Containing 20g w/w PS in distilled water)

Observations after two days indicate the antimicrobial activity of the three mono-layered films obtained by measuring the diameter of the zones with a veneer caliper. The inhibition zones were, 0.92cm, 1.16cm and 1.3cm for films M_{15}^{+}, M_{13}^{+} and M_{10}^{+} respectively as shown in the figure 4.6.2. After five days the zones were observed to be closing up to approximately about 0.46 cm, 0.58 cm and 0.65 cm, for samples M_{15}^{+}, M_{13}^{+} and M_{10}^{+} respectively. Hence the rate of diffusion of PS to the agar medium

in the petri dish reduced with time. By the seventh day there was no visible clear zone as shown below in figure 4.6.4.

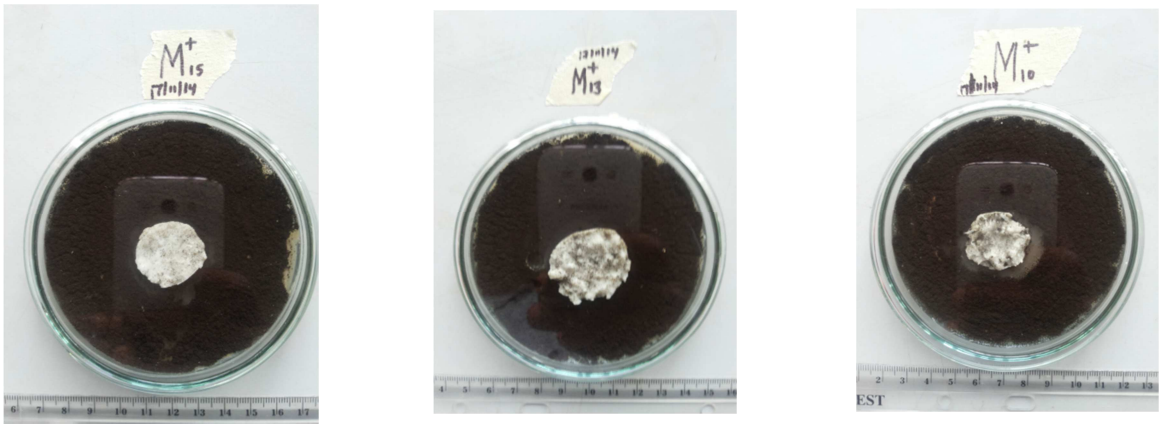


Figure 4.6.3 Antimicrobial activity of mono layer CA films on Aspergillus niger. At room temperature of 24°C with (a) M_{15}^{+} with 15 wt% CA (b) M_{13}^{+} with 13 wt% CA (c) M_{10}^{+} with 15 wt% CA, all in 8 ml PS (Containing 20g w/w PS in distilled water)

4.7 Industrial application of antimicrobial film

After subjecting the viscous antimicrobial polymer through blow molding it turns out that the finished product were all defective as seen in the figure 4.5.2 (a) compared with the good product in (b). Hence this shows that antimicrobial agents cannot be added to the industrial master batch as this will amount to defective plastic containers. The solution will be to incorporate the antimicrobial agent directly into polymer pellets from the chemical industry while forming the pellets. Hence, antimicrobial agent cannot be added to the polymer pellets directly because the machines used in producing the plastic containers are hydrophobic in nature, doing that will amount to the production of defective containers with much porosity.

(a) (b)

Figure 4.5.2 Showing (a) defective and (b) good antimicrobial HDPE polymer containers formed from First drops Nigerian Limited.

CHAPTER FIVE

5.1 Conclusion

In this work, mono layer fungicidal antimicrobial films were formed with potassium sorbate (PS) incorporated into cellulose acetate (CA) polymers to form antimicrobial films with different amount of CA in them. The films were poured into a petri dish mold. The film structure that was formed has variation in its porosity depending on the amount of Cellulose acetate in the film forming material that was added in the film formation. The result shows that the rate of release of the antimicrobial agent from the film is not controlled by Fick's law because of the inhomogeneity in the films that were formed by manually pouring the antimicrobial mixture in a petri dish, hence the films exhibits an anomalous fluid release from the film to the target food substance. The fractional release exponent n-values for the film ranges from 0.16 – 0.54 at 24˚C and 0.5 - 1.1 at 37˚C. The geometrical constant (k) ranges from 0.0063 – 0.19 at 24˚C and 0.000041 – 0.0057 at 37˚C The diffusion coefficient of the film varies from 1.97×10^{-8} m^2s^{-1} – 5.29×10^{-7} m^2s^{-1} at 24˚C and 1.3×10^{-10} m^2s^{-1} – 1.80×10^{-8} m^2s^{-1} at 37˚C. The average swelling ratio also showed that it ranges from 1.026 - 1.56 at 24˚C and 0.71 - 1.51 at 37˚C. SEM images were obtained to observe the morphology of the films and UV-Vis spectrophotometer was also used to study the amounts of PS released from the films.

The monolayer antimicrobial films containing 2g wt% potassium sorbate (PS) was able to restrain the growth of the target microorganism, *Aspergillus niger,* in peanut and bread but it could not prevent the growth of *Aspergillus niger* cultured in a Potato Dextrose Ager (PDA) medium, with gradual increment in the amount of PS to 20g wt%, it was observed that the antimicrobial growth of, *Aspergillus niger,* was prevented for about eight days which is seen from the visible zone of inhibition created by the release of the antimicrobial agent from the film.

The efficacy of the antimicrobial films formed containing different amount of cellulose acetate was tested on peanut and bread. It turns out that fungicidal antimicrobial film was able to prevent the growth of mould on the bread for eight days and *Aspagillus niger* was also prevented from growing on both the bread and the peanut for the same time frame. It was also observed that the antimicrobial agent cannot be mixed directly into the polymer master batch used in making food preservation containers except if it is incorporated in forming polymer pellets.

Hence the fungicidal antimicrobial films used in this project can be effective in controlled release food packaging to preserve foods such as peanuts and bread from microbial attack.

5.2 Recommendations

Further research could be done to produce hydrophobic antimicrobial mater batch in form of pellets that will contain polymer and the antimicrobial agents. To proffer ways of obtaining films of uniform thickness is another challenging issue that calls for more research work.