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JUDUL : CHARACTERIZATION AND ANTIMICROBIAL ANALYSIS OF CHITOSAN COMPOSITE BIODEGRADABLE FILMS WITH ADDITION OF GINGER ESSENTIAL OIL.

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CHARACTERIZATION AND ANTIMICROBIAL ANALYSIS OF CHITOSAN COMPOSITE BIODEGRADABLE FILMS WITH ADDITION OF GINGER ESSENTIAL OIL

NUR 'ADILAH BINTI ISMAIL

A thesis submitted in fulfillment
of the requirements for the award of the degree of
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DECLARATION

I declare that this thesis entitled "Characterization and Antimicrobial Analysis of Chitosan Composite Biodegradable Films with Addittion of Ginger Essential Oil" is the result of my own research except as cited in references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

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DEDICATION

To my beloved Mom and Dad,
My beloved supervisor,
My family members that always love me,
My research project teammate that always supporting each other,
My fellow colleague,
And all faculty members,

For all your care, support and believe in me.

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ABSTRACT

Innovative techniques of preserving food safety and structural integrity as well as complete biodegradability must be adopted. This research involves the development process in making the antimicrobial biodegradable film in order to restrain and inhibit the growth of spoilage and pathogenic bacteria that are contaminating food. The main objective of this research is to formulate the best film for food packaging, which has high thermal, antimicrobial and mechanical properties. The films were prepared by casting method. Chitosan were dissolved in 1% v/v acetic acid and yam starch by heat gelatinized, then PEG and ginger essential oil was added to the mix solution. After that, six analyses were tests for those films. The antimicrobial activity was determined by liquid culture test and agar plate test. The results showed that Sample C has highly antimicrobial properties in order to inhibit more *Bacillus subtilis* and *Escherichia coli* in liquid culture medium and has greatest clear zone on agar plate. The films morphology structure was observed using scanning electron microscopy (SEM). The results revealed that Sample C more smooth surface and compact structure. Chemical composition of the films was investigated using fourier transform infrared spectroscopy (FTIR) and revealed that starch, chitosan, essential oil and additives presence in the films. The thermal stability characterization using thermal gravimetric analysis (TGA) and differential scanning calorimetric (DSC) showed that Sample C has higher melting point and high heat resistance. In conclusion, Sample C is the best among three samples, prove that the addition of antimicrobial agent such as essential oil will give a better performance in film making.

ABSTRAK

Teknik inovatif untuk memelihara keselamatan makanan dan integriti struktur serta biodegradasi lengkap harus diambil. Penyelidikan ini melibatkan proses pembangunan dalam pembuatan filem biodegradasi antimikrob untuk menahan dan menghalang pertumbuhan pembusukan dan bakteria patogen yang mencemarkan makanan. Objektif utama penyelidikan ini adalah memformulasikan filem terbaik untuk bungkusan makanan, yang tinggi kestabilan terma, antimikrob dan sifat mekanikal. Filem-filem itu dibuat melalui kaedah *casting*. Chitosan dilarutkan di dalam 1% v / v asid asetat dan larutan tepung keladi dipanaskan, kemudian PEG dan minyak pati halia ditambah kepada larutan campuran. Kemudian, enam analisis diuji ke atas filem-filem tersebut. Aktiviti antimikrob ditentukan oleh *liqiud culture test* dan *agar plate test*. Keputusan menunjukkan Sampel C sangat antimikrob untuk menghalang pembiakan *Bacillus subtilis* dan *Escherichia coli* dalam medium kultur cecair dan mempunyai zon jelas terbesar di piring agar. Struktur morfologi filem diamati dengan *scanning electron microscopy* (SEM). Keputusan menunjukkan bahawa sampel C lebih halus permukaan dan berstruktur padat. Komposisi kimia filem diselidiki menggunakan *fourier transform infrared spectroscopy* (FTIR) dan mendedahkan keladi, chitosan, minyak pati halia dan aditif dalam filem. Karakterisasi kestabilan terma menggunakan *thermal gravimetric analysis* (TGA) dan *differential scanning calorimetric* (DSC), menunjukkan bahawa Sampel C mempunyai takat lebur yang lebih tinggi dan tahan haba. Kesimpulannya, Sampel C adalah yang terbaik di antara tiga sampel, membuktikan bahawa penambahan agen antimikrob seperti minyak pati halia akan memberikan prestasi yang lebih baik dalam pembuatan filem.

TABLE OF CONTENT

LIST OF SYMBOLS

PEG	Polyethylene glycol
SEM	Scanning electron microscope
FTIR	Fourier transform infrared spectroscopy
TGA	Thermo gravimetric analysis
DSC	Differential scanning calorimeter
% v/v	Percent volume per volume
λ	Wavelength
β	Heating rate
T	Temperature
T_M	Melting temperature

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Packaging is a modern technique for protecting food, increasing shelf life and safety. Furthermore, it facilities the sale and distribution of agricultural, industrial and consumer products, making distribution also possible over long distances. The package must prevent any contact between items and the surrounding, avoiding any undesired alterations and maintaining the purity and freshness of its contents. Moreover, it has the role of a communicative link between consumer and manufacturer, it must identify the contents, their quantity, the price, warning and other information that are carried on an applied label or directly imprinted on the packaging. Today, the basic materials of packages include, paper, paperboard, cellophane, steel, glass, wood, textiles and plastics. With 74% of the 13 billion m^2 market, plastic is the dominant flexible packaging material in Western Europe, according to a new Market power report. Total consumption of flexible packaging grew by 2.9% per year in 1992–1997, with the strongest growth in processed food and above average growth in chilled foods, fresh foods, detergent and pet foods. In this field plastics allow packaging to perform many necessary tasks providing many important properties such as strength and stiffness, barrier to oxygen transmission and moisture, resistance to food component attack and flexibility. Future growth will be 2% per year, as some markets reach maturity. The share of plastics in Western Europe will increase to 75.8% in 2002 (Avella *et al.*, 2001*).*

Nowadays, the largest parts of materials used in packaging industries are produced from fossil fuels and are practically nondegradable. For this, packaging materials for foodstuff, like any other short-term storage packaging material, represent a serious global environmental problem (Kirwan and Strawbridge, 2003). A big effort to extend the shelf life and enhance food quality while reducing packaging waste has encouraged the exploration of new bio-based packaging materials, such as edible and biodegradable films from renewable resources (Tharanathan, 2003). The use of these materials, due to their biodegradable nature, could at least to some extent solve the waste problem (Sorrentino *et al*., 2007). The escalating problems caused by non-degradable plastics have led to development of biodegradable plastics. The major advantages of biodegradable plastics are they can be composted with organic wastes and returned to enrich the soil; their use will not only reduce injuries to wild animals caused by dumping of conventional plastics but will also lessen the labor cost for the removal of plastic wastes in the environment because they are degraded naturally; their decomposition will help increase the longevity and stability of landfills by reducing the volume of garbage; and they could be recycled to useful monomers and oligomers by microbial and enzymatic treatment (Tokiwa and Calabia ,2007).

Antimicrobial packaging is gaining interest from researchers and industry due to its potential to provide quality and safety benefits. By means of the correct selection of materials and packaging technologies, it is possible to keep the product quality and freshness during the time required for its commercialization and consumption (Brown, 1992; Stewart, *et al*., 2002; Sorrentino *et al*., 2007). Currently, development is limited due to availability of antimicrobials and new polymer materials, regulatory concerns, and appropriate testing methods. With the advent of new materials and more information this may change. New coating/binder materials compatible with polymers and antimicrobials, functionalized surfaces for ionic and covalent links and new printing methods combined with encapsulation are examples of the technologies that will play a role in the development of antimicrobial packaging. Antimicrobials that can be attached or coated to films and rigid containers after forming to avoid high temperature and other processing issues will allow a wide range of compounds to be incorporated into

polymers. These developments will require surfaces containing functional groups available for attachment. Physical methods to modify polymer surface (electron beam, ion beam, plasma and laser treatments) are emerging and pose potential for functionalizing inert surfaces such as those of PE, PET, PP and PS (Ozdemir, *et al*., 1999) HDPE and LLDPE have already been functionalized by graft polymerization with amide, amino and carboxyl groups in order to immobilize proteins and enzymes (Hayat *et al*., 1992; Sano *et al*., 1993; Wang and Hsiue, 1993). It has been suggested also that cross-linking edible films like calcium caseinate by gamma irradiation will find applications as supports for the immobilization of antimicrobials and other additives (Lacroix and Ouattara, 2000).

Future work will focus on the use of biologically active derived antimicrobial compounds bound to polymers. The need for new antimicrobials with wide spectrum of activity and low toxicity will increase. It is possible that research and development of 'intelligent' or 'smart' antimicrobial packages will follow. These will be materials that sense the presence of microorganism in the food, triggering antimicrobial mechanisms as a response, in a controlled manner. Antimicrobial packaging can play an important role in reducing the risk of pathogen contamination, as well as extending the shelf-life of foods; it should never substitute for good quality raw materials, properly processed foods and good manufacturing practices. It should be considered as a hurdle technology that in addition with other non-thermal processes such as pulsed light, high pressure and irradiation could reduce the risk of pathogen contamination and extend the shelf-life of perishable food products. Participation and collaboration of research institutions, industry and government regulatory agencies will be key on the success of antimicrobial packaging technologies for food applications (Appendini and Hotchkiss, 2002).

1.2 Problem Statement

Nowadays, about 150 million tons of plastic are produced annually all over the world, and the production and consumption continue to increase (Parra *et al.*, 2004). Most of these plastics are crude oil based. In addition, handling of plastic waste associated with serious environmental pollution problem due to waste disposal and nondegraded polymers. Therefore, the use of agricultural biopolymers that are easily biodegradable not only would solve these problems, but would also provide a potential new use for surplus farm production (Okada, 2002; Pavlath and Robertson, 1999; Scott, 2002 and Salleh *et al*. 2007). The environmental impact caused by the excessive quantity of non-degradable waste materials discarded every day is a matter of great concern. This reality is stimulating a great R&D effort to develop new biodegradable packing materials that can be manufactured with the utilization of environmentally friendly raw materials (Avérous *et al.*, 2001 and Galdeano *et al*. 2009).

The use of protective coatings and suitable packaging by the food industry has become a topic of great interest because of their potentiality for increasing the shelf life of many food products (Ahvenainen, 2003; Coles *et al*., 2003; Giles and Bain, 2001; Hernandez *et al*., 2000). By means of the correct selection of materials and packaging technologies, it is possible to keep the product quality and freshness during the time required for its commercialization and consumption (Brown, 1992; Stewart *et al*., 2002). A big effort to extend the shelf life and enhance food quality while reducing packaging waste has encouraged the exploration of new bio-based packaging materials, such as edible and biodegradable films from renewable resources (Tharanathan, 2003). The use of these materials, due to their biodegradable nature, could at least to some extent solve the waste problem. However, like conventional packaging, bio-based packaging must serve a number of important functions, including containment and protection of food, maintaining its sensory quality and safety, and communicating information to consumers (Robertson, 1993 and Sorrentino *et al*., 2007).

The performance expected from bioplastic materials used in food packaging application is containing the food and protecting it from the environment and maintaining food quality (Arvanitoyannis, 1999). It is obvious that to perform these functions is important to control and modify their mechanical and barrier properties that consequently depend on the structure of the polymeric packaging material. In addition, it is important to study the change that can occur on the characteristics of the bioplastics during the time of interaction with the food (Scott, 2000 and Siracusa *et al*., 2008).

So, the development of this research is due to the handling problem of non-degradable plastics packaging waste in the world and the high production cost of the biodegradable plastics packaging that consumes to global warming and non-friendly environment issues. In order to preserve food safety and to sustain the environment, the advances in film making have been study. This research involves the development process in making the antimicrobial biodegradable film in order to restrain and inhibit the growth of spoilage and pathogenic microorganisms that are contaminating food.

The significant of the research is to use an alternative method to petroleum-based plastic for plastic packaging material. In consequent, it will decrease the soil pollution and environment problem by producing the biodegradable plastics. By applying antimicrobial agent, it will give the effect to the extension of the shelf-life of the food and the maintenance or even improvement of its quality. These advances in the technology of film production will be future interest and the global market demand with a special emphasis on safety concerns and assessment.

1.3 Research Objective

The objective of this research are to formulate the best film for food packaging, which is high thermal stability, highly antimicrobial, highly mechanical properties and smooth film’s surface from the mixture of chitosan and yam starch with addition of

polyethylene glycol (PEG) and ginger essential oil. Another objective is characterization of film fabricated by various methods which are:

a. Scanning Electron Microscopy (SEM)
b. Fourier Transform Infrared (FTIR)
c. Thermo Gravimetric Analysis (TGA)
d. Differential Scanning Calorimeter (DSC)
e. Antimicrobial Analysis

1.4 Scope of Study

In order to achieve the objective, scopes have been identified in this research. The scopes of this research are list as below:

a. Film preparation from the chitosan-yam starch solution by casting method.
b. Evaluation the antimicrobial effectiveness toward *Bacillus subtilis* and *Escherichia coli* on the film packaging which representing gram positive and gram negative bacteria.
c. Characterize the film by using various methods which are Scanning Electron Microscopy (SEM), Fourier Transform Infrared (FTIR), Thermo Gravimetric Analysis (TGA), and Differential Scanning Calorimeter (DSC).

CHAPTER 2

LITERATURE REVIEW

2.1 Biodegradable Film

The materials most used for food packaging are the petrochemical-based polymers, due to their availability in large quantities at low cost and favourable functionality characteristics, such as, good tensile and tear strength, good barrier properties to O_2 and heat sealability (Tharanathan, 2003). However, these materials are totally non-biodegradable, leading to serious ecological problems. As a consequence, the consumer demand has shifted to eco-friendly biodegradable materials, especially from renewable agriculture by-products, food processing industry wastes and low cost natural resources. The biopolymers commonly used to produce films are carbohydrates, often vegetal starchy and pectic materials and proteins, vegetal and animal (Vermeiren *et al*., 1999 and Alvarez, 2000). Usually, these biopolymers require that their mechanical and rheological properties be improved by molecular restructuring or by the inclusion of food grade additives. In addition to the appropriate mechanical properties, the films must have also the adequate permeability to water vapour and gases. The specific barrier requirements of the packaging depend upon the products characteristics and the intended end-use application. In the case of a packaged product whose deterioration is related to its moisture content, the barrier properties of the package relating to water vapour will be of major importance in extending shelf life. Similarly, the oxygen concentration in a permeable package will affect the rate of oxidation of nutrients such as vitamins, proteins and fatty acids. The required specific permeability properties of the films can be

obtained by inclusion of inert impermeable barriers and/or reactive compounds in the polymer matrix. The inert barriers can reduce permeability by increasing the diffusion path, while the reactive compounds interact selectively with the diffusing species increasing the time before a significant permeability occurs (Alves *et al*., 2006).

2.2 Biocomposite

Ecological concerns have resulted in a renewed interest in natural and compostable materials, and therefore issues such as biodegradability and environmental safety are becoming important. Tailoring new products within a perspective of sustainable development or eco-design is a philosophy that is applied to more and more materials. It is the reason why material components such as natural fibres, biodegradable polymers can be considered as interesting and environmentally safe and can be used as alternatives for the development of new biodegradable composites. When it comes to improvements in edible film technologies, most research has addressed film formulations using various combinations of edible materials. Two or more materials can be combined to improve gas exchange, adherence to coated products, or moisture vapor permeability properties (Baldwin et al., 1995). Biodegradable composites consist of biodegradable polymers as the matrix material and biodegradable fillers, usually biofibres. Since both components are biodegradable, the composite as the integral part is also expected to be biodegradable (Mohanty *et al*., 2000). (Averous and Boquillon, 2004).

Composite films consisting of lipids and a mixture of proteins or polysaccharides take advantage of the individual component properties. In doing so, these individual or combined films can be applied as emulsions or bilayer films (Cutter and Sumner, 2002). Additionally, plasticizers can be used to modify film mechanical properties, thereby imparting desirable flexibility, permeability, or solubility to the resulting film (Ben and Kurth, 1995). For example, adding glycerol, polyethylene glycol, or sorbitol to a film

composition can reduce brittleness (Ben and Kurth, 1995). In another example of composite films, a combination of vegetable oils, glycerin, citric acid, and antioxidants prevented rancidity by acting as a moisture barrier, restricting oxygen transport, and serving as a carrier for antioxidants to various foods (Baldwin *et al*., 1995; Cutter and Sumner, 2002). In another study, barrier properties were determined for caseinate films that were treated with a lipid or an enzyme and held at 4°C and 90% relative humidity (Ben and Kurth, 1995). Lipid addition notably improved moisture barrier properties, but the films appeared slightly cloudy, such that when these particular films were applied to meat surfaces, the appearance of the meat surface was unacceptable (Ben and Kurth, 1995, Cutter, 2006).

2.3 Biopolymer or Bio-based Polymer

The biopolymers commonly used to produce films are carbohydrates, often vegetal starchy and pectic materials and proteins, vegetal and animal (Vermeiren *et al*., 1999 and Alvarez *et al*., 2000). Usually, these biopolymers require that their mechanical and rheological properties be improved by molecular restructuring or by the inclusion of food grade additives. In addition to the appropriate mechanical properties, the films must have also the adequate permeability to water vapour and gases. The specific barrier requirements of the packaging depend upon the products characteristics and the intended end-use application. In the case of a packaged product whose deterioration is related to its moisture content, the barrier properties of the package relating to water vapour will be of major importance in extending shelf life. Similarly, the oxygen concentration in a permeable package will affect the rate of oxidation of nutrients such as vitamins, proteins and fatty acids (Alves *et al*., 2006).

Typically, bio-based polymers or biopolymers are developed from renewable resources (Comstock *et al*., 2004; Weber *et al*., 2002). Examples of renewable resources used in the manufacture of these types of polymers include polysaccharides such as

starch, alginates, pectin, carrageenans, and chitosan/chitin, proteins such as casein, whey, collagen, gelatin, corn, soy, and wheat, and lipids such as fats, waxes, or oils (Comstock et al., 2004; Cutter and Sumner, 2002). Polymers, such as polylactate (PLA) or polyesters also may be synthesized from biologically-derived monomers, while microorganisms also can produce polymers such as cellulose, xanthan, curlan, or pullulan (Comstock *et al*., 2004; Kandemir *et al.,* 2005). Researchers also have further categorized biopolymers based on the ability to be compostable or biodegradable (Comstock *et al.,* 2004). It is important to note that while some bio-based packaging materials may be biodegradable, not all biodegradable materials are bio-based (Weber *et al*., 2002 and Cutter, 2006).

Recent technological advances also have allowed biopolymers to be processed similarly to petroleum-based plastics, whether in sheets, by extrusion, spinning, injection molding, or thermoforming (Comstock *et al*., 2004). Notable advances in biopolymer production, consumer demand for more environmentally-friendly packaging, and technologies that allow packaging to do more than just encompass the food are driving new and novel research and developments in the area of packaging for muscle foods (Cutter, 2006).

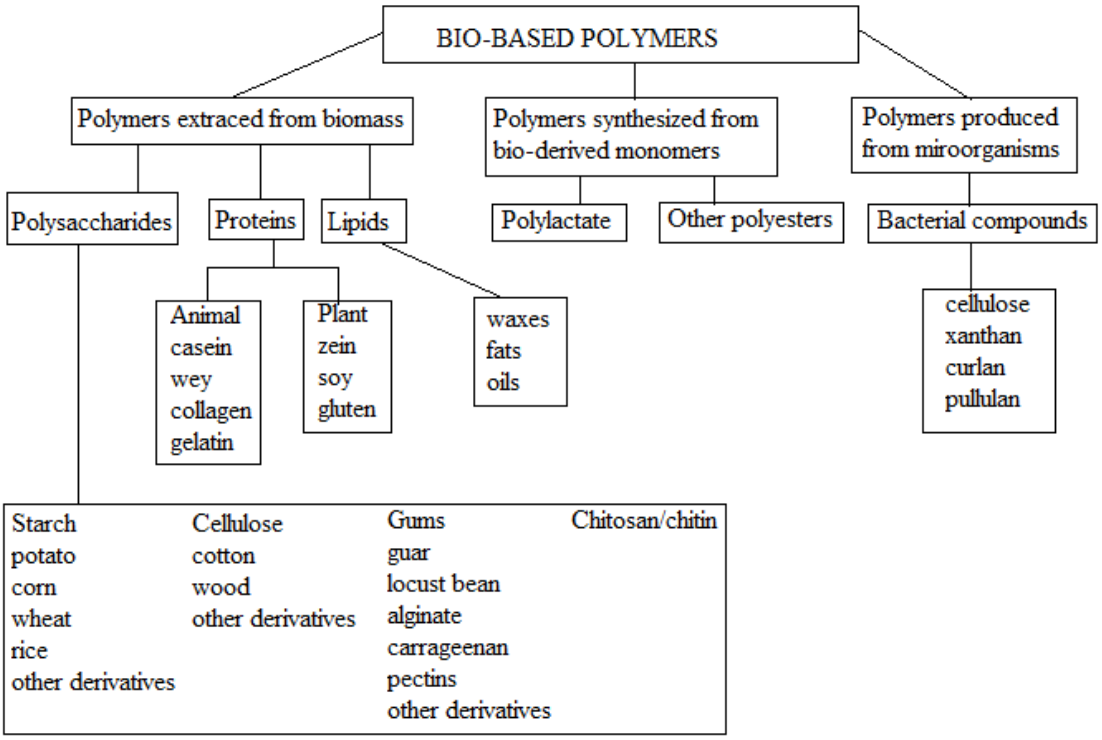


Figure 2.1 Different categories of bio-based materials (adapted from Weber *et al.*, 2002).

2.4 Antimicrobial Packaging

The demand for minimally processed, easily prepared and ready-to-eat 'fresh' food products, globalization of food trade, and distribution from centralized processing pose major challenges for food safety and quality. Recent food-borne microbial outbreaks are driving a search for innovative ways to inhibit microbial growth in the foods while maintaining quality, freshness, and safety. One option is to use packaging to provide an increased margin of safety and quality. The next generation of food packaging may include materials with antimicrobial properties. These packaging technologies could play a role in extending shelf-life of foods and reduce the risk from pathogens. Antimicrobial polymers may find use in other food contact applications as well.

The use of bio-based, polymer-based films as antimicrobial delivery systems to reduce undesirable bacteria in foodstuffs is not a novel concept. Various approaches have been proposed and demonstrated for the use of these films to deliver compounds to a variety of food surfaces, including muscle foods. As mentioned previously, these types of films, gels or coatings are receiving considerable attention since they satisfy consumers' demands for products made from sustainable materials and/or recyclability (Durango et al., 2006 and Cutter, 2006).

Antimicrobial packaging is a form of active packaging. Active packaging interacts with the product or the headspace between the package and the food system, to obtain a desired outcome (Labuza and Breene, 1989; Rooney, 1995; Brody, Strupinsky and Kline, 2001). Likewise, antimicrobial food packaging acts to reduce inhibit or retard the growth of microorganisms that may be present in the packed food or packaging material itself (Appendinia *et. al*, 2002). Direct addition of antimicrobial substances into food formulations or onto food surfaces may not be sufficient to prevent the growth of pathogenic and spoilage microorganisms as antimicrobial substances applied could be partially inactivated or absorbed by the food systems (Ouattara *et al.,* 2000). Antimicrobial films render sustained release of antimicrobial substances onto the food surface and compensate for the partial inactivation or absorption of them by food systems (Siragusa and Dickson, 1992).

2.4.1 Type of Antimicrobial Packaging

From the Journal of Review of Antimicrobial Food Packaging (2002), the writers had determined the form of Antimicrobial packaging. Below are several forms of Antimicrobial packaging which are:

a. Addition of sachets/pads containing volatile antimicrobial agents into packages.

b. Incorporation of volatile and non-volatile antimicrobial agents directly into polymers.
c. Coating or adsorbing antimicrobial agents into polymer surfaces.
d. Immobilization of antimicrobial agents to polymers by ion or covalent linkages.
e. Use of polymers that are inherently antimicrobial.

In this research, I will focus on incorporation of volatile and non-volatile antimicrobial agents directly into polymers. The rationale for incorporating antimicrobials into the packaging is to prevent surface growth in foods were a large portion of spoilage and contamination occurs. For example, intact meat from healthy animals is essentially sterile and spoilage occurs primarily at the surface. This approach can reduce the addition of larger quantities of antimicrobials that are usually incorporated into the bulk of the food. Table below shows the different antimicrobial agents directly incorporate with different polymers for antimicrobial food packaging.

Table 2.1: Antimicrobial incorporated directly into polymers used for food packaging (Appendini and Hotchkiss, 2002).

Antimicrobials incorporated directly into polymers used for food packaging			
Antimicrobials	Polymer/ carrier	Main target microorganisms	References
Organic acids / anhydrides: Propionic, benzoic, sorbic, acetic, lactic, malic	Edible films, EVA, LLDPE	Molds	Guilbert(1988), Baron & Summer(1993), Torres & Karel(1985), Devlieghre, Vermeiren, Hockstal & Debevere (2000), Weng & Hotchkiss(1993)
Inorganic gases: Sulfure dioxide, chlorine	Various polyolefins	Molds, Bacteria, Yeasts	CSIRO (1994) Wellinghoff (1995)

dioxide			
Metals: Silver	Various polyolefins	Bacteria	Ishitani (1995)
Fungicide: Benomyl, imazalil	LDPE	Molds	Weng(1992) Padgett, Han & Dawson (1998)
Bacteriocius: Nisin, pediocins, lacticin	Edible films, cellulose, LDPE	Gram-positive bacteria	Siragusa, Cutter & Willet (1999) Scanell, Hill, Ross, Mars, Hartmeier & Areadt (2000)
Enzymes: Lysozyme, glucose oxidase	Cellulose acetate, PS	Gram-positive bacteria	Appendini and Hotchkiss (1997) Padget et. Al (1998)
Chelating agents: EDTA	Edible films	Gram-negative bacteria	Padget et. Al (1998)
Spices: Cinamic, caffeic, p-coumaic acids Horseradish (allylisothiocynate)	Nylon/PE, cellulose	Molds, Bacteria, Yeast	Hoshino, Ijima, Hayashi & Shibata (1998) Anon (1995), Nielsen & Rios (2000)
Essential oils(plant extracts): Grapefruit seed extract, bamboo powder, Rheum palmatum, Coptis chinesis extracts	LDPE, cellulose	Molds, Bacteria, Yeast	Lee, Hwang & Cho (1998) Imakura, Yamada & Fukuzawa (1992) Oki(1998), Chung, Cho & lee (1998) Hong et al. (2000)
Parabens: Propylparaben, ethylparaben	Clay-coated cellulose LDPE	Molds	Katz (1998) Dobies et al. (1998)
Miscellaneous: Hexamethyl-eneteteamin	LDPE	Yeasts, anaerobes and acrobes	Devlieghere et al. (2000)
Abbreviations: EVA(ethylene vinyl acetate); LLDPE (linear low density polyethylene); LDPE (low density polyethylene); PS (polystyrene); PE (polyethylene)			

2.5 Chitosan

Chitosan is a carbohydrate polymer that can be derived from crustacean seafood wastes such as shells of crabs, shrimps and crawfish. Chitosan has a wide range of applications in diverse fields ranging from medical sutures and seed coatings to dietary supplements and coagulants for waste treatment. Physicochemical properties of chitosans and their functionalities are affected by their sources (Rhazi *et al*., 2004). Chitosan is the N-deacetylated derivative of chitin; although this N-deacetylation is almost never complete, this could be defined as chitin sufficiently deacetylated to form soluble amine salts. The required degree of deacetylation to obtain a soluble product must be 80–85% or higher. Chitosan products are highly viscous, resembling natural gums (Peniston and Johnson, 1980). The physico-chemical and biological properties of chitosan justify its introduction in food formulations once it could improve nutritional, hygienic and/or sensory properties, because of its emulsifying, antimicrobial, antioxidant and gelling properties, while also acting as a functional fiber. Chitosan's safety can be evaluated by its remarkably high lethal doses (1.6 g/kg of body weight in rats), being comparable to those of sugar and even less toxic than salt. For all these reasons, chitosan has been accepted as a dietary supplement or a food additive in many countries (e.g. Italy, France, Norway, Poland, United States of America, Argentine, Japan and Korea) (Argullo´ *et al*., 2004 and Park *et al*., 2002). (Nadarajah, 2005).

2.5.1 Sources of Chitosan

Chitosan is converted from chitin, which is a structural polysaccharide found in the skeleton of marine invertebrates, insects and some algae. Chitin is perhaps the second most important polysaccharide after cellulose and is an abundantly available renewable natural resource. The aquatic species that are rich in chitinous material (10-55 % on a dry weight basis) include squids, crabs, shrimps, cuttlefish and oysters. Mucoraceous fungi, which are known to contain chitin and the deacetylated derivate, chitosan, in cell walls (22 to 44%), have been used for commercial chitin production

(Muzzarelli, 1977; Muzzarelli *et al.,* 1994). However, in comparison with marine sources, which yield more than 80,000 metric tons of chitin per year (Muzzarelli ,1977; Subasingle, 1995), chitin production from fungal waste is negligible (Nadarajah, 2005).

Depending on the sources, the physicochemical properties and functionalities of chitosan differ (Rhazi *et al.,* 2004). For example, chitosan prepared from squid contains β-chitin (amine group aligned with the OH and CH_2OH groups) and those prepared from crustaceans contain α-chitin (anti-parallel chain alignment) (Shepherd *et al.,* 1997; Felt *et al.,* 1999). Despite a wide range of available sources, chitosan is commercially manufactured only from crustaceans (crab, shrimp, krill, and crayfish) primarily because a large amount of crustacean exoskeleton is available as a byproduct of food processing. Disposal of crustacean shell waste has been a challenge for seafood processors.

Therefore, production of value-added products, such as chitin, chitosan and their derivatives, and utilization of these values added 13 products in different fields are of utmost interest to food industries. Continual use of new raw materials as a source of chitin would enable production to be significantly increased. Major progress is being made in the development of profitable technology for isolation of chitin and its derivatives (Rashidova *et al.,* 2004). However, commercial extraction has been hampered by the corrosive nature of the strong acids and bases used in the manufacture of chitosan, which destroys equipment, requires careful handling by workers, and presents potential environmental hazards (Peniston and Johnson 1980; Leffler 1997; Nadarajah, 2005).

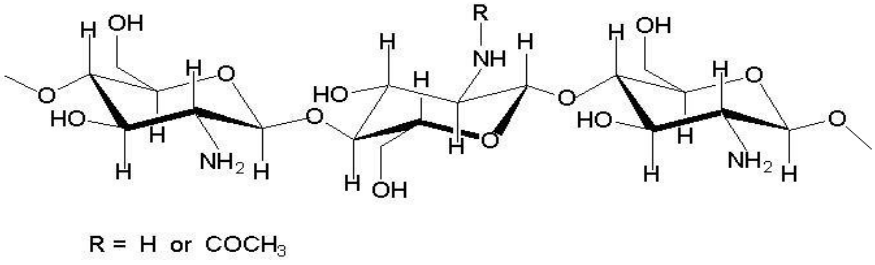


Figure 2.2 Molecular formula of chitosan

2.5.2 The Properties of Chitosan

At room temperature, chitosan forms aldimines and ketimines with aldehydes and ketones, respectively. Reaction with ketoacids followed by reaction with sodium borohydride produces glucans carrying proteic and non-proteic amino groups. *N*-Carboxymethyl chitosan is obtained from glyoxylic acid. Examples of non-proteic amine acid glucans derived from chitosan are the *N*-carboxybenzyl chitosans obtained from *o*- and *p*-phthalal-dehydic acids (Madhavan *et al*., 1993 and Muzzarelli *et al*., 1984). Chitosan and simple aldehydes produce *N*-alkyl chitosan upon hydro-genation. The presence of the more or less bulky substituent weakens the hydrogen bonds of chitosan; therefore *N*-alkyl chitosans swell in water in spite of the hydrophobicity of the alkyl chains, but they retain the film forming property of chitosan (Ravi Kumar, 2000 and Nadarajah, 2005).

2.5.3 Antimicrobial Properties of Chitosan and Chitosan Films

Such packaging or coating can maintain a high concentration of antimicrobial agents on the food surface and allow low migration into food (Torres *et al.,* 1985; Siragusa and Dickson 1992; Ouattara *et al.,* 2000). Chitosan films having the ability for controlled release of added substances would help in this context. Chitosan possesses unique properties that make it an ideal ingredient for development of antimicrobial edible film. Chitosan possesses film-forming properties (Averbach, 1978), greater and broader spectra of antibacterial activity compared to disinfectants, a higher bacterial/fungal killing rate, and lower toxicity toward mammalian cells (Franklin and Snow, 1981; Takemono *et al.,* 1989). Further, Rhoades and Roller (2000) reported that the interaction (binding or chelation) of chitosan with endotoxins of gram-negative bacteria decreased their acute toxicity. Because of the strong chelating ability of chitosan, external chelating agents such as EDTA may not be required, when antimicrobial agents such as nisin are added to chitosan to control gram-negative bacteria (Nadarajah, 2005).

Antimicrobial properties of chitosan have been reported by many investigators. Chitosan's ability to inhibit a wide variety of bacteria (Sudarshan *et al.*, 1992; Yalpani *et al.*, 1992), fungi (Allan and Hadwiger, 1979; Stossel and Leuba, 1984; Kendra *et al.*, 1989; Fang *et al.*, 1994), yeasts (Ralston *et al.*, 1964), and viruses (Kochkina *et al.*,; 1995; Pospieszny, 1997; Chirkov, 2002) make it a broad spectrum antimicrobial agent. A variety of research has been conducted to assess inhibitory effects of chitosan in a solution state or its oligosaccharides in terms of minimum inhibitory concentration (MIC). Chitosan is more effective in inhibiting bacteria than chitosan oligomers (Jeon *et al.*, 2001). Antimicrobial activity of chitosan is influenced by its molecular weight, degree of deacetylation, concentration in solution, and pH of the medium (Lim and Hudson, 2003). No *et al.*, (2002), reported that chitosan with different organic acid solvents exhibited varying inhibitory effects on bacteria. In general, acetic acid, lactic acid, and formic acids were more effective in inhibiting bacterial growth than propionic and ascorbic acids. Chitosan shows stronger antimicrobial activity for gram-positive than gram-negative bacteria (Jeon *et al.*, 2001). Chitosan has been observed to act more quickly on fungi and algae than on bacteria (Cuero, 1999); however, like other properties of chitosan, this activity may be dependent on the type of chitosan, chitosan molecular weight, and degree of deacetylation, among other factors influencing the environment in which the chitosan is stored (Nadarajah, 2005).

Antimicrobial property of chitosan can be enhanced by irradiation (Matsuhashi and Kume, 1997), ultra violet radiation treatment, partial hydrolyzation (Davydova *et al.*,2000), using different organic solvents (No *et al.*,2002), chemical modifications (Nishimura *et al.*,1984; Tanigawa *et al.*,1992), synergistic enhancement with preservatives (Chen *et al.*,1996; Roller *et al.*,2002), synergistic enhancement with antimicrobial agents (Lee *et al.*,2003; Song *et al.*,2002), or in combination with other hurdle technologies (Nadarajah,2005).

2.5.4 Application of Chitosan

Chitosan possesses many desirable properties for use in food systems. The film-forming ability of chitosan and gas-barrier properties of chitosan film favor its use as an edible food packaging material. Their inherent antimicrobial properties along with non-toxicity and biocompatibility offer their use as antimicrobial additives. Further, as an additive chitosan can offer a variety of functionalities (Nadarajah, 2005). Chitosan is one of the most abundant natural amino polysaccharides. It has been widely used in food production for clarification and deacidification of fruit juices (fining agent), for purification of water, for antioxidative maintenance in muscle foods, etc., (Shahidi, Arachchi, and Jeon, 1999) and in pharmaceutical areas, e.g. for drug-delivery systems (Vilivalam and Dodane, 1998) because it has good biocompatibility, biodegradability, film-forming property and antimicrobial activity (Ravi and Majeti, 2000).

In order to widen its application, many attempts have been made to optimize its properties, such as by cross-linking (Lopez and Bodmeier, 1997; Wei, Hudson, and Mayer, 1992), blending (Perugini, Genta, Conti, Modena, and Pavanetto, 2003; Rujiravanit, Kruaykitanon, Jamieson, and Tokura, 2003; Wang, Du, and Fan, 2005) and formation into chitosan based composites (Li, Du, Zhang, and Pang, 2003; X. Wang *et al*., 2007). Relatively low cost, widespread availability from a stable renewable source, that is, shellfish waste of the sea food industry, along with chitosan's ability to form a good film, are primary reasons to seek new applications of this polymer (Bangyekan, Aht-Ong, and Srikulkit, 2005; Bourtoom and Chinnan, 2007).

2.6 Starch

Starch, composed of amylose and amylopectin, is primarily derived from cereal grains, potatoes, tapioca, or arrowroot (Baldwin *et al*., 1995; Cutter and Sumner, 2002; Nisperos-Carriedo, 1994). Starch-based films exhibit physical haracteristics similar to

plastic films in that they can be odorless, tasteless, colorless, non-toxic, biologically absorbable, semi-permeable to carbon dioxide, and resistant to passage of oxygen (Nisperos-Carriedo, 1994; Rankin, Wolff, Davis, and Rist, 1958). High amylose starch films have been made that are flexible, oxygen impermeable, oil resistant, heat-sealable, and water soluble (Gennadios *et al*., 1997). Not only did the films protect meat products during frozen storage, but also dissolved during thawing and cooking (Gennadios *et al*., 1997).

Starch has been used to produce biodegradable films to partially or entirely replace plastic polymers because of its low cost and renewability. However, wide application of starch film is limited by its water solubility and brittleness (Wu and Zhang, 2001). In order to overcome these shortcomings (Jagannath *et al*., 2003) blended starch with different proteins to decrease the water vapor permeability of the films and to increase their tensile strength (Xu *et al*., 2004).

Among the natural polymers, starch has been considered as one of the most promising candidates for future materials because of its attractive combination of price, abundance and thermoplastic behavior, in addition to biodegradability. Starch-based materials have poor mechanical properties, however, and particularly poor elongation (around 6%) at ambient conditions. Thus, the incorporation of a plasticizer is required to overcome the brittleness of these materials (Galdeano *et al*. 2009). In order to improve the physical and functional properties of starch films, blending with other biopolymers, hydrophobic substances and/or antimicrobial compounds has been proposed (Anker, 2001; Ayranci and Tunc, 2003; Flores, Haedo, Campos, and Gerschenson, 2007; Garcia, Martino, and Zaritzky, 2000; Vásconez *et al*., 2009).

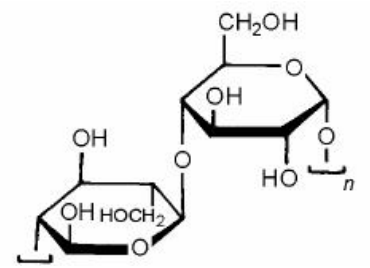


Figure 2.3 Chemical Structure of Starch

2.6.1 Yam

Yam (Discorea spp.) tubers are widely consumed as cooked slices or purees in the Asian sub-continent including Taiwan, Japan and China. Its health effects are attributed to the presence of sapogenins (Degras, 1993). The major constituent in yam is starch (18–25% based on fresh weight (Scott *et al.*, 2000)), which affects the physico-chemical characteristics (such as gelatinization temperature and gelation property) of products. Mucilage, composed mainly of mannan–protein, that plays an important role on the functionality including immunostimulatory activities (Shang *et al*., 2007), antioxidative activity (Hou *et al*., 2001), and hypolipidaemic effect (Boban *et al*., 2006). Although the concentration of mucilage is about 5% d.b. only (Tsai and Tai, 1984), it is also an important component in yam (Yeh *et al.*, 2009).

Yam starch is a promising polymer for biofilm production because it contains about 30% of amylose, and this is important for film production because amylose is responsible for the film-forming capacity of starches. Yam starch films were described as films with a homogeneous matrix, with stable structure at ambient conditions and a poor water vapor barrier compared with synthetic materials, which could be promising in the postharvest conservation of fruits and vegetables (Mali and Grossmann, 2003 and Mali *et al*., 2002, 2005).

Yam tubers show strong enzymatic browning reactions when cut and exposed to the air. This browning has been attributed to the oxidation of phenolic compounds (Ozo *et al*., 1984). Farombi, Britton, and Emerole (2000) reported that yam tubers contain polyphenols, such as catechins, chlorogenic acids, proanthocyanidinis, and anthocyanins. The total phenolic content and the presence of various phenolic compounds have been reported in some species of domesticated yam tubers (Martin and Ruberte, 1976; Muzac-Tucker *et al*., 1993 and Ozo *et al*., 1984), but very scarce data on the quantity of phenolics and antioxidant activity have been reported. Phenolic compounds are reported to have multiple biological effects, including antioxidant activity, antitumor, antimutagenic and antibacterial properties (Shui and Leong, 2002 and Bhandari and Kawabata, 2004).

2.7 Antimicrobial Agent and Its Type

2.7.1 Essential Oil

Essential oils have been used for centuries by populations around the world for medicinal, cosmetic, and spiritual purposes. Although the antimicrobial activities of a number of essential oils have been studied extensively with respect to gastrointestinal pathogens, few scientific reports are available on the dermatologic application of essential oils other than tea tree oil (Kassim, 2008). Essential oils or the so-called volatile or ethereal oils (Guenther, 1948) are aromatic oily liquids obtained from plant organs: flower, bud, seed, leaf, twig, bark, herb, wood, fruit and root. The term of ‘‘essential oil’’ is thought to derive from the word Quinta essential with medical use attributed to Paracelsus.

Until recently, essential oils have been used as food flavourings due to their flavour and fragrance, but nowadays essential oils and their pure components are gaining

increasing interest from the point of view of their safe status, wide acceptance by consumers and their exploitation for multi-purpose uses (Cowan, 1999). The utilisation of natural antioxidants as substitutes for those from chemical synthesis has encouraged the search of new sources including essential oils (Capecka, Mareczek, and Leja, 2005; Ruberto and Baratta, 2000).

In addition, the antimicrobial properties of essential oils derived from many plant organs have been empirically recognized for centuries, but only came to scientific attention recently (Appendini and Hotchkiss, 2002; Burt, 2004). Table 2.2 shows some of the most common essential oils as well as the major component used in the food industry with description of antioxidant or antimicrobial properties in vitro. These natural compounds belong to genus *Thymus*, *Origanum*, *Syzygium*, *Mentha* and *Eucalyptus* (Serrano *et al*., 2008)

Table 2.2: Some common essential oils and their components used as flavouring in the food industry that exhibit antioxidant, antifungal and antibacterial activity *in vitro* systems (Serrano *et al*., 2008).

Common name	Latin Name	Source	Major component	Antioxidant	Antifungal	Antibacterial	Reference
Clove	*Syzygium aromaticum*	Bud Leaf	Eugenol	Lipid	*Penicillium* *Aspergillus*	*Listeria monocytogenes* *Lactobacillus sakei*	Gill and Holley (2004), Lee and Shibamoto (2001) and Suhr and Nielsen (2004)
Eucalyptus	*Eucalyptus globulus*	Leaf Wood	Eucalyptol	Thiobarbituric acid DPPH	Moulds and yeasts *in vivo*	Pathogenic bacteria	Amakura *et al.* (2002), Gonzalez, Cruz, Dominguez, and Parajo (2004) and Ponce, Del Valle, and Roura (2004)
Mint	*Mentha canadensis*	Leaf	Menthol	$ABTS^{++}$	*Botrytis*	Pathogenic bacteria	Bouchra,Achouri, Hassani, and Hmamouchi (2003), Iscan, Kirimer, Korkcooglu, Baset, and Deminci (2002), Ozkan, Sagdic and Ozcan (2002) and Shan *et al.* (2005)
Oregano	*Origanum vulgare*	Leaf Flower	Eugenol Carvacrol Thymol	Peroxidase	*Botrytis* *Fusarium* *Clavibacter*	Shigella sp.	Bagamboula *et al.* (2004), Daferera, Ziogas, and Polission (2003) and Milos *et al.* (2000)
Thyme	*Thymus vulgaris*	Leaf	Carvacrol *p*-Cymene Thymol	Aldehyde/ Carboxylic acid	*Aspergillus*	Pathogenic bacteria	Lee *et al.* (2005), Ozkan *et al.* (2002) and Rasooli and Abyaneh (2004)

2.7.2 Herbs Essential Oil as Antimicrobial Agent

Plant essences have been used for thousands of years to promote both health and beauty. The essential oils are absolutely pure and can be used for aromatherapy or as perfumes. They can also be added to baths or lotions as they do not contain artificial scents or dyes. Even essential oils from some herbs commonly used in cooking, such as rosemary and sage, contain concentrated amounts of certain compounds that can cause seizures. Many of these compounds can be harmful when absorbed through the skin, not just when they are taken by mouth.

In Ayurveda, the aerial plant parts are thought to be stimulating, the roots grounding, bark and resins heating, and leaves and stems cooling. The many spices or aromatic herbs used to give special aromas or flavours to foods and at the same time known to have antimicrobial properties include garlic, onion, cinnamon, nutmeg, curry, mustard, black pepper, thyme, oregano, sage, rosemary, mint, Jamaican pepper, aniseed, basil, paprika, turmeric, bay, cardamom, cassia, Cayenne pepper, celery, chives, clover, coriander, dill, ginger, savory and marjoram (Beuchat, 1976; Zaika and Kissinger, 1981; Aureli *et al*., 1992; Pandit and Shelef, 1994; Sivropoulou *et al*., 1996; Marino *et al*., 1999; Tassou *et al*., 2000).

2.7.3 Ginger Essential Oil

2.7.3.1 General Description

Ginger is one of the oldest herbs known by the people and is one of the earliest spices to be known in the east. Ginger of the commerce consists of thick scaly rhizomes of the plant *Zingiber officinale*, belonging to the family *Zingiberaceae*. The plant is indigenous to warm tropical climates, particularly southeastern Asia. It is now extensively cultivated in India, China, Africa, Jamaica, Mexico and Hawaii (Evans,

1989). Indian ginger plant is an erect perennial, growing from 1–3 ft. in height. Mostly gingers in cultivation are sterile cultivars grown for the edible rhizomes and flowers are rarely seen. The rhizomes (spice of commerce) are aromatic, thick lobed, branched and scaly structures with a spicy lemon-like scent. It is well known that ginger rhizomes contain both aromatic and pungent components. The essential oil and oleoresins extracted from ginger rhizomes are very valuable products responsible for the characteristic ginger flavor and pungency (Singh, 2008).

Z. officinale and its constituents have been reported to exhibit a wide range of pharmacological activities, such as antibacterial (Yamada *et al*., 1992), antioxidant (Jitoe *et al*., 1992; Kikuzaki and Nakatani, 1993), analgesic, anti-inflammatory, carminative, diuretic and stimulatory (Tanabe *et al*., 1993 and Langner *et al*., 1998), and antifungal properties (Singh *et al*., 2005) attributed to its pungent principles (Yamahara *et al*., 1989). For centuries, *Z. officinale* has been known in Asia, Africa and other folk medicines as a most effective remedy for rheumatic diseases, respiratory diseases, loss of appetite, vomiting, nausea, and convulsion in children (Langner *et al*., 1998 , Ali *et al*., 2008 and Ukeh *et al*., 2009).

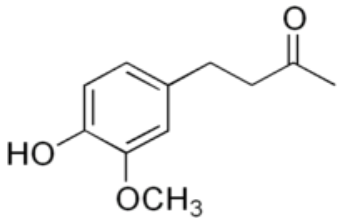


Figure 2.4 Chemical formula of ginger.

2.8 Additives

Plasticizers reduce intermolecular forces and increase themobility of polymer chains, decreasing the glass transition temperature (Tg) and improving flexibility (Garcia *et al*., 2000 and Galdeano *et al*. 2009).

2.8.1 Function of Plasticizer in Film Formation

Films prepared from pure polymers tend to be brittle and often crack upon drying. Addition of food-grade plasticizers to film-forming solution alleviates this problem (McHugh and Krochta, 1994). When a plasticizer is added, the molecular rigidity of a polymer is relieved by reducing the intermolecular forces along the polymer chain. Plasticizer molecules interpose themselves between the individual polymer chains, thus breaking down polymer-polymer interactions, making it easier for the polymer chains to move past each other. The plasticizer improves flexibility and reduces brittleness of the film. Polyethylene glycol, glycerol, propylene glycol, and sorbitol are the most commonly used plasticizers in edible film production (Aydinli and Tutas 2000). The amount of plasticizer added can cause adverse effects on film properties such as increasing mass transfer through the films. Hence, plasticizers must be used with caution. When the plasticizer concentration exceeds its compatibility limit in the polymer, it causes phase separation and physical exclusion of the plasticizer (Aulton and others 1981). This leads to development of a white residue on edible films which have been referred to as “blooming” (Aulton and others 1981) or “blushing” (Sakellariou and others 1986). The amount of plasticizer used in film formation should also be small enough to avoid probable toxic effects (Nisperos-Carriedo, 1994 and Nadarajah, 2005).

2.8.2 Polyethylene Glycol (PEG)

The synonyms and trade names are Carbowaxes; 1,2-ethanediol, homopolymers; ethylene glycol, homopolymers; ethylene oxide, polymers; ethylene polyoxide; lutrol; oxyethylene polymer; polyethylene oxide polymers; Poly(ethyleneoxide)s; polyhdroxyethylene; polyoxyethylene; polyoxyethylenediol; poly(oxyethylene) glycols and poly(vinyl oxide). It is clear, viscous liquids with molecular weight from 200 to 600 or waxiform products with molecular weight from 1000 to 6000. Solubility in water is inversely proportional to molecular mass. Liquid PEG is colorless, almost odorless, and

miscible with water. Meanwhile, Waxiform PEGs (carbowaxes) are soluble in water (50 to 73%). At a concentration of 1.0 g/1, they do not alter the color, odor, or taste of water.

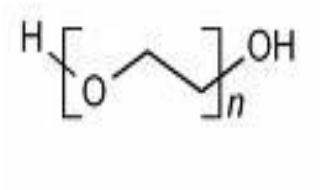


Figure 2.5 Chemical formula of polyethylene glycols (PEG).

Polyethylene Glycol (PEG) is used in food and food packaging. It used as plasticizers, solvents, water-soluble lubricants for rubber molds; wetting or softening agents, antistatic in the production of urethane rubber, components of detergents and others. In medicine, PEGs are used in cosmetics, ointments, suppositories, in ophthalmic solutions and sustained-released oral pharmaceutical applications (Sheftel, 2000).

2.9 Acetic Acid

2.9.1 Production of Acetic acid

Acetic acid is an important feedstock for various industrial products and also used in food and pharmaceutical industries. Acetic acid is produced by chemosynthesis from butane, methanol and other petroleum products (Ghose and Bhadra, 1985; Kirk-Othmer, 1991; Cheryan *et al*., 1997) and also by microbial fermentation (Sugaya *et al*., 1986; Ebner *et al*., 1996). Due to depleting natural resources more emphasis is given to fermentative production of acetic acid using cheap and abundantly available biomass as substrate (Slapack *et al.,* 1985). The conversion of cellulosic biomass to acetic acid is mainly carried out either by the conventional multi-step approach (Busche, 1985; Slapack *et al.,* 1985; Sugaya *et al.,* 1986; Ebner *et al.,* 1996) or by a novel single-step

process (Ruyet *et al.*, 1984; Miller and Wolin, 1995; Lee, 1997). The conventional multi-step approach includes acid or enzymatic hydrolysis of the substrate, followed by yeast fermentation and oxidation to acetic acid by *Acetobacter sp.* Acid hydrolysis is hindered mainly by glucose yields and corrosion of the equipment. Enzymatic hydrolysis, which usually employs cellulases from *Trichoderma reesei*, achieves higher substrate conversion yields, but its production is very expensive (Parisi, 1989; Vallender and Eriksson, 1990). Therefore, direct conversion of cellulosic biomass to acetic acid by a single (in situ) fermenting organism is economical, which eliminates the need for separate fomenters. In this direction, we have earlier reported the isolation of various cellulolytic mesophilic , anaerobic, acetogenic *Clostridium sp*. From faecal dropping of various herbivorous animals and birds (Ravinder *et al.,* 2001).

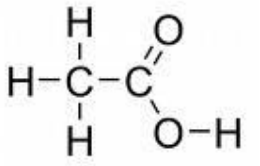


Figure 2.6 Chemical formula of acetic acid

2.10 Bacteria Strain

2.10.1 *Bacillus subtilis*

2.10.1.1 Strain *Bacillus subtilis* for Biofilm Fermentation

Bacillus subtilis recently has received a huge interest for its nature to develop into biofilm. In biofilm *Bacillus* shows significantly different genetic and morphological development compared to its planktonic state in submerged culture (Pratt and Kolter, 1999; Kuchma and O'Toole, 2000; Branda *et al*., 2001, 2004, 2005; Oosthuizen *et al*., 2002). The ubiquity of biofilm has been introduced in the studies of community of microorganisms with a definition that biofilm is the natural state of the microorganisms

where they remain in a community exhibiting high degree of structure (Costerton *et al*., 1978; Davey and O'Toole, 2000). When the culture is incubated on a liquid medium, wild type strain of *B. subtilis* produces biofilms in the liquid–air surface of the culture medium. However, the domesticated or laboratory strains such as *B. subtilis* 168 produce thin and fragile biofilm (Branda *et al*., 2001). *B. subtilis* 168 was converted into iturin A producer by horizontal transfer of the significantly large 42 kb fragment containing *itu* operon (Kunst *et al*., 1997 and Tsuge *et al*., 2001, 2005). Introduction of the *degQ* improved the productivity of iturin A in submerged fermentation (Tsuge *et al*., 2005). Transformation of large operons in *Bacillus* is practically difficult; however, strain 168 shows stability of the introduced operon and successful expression of the introduced genes. The transformant strain produced several lipopeptide antibiotics including iturin A, surfactin and plipastatin (Tsuge *et al*., 2005). These findings are notably important for introduction of sufficiently large gene fragments for the production of desired antibiotics (Rahman *et al*, 2007).

2.10.2 *Escherichia coli*

Escherichia coli are a common cause of bacteraemia. It is often precipitated by bile and urinary tract infections, sterile fluids in close proximity to the gastrointestinal tract. A small number of serotypes cause disease and virulence factors (e.g. antiphagocytic capsules, adhesions, invasins, lipopolysaccharide and toxins) are well described. Clinical studies of patients with *E. coli* infection and bacteraemia have demonstrated a mortality rate of 10 to 30% with chances of survival increasing with early administration of an effective antibiotic (Gransden *et.a l*., 1990). Cephalosporins are the mainstay of empirical antibiotic therapy and, until now, have effectively treated most *E. coli* infections (Melzer and Petersen, 2007).

In 2003, the UK Health Protection Agency (HPA) confirmed reports that extended-spectrum beta-lactamase (ESBL) producing *E. coli* was causing infections in

different parts of the UK. These were predominantly of the type CTX-M-15. Most strains were resistant to beta-lactams and other classes of antibiotics and, in some cases, only carbapenems and aminoglycosides (eg. amikacin) could reliably be used to treat infections (Woodford *et al*., 2004). The mechanism of multiple-drug resistance via ESBL production is well elucidated (Jacoby and Monez-Price, 2005) and recently, the HPA, in collaboration with the British Society for Antimicrobial Chemotherapy, issued laboratory guidelines for detection of ESBL producers (Melzer and Petersen, 2007).

In patients with *E. coli* infection and bacteraemia, quinolone resistance has been reported to be associated with a higher incidence of death (Lautenbach *et al.*, 2005). The effect of ESBL production on clinical outcomes is less well described (Ramphal and Ambrose, 2006) although one small Scandinavian study reported a high death rate in bacteraemic patients treated with ceftazidime (Ho *et al.*, 2005). Smaller studies have examined the effect of ESBL production in several Enterobacteriaceae species together (Schwaber *et al.*,2006; Zaoutis *et al.*,2005; Kim *et al.*,2002; Blomberg *et al.*,2005; Kang *et al.*,2004; Du *et al.*,2002 ; Henshke-Bar-Meir et al.,2006 and Skippen *et al.*,2006) but rarely in *E. coli* where one ESBL predominates (Meton *et al*.,2005; Bin *et al*.,2006; Rodriguez-Bano *et al.*,2006 and Calbo *et al*.,2006). The primary aim of this study was to compare mortality in those with infection and bacteraemia caused by ESBL and non-ESBL producing *E. coli*. Secondary aims were to determine time to death following bacteraemia and, in those who survived length of inpatient stay (Melzer and Petersen, 2007).

2.10.2.1 History of *Escherichia coli* as a Pathogen

Escherichia coli O157 was first conclusively identified as a pathogen in 1982 when associated with 2 clusters of food-borne illness in Oregon USA (Riley, Remis and Helgerson, 1983). Since then, there have been several more episodes where food has been strongly implicated not least recent outbreaks in Japan (Eley, 1997) and Scotland

(Scottish Centre for Infection and Environmental Health, 1996) which have received widespread media coverage due to the severity of the illness which can be caused by this organism (Chapman, 1995; Neill, 1989). In 1992, due to increasing UK Government concern over the number of food-related outbreaks associated with *E. coli* O157, a Working Group on Verocytotoxin-producing *E. coli* (VTEC) was set up by the Advisory Committee on the Microbiological Safety of Food (ACMSF) with the remit 'To assess the significance of VTEC as a foodborne pathogen and to advise on any action which could be taken to reduce foodborne disease associated with it. In 1995, the ACMSF produced its 'Report on Verocytotoxin-producing *Escherichia coli* which provides a comprehensive account of current knowledge (ACMSF, 1995). The ACMSF recommended specifically that Government fund (i) research into the development and evaluation of different solid selective media for O157 VTEC and (ii) evaluate conventional and rapid methods for the examination of foods and environmental samples. A useful review of methods for the detection of VTEC of the O157 serogroup has been carried out by Vernozy-Rozand (1997). (Scotter *et al.*, 2000).

Figure 2.7 3D structure of *Escherichia coli*

2.11 Mechanism of Antimicrobial Film

Active packaging can be done based on two main concepts that is active releasing and active scavenging. The forms of these two types of concept are as shown in Table 2.3.

Table 2.3: Concept of active packaging

Concept	Formed
Active releasing	Antimicrobial film Anti-oxidant film
Active scavenging	Oxygen scavenging Ethylene scavenging

As stated in Table 2.3, Antimicrobial film is a form of active packaging that apply active releasing concept. Boumans (2003) have developed the latest technology of this packaging by applying the bio-switch concept. The general concept of bioswitch (Figure 2.7) describes a system that is able to detect and automatically give responses if there is any a change or external stimulus in the environment. The bioswitch will convert the stimulus into a particular functionality.

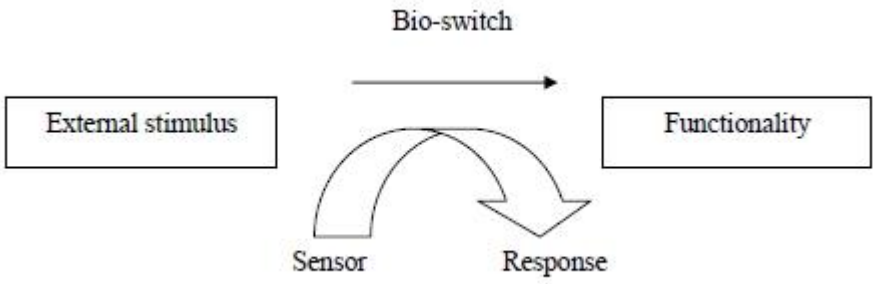


Figure 2.8 General concept of bio-switch

Figure 2.8 represent the mechanism of how the antimicrobial active packaging inhibits the microbial growth. Microorganism will first try to hydrolyze the starch-based

particles causing the release of the antimicrobial compound which finally resulting inhibition of the microbial growth.

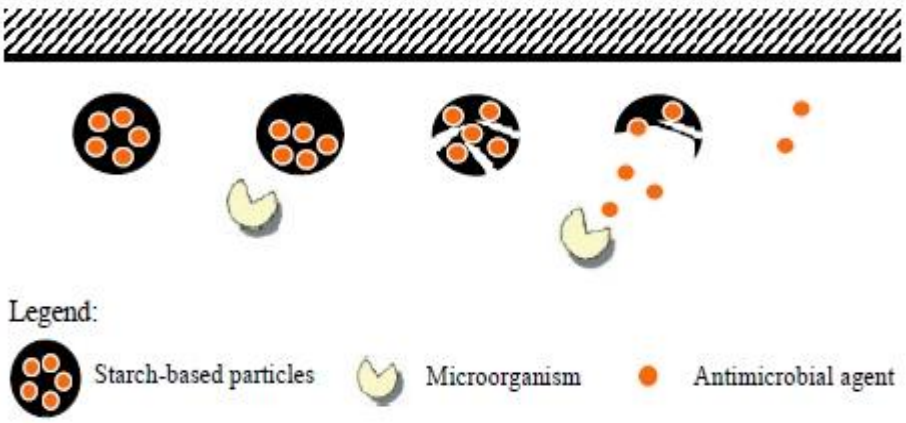


Figure 2.9 The anti-microbial active packaging action applying bio-switch concept. (Muhammad, 2005)

2.12 Antimicrobial Technologies

In recent years, a whole range of antimicrobial technologies has been developed. Applications are mainly situated in the medical sector such as medical devices, healthcare products, personal hygiene products and dental care products and also in the food-contact and household area such as cutting boards, knife handles, protective clothing and others. Other applications are biofilm-sensitive systems such as pipe lines, laboratory, and other scientific equipment and materials of construction for food equipment (Kane 1999). Until now, however, very few of these concepts have applications as food packaging materials. (Vermeiren *et al.*, 2001).

The expected growth of the global plastic antimicrobial industry is 4% per year up to 2005. There are a number of key drivers for this growth. These include:

a. The marketing advantages of value added antibacterial products, which have generally met with consumer favour, and the need to ensure hygienic conditions in industrial, commercial, medical and other institutional settings will support further gains. Regulatory pressures on traditional antimicrobials, such as OBPA, PCP, TBTO, for example.
b. Increasing concerns related to disease transmission will drive demand for surfaces treated with antimicrobials.
c. High end-industry growth in particular geographical regions, such as Asia.
d. Growing use of antimicrobials as hygiene aids.
e. Increasing use of plastics in new applications, including decking.

2.13 Several Methods to Characterize the Films

2.13.1 Scanning Electron Microscope (SEM)

Scanning electron microscope (SEM) is one of widely used microscopes. High resolution images of surface topography are produced using a highly focused, scanning electron beam. SEM accompanied by energy dispersive X-ray analysis (EDX) is a rapid, easy and non-destructive approach to surface elemental analysis. SEM-EDX is capable of detecting all elements from carbon to uranium, with a detection limit of ~0.5 wt. % for most elements (Goldstain *et al*., 2003 and Williams *et al*., 1995). High spatial resolution owing to the ability of focusing electron beam to a very small spot (<10 nm) realizes to analyze the local area. It has the following characteristics: sensitive to lighter elements, not usable in atmospheric pressure but not necessary for ultra high vacuum, and capable of analyzing elements of local area with observing the target area by secondary electron images. The shortcomings are: difficult to detect with high sensitivity the heavier elements, and inadequate to analyze trace elements (Toyoda *et al.,* 2004).

If the SEM is equipped with a cold stage for cryo-microscopy, cryofixation may be used and low-temperature scanning electron microscopy performed on the cryogenically fixed specimens. Nano-devices can be built from the bottom by directly assembling atoms, molecules, or other nano-objects. The bottom-up technology can be implemented using two different approaches. The first one is based on self-assembly. Self-assembly is the autonomous placement of components in predefined locations. It is driven by the tendency of physical systems to minimize their potential energy. The second bottom-up approach is characterized by the controlled manipulation and positioning of atoms, molecules, clusters or nanometer-sized objects, one-by-one, with the aid of elaborate equipment and tools, such as atomic force microscopes (AFM) or dedicated nanomanipulators in scanning electron microscopes (SEM). The work described hereinafter focuses on the SEM-based approach. SEM-based nanomanipulation systems must be adaptable to various applications, not only in manipulation and assembly but also in the characterisation of materials, small-scale structures and devices (Kraft and Volkert, 2001).Thus, a modular design is required to ensure a high flexibility, and an easy reconfigurability. Particularly with regard to semi-automated nanomanipulation, complex and repetitive tests without the need to open the SEM chamber have to be accomplished. This will reduce the handling time, increase the reliability and quality and generate a user-friendly process. One of the biggest challenges evolves from the fact that nano-scale objects require new strategies for their visualization, analysis and manipulation both as single objects and in a batch. It is obvious that to attain further advances in nanotechnology, focused effort is necessary in developing new measurement and manipulation tools that can be integrated into high spatial resolution SEMs.

Micromanipulation inside an SEM was for the first time reported and pioneered at the University of Tokyo by the group of Hatamura and Morishita and Sato *et al.* , respectively. The aim was to manipulate micro-objects smaller than 100 μm in order to construct micro-devices and micro-machines. The design of their micromanipulator was inspired by manual production in the macro-world. A craftsman uses both of his hands, one to hold the workpiece and one to work on it with a tool, while watching the

processing with his eyes. Thus, the micromanipulator developed consists of two so-called sub-manipulators and a worktable, and it is operated inside an SEM for visual supervision. The manipulators and the worktable have several translational and rotational degrees of freedom. They are driven by ultrasonic motors and balls screws for coarse positioning with a resolution of 70 nm. To achieve a higher accuracy, fine positioning mechanisms made of piezoelectric actuators and parallel plate structures have been integrated. An image processing system has been implemented to automatically place micro-objects based on the SEM image. Sato et al. finally succeeded in an automated pick-and-place arrangement of several micro-spheres with a diameter of 30 μm (Kasaya *et al*., 1998).

The SEM magnification was fixed to 1000 times; the tolerable error range of the objects' position was 10 μm. Though the authors describe an automated pick-and-place operation inside an SEM for the first time, the application of this micromanipulation system is clearly limited to the handling of relatively large micro-objects. The assembly and fixing of micro- or even nano-objects, as required for a nanomanufacturing system, has not been achieved. In view of sensor-based nano-scale automation, SEM nanomanipulators offer several advantages compared to AFM-based manipulators. These are, for example, a larger workspace, greater flexibility through more degrees of freedom (DOF), dedicated control systems. However, up to now, only a small degree of automation has been implemented with these systems. SEM image processing, for instance, is hardly used for semi automated positioning or pick-and-place operations. Most of the systems still rely on the operator's attention to the positioning tables' movements as shown on the SEM screen. Sensor information from position sensors is only available within a few systems; closed-loop nano positioning is still an exception.

2.13.2 Fourier Transform Infrared Spectroscopy (FTIR)

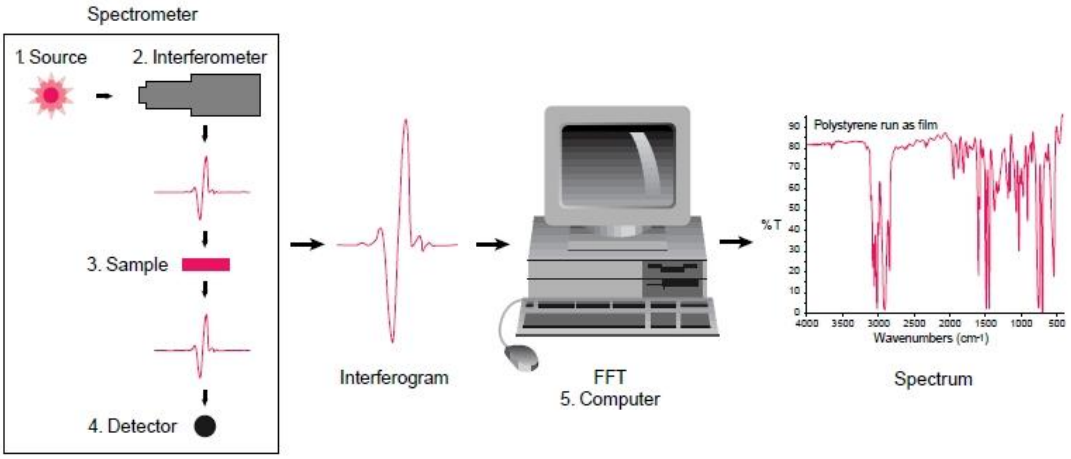
FTIR spectroscopy is an analytical technique that utilizes the infrared radiation of the electromagnetic spectrum to excite molecular motions. Among of a broad variety of methods Fourier transform infrared (FT-IR) spectroscopy has proved to be a very promising way to get information on the molecular level about the structure and electronic properties of molecules (Griffiths, 1975). The use of FT-IR technique in emission spectroscopic measurements has great potential, however, relatively few works have been reported (Willis *et al.,* 1987; Mink and Keresztury, 1993) Perhaps, and this is due to the greatest problem associated with this method, which is that the sample needs to be strongly heated. Besides the possible destruction of the sample, the background emission from different surfaces creates several technical problems in the processing of data (Griffiths, 1975; Willis *et al.,* 1987).

The determination of the texture such as the preferential growth of certain crystallographic planes parallel to the surface) and microstructure in terms of porosity, the development of columnar or globular structures of thin films has been traditionally carried out by means of X-ray diffraction (XRD) and electron microscopy, respectively. In this context, Fourier transform infrared spectroscopy (FT-IR) has not been so widely used, very likely because of the common belief that this technique is only useful to study substances at an atomic scale, with little potential to get information about characteristics of thin films associated with morphology. Thus, when dealing with thin films, the use of FT-IR spectroscopy has been typically limited to assess bond structures in relatively covalent materials (Ristein *et al*.,1998) that present sharp and almost invariable IR absorption peaks.

In the present investigation we have pursued FT-IR studies of anatase thin films having a significant porous microstructure. The theoretical analysis shows that both anatase texture and microstructure may have a large influence on the shape and behaviour of infrared spectra and that, therefore FT-IR can be used to investigate these thin film properties. For this purpose, a generalized formulation of the previous theoretical model has been developed to account for the influence of void

microstructures in the shape of FT-IR spectra of anatase thin films (Terpugov and Degtyareva, 2001).

Figure 2.10 The sample analysis process of FTIR



2.13.3 Thermo Gravimetric Analysis (TGA)

Thermo Gravimetric Analysis (TGA) is an analytical technique for measuring the weight loss of a material as a function of temperature. When the materials are heated, they can lose weight from a simple process. For example drying process and from chemical reactions that liberate gasses. But, some materials can gain weight when it reacts with the atmosphere in the testing environment. Since weight loss and gain are disruptive processes to the sample material or batch, knowledge of the magnitude and temperature range of those reactions are necessary in order to design adequate thermal ramps and holds during those critical reaction periods. Thermal degradation during the processing of starch is an important issue. Thermo Gravimetric Analysis (TGA) has been the conventional and most popular technique used to study the thermal stability and decomposition of starches, and many methods of kinetic analysis have been developed to describe and investigate decomposition rates based on TGA measurements (Liu *et al.*, 2009).

2.13.4 Differential Scanning Calorimeter (DSC)

The main application of DSC is in studying phase transitions, such as melting, glass transitions, or exothermic decompositions. These transitions involve energy changes or heat capacity changes that can be detected by DSC with great sensitivity. Differential scanning calorimeters (DSC) provide a widely used and rapid method to determine, as thermometers, temperatures of phase transition or chemical reaction, or, as calorimeters, heat capacities or enthalpies of transition or chemical reaction in the temperature range from about 100 to 1800 K (Hemminger and Höhne, 1984; Wunderlich, 1990; Hemminger and Cammenga,1989; Richardson and Charsley,1998) . However, the quality of measurements is influenced by instrumental, sample and operator-related parameters and critically depends on the accuracy of the temperature and caloric calibration of the instrument.

DSC instruments are no absolute measuring devices, the measurements are not made in thermal equilibrium and, therefore, the *relative* data obtained must be related to absolute thermodynamic values by calibration (Gmelin and Sarge, 1995). The dynamic working principle of the various instruments is complex and theoretically not yet fully understood in every detail. The calibration factors for heat and heat flow rate calibration differ and are, in the first place, dependent on temperature, sample mass and heat flow rate. In addition, the result may depend on numerous boundary conditions, e.g. parameter setting, emissivity and thermal resistance of the sample, sample geometry. The accuracy of DSC experiments is also intimately correlated with the individual knowledge, skill and working style of the operator (Gmelin and Sarge, 1995; Callanan and Sullivan, 1986; Hohne and Gloggler, 1989). Finally, the reliability of the calibration performed depends on the degree to which cell asymmetry can be corrected.

Calibration of these instruments is necessary for several reasons which are to convert the indicated relative scales (temperature and caloric scales) to an absolute scale,

to establish the traceability of the measured values to SI units; and to assess the uncertainty of measurement (Gmelin and Sarge, 2000).

CHAPTER 3

METHODOLOGY

3.1 Introduction

In general, the edible film based was the combination of chitosan and yam starch. The coating film were prepared and furthered with film casting procedure. After that, the several characterization and antimicrobial analysis were made on the casted films. For antimicrobial analysis, the culture medium for bacteria growth which was nutrient broth and agar plate were prepared.

3.2 Materials

There were several materials and chemicals were used in this experiment. The table 3.1 below lists the chemicals and materials used in the experiment and their function. Commercial low molecular weight chitosan purchased from Aldrich Chemistry in fine powder form. Polyethylene glycols (PEG) with molecular weight of 400 supplied by R & M Chemical Malaysia Sdn. Bhd. in constant concentration used as plasticizer. Yam flour purchased from Happy Grass marketing Sdn. Bhd., Pontian, Johor.

Table 3.1: Chemical and material used in this experiment and their functions

Chemicals/Materials	Function
Yam Starch	Edible Film Based
Chitosan	
Ginger Essential Oil	Antimicrobial Agent
PEG (polyethylene glycol)	Plasticizer
Glacial Acetic Acid	Additives
Bacillus subtilis	Bacteria Strain
Escherichia coli	

3.3 Equipments

The equipments used for running this experiment were beaker, glass plate, casting knife, electric stirrer, magnetic stirrer, Bunsen burner, inoculating loop, broth bottle, test tube, pipette and petri dishes.

3.4 Bacteria Culture Preparation

Bacteria must be cultured in order to facilitate identification and to examine their growth and metabolism. Bacteria were inoculated or introduced into various forms of culture media in order to keep them alive and to study their growth. Inoculations must be done without introducing unwanted microbes or contaminants into the media. Aseptic technique was used in microbiology to exclude contaminants. This procedure was referred to Biochemistry and Microbiology Lab Manual (BKB2761).

3.5 Edible Film Preparation

The films of yam starch and chitosan was prepared by casting method. The first control film, without ginger essential oil using the mixtures of yam starch, chitosan and PEG (polyethylene glycol) was formed. Then, the second control film, without ginger

essential oil using the mixtures of yam starch, gelatin, chitosan and PEG (polyethylene glycol) was formed.

Chitosan was dissolved in 1.0% (v/v) glacial acetic acid. The yam starch-chitosan films were prepared then by heat-gelatinization using suspension of yam starch (w/w). Then, mixed the both solution with addition of 3ml of PEG (polyethylene glycol) and stirred it about 10 hours and 300 rpm. Then, the ginger essential oil was added to the suspension with 0.5 volumes (ml).

Table 3.2: The amount of each material added for several solutions

Sample	Yam Starch (g)	Gelatin (g)	Chitosan (g)	Ginger Essential Oil (ml)	PEG(ml)
Sample A (control)	2.0	0.0	2.0	0.0	3.0
Sample B (control)	1.0	1.0	2.0	0.0	3.0
Sample C	1.0	1.0	2.0	0.5	3.0

3.6 Film Casting

Ten ml of the prepared solution was pipette and casted on a smooth plane in order to produce a film with smooth surface. The degassed solutions were poured on glass plates thoroughly. The layer thickness of the solution during casting process can be adjusted with the use of certain equipment such as the casting knife. The casted films were left for overnight under ambient temperature. Lastly, the casted films were peeled from the glass plate after they were completely dry. After that, six analysis and measurements were made on each sample by using agar diffusion test, liquid culture test, SEM, FTIR, DSC and TGA.

3.7 Characterization and Analysis of Yam Starch - Chitosan Film with Combination of Ginger Essential Oil

In this experiment, several analysis and characterization must be test on the film after the dried films were obtained in order to define its chemical, physical, thermal stability and mechanical properties.

3.7.1 Testing Antimicrobial Effectiveness

The antimicrobial analysis test towards *Bacillus subtilis* and *Escherichia coli* by two different methods which were agar diffusion test and liquid culture test.

3.7.1.1 Agar Diffusion Test (Zone Inhibition Assay)

Antimicrobial activity test was carried out using agar diffusion method. Indicator cultures were *Bacillus subtilis* representing Gram-positive bacteria and *Escherichia coli* representing Gram-negative bacteria. One hundred microliters of the inoculums solution was added to 5 ml of the appropriate soft agar, which was overlaid onto hard agar plate. Each film was cut into squares (1cm x 1cm) and was placed on the bacterial lawns. Duplicate agar plates were prepared for each type of film and control film. The plates were incubated for 48 h at 37 ^{o}C in the appropriate incubation chamber (aerobic chamber for *E. coli*). The plates were visually examined for zones of inhibition around the film disc, and the size of the zone diameter was measured at two cross sectional points and the average was taken as the inhibition zone (Salleh *et al*., 2007).

3.7.1.2 Liquid Culture Test (OD_{600nm} Measurement)

For the liquid culture test (Chung, Papadakis and Yam, 2002), each film was cut into squares (1cm x 1cm). Three sample squares were immersed in 20 ml nutrient broth

(Merck, Germany) in a 25 ml universal bottle. The medium was inoculated with 200µl of *Escherichia coli* / *Bacillus subtilis* in its late exponential phase, and then transferred to an orbital shaker and rotated at 37ºC at 200 r.p.m. The culture was sampled periodically (0, 2, 4, 8, 12 and 24 hours) during the incubation to obtain microbial growth profiles. The same procedure was repeated for the control starch-based film. The optical density (O.D. 600) was measured at λ = 600nm using a spectrophotometer (Model UV-160, Shimadzu, Japan) (Salleh *et al*., 2007).

3.7.2 Morphology Analysis of Yam Starch - Chitosan Film with Combination of Ginger Essential Oil

The surface morphology analysis of the film was defined by scanning electron microscopy (SEM). Scanning electron microscopy (SEM) was analyzed in details morphology of the film which were surface and cross-section of the films' study.

3.7.2.1 Microstructure Studies by Scanning Electron Microscopy (SEM)

Film surface morphology was examined using scanning electron microscopy. The dried film samples were mounted on a metal stub with double-sided adhesive tape. The morphological structures of the films were studied by a JSM-5600 LV scanning electron microscope of JEOL, Tokyo, Japan and the images were taken at accelerating voltage 5 kV and a magnification 100, 500 and 1000 times of origin specimen size. (Salleh and Muhamad, 2007).

3.7.3 Others Analysis

Others analysis for this film were fourier transform infrared spectroscopy (FTIR), thermo gravimetric analysis (TGA), differential scanning calorimeter (DSC), and antimicrobial activity.

3.7.3.1 Fourier Transform Infrared Spectroscopy (FTIR)

The information on structural changes which took place during the degradation was collected in the FTIR analysis using FTIR Nicolet Avatar 370 DTGS. All spectra were recorded at ambient temperature at the resolution of 4 cm^{-1} and 16-times scanning. Films used in the infrared tests were about 2 cm in diameter and the wavenumbers range between 500 to 4000 cm^{-1}. The FTIR analysis was based on the identification of absorption bands concerned with the vibrations of functional groups present in the films. The bands wave numbers (cm^{-1}) were as follows:

a. 3450: OH hydroxyl group.
b. 3360: NH group-stretching vibration.
c. 2920, 2880, 1430, 1320, 1275, 1245: symmetric or asymmetric CH_2 stretching vibration attributed to pyranose ring.
d. 1730: carbonyl group vibration.
e. 1660: C=O in amide group (amide I band).
f. 1560: NH-bending vibration in amide group.
g. 1590: NH_2 in amino group.
h. 1415, 1320: vibrations of OH, CH in the ring.
i. 1380: CH_3 in amide group.
j. 1255: C–O group.
k. 1150–1040: –C–O–C– in glycosidic linkage.
l. 850, 838: CH_3COH group.
(Pawlak and Mucha, 2003)

3.7.3.2 Thermo Gravimetric Analysis (TGA)

Changes which take place during thermal degradation of the investigated polymer systems were observed while performing thermogravimetric measurements. For this purpose a TGA Q500 series Thermogravimetric Analyzer (TA Instruments) was used to carry out measurements in the air, at a constant heating rate β = 15 °C /min in the range of temperature from 100°C to 450 °C. The CH blends were also subjected to

thermo degradation in the air at different temperatures from 100°C to 200 °C (isothermal TG in a special oven) while analyzing mass loss and structural changes (Pawlak and Mucha, 2003).

3.7.3.3 Differential Scanning Calorimeter (DSC)

DSC was used to measure the thermodynamic properties of the materials. All the measurements were carried out with a model DSC Q1000 series (TA Instruments) operating under Nitrogen atmosphere. Tiny portions of the samples, weighing about 5 mg, were closed in aluminum pans. A heating rate of 10°C/min up to 200°C was set in order to observe the polymer melting peak. After erasure of thermal history by a 5 min isotherm at 200°C, the sample was cooled down to room temperature at 10°C/min and heated again to 200°C at the same rate. Indium of high purity was used for calibrating the DSC temperature and enthalpy scales. The choice of the baseline in enthalpy evaluations has been standardized for all samples. In order to quantify the repeatability of the measurements, five replicates were recorded for selected samples and their standard deviations were computed. A 3% error on the enthalpy associated to melting endotherms and a 2% error on the enthalpy of crystallization was obtained. All the remaining samples were analyzed twice, a third confirmation replicate was performed if the two previous measurements differed by more than 3% in case of melting peaks or 2% in case of crystallization exotherms (Causin *et al*. ,2006).

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 Antimicrobial Activity

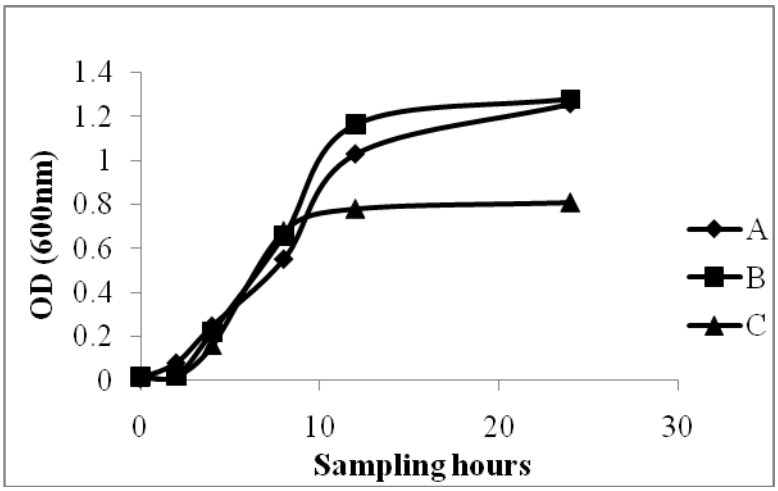
4.1.1 Liquid Culture Test (OD_{600nm} Measurement)

Table 4.1 below shows the OD value for Sample A, B and C against *Bacillus subtilis* and *Escherichia coli* at 0, 2, 4, 8, 12, and 24 period hours. The samples are reading via UV-VIS spectrophotometer at wavelength of 600 nm.

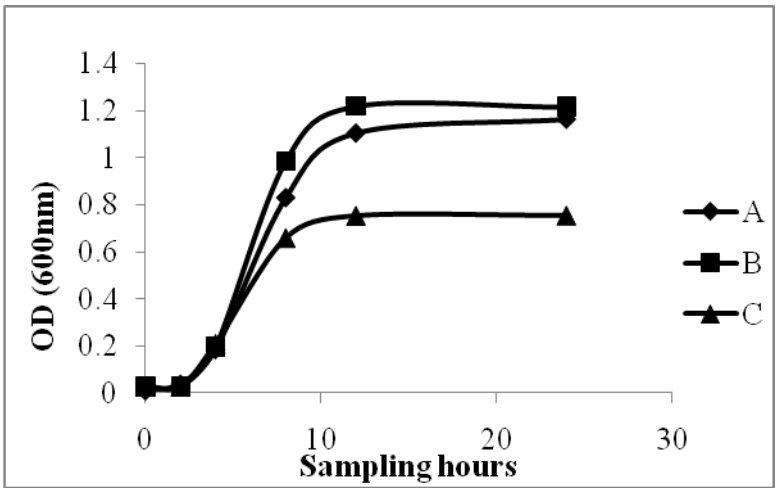
Table 4.1: OD value for Sample A, B and C against *Bacillus subtilis* and *Escherichia coli* at 0, 2, 4, 8, 12, and 24 period hours.

Bacteria Strain	*Bacillus subtilis*			*Escherichia coli*		
Period (hrs)	A	B	C	A	B	C
0	0.010	0.018	0.012	0.010	0.026	0.028
2	0.080	0.021	0.018	0.038	0.026	0.029
4	0.247	0.221	0.158	0.185	0.195	0.210
8	0.551	0.658	0.677	0.830	0.986	0.659
12	1.028	1.162	0.778	1.104	1.219	0.753
24	1.256	1.278	0.807	1.163	1.216	0.754

From the result obtained, the graph of OD measurement versus period hours for Sample A, B, and C against *Bacillus subtilis* and *Escherichia coli* were plotted to see the microbial growth of both bacteria strain in the liquid culture medium. Figure 4.1 below show the graph of OD measurement versus period hours for Sample A, B, and C (a) against *Bacillus subtilis* and (b) against *Escherichia coli*.



(a)



(b)

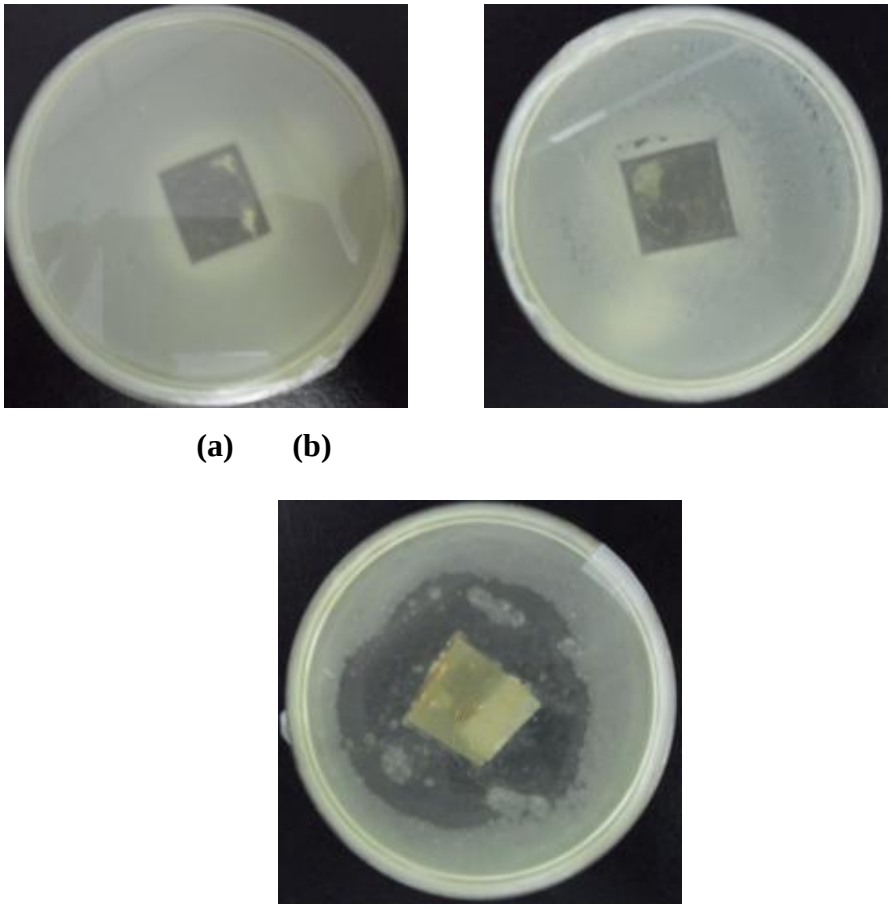
Figure 4.1 Graph of OD measurement versus period hours for Sample A, B, and C (a) against *Bacillus subtilis* and (b) against *Escherichia coli*.

Figure 4.1(a) show the inhibition of *Bacillus subtilis* by the Sample A, B, and C in liquid culture test. Clearly, Sample C shows the largest reduction of stationary growth phase. The effect of ginger essential oil on the inhibition is distinguished in Figure 4.1(a). The effects keep increase until it become almost consistent after 12 hour. It is shown that the addition of ginger essential oil in a film will decreased the reading of OD.

Figure 4.1(b) show the inhibition of *Escherichia coli* by the Sample A, B, and C in liquid culture test. Clearly, Sample C shows the largest reduction of stationary growth phase. The effect of ginger essential oil on the inhibition is distinguished in Figure 4.1(b). The effects keep increase until it become almost consistent after 12 hour. It is shown that the addition of ginger essential oil in a film will decreased the reading of OD.

Sample C is the most effective formulation to inhibit *Bacillus subtilis* and *Escherichia coli* as can be seen figure 4.1. However, Sample C with addition of ginger essential oil was more effective against Gram-positive bacteria than Gram-negative bacteria. As well as incorporation of chitosan, besides inhibit *E. coli* and increase the film effect on *B.subtilis* inhibition, it helps to enhance the antimicrobial starch-based film strength (Ban *et. al*., 2005 and Salleh *et al*., 2007). Most studies investigating the action of whole essentials oils (EOs) against food spoilage organisms and food-borne pathogens agree that, generally, EOs are slightly more active against Gram-positive than Gram-negative bacteria (Burt, 2004 and Zivanovic, 2007). This result may be related to the presence of an additional external membrane surrounding the cell wall in Gram-negative bacteria, which restricts diffusion of hydrophobic compounds through its lipopolysaccharide covering (Burt, 2004). According to Zivanovic and Draughon (Zivanovic, 2007), the proposed mechanism of antimicrobial activity of phenolic compounds of EOs is in their attack on the phospholipid cell membrane, which causes increased permeability and leakage of cytoplasm, or in their interaction with enzymes located on the cell wall. Thus, the resistance of Gram-negative bacteria to the essential oils likely lies in the protective role of their cell wall lipopolysaccharides or outer membrane proteins (Pelissari, 2009).

4.1.2 Agar Plate Test (Zone Inhibition Assays)



(a) **(b)**

(c)

Figure 4.2 Zone Inhibition of (a) Sample A, (b) Sample B, and (c) Sample C against *Bacillus subtilis*

The agar diffusion test provides the rapid assessment for microbial activity. Figure 4.2 shows the zone inhibition of Sample A, B and C towards *Bacillus subtilis.* From figure 4.2 (a) is shows that the clear zone only covered on the chitosan biocomposite film. Meaning that the bacteria is inhibit only on the surface of chitosan film, as we know chitosan has antimicrobial properties itself. As well as incorporation of chitosan, besides inhibit *E. coli* and increase the film effect on *B.subtilis* inhibition, it helps to enhance the antimicrobial starch-based film strength (Ban *et. al*., 2005 and Salleh *et al*., 2007). The result is same for Sample B as it has chitosan itself. Figure 4.2

(c) shows the clear zone formed around the disc film after in contact with microbe colonies. It's indicated that ginger essential oil works better to inhibit the growth of *B. Subtilis*.

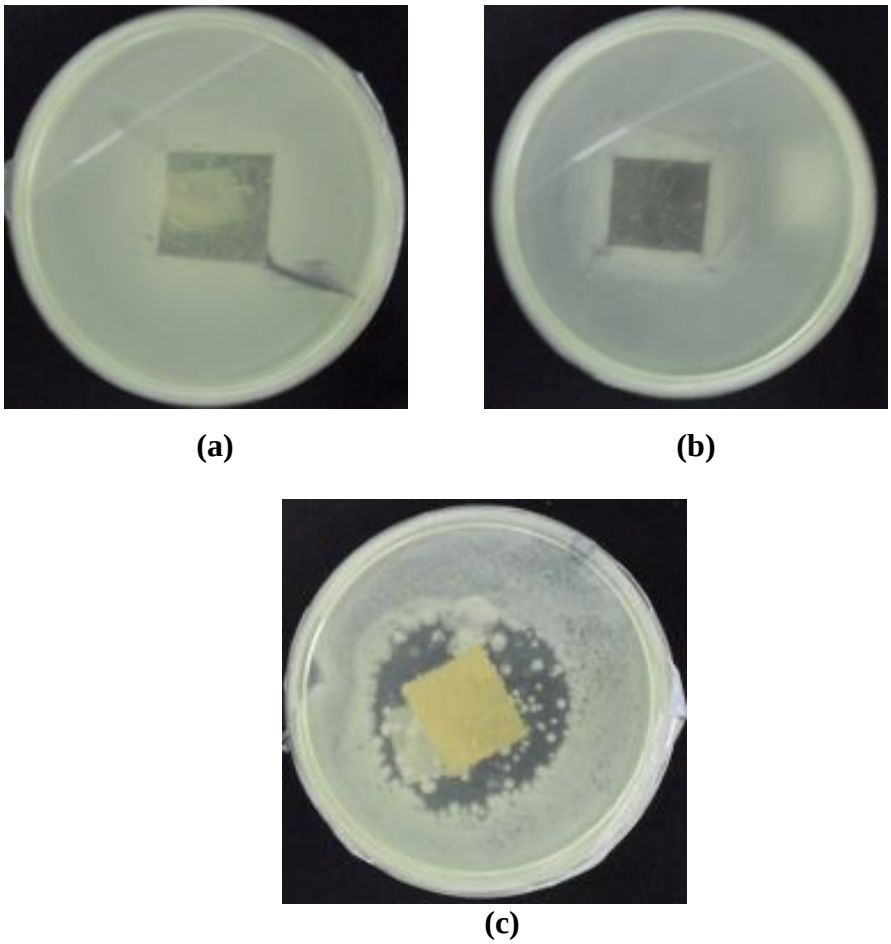


Figure 4.3 Zone Inhibition of (a) Sample A, (b) Sample B, and (c) Sample C against *Escherichia coli*

Figure 4.3 shows the zone inhibition of Sample A, B and C towards *Escherichia coli.* From figure 4.3 (a) is shows that the clear zone only covered on the chitosan biocomposite film. Meaning that the bacteria is inhibit only on the surface of chitosan film, as we know chitosan has antimicrobial properties itself. As well as incorporation of chitosan, besides inhibit *E. coli* and increase the film effect on *B.subtilis* inhibition, it helps to enhance the antimicrobial starch-based film strength (Ban *et. al*., 2005 and

Salleh *et al*., 2007). The result is same for Sample B as it has chitosan itself. Figure 4.3 (c) shows the clear zone formed around the disc film after in contact with microbe colonies. It's indicated that ginger essential oil works better to inhibit the growth of *Escherichia coli*.

Table 4.2: Diameter of Zone Inhibition Assays of Sample A, B and C against *Bacillus subtilis* and *Escherichia coli.*

Sample	Diameter (cm) against *B. subtilis*	Diameter (cm) against *E. coli*
A	2.0	2.0
B	2.0	2.0
C	5.3	4.8

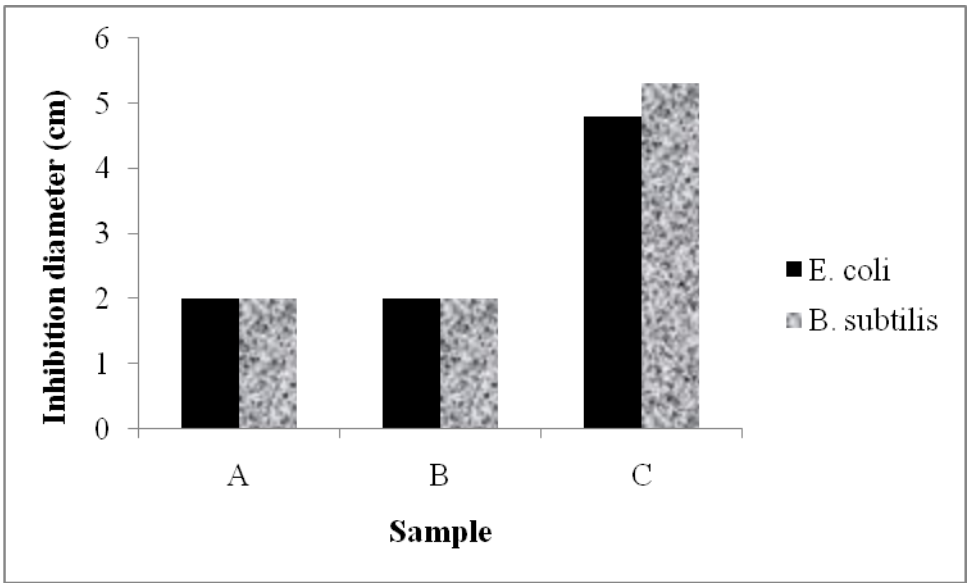
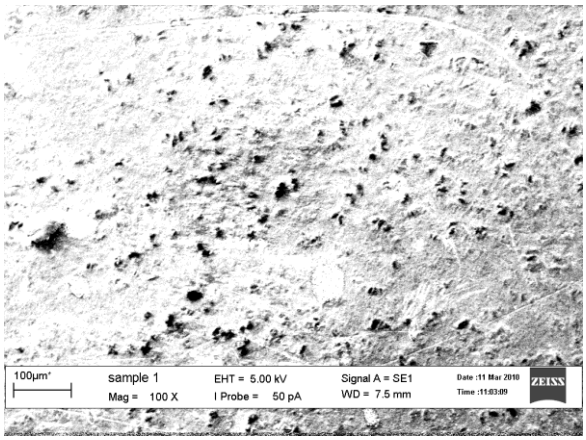


Figure 4.4 Bar chart of Inhibition diameter (cm) vs sample A, B & C against *B. subtilis* and *E.coli*

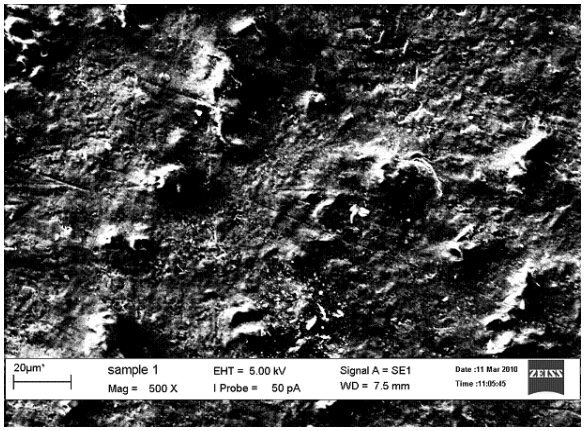
The diameter of clear zone of sample A, B and C against *B. subtilis* and *E.coli* was determined and record in the Table 4.2. Then, the data recorded are transfer to the cone chart as shown above. From Figure 4.4, it can conclude that Sample A and B give the same inhibitory effect towards *B. subtilis* and *E.coli*. Chitosan has the advantage of being able to incorporate functional substances such as minerals or vitamins and possesses antibacterial activity (Chen *et al*., 2002; Jeon *et al*., 2002, Möller *et al*., 2004 and Dutta *et al*., 2009). Sample C shows the clear zone surrounding the film towards both *B. subtilis* and *E.coli*. It's indicated that ginger essential oil works better to inhibit the growth of *B. subtilis* and *E.coli*. However, the largest zone inhibition of Sample C is towards *B. subtilis* as the Gram-positive bacteria. Dutta *et al*. reported that in the Gram-positive bacteria, the major constituent of their cell wall is peptidoglycan and a little amount of protein. The cell wall of Gram-negative bacteria on the other hand is thinner but more complex and contains various polysaccharides, proteins and lipids beside peptidoglycan. Also, the cell wall of Gram negative bacteria has an outer membrane which constitutes the outer surface of the wall (Black, 1996 and Dutta *et al*., 2009).

4.2 Scanning Electron Microscopy (SEM)

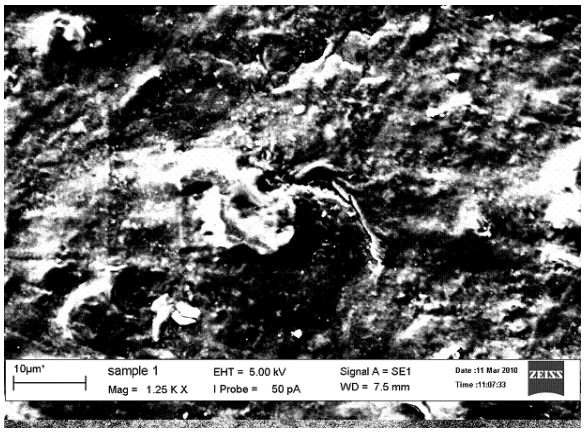
There are two parameter are analyzed in Scanning Electron Microscopy (SEM) which are surface morphology and cross-section of the film at different magnification of 100x, 500x and 1000x. Below are the figure of are surface morphology and cross-section of the film at 100x, 500x and 1000x magnification for Sample A, B and C.



(a)

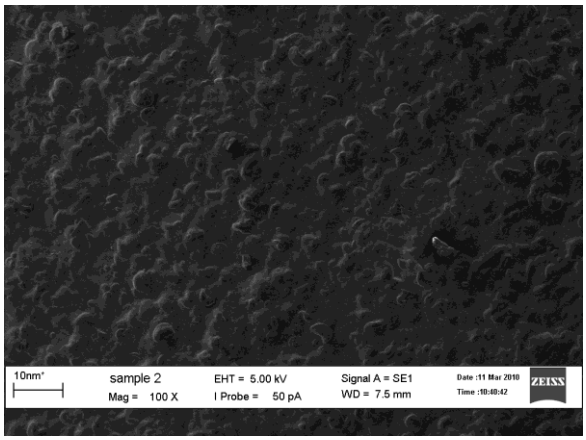


(b)

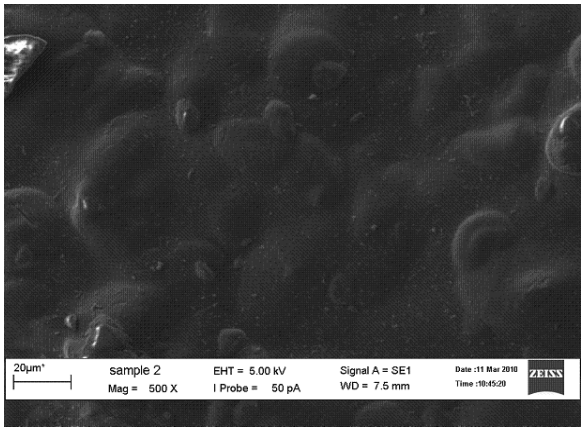


(c)

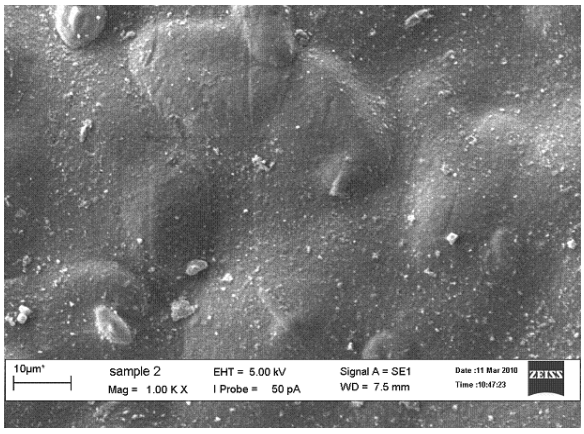
Figure 4.5 Surface of Sample A at (a) 100x magnification, (b) 500x magnification and (c) 1000x magnification



(a)

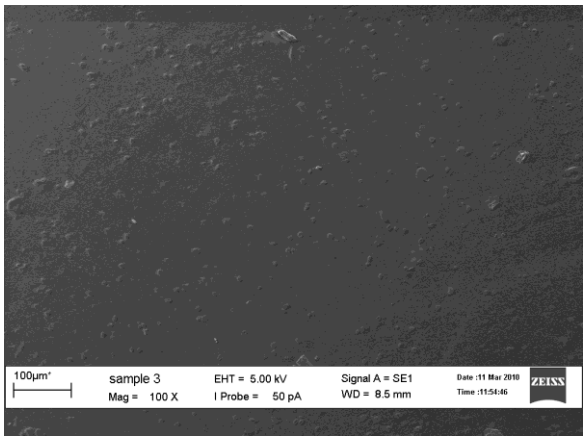


(b)

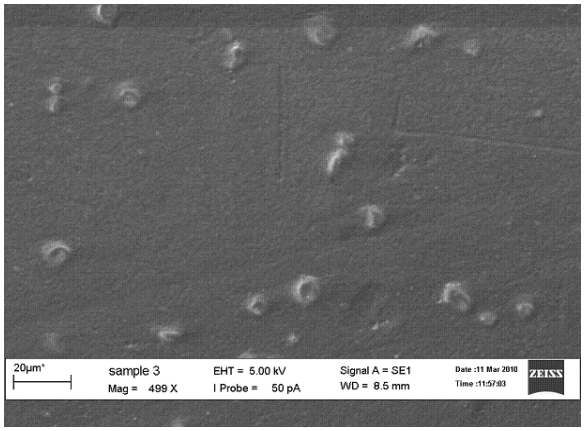


(c)

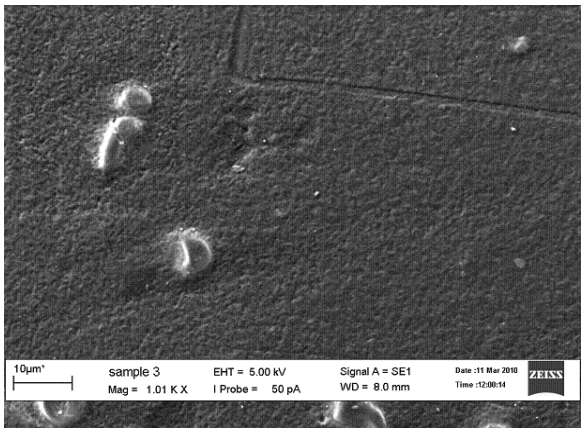
Figure 4.6 Surface of Sample B at (a) 100x magnification, (b) 500x magnification and (c) 1000x magnification



(a)

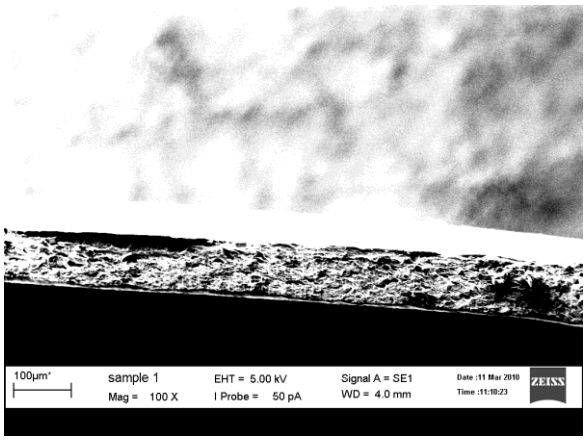


(b)

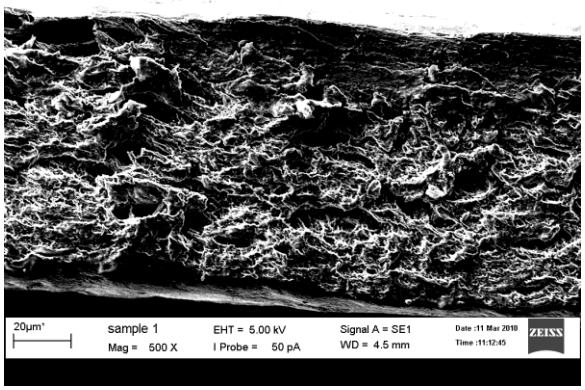


(c)

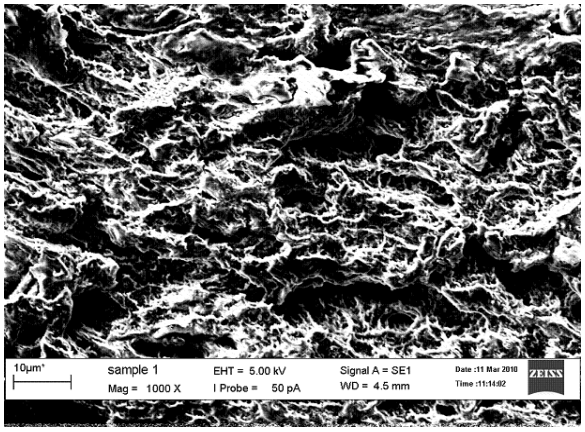
Figure 4.7 Surface of Sample C at (a) 100x magnification, (b) 500x magnification and (c) 1000x magnification



(a)

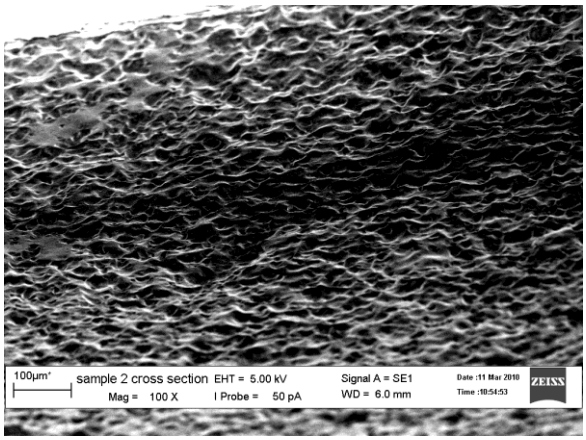


(b)

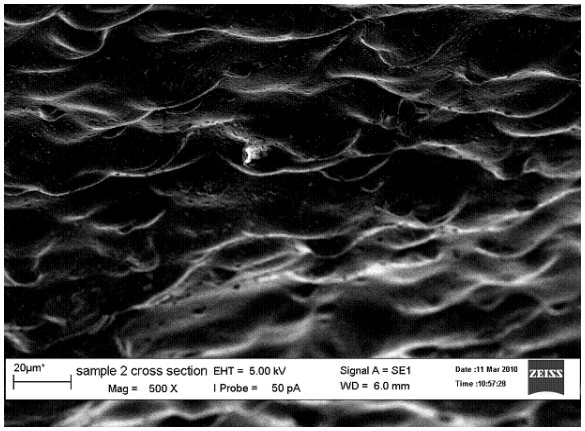


(c)

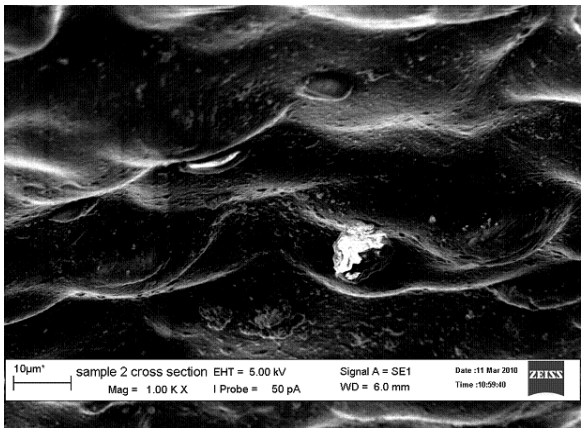
Figure 4.8 Cross-sectional of Sample A at (a) 100x magnification, (b) 500x magnification and (c) 1000x magnification



(a)

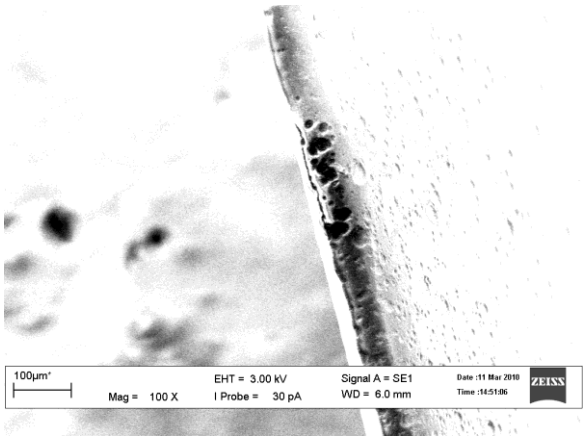


(b)

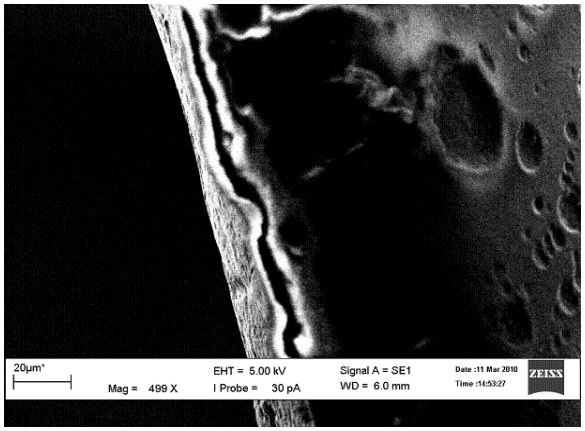


(c)

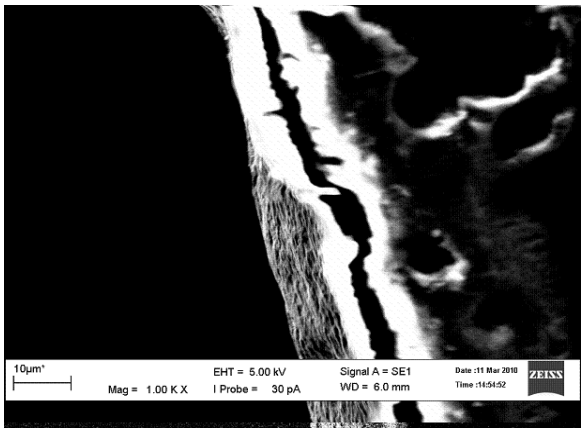
Figure 4.9 Cross-sectional of Sample B at (a) 100x magnification, (b) 500x magnification and (c) 1000x magnification



(a)



(b)



(c)

Figure 4.10 Cross-sectional of Sample C at (a) 100x magnification, (b) 500x magnification and (c) 1000x magnification

Figure 4.5 show the surface of Sample A at (a) 100x magnification, (b) 500x magnification and (c) 1000x magnification. Meanwhile, figure 4.6 show the surface of Sample B at (a) 100x magnification, (b) 500x magnification and (c) 1000x magnification. From the figure 4.5 and 4.6, it shown that Sample A and B have rough surface morphology. This is due to the chemical composition and interaction of molecule. There are residual granular structures and starch is not completely break-up and not completely mix during the blending hour for both solutions. Clearly, Sample A has rougher surface than Sample B. This is because Sample A has more composition of yam starch than Sample B. According to Riley *et al.*, (2008), most of the yam starch granules were of oval to ellipsoid in shape with a few spherical ones. Figure 4.7 show the surface of Sample C at (a) 100x magnification, (b) 500x magnification and (c) 1000x magnification. Among the samples, it shows that Sample C has relatively smooth surface of film morphology. It is homogenous and continuous matrix without any pores. This is shown that there are good and strong interaction between each component. It can say here, the incorporation of ginger essential oil into the film give the make the miscibility and compability of each component increased.

Figure 4.8 shows the cross-sectional of Sample A at (a) 100x magnification, (b) 500x magnification and (c) 1000x magnification. Figure 4.9 shows the cross-sectional of Sample B at (a) 100x magnification, (b) 500x magnification and (c) 1000x magnification. Figure 4.10 shows the cross-sectional of Sample C at (a) 100x magnification, (b) 500x magnification and (c) 1000x magnification. For overall shows all the samples have compact structure of cross-section. It shows the inner interaction between compositions of the samples. But, the most compact is Sample C. This is due to addition of ginger essential oil resulting in the strong hydrogen bonding in the sample. So, Sample C has highest heat resistance.

In conclusion, Sample C is the best sample in term of better surface morphology, compact cross section and higher heat resistant.

4.3 Fourier Transform Infrared Spectroscopy (FTIR)

The function of Fourier Transform Infrared (FTIR) is to determine the functional group presences in the film. Table 4.3 below shows the functional group of chemical substances according to wavenumber (Li *et al.*).

Table 4.3: Functional group according to wavenumber (Li *et al.*, 2008)

Wavenumber (cm^{-1})	Functional group
1030 - 1155	C-O
1550 - 1650	NH_2
600 - 400	Aromatic ring
2500 - 3500	O-H
2880 - 2500	C-H
3300 - 3500	N-H

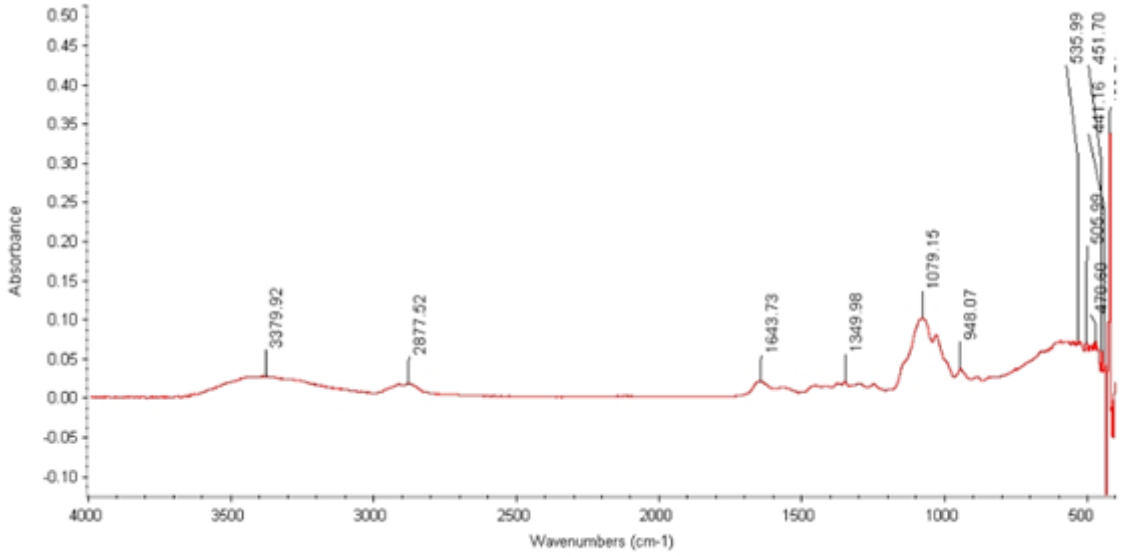


Figure 4.11 Graph of absorbance vs wavenumber (cm^{-1}) for Sample A

Figure 4.11 above shown the FTIR spectrum for Sample A. The peaks at 3379.92 cm^{-1} is indicates the functional group of N-H and O-H stretching of polysaccharide. Next, the peaks at 2877.52 cm^{-1} suggested the presence of C-H functional group in the film. The peaks at 1643.73 cm^{-1} represent the NH_2 functional group of chitosan in the film. The peaks at 1079.15 cm^{-1} suggested the presence of C-O group in the film and 600 to 400 cm^{-1} is the fingerprint region. Fingerprint region is the region consists of absorptions due to all other single bonds (except H-Z), making it often a complex region that is very difficult to analyze. When two components are mixed, the physical blends versus chemical interactions are affected by changes in the characteristic spectra peaks (Guan *et al.,* 1998 and Yin *et al.,* 1999).

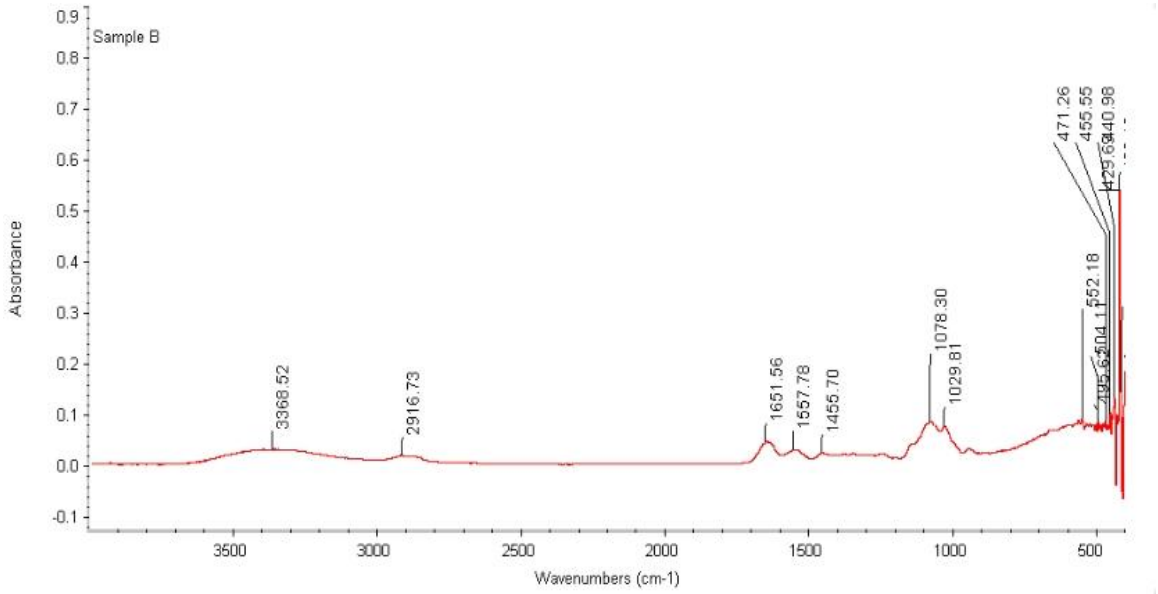


Figure 4.12 Graph of absorbance vs wavenumber (cm^{-1}) for Sample B

Figure 4.12 above shown the FTIR spectrum for Sample B. The peaks at 3368.52 cm^{-1} is indicates the functional group of N-H in gelatin and O-H stretching of polysaccharide. Next, the peaks at 2916.73 cm^{-1} suggested the presence of C-H functional group in the film. The peaks at 1651.56 cm^{-1} and 1557.78 cm^{-1} represent the NH_2 functional group of chitosan in the film. The peaks at 1078.30 cm^{-1} and 1029.61 cm^{-1} suggested the presence of C-O group in the film and 600 to 400 cm^{-1} is the fingerprint region. When two components are mixed, the physical blends versus chemical interactions are affected by changes in the characteristic spectra peaks (Guan *et al.*, 1998 and Yin *et al.*, 1999).

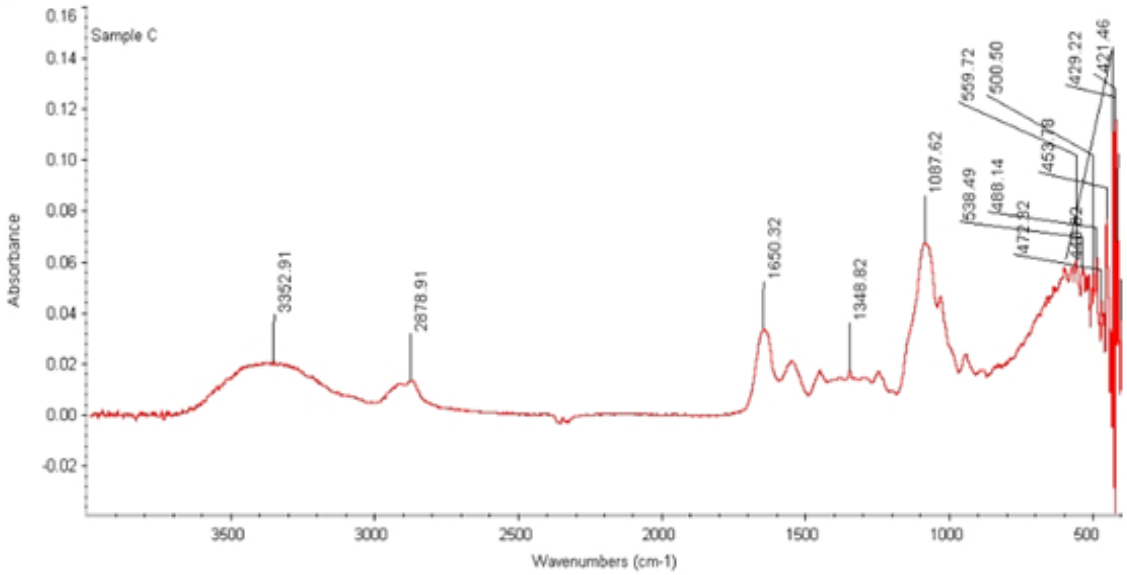


Figure 4.13 Graph of absorbance vs wavenumber (cm^{-1}) for Sample C

Figure 4.13 above shown the FTIR spectrum for Sample C. The peaks at 3352.91 cm^{-1} is indicates the functional group of N-H in gelatin, and O-H stretching of polysaccharides. Next, the peaks at 2878.91 cm^{-1} suggested the presence of C-H functional group in the film. The peaks at 1650.32 cm^{-1} represent the NH_2 functional group of chitosan in the film. The peaks at 1087.62 cm^{-1} suggested the presence of C-O group in the film indicates the ginger essential oil and 600 to 400 cm^{-1} is the presence of aromatic ring in ginger essential oil. When two components are mixed, the physical blends versus chemical interactions are affected by changes in the characteristic spectra peaks (Guan *et al.*, 1998 and Yin *et al.*, 1999).

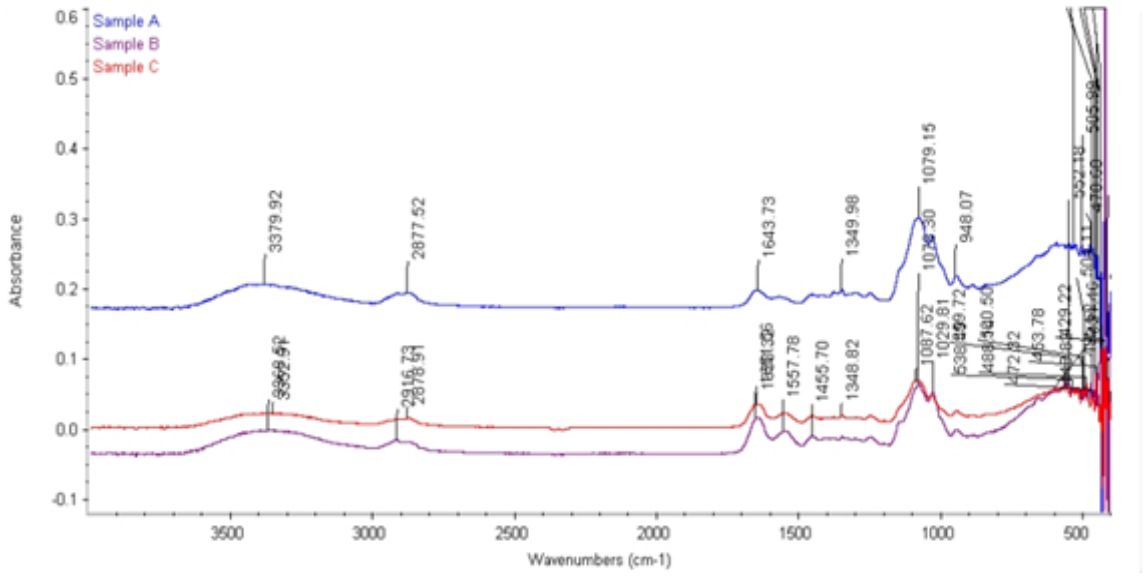


Figure 4.14 Graph of absorbance vs wavenumber (cm^{-1}) for Sample A, B and C

Figure 4.14 below show Fourier Transform Infrared (FTIR) spectrum of Sample A, B and C. For overall, the broadband area shown for all three sample are at the same broadband range represent the functional group of N-H, NH_2, C-O, C-H, O-H and aromatic ring presence in the sample. The broadband range between 600 cm^{-1} to 400 $cm^{-1}$is indicates as the fingerprint region. Fingerprint region is the region consists of absorptions due to all other single bonds (except H-Z), making it often a complex region that is very difficult to analyze.

4.4 Thermo Gravimetric Analysis (TGA)

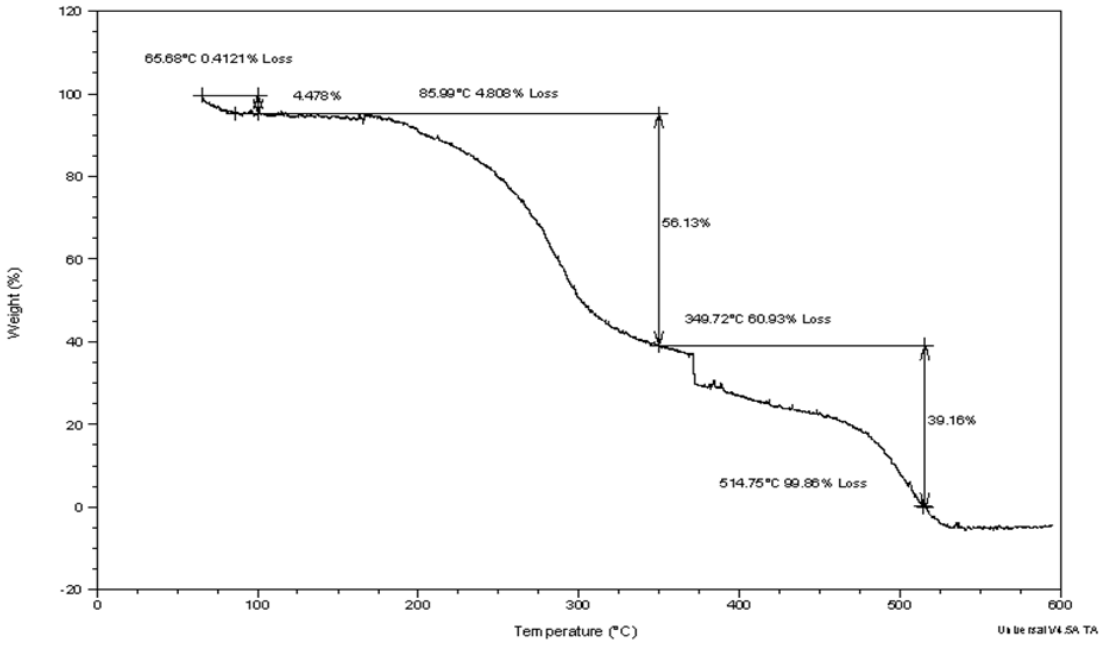


Figure 4.15 Graph of Weight (%) vs Temperature (°C) for Sample A

Figure 4.15 shows the TGA thermogram of Sample A. The Sample A begins weight to be loss of 0.4121 % weight loss at 65.68˚C. There are three stage of weight loss for Sample A. The first stage represent the weight loss of 4.478 % observed up to 100 °C is due to loss of water from chitosan composites (Chan *et al.*, 1989; Yue, Epstein, Zhong, 1991). For the second stage, 56.13% of weight loss due to decomposition of starch and chitosan occur approxiamately 250˚C to 350˚C. At third stage, the weight loss of 39.16% is representing the weight loss of other component in chitosan composite film. After the temperature of 514.75˚C, Sample A will undergo 100% weight loss.

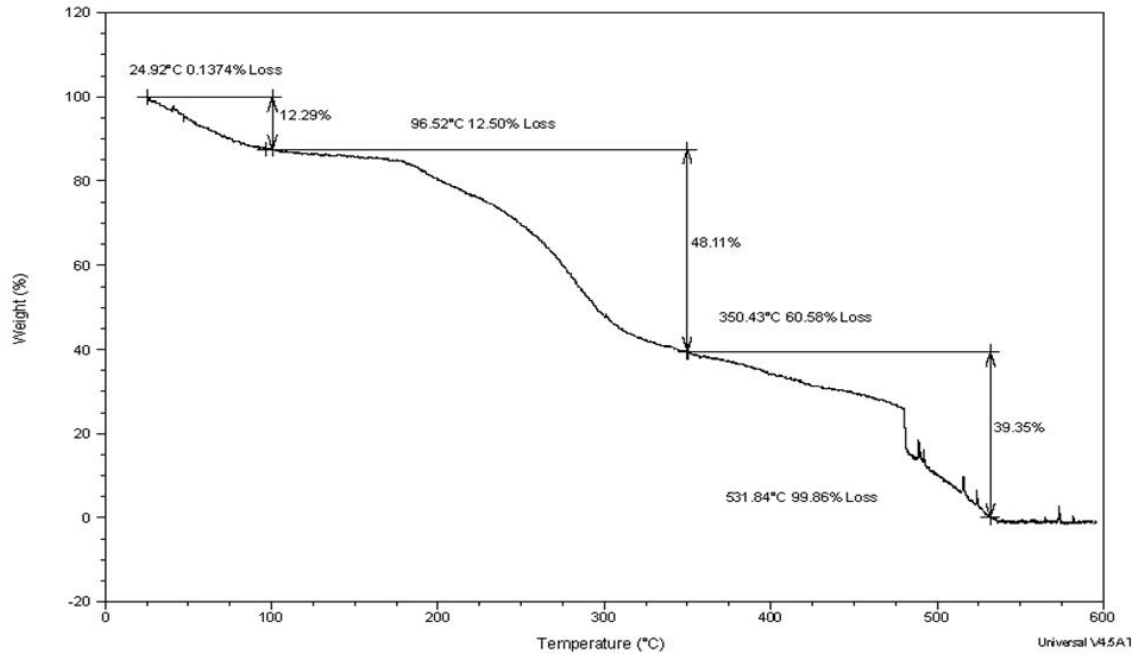


Figure 4.16 Graph of Weight (%) vs Temperature (°C) for Sample B

Figure 4.16 shows the TGA thermogram of Sample B. The Sample B begins weight to be loss of 0.1374% weight loss at 24.92˚C. There are three stage of weight loss for Sample B. The first stage represent the weight loss of 12.29 % observed up to 100 °C is due to loss of water from chitosan composites (Chan *et al.*, 1989; Yue, Epstein, Zhong, 1991). For the second stage, 48.11% of weight loss due to due to decomposition of starch and chitosan occur approxiamately 250˚C to 350˚C. At third stage, the weight loss of 39.35% is representing the weight loss of other component in chitosan composite film. After the temperature of 531.84˚C, Sample B will undergo 100% weight loss.

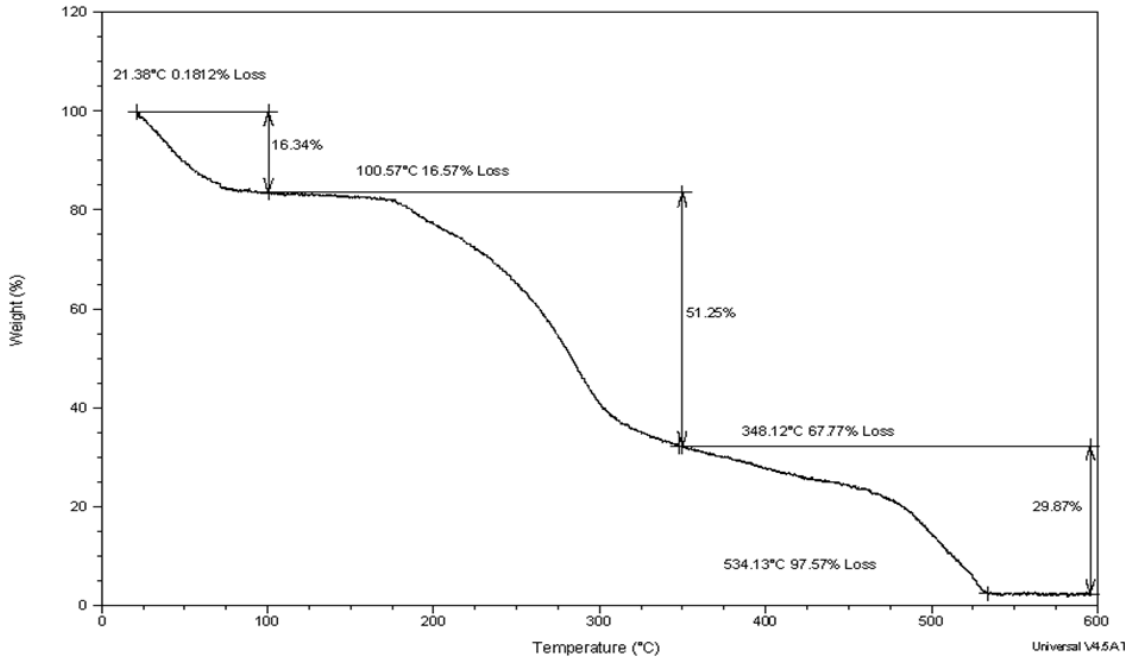
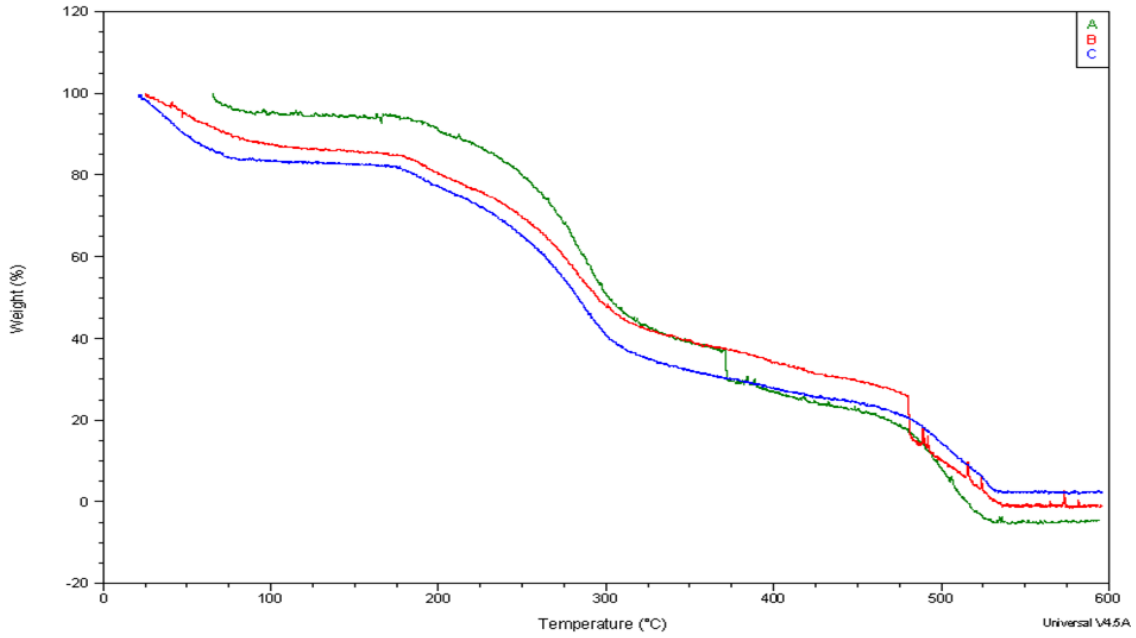


Figure 4.17 Graph of Weight (%) vs Temperature (°C) for Sample C

Figure 4.17 shows the TGA thermogram of Sample C. The Sample C begins weight to be loss of 0.1812% weight loss at 21.38˚C. There are three stage of weight loss for Sample C. The first stage represent the weight loss of 16.34 % observed up to 100 °C is due to loss of water from chitosan composites (Chan *et al.*, 1989; Yue, Epstein, Zhong, 1991). For the second stage, 51.25% of weight loss due to due to decomposition of starch and chitosan occur approxiamately 250˚C to 350˚C. At third stage, the weight loss of 29.87% is representing the weight loss of other component in chitosan composite film. At the temperature of 534.13˚C, Sample C will completely 97.57% weight loss. Sample C cannot 100% weight loss because the group of aromatic ring presents in ginger essential oil. The aromatic structures present in the ginger essential oil are highly stable due to the resonance of the benzene ring, which results in the decomposition of these compounds at higher temperatures. It is also observed that an increase in percent residue after the incorporation of the ginger essential oil (Pelissari, 2009).



(d)

Figure 4.18 Graph of Weight (%) vs Temperature (°C) for Sample A, B and C

Figure 4.18 shows the combine TGA thermogram of Sample A, B and C. the first stage before 100˚C is the evaporation of water absorbed by starch, chitosan, and PEG along with the evaporation of low molecular weight compounds. Second stage is weight loss due to decomposition of polysaccharides occur approximately 250˚C to 350˚C. Third stage is the weight loss due to others component occurs at 350˚C to 500˚C.From the figure, it shows the 100% weight loss of Sample A at 515˚C, Sample B at 526˚C and Sample C at 533˚C. So, Sample C has the most highest of heat resistant among the three samples. It concludes here, Sample C is the best sample compare to the Sample A and Sample B.

4.5 Differential Scanning Calorimeter (DSC)

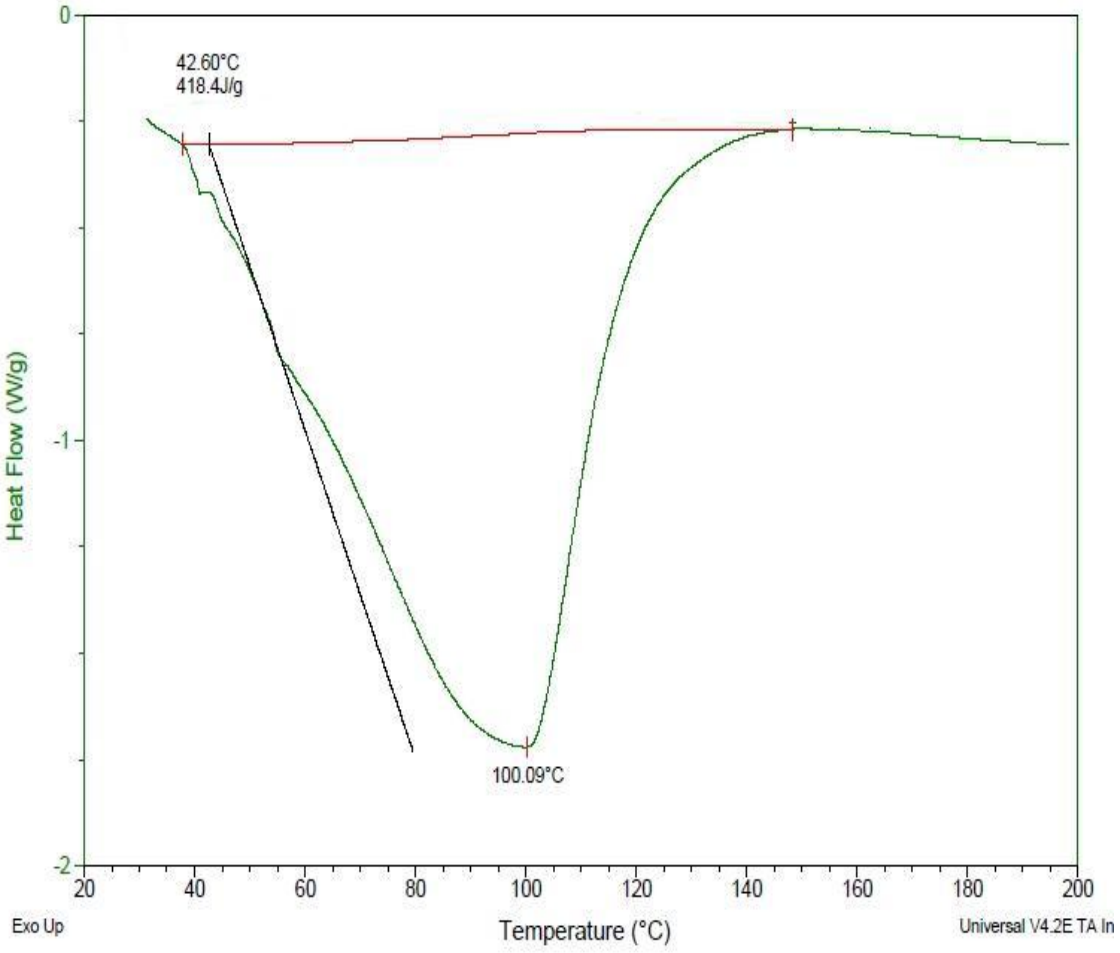


Figure 4.19 Graph of Heat Flow (W/g) vs Temperature (°C) for Sample A

From the DSC thermogram of Sample A, which is shown in Figure 4.19, the melting point of Sample A is 42.60˚C. Meanwhile, at 100.09˚C, Sample A will completely melt.

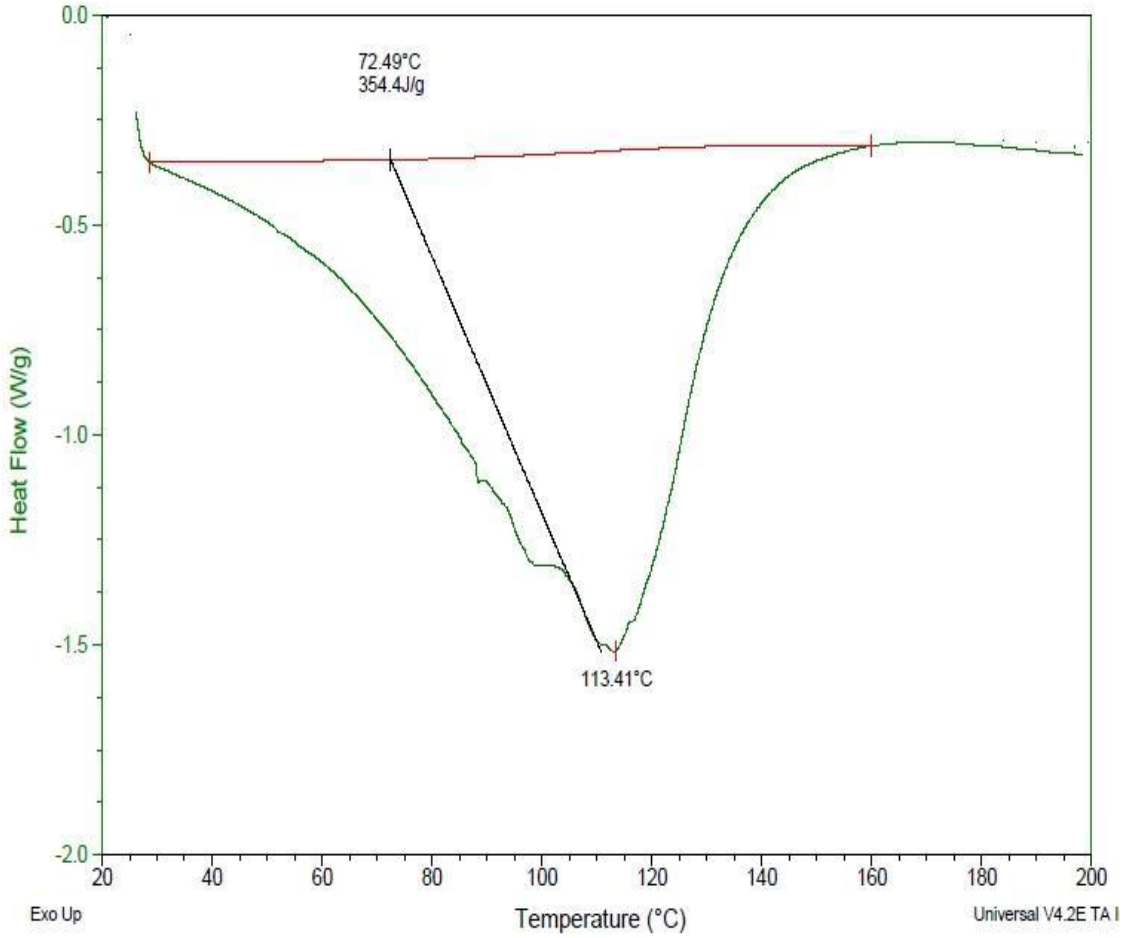


Figure 4.20 Graph of Heat Flow (W/g) vs Temperature (°C) for Sample B

From the DSC thermogram of Sample B, which is shown in Figure 4.20, the melting point of Sample B is 72.49˚C. Meanwhile, at 113.41˚C, Sample B will completely melt.

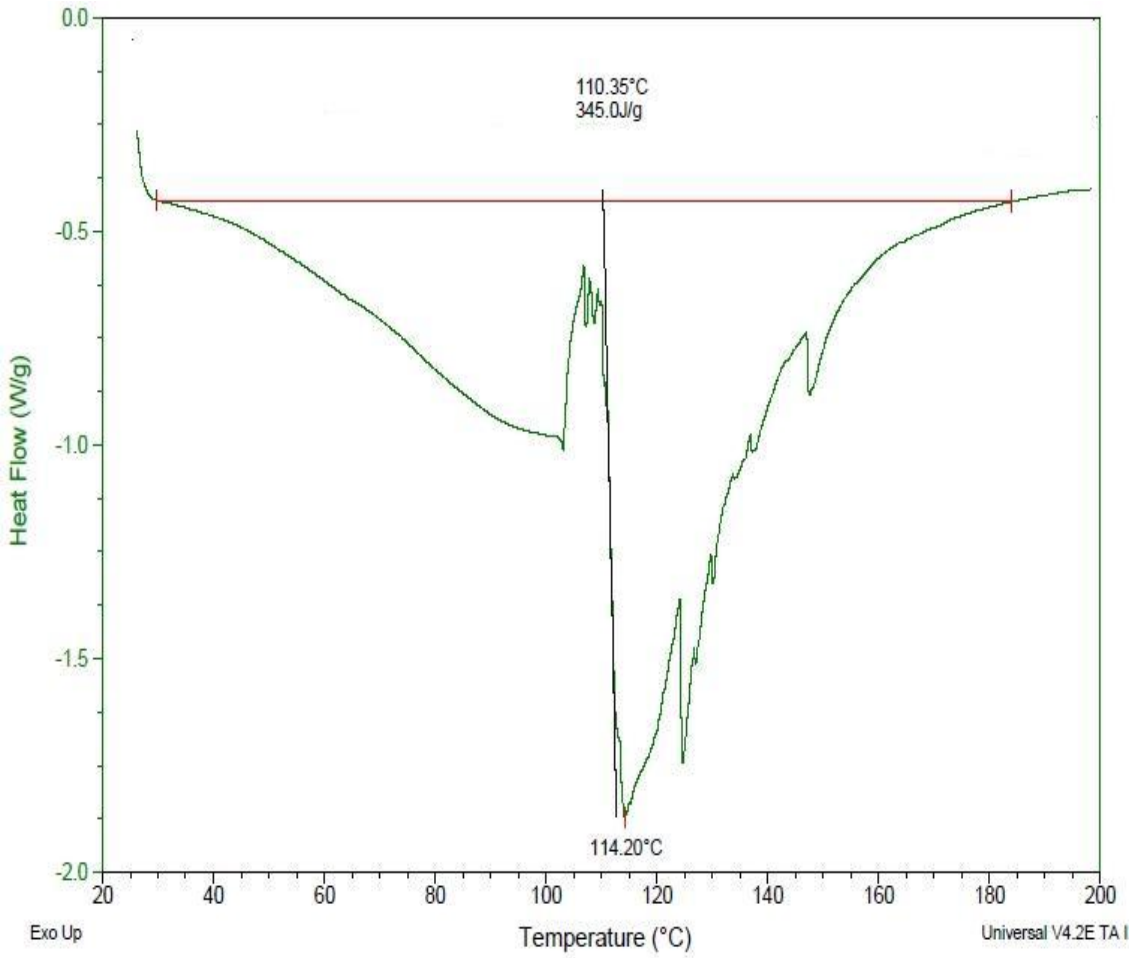


Figure 4.21 Graph of Heat Flow (W/g) vs Temperature (°C) for Sample C

From the DSC thermogram of Sample C, which is shown in Figure 4.21, the melting point of Sample C is 110.35˚C. Meanwhile, at 114.20˚C, Sample C will completely melt.

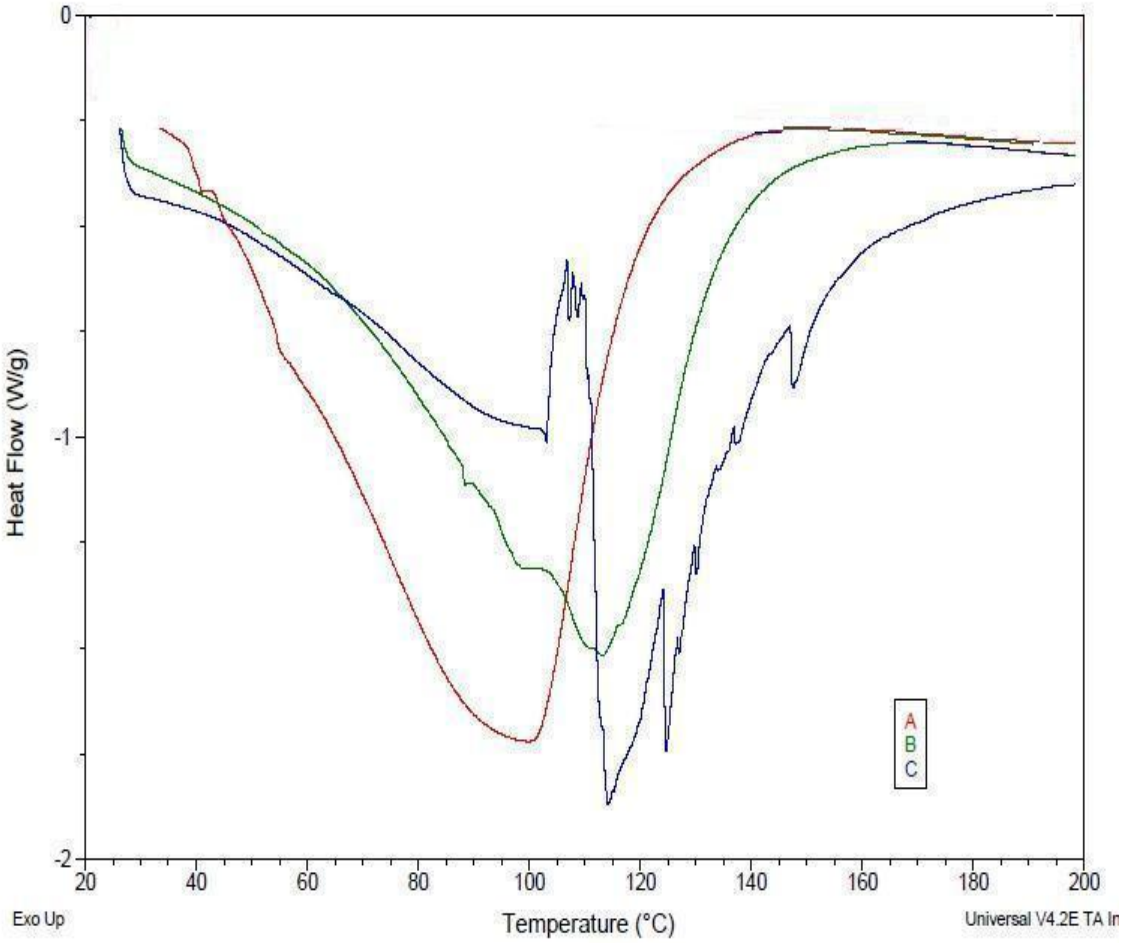


Figure 4.22 Graph of Heat Flow (W/g) vs Temperature (°C) for Sample A, B and C

From the DSC thermogram above is shown that Sample C has the highest melting point (110.35˚C) than Sample A and Sample B. It is because the presence of ginger essential oil in Sample C. So, it can conclude here, Sample C has highest heat resistant among the three samples. Thus, Sample C is the best sample for DSC analysis.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

In this undergraduate research project, the experimental study of chitosan composite biodegradable film was successfully fabricated. Several analyses had been tested on the film to characterize their properties in term of thermal stability, chemical properties, physical properties, mechanical properties and antimicrobial properties. Therefore, their usability of biocomposite film application's can be determined. Sample C is the best sample among three samples in term of highest heat resistant, highest melting point, highly antimicrobial effectiveness toward *Bacillus subtilis* and *Escherichia coli*, smooth film surface morphology and has highest compact cross section. For overall, both research objectives have been achieved successfully. As a conclusion, antimicrobial properties is important in food packaging has in order to reduce the risk of pathogen microorganism contamination and at the same time it can extend the food shelf life.

5.2 Recommendation

It is recommended that this research will be extends to the further study for a new antimicrobial agents with wide spectrum of activity. It also that this research

will be further on the development of starch-based film from new alternatives starch sources such as breadfruit starch. I also suggested that this research will be extends to a new application of film making such as wound dressing.

It should use the good raw materials which are non-toxicity, plant resources, economical and availability. Thus, the product is safe to the consumer, less cost and it can sustain the environmental problems.

LIST OF REFERENCE

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APPENDICES A

MATERIALS AND METHODOLOGY

Figure 1: Yam flour

Figure 2: Ginger essential oil

Figure 3: Chitosan was weighting

Figure 4: Preparation of 1% (v/v) of acetic acid

Figure 5: Heat gelatinized of yam starch

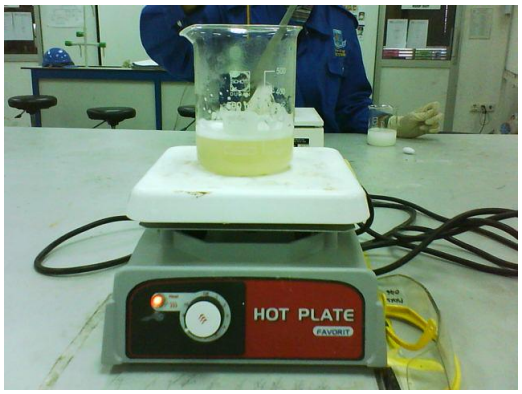

Figure 6: Chitosan dissolves in 1% (v/v) of acetic acid

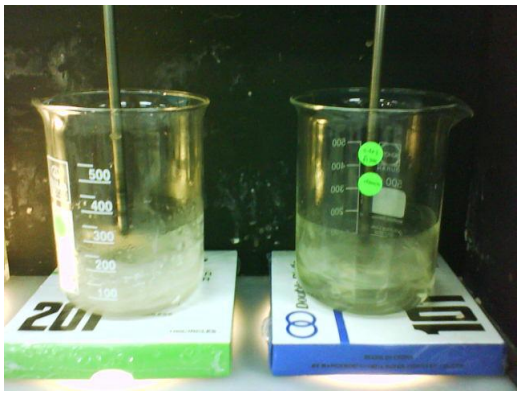

Figure 7: Stir the mixture about 10 hrs and 300 rpm

Figure 8: Dried film at ambient room temperature

Figure 9: Peeled the film from glass plate

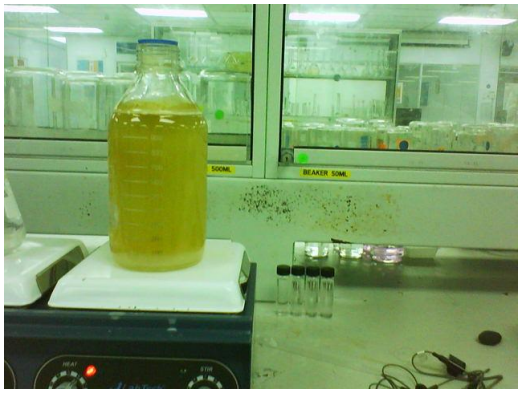

Figure 10: Broth medium preparation

Figure 11: Liquid culture medium preparation

Figure 12: Agar medium preparation

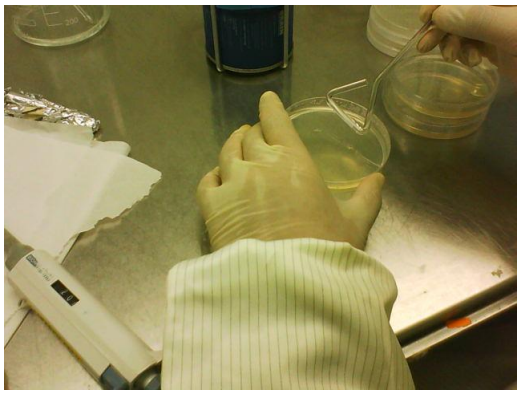

Figure 13: Spread method

Figure 14: Streaking method

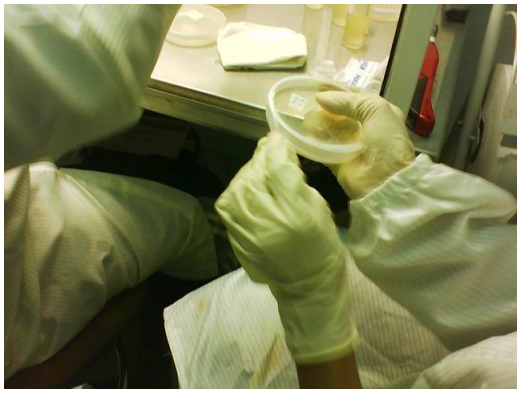

Figure 15: Agar Plate Test

Figure 16: Liquid Culture Test

Figure 17: Sample reading via UV-VIS spectrophotometer

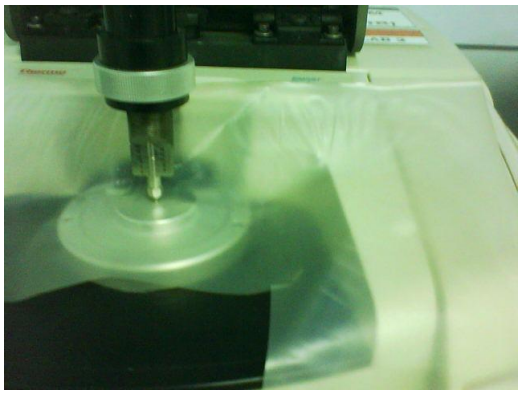

Figure 18: Sample lawns on FTIR for analyze

Figure 19: TGA Q500 series Thermo gravimetric analyzer (TA Instruments)

Figure 20: DSC Q1000 series (TA Instrument)