

CHARACTERIZATION AND ANTIMICROBIAL ANALYSIS OF  
CHITOSAN COMPOSITE BIODEGRADABLE FILMS WITH ADDITION  
OF CLOVE ESSENTIAL OILS

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COMPOSITE BIODEGRADABLE FILMS WITH ADDITION OF CLOVE  
ESSENTIAL OILS

**SESI PENGAJIAN** : 2009/2010

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Date : 30 April 2010

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COMPOSITE BIODEGRADABLE FILMS WITH ADDITION OF CLOVE  
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**NUR LAILI BINTI MUHAMMAD LOKMAN**

**A thesis submitted in fulfillment  
of the requirements for the award of the degree of  
Bachelor of Chemical Engineering (Biotechnology)**

**Faculty of Chemical & Natural Resources Engineering  
Universiti Malaysia Pahang**

**APRIL 2010**

## DECLARATION

“I declare that this thesis entitled *Characterization and Antimicrobial Analysis of Chitosan Composite Biodegradable Films with Addition of Clove Essential Oils* is the result of my own research except as cited in the 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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Date: 30 April 2010

To my beloved mother and father

## **ACKNOWLEDGEMENT**

In the Name of Allah, the Most Gracious, the Most Merciful

First and foremost, thanks to God Almighty for the guidance and aid in giving me the strength to complete this project research. I would also like to take this opportunity to express my utmost gratitude to my supervisor, Madam Norashikin Binti Mat Zain for her valuable guidance, inspiration, patience and support throughout this thesis study.

Appreciation is also to Mr. Mohd Zaki Bin Sahad, the lab owner of Basic Engineering Lab, for his kindness in supporting this study. I would like to express my sincere appreciation to technicians in Chemical Engineering Laboratory University Malaysia Pahang (UMP) for their help during the various laboratory tasks. A word of thanks also goes to all personnel and UMP staff due to their full support in my research experiments especially to my friends Mohd Fouzzi, Maa Ajmala Isyatu Illani, Nur 'Adilah and Nabilah who accompanied me throughout this research.

I would like to express my deepest appreciation to my father, Muhammad Lokman Bin Razali and mother Mardiana Binti Shamsudin for their long continuous encouragement and support throughout the research done. Finally, special thanks to my beloved friend Muhammad Khairul Hamizan for his support.

## ABSTRACT

Synthetic films materials mostly used to preserve foodstuffs. Recently, attention has turned to biodegradable films because of environmental reasons. The development of new biodegradable packaging material films, for example, chitosan film, is needed to find alternatives to petroleum-based plastics. Chitosan has antimicrobial activities against different groups of microorganisms, such as bacteria, fungus, and yeast. In this project, gelatin is used as the biopolymers in producing the biodegradable film. Gelatin is an attractive alternative to synthetic plastic materials made from non-renewable resources that can be a hazard to the environment. Since gelatin and chitosan are hydrophilic biopolymers with good affinity and compatibility, they are expected to form composite films with good properties. The film preparation process consist by hydrating gelatin powder with distilled water for 4 hour and then the samples were heated at 60°C before mixed with chitosan based solution. Then, the solution was poured on a glass plate and left it dried. Lastly, the film was peeled off the glass plate after it completely dried. This work also aimed on the antimicrobial analysis of films against *E. coli* and *B. subtilis*. Method used in this analysis is zone inhibition assay where the inhibition zone diameter is measured and also liquid culture test where the OD reading was measured using spectrophotometer. Based on the study, the clove essential oil has its own antimicrobial activity onto the films. Thus, from the results, it can be concluded that the biodegradable film has smoother surface, higher thermal stability and slightly higher melting point which obtain from SEM, FTIR, TGA and DSC. Gelatin has great potential for being an additive for the composite film and the amount of plasticizer added in the composite film helps to enhance the properties of the film.



## ABSTRAK

Filem yang digunakan sebagai bahan pembungkus makanan kebanyakannya diperbuat daripada plastik sintetik. Walaubagaimanapun, disebabkan isu alam sekitar plastik sintetik telah diperbaharui kepada filem biodegradasi. Filem komposit boleh biodegradasi diperbuat daripada chitosan adalah sebagai alternatif. Tambahan pula, chitosan mempunyai antimikroorganisma aktiviti keatas pelbagai kumpulan mikroorganisma sebagai contoh bakteria, kulat dan yis. Di dalam kajian ini, gelatin digunakan sebagai bahan biopolimer di dalam penghasilan filem biodegradasi ini. Gelatin merupakan alternatif yang menarik memandangkan sumber gelatin adalah dari bahan yang tidak membahayakan alam sekitar. Memandangkan gelatin dan chitosan merupakan hidrofilik biopolimer, maka filem komposit ini dijangkakan mempunyai sifat-sifat yang bagus. Penyediaan filem ini merangkumi hidrolisis serbuk gelatin beserta air suling sehingga 4 jam pada suhu sekitar 60°C sebelum ia disebatikan dengan larutan berasaskan chitosan. Larutan yang telah sebatu dituang pada kepingan kaca dan dibiarkan pada suhu persekitaran sehingga membentuk filem. Akhir sekali filem dikupas dari kepingan kaca selepas ia telah kering dengan sepenuhnya. Kajian ini juga fokus ke atas antimikroorganisma analisis oleh filem komposit terhadap *E. coli* dan *B. subtilis*. Kaedah yang digunakan di dalam analisis ini adalah kaedah kawasan sisihan oleh bakteria di mana diameter sisihan diukur. Kaedah kedua adalah ujian kultur cecair dimana bacaan OD diukur menggunakan UV-Vis. Berdasarkan keatas kajian tersebut, boleh disimpulkan bahawa filem boleh biodegradasi ini mempunyai permukaan yang licin, kestabilan haba dan takat pelakuran yang tinggi yang diperolehi daripada SEM, FTIR, TGA and DSC. Ini menunjukkan gelatin mempunyai potensi sebagai ejen penambah terhadap filem komposit dan kuantiti ejen plastik juga mampu meningkatkan sifat filem.

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## LIST OF ABBREVIATIONS

PVC	Polyvinyl chloride
PEO	Poly ethylene oxide
PEG	Poly ethylene glycol
SEM	Scanning electron microscopy
FTIR	Fourier transform infrared
TGA	Thermo gravimetric analyzer
DSC	Differential scanning calorimeter
Mr	Molecular weight
% v/v	volume percentage for chemical per basis
%w/w	weight percentage for chemical per basis
<i>E. coli</i>	Escherichia Coli
<i>B. subtilis</i>	Bacillus subtilis



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## **CHAPTER 1**

### **INTRODUCTION**

#### **1.1 General Background**

Plastic is the general common term for a wide range of synthetic that suitable for the manufacture of industrial products. Plastics are durable and degrade very slowly because the molecular bonds that make plastic so durable make it equally resistant to natural processes of degradation. Since the 1950s, one billion tons of plastic has been discarded and may persist for hundreds or even thousands of years. In some cases, burning plastic can release toxic fumes. Burning the plastic polyvinyl chloride (PVC) may create dioxin. The biggest threat to the conventional plastics industry is most likely to be environmental concerns, including the release of toxic pollutants, greenhouse gas, litter, biodegradable and non-biodegradable landfill impact as a result of the production and disposal of petroleum and petroleum-based plastics (Chen *et al*, 2007).

Nevertheless, for environmental reasons, attention has lately been turned towards biodegradable and edible films to preserve foodstuffs. The materials used to make films are ordinarily waste products from food processing, thereby enhancing processing sustainability. The biodegradable, edible nature of these films meant that they can be employed in both food and agricultural applications (Perez *et al*, 2007). The interest in biodegradable edible films in recent decades can be explained by consumer demand for high quality foods, health factors and environmental concerns over the disposal of non-renewable food packaging materials, and

opportunities for creating new market outlets for film e forming ingredients derived from agricultural products (Soares *et al*, 2005). So, biodegradable plastic is better alternatives to petroleum-based plastics. Biodegradable plastics that are break down with exposure to sunlight, water or dampness, bacteria, enzymes or environmental degradation. Starch powder has been mixed with plastic as a filler to allow it to degrade more easily.

In this project the biodegradable film is made from Chitosan and Gelatin. Chitosan is a valuable component of natural packaging films. It is generally obtained from natural chitin after its N-deacetylation by an alkaline treatment. Chitosan is a biodegradable and non-toxic polymer. (Ilona and Barbara, 2006). Chitosan is readily soluble in various acidic solutions such as formic and acetic acids (Chen *et al*, 2007). Chitosan has been found to be nontoxic, biodegradable, biofunctional, biocompatible in addition to having antimicrobial characteristics. In view of these qualities, chitosan films have been used as packaging material for the quality preservation of variety of food (Dutta *et al*, 2008).

Chitosans are described in terms of the degree of deacetylation and average molecular weight and their importance resides in their antimicrobial properties in conjunction with their cationicity and their film-forming properties. Film-making conditions, including solvent pH, ionic strength, type of solvent (acid) used and annealing treatment, are parameters often manipulated to alter the mechanical properties and membrane porosity. Ionic strength or pH can be manipulated in order to reduce inter- and intramolecular electrostatic repulsion between chitosan chains, thus allowing the chains to approach each other and enhance the inter- and intramolecular hydrogen bonding. Chitosan has been extensively used over a wide range of applications, such as a biomaterial in medicine either on its own or as a blend component, a membrane filter for water treatment, a biodegradable, edible coating or film in food packaging a dietary fiber, and a medicine against hypertension because of its scavenging action for chloride ions (Arvanitoyannis *et al*, 1998)

However, in recent years carbohydrates and proteins have been extensively tested to develop biodegradable films having more and more versatile properties. Protein based films offer better mechanical and barrier properties due both to the specific structure of the proteins and the ability of proteins to form stronger intermolecular covalent bonds than carbohydrates (Mateos *et al*, 2007).

In this project, gelatin is used as the biopolymers in producing the biodegradable film. Gelatin is an attractive alternative to synthetic plastic materials made from non-renewable resources that can be a hazard to the environment. Gelatin obtained by partial degradation of collagen has gained more attention as edible films for its abundance and biodegradability. Gelatin has relatively low cost and excellent functional and filmogenic properties. Whereas biopolymers, such as proteins and polysaccharides, provide the supporting matrix, lipids provide a good barrier to water vapor. Since gelatin and chitosan are hydrophilic biopolymers with good affinity and compatibility, they are expected to form composite films with good properties (Rivero *et al*, 2008).

Like the collagen, the gelatin chains are macromolecules with a tendency mainly to interchain, rather than intrachain and hydrogen bonding. Gelatin forms a three-dimensional network with zones of intermolecular microcrystalline junctions and the dehydration of this system may produce brittle films. Thus, plasticizers must be added to reduce interchain interactions improving film flexibility (Vanin *et al*, 2005).

Furthermore, in this project there is combination of clove essential oils in producing the biodegradable film. One specific application is to incorporate essential oil into the packaging as antimicrobial agent to prevent the growth of microorganism. The antimicrobial and antioxidant properties of essential oils have been known for a long time, and a number of investigations have been conducted into their antimicrobial activities using various bacteria, viruses and fungi (Dunan *et al*, 2006). Clove oil is a natural preservative and flavoring substances that are not harmful when consumed in food products (Matan *et al*, 2005). Clove oils have biological activities, such as antibacterial, antifungal, insecticidal and antioxidant properties, and are used traditionally as flavoring agent and antimicrobial material in food.

The high levels of eugenol contained in clove essential oil give it strong biological activity and antimicrobial activity (Wenqiang, 2006).

## **1.2 Problem Statement**

The most common materials used for packaging are paper, fiberboard, plastic, glass, steel, and aluminum. However, they pose a serious global environmental problem by generating large volumes of non-biodegradable waste. Moreover, in addition to safety and environmental issues, recycling of plastics is complicated for technical and economic reasons. Thus, new biodegradable films made from edible biopolymers from renewable sources could become an important factor in reducing the environmental impact of plastic waste. Proteins, lipids, and polysaccharides are the main biopolymers employed to make edible films and coatings (Guille'n *et al*, 2009).

In this project, chitosan is one of the raw materials. From the previous work, it reported that films made from chitosan lack of water resistance and has poor mechanical properties. Forming biodegradable film from chitosan with other biopolymers is an alternative. So, gelatin is added into chitosan to help the film has good mechanical properties and high tensile strength since gelatin and chitosan are hydrophilic biopolymers with good affinity and compatibility).

P.K.Dutta *et al*, 2008 reported that chitosan has received a significant attention as antimicrobial film-forming agent for food preservation to the researchers due to its biodegradability and antimicrobial activity. Clove essential oil is added in enhancing the antimicrobial analysis of the biodegradable film. The high levels of eugenol contained in clove essential oil inhibit production of an essential enzyme by the bacteria or cause damage to the cell wall of bacteria (Guan *et al*, 2006).

### 1.3 Objective

The objectives of this study are listed as following;

- a. To fabricate composite biodegradable film from chitosan and gelatin.
- b. To characterize composite biodegradable film in terms of antimicrobial analysis, morphology and others.

### 1.4 Scope of Study

The scopes of this study are listed as following;

- a. Fabrication of composite biodegradable film from gelatin with chitosan and PEG 400 as additives.
- b. The characterization of the composite biodegradable film using various analysis method:-
  - i. Fourier Transform Infrared (FTIR) Spectroscopy
  - ii. Differential Scanning Calorimetry (DSC)
  - iii. Thermogravimetric Analysis (TGA)
  - iv. Scanning Electron Microscopy (SEM)
- c. The antimicrobial analysis of composite biodegradable film against *E. coli* and *B. subtilis* using these method;
  - i. Zone Inhibition Assay
  - ii. Liquid Culture Test

## **CHAPTER 2**

### **LITERATURE REVIEW**

#### **2.1 Biodegradable Film**

The use of plastic for packaging has grown extensively in recent years and the use of biodegradable films might be effective in environmental protection. Edible, biodegradable films and coatings, by acting as barriers to control the transfer of moisture, oxygen, carbon dioxide, lipids, and flavor components, can prevent quality deterioration and increase the shelf life of food products . In addition, edible films or coatings may provide mechanical integrity and improve the handling characteristics of the food. They can be effective carriers of many functional ingredients, such as antimicrobial agents to improve safety and stability of foods, antioxidants to prevent lipid oxidation, and flavorings and pigments to improve quality of foods. Materials that can be used for film making include polysaccharides, proteins, lipids and polyesters or combinations of them (Babak and Oromiehi , 2008).

In recent years, development of biodegradable packaging materials from renewable natural resources has received widespread government support in EU countries and many national or international organizations have been established to facilitate the development in this area. The UK Government-Industry Forum has strongly recommended greater use of nonfood crops, particularly for biodegradable packaging applications. The objectives in the development of biodegradable packaging are two-fold: to utilize renewable and potentially

more sustainable sources of raw materials (crops instead of crude oil) and to facilitate integrated waste management approaches so as to reduce landfill. To date, significant technological development has been achieved to produce biodegradable materials for packaging applications with comparable functionalities to those of traditional oil-based plastic packaging (Davis and Song, 2006).

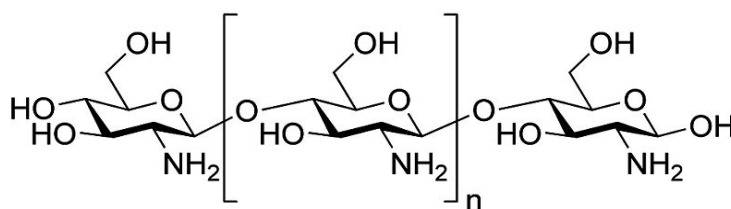
Biodegradable film is the biopolymer films. It is environmentally friendly and could be degraded by microorganisms without further assistance. The films may contain of polysaccharides, protein, lipids and etc. the films easy to degrade due to it's polymer that built in it is made of organic materials. The biodegradable may consist of one or two organic materials. If the films consist of two organic materials that can be easily biodegrade, it called as biocomposite degradable films. Films based on biopolymers are generally sensitive to the relative humidity of the air since they are normally hygroscopic and have limited mechanical resistance compared with synthetic films. Nevertheless protein-based films display high deformability. A possible solution to improve the mechanical characteristics of protein-based films could be the mixing of these biopolymers with synthetic polymers (Tharanathan, 2003).

Except for plastics related to petrochemical polymers, there are also biodegradable plastics like polylactic acid, but even those could be replaced by polysaccharides due to lower costs and better properties of final composite. Mixing of polysaccharide based materials with plastics means mixing hydrophilic and hydrophobic materials, which requires energy. The goal to replace only a part of the higher-cost plastics with lower-cost polysaccharides is not the best strategy (Rowell *et al.*, 2007).



## 2.2 Chitosan (Raw Material)

Chitosan, a linear  $\beta$ -1,4-D-glucosamine, is a biocompatible, nontoxic compound mainly obtained by deacetylation of chitin, a natural structural component present for instance in crustaceans such as crabs shells. Several works exist in the literature that demonstrate the inherent biocide properties of this natural carbohydrate polymer against a wide range of microorganisms such as filamentous fungi, yeast and bacteria (Coma *et al.*, 2003 and Moller *et al.*, 2004).



**Figure 2.1:** Chemical structure of chitosan

Chitosan is a natural polymer, nontoxic, edible, and biodegradable, derived by deacetylation of chitin, which is the second most abundant biopolymer in nature after cellulose. In general, chitin presents in the exoskeleton of arthropods such as insects, crabs, shrimps, lobsters, and certain fungal cell walls. It has a potential as a packaging polymer and, more particularly, as an edible packaging or coating because of its ability to form a film without any use of additives. Moreover, chitosan film has good oxygen and carbon dioxide permeability, which is lower than that of polyethylene film, and good mechanical properties, which are comparable with those of many medium-strength commercial polymers. Furthermore, chitosan has antimicrobial activities against different groups of microorganisms, such as bacteria, fungus, and yeast. Therefore, chitosan has been used in edible coatings or films to extend shelf life of foodstuffs; for example, fruits, meat, and fish and seafood. These studies justify that chitosan might be used as antimicrobial packaging, which is a promising form of active food packaging. Furthermore, the development of new biodegradable

packaging material films, for example, chitosan film, is needed to find alternatives to petroleum-based plastics because of environmental concerns (Nugraha *et al*, 2004).

The previous studies have highlighted that several characteristics such as degree of deacetylation, film-forming and storage conditions, molecular weight or the type and concentration of the organic solvent employed, determine the antibacterial effectiveness of the compound. Although, there are over 22,600 publications related to chitin and chitosan since 1907, there is still great controversy regarding the phenomenology and mechanisms of the biocide properties of this natural component. For this reason, there is a critical need to establish more reliable analytical methods for a proper quality control in the chitosan production, especially regarding molecular weight and degree of deacetylation (No *et al.*, 2007).

Chitosan is presently under investigation for a wide range of therapeutic applications, such as burn and wound dressings, sutures, bone fillers, engineered tissue scaffolds, and drug and gene delivery vehicles. Chitosan also has its own advantages such as water binding capacity, fat binding capacity, bioactivity, biodegradability, nontoxicity, biocompatibility and antifungal activity (Yang *et al*, 2004). Chitosan possesses repeating units of 1,4 linked 2-deoxy-2-aminoglucose. The amino group  $\text{NH}_2$  can be protonated to  $\text{NH}_3$  and readily form electrostatic interactions with anionic groups in an acid environment. This property has been applied on edible films (Xu *et al*, 2005).

### 2.3 Gelatin (Raw Material)

Gelatin is a protein substance derived from collagen, a natural protein present in the tendons, ligaments, and tissues of mammals. It is produced by boiling the connective tissues, bones and skins of animals, usually cows and pigs. Gelatin's ability to form strong, transparent gels and flexible films that are easily digested, soluble in hot water, and capable of forming a positive binding action have made it a valuable commodity in food processing, pharmaceuticals, photography, and paper production.

Gelatin is obtained from collagen by thermal denaturation or physical and chemical degradation (Valeria 2007). Gelatin was one of the first materials employed in formation of biomaterials, and has been subjected in many patents. Gelatin continues to be used in studies on edible films because it is an abundant raw material, produced in the whole world at low cost and has excellent film forming properties. Good revisions on the gelatin structure and its functional properties related to filmogenic abilities have been recently published (Vanin *et al*, 2004).

Gelatin's ability to form thermoreversible gels with a melting point close to body temperature has contributed substantially to an increase in its applications. Gelatin's largest single food use is in gel desserts because of the unique 'melt at mouth temperature, in frozen foods and in dairy products as a protective colloid or stabilizer, i.e. ice crystal inhibitor. Gelatin has also been used in photographic emulsions, playing a multipurpose role such as a protective colloid, ripening agent and binder, in the textile industry as an adhesive and in the pharmaceutical industry for the production of tablets and hard capsules. Food coating and casing applications such as sausage casings and poultry coatings, with or without the presence of antimicrobial compounds, are envisaged as another important and promising issue which has primarily received attention by the meat industry. Occasionally, gelatin has been used in conjunction with other hydrocolloids such as acacia (gum arabic), alginate and pectate esters, soluble and hydroxyl propyl starch (Ioannis *et al*, 1998)

It has gained more attention as edible films for its abundance and biodegradability. Gelatin has relatively low cost and excellent functional and filmogenic properties. Gelatin film itself, as most protein films, does not have ideal water vapor barrier properties. Thus, some chemical treatments can be applied to modify the polymer network through cross-linking of the polymer chains to improve the hydrocolloid film functionality. Food, pharmaceutical and industries are the main users of gelatin, which has several other technical applications. Their most frequent uses in the biomedical field include hard and soft capsules, wound dressings and adsorbent pads for surgical uses, as well as three-dimensional tissue regeneration (Rivero *et al*, 2008).

Like the collagen, the gelatin chains are macromolecules with a tendency mainly to interchain, rather than intrachain and hydrogen bonding. Gelatin forms a three-dimensional network with zones of intermolecular microcrystalline junctions, and the dehydration of this system may produce brittle films. Thus, plasticizers must be added to reduce interchain interactions improving film flexibility (Vanin *et al*, 2005).

## 2.4 Polyethylene Glycol (PEG)

PEGs are prepared by polymerization of ethylene oxide and are commercially available over a wide range of molecular weights from 300 g/mol to 10,000,000 g/mol. While PEG and PEO with different molecular weights find use in different applications and have different physical properties due to chain length effects, their chemical properties are nearly identical. The two PEG and PEO are chemically synonymous, but historically PEG has tended to refer to shorter polymers with molecular weight  $M_r < 20,000$ , polyethylene oxide to high-molecular adducts. PEG undergoes thermo-oxidative and oxidative destruction at the temperature above 310°C, also it is destructed by action of high-speed stirring (Fernandez *et al*, 2006).

The numbers that are often included in the names of PEGs indicate their average molecular weights, e.g. a PEG with  $n=80$  would have an average molecular weight of approximately 3500 daltons and would be labeled PEG 3500. Most PEGs include molecules with a distribution of molecular weights, i.e. they are polydisperse. The size distribution can be characterized statistically by its weight average molecular weight ( $M_w$ ) and its number average molecular weight ( $M_n$ ), the ratio of which is called the polydispersity index ( $M_w/M_n$ ).  $M_w$  and  $M_n$  can be measured by mass spectroscopy. PEGylation is the act of covalently coupling a PEG structure to another larger molecule, for example, a therapeutic protein (which is then referred to as PEGylated). PEGylated interferon alfa-2a or -2b is a commonly used injectable treatment for Hepatitis C infection (Fernandez *et al.*, 2006).

The composite biofilms of chitosan-polylactic acid incorporated with Polyethylene glycol will make the product more flexible and ‘easy to recover’ materials. The chitosan-polylactic acid films will have variable thickness depend on their blending mixture and the concentration of polyethylene glycol. The higher the polyethylene glycol, the easier the films of chitosan were removed from polypropylene support and more flexible there were (Se’bastian *et al*, 2006).

The higher concentration of polyethylene glycol in the composite films, the more higher the water vapor transmission rate will be, it is due to progressive film plasticization which is associated with modification of the hydrophilic character of polylactic acid film. The polyethylene glycol, thus decrease the material cohesion by creating intermolecular spaces and increasing water molecule diffusion coefficient or the easier separation of polyethylene glycol with the amorphous phase of polylactic acid which significantly explain the result (Glauser *et al*, 2005). For this research, PEG 400 will be used as the plasticizer.

## 2.5 Clove Essential Oils

Essential oils also called volatile or ethereal oils are aromatic oily liquids obtained from plant material (flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits and roots). They can be obtained by expression, fermentation, effleurage or extraction but the method of steam distillation is most commonly used for commercial production of EOs. The term ‘essential oil’ is thought to derive from the name coined in the 16th century by the Swiss reformer of medicine, Paracelsus von Hohenheim; he named the effective component of a drug Quinta essential. An estimated 3000 EOs are known, of which about 300 are commercially important—destined chiefly for the flavors and fragrances market. It has long been recognized that some EOs have antimicrobial properties and these have been reviewed in the past as have the antimicrobial properties of spices but the relatively recent enhancement of interest in ‘green’ consumerism has lead to a renewal of scientific interest in these substances. Besides antibacterial properties, EOs or their components have been shown to exhibit antiviral, antifungal, antitoxigenic, antiparasitic, and insecticidal properties. These characteristics are possibly related to the function of these compounds in plants (Sara, 2004).

Clove (*Eugenia caryophyllata* Thunb.) is widely cultivated in Madagascar, Sri Lanka, Indonesia and the south of China (Bureau of Drug Administration of China, 1989). Clove bud oils have biological activities, such as antibacterial, antifungal, insecticidal and antioxidant properties, and are used traditionally as flavoring agent and antimicrobial material in food.

The high levels of eugenol contained in clove essential oil give it strong biological activity and antimicrobial activity (Guan 2006).

## 2.6 Characterization

### 2.6.1 Scanning Electron Microscope (SEM)

Scanning electron microscope (SEM) is a type of electron microscope that can take images of a sample surface by scanning it with a high-energy beam of electrons in a raster scan pattern. Electrons from the SEM interact with the atoms of the sample that make up the sample producing signals. These signals contain information about the sample's surface topography, composition and other properties such as electrical conductivity (Kalsom *et al*, 2003).



**Figure 2.2:** Scanning Electron Microcopy

In order to make it functional the electron microscope must of course have a source of electrons which comprises its illumination system. These illumination electrons are produced by the electron gun. The electron gun consists of three parts, the filament, the shield and the anode. Some of the alternative names for the filament include cathode or emitter (Yakimets *et al*, 2005).

Basically there are two major categories of electron emitters used in SEMs. The first of these represents a class of electron sources that emit electrons as they are heated. These thermionic emitters operate on principal that as certain materials are heated the electrons in the outer orbital become unstable and are more likely to fly free of their atoms.

To create an SEM image, the incident electron beam is scanned in a raster pattern across the sample's surface. The emitted electrons are detected for each position in the scanned area by an electron detector. The intensity of the emitted electron signal is displayed as brightness on a cathode ray tube (CRT). By synchronizing the CRT scan to that of the scan of the incident electron beam, the CRT display represents the morphology of the sample surface area scanned by the beam. Magnification of the CRT image is the ratio of the image display size to the sample area scanned by the electron beam. The SEM column and sample chamber are at a moderate vacuum to allow the electrons to travel freely from the electron beam source to the sample and then to the detectors. This mode provides high-resolution imaging of fine surface morphology. Inelastic electron scattering caused by the interaction between the sample's electrons and the incident electrons results in the emission of low-energy electrons from near the sample's surface. The topography of surface features influences the number of electrons that reach the secondary electron detector from any point on the scanned surface. This local variation in electron intensity creates the image contrast that reveals the surface morphology. The secondary electron image resolution for an ideal sample is about 3.5 nm for a tungsten-filament electron source SEM or 1.5 nm for field emission SEM (Beacom *et al*, 2001).



### 2.6.2 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR can be used to identify chemicals from spills, paints, coatings, drugs, and contaminants. FTIR is perhaps the most powerful tool for identifying types of chemical bonds (functional groups). The wavelength of light absorbed is characteristic of the chemical bond as can be seen in this annotated spectrum (Yakimets *et al*, 2005).



**Figure 2.3:** Fourier Transform Infrared

By interpreting the infrared absorption spectrum, the chemical bonds in a molecule can be determined. FTIR spectra of pure compounds are generally so unique that they are like a molecular “fingerprint”. While organic compounds have very rich, detailed spectra, inorganic compounds are usually much simpler. For most common materials, the spectrum of an unknown can be identified by comparison to a library of known compounds. WCAS has several infrared spectral libraries including on-line computer libraries. To identify less common materials, IR will need to be combined with nuclear magnetic resonance, mass spectrometry, emission spectroscopy, X-ray diffraction, and/or other techniques (Chien *et al*, 2003).

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compounds are usually much simpler. For most common materials, the spectrum of an unknown can be identified by comparison to a library of known compounds. WCAS has several infrared spectral libraries including on-line computer libraries. To identify less common materials, IR will need to be combined with nuclear magnetic resonance, mass spectrometry, emission spectroscopy, X-ray diffraction, and/or other techniques (Skoog *et al*, 2003).

Fourier Transform-Infrared Spectroscopy (FTIR) is an analytical technique used to identify organic (and in some cases inorganic) materials. This technique measures the absorption of infrared radiation by the sample material versus wavelength. The infrared absorption bands identify molecular components and structures. When a material is irradiated with infrared radiation, absorbed IR radiation usually excites molecules into a higher vibrational state. The wavelength of light absorbed by a particular molecule is a function of the energy difference between the at-rest and excited vibrational states. The wavelengths that are absorbed by the sample are characteristic of its molecular structure. The FTIR spectrometer uses an interferometer to modulate the wavelength from a broadband infrared source. A detector measures the intensity of transmitted or reflected light as a function of its wavelength. The signal obtained from the detector is an interferogram, which must be analyzed with a computer using Fourier transforms to obtain a single-beam infrared spectrum. The FTIR spectra are usually presented as plots of intensity versus wavenumber (in  $\text{cm}^{-1}$ ). Wavenumber is the reciprocal of the wavelength. The intensity can be plotted as the percentage of light transmittance or absorbance at each wavenumber. To identify the material being analyzed, the unknown IR absorption spectrum is compared with standard spectra in computer databases or with a spectrum obtained from a known material. Spectrum matches identify the polymer or other constituent(s) in the sample. Absorption bands in the range of 4000 - 1500 wavenumbers are typically due to functional groups (such as, -OH, C=O, N-H, CH<sub>3</sub>). The region from 1500 - 400 wavenumbers is referred to as the fingerprint region. Absorption bands in this region are generally due to intramolecular phenomena and are highly specific to each material. The specificity of these bands allows computerized data searches within reference libraries to identify a material (Karnnet *et al*, 2005).

### 2.6.3 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. The technique was developed by E.S Watson and M.J. O'Neill in 1960, and introduced commercially at the 1963 Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy. DSC is used widely for examining polymers to check their composition. Melting points and glass transition temperatures for most polymers are available from standard compilations, and the method can show up possible polymer degradation by the lowering of the expected melting point,  $T_m$ , for example.  $T_m$  depends on the molecular weight of the polymer, so lower grades will have lower melting points than expected (Bourtoom and Chinnan, 2008)



**Figure 2.4:** Differential Scanning Calorimetry

Differential Scanning Calorimeter (DSC) is a thermo analytical technique in which the difference in the amount of heat required to increase the temperature of the sample and reference are measured as a function of temperature. Both the sample and reference are maintained at nearly the same temperature throughout the experiment. Generally, the temperature program for a DSC analysis is designed such that the sample holder temperature increases linearly as a function of time (Yakimets *et al*, 2005).

Two methods can be used for the calorimetric measurements. Differences in temperature between the sample and reference material can be measured as the same amount of heat energy (calories) is added to both. Or, differences in the amount of heat energy added to both are measured as the temperature for both the sample and reference are kept constant. In both cases, the heat flow and temperature of the sample are monitored in comparison to the reference material. The analysis is usually performed in an inert gas atmosphere, such as nitrogen. The amount of energy absorbed (endotherm) or evolved (exotherm) as the sample undergoes physical or chemical changes (such as melting, crystallization and curing) is measured in calories as a function of the temperature change. Any material reactions involving changes in heat capacity (e.g. glass transition) are also detected. By closely monitoring the heat flow and temperature, DSC can provide abundant information regarding a polymer material including: melting temperature, heat of fusion, glass transition temperature, curing temperature, heat of reaction, thermal history, and others. DSC is ideal for studying reversible reactions of thermoplastics such as melting-crystallization points and glass transition temperature. It is also used in the study of the kinetics of thermoset curing reactions, purity, heat capacities, and the effects of additives. Similarly, DTA analysis is used for determining the temperatures for melting and solid state phase transformations in metals, minerals, and ceramics (Maria *et al*, 2001).

#### **2.6.4 Thermo Gravitation Analysis (TGA)**

The TGA data gives information about such properties as thermal stability, moisture or solvent content, additive or filler content, oxidation and decomposition temperature and rate. Thermal events such as melting, glass transition and other changes are not detected because there are no changes in the sample mass associated with this events. Any physical and chemical change involving mass may be studied. Thermal methods investigate changes that occur upon heating a sample. Thermo gravimetric measures changes in the mass of a sample that occur when it is heated. These changes relate to the reactions during decomposition, the loss of volatile material and the reactions with the surrounding atmosphere. Thermo gravimetric analysis continuously measures a weight of samples as a function of temperature and time (Bourtoom and Chinnan, 2008).

Thermo gravimetric analysis (TGA) is one of the members of the family of thermal analysis techniques used to characterize a wide variety of materials. TGA provides complimentary and supplementary characterization information to the most commonly used thermal technique, DSC. TGA measures the amount and rate (velocity) of change in the mass of a sample as a function of temperature or time in a controlled atmosphere. The measurements are used primarily to determine the thermal and/or oxidative stabilities of materials as well as their compositional properties. The technique can analyze materials that exhibit either mass loss or gain due to decomposition, oxidation or loss of volatiles (such as moisture). It is especially useful for the study of polymeric materials, including thermoplastics, thermosets, elastomers, composites, films, fibers, coatings and paints. TGA measurements provide valuable information that can be used to select materials for certain end-use applications predict product performance and improve product quality (Sichina, 2003).

Thermo gravimetric analysis continuously measures the weight of a sample as a function of temperature and time. The sample is placed in a small pan connected to a microbalance and heated in a controlled manner and/or held isothermally for a specified time. The atmosphere around the sample may consist of an inert gas, such as nitrogen, or a reactive

gas, such as air or oxygen. The heating program may start in an inert atmosphere and then be switched to air at a certain point to complete the analysis. Weight changes observed at specific temperatures correlate to volatilization of sample components, decomposition, oxidation/reduction reactions, or other reactions or changes. Fourier transform infrared spectroscopy (FTIR) or mass spectroscopy (MS) may be used in conjunction with TGA to analyze and identify the evolved gases from constituents volatilized from the sample at specific temperatures. As the TGA instrument measures the temperature and weight of the sample, thermally activated events are recorded. These events are expressed as weight loss or weight change for a given time or temperature. They may also be expressed as a rate of weight loss. The onset temperature for the weight loss is also recorded. These data correlate to and give information about such properties as: thermal stability, moisture or solvent content, additive or filler content, oxidation or decomposition temperatures and rate. Thermal events such as melting, glass transition, and other changes are not detected because there is no change in sample mass associated with these events (Wang *et al*, 2001).

## **CHAPTER 3**

### **METHODOLOGY**

#### **3.1 Materials and Equipment**

In this project, the materials used are chitosan and gelatin. The chitosan used was chitosan, from crab shell. The gelatin used from bovine sources. Both of these materials were used as the polymer in fabricating the biodegradable film. For the preparation of composite solution, polyethylene glycol (PEG) with the molecular weight of 400 was used as the plasticizer. Furthermore, clove essential oil is added as an antimicrobial agent. Different volumes of clove essential oil were used in order to observe whether the antimicrobial agent does or doesn't affect the antimicrobial analysis of the biodegradable film.

Three solutions were prepared for this research. Sample A is the solution with the mixture of chitosan, gelatin and PEG 400. Sample B is the solution with the mixture of chitosan, gelatin, PEG 400 and clove essential oils. Meanwhile, Sample C is almost the same as Sample B but different amount of clove essential oils.

**Table 3.1:** The amount of each material.

<b>Materials</b>	<b>Sample A</b>	<b>Sample B</b>	<b>Sample C</b>
<b>Chitosan</b>	2 g	2 g	2 g
<b>Gelatin</b>	2 g	2 g	2 g
<b>PEG 400</b>	2 ml	2 ml	2 ml
<b>Acetic acid (1% v/v)</b>	100 ml	100 ml	100 ml
<b>Clove essential oils</b>	-	0.25 ml	0.5 ml

### **3.2 Fabrication of the Biodegradable film**

#### **3.2.1 Hydrocolloids solutions**

Gelatin solution was prepared, using a concentration of 7.5% (w/w) by hydrating gelatin powder with distilled water for 4 h and then samples were heated at 60 °C until complete dissolution. Chitosan solution of 1% (w/w) was prepared by solubilizing in 1% (v/v) acetic acid solution. PEG 400 was added as plasticizer, the concentration used was 0.75% (w/w).

#### **3.2.2 Composite solution preparation**

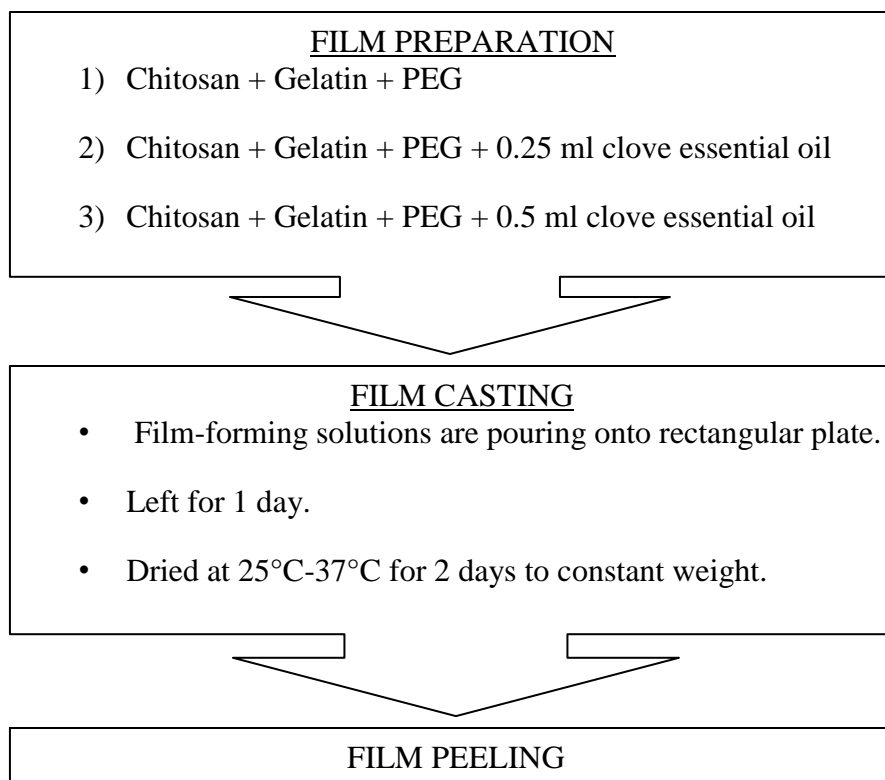
The composite solution was prepared in order to cast the biodegradable film properly. 2 g of gelatin was mixed with 100 ml distilled water. 2 g of chitosan was mixed with 100 ml of 1% v/v acetic acid. This mixture was stirred and heated until completely mix. The solution was the mixed together with the solution that prepared earlier. Then PEG was added into the mixed solution and stirred for 4 hours or more until it is well mixed. After the solutions well mixed, the solution was degassed for 24 hours until no bubbles are visible. For sample B and



sample C same amount of materials and chemical must be made. But for sample B and sample C clove essential oils was added after PEG was adding into the mixed solution with the respective amounts for both sample that is 0.25 ml of clove essential oils and 0.5 ml of clove essential oils.

### 3.2.3 Film Casting

The prepared composite solution was 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. Under ambient temperature, the casted films were left for 1 day. The films were peeled from the glass plate after they are completely dry. Process flow to produce biodegradable film is presented as following;



**Figure 3.1:** Process flow biodegradable film fabrication

### **3.3 Film Characterization**

For film characterization, four methods of characterization were done which are Scanning Electron Microscopy (SEM) to observe the surface morphology and roughness analysis, Fourier Transform Infrared (FTIR) to identify chemical bonds in a molecule by the infrared absorption spectrum produced by the bonds, Differential Scanning Calorimeter (DSC) to identify the melting temperature of the composite biodegradable film and Thermo Gravimetric Analysis (TGA) to measure thermal degradation. As for the film antimicrobial analysis, two methods were done which are Zone Inhibition Assay and OD Measurement.

#### **3.3.1 Scanning Electron Microscopy (SEM)**

Morphology of films was analyzed using a scanning electron microscopy (SEM EDX Spectrometer EVO 50). Fractured films were rested vertically on the sides of an aluminium sheet and fixed on stubs using double sided adhesive carbon tape. Mounted samples were gold coated with a layer of 40-50 nm of thickness to permit the observation of the surface and cross section of the films. SEM observations were performed using the following magnifications: 500 and 1000.

#### **3.3.2 Fourier Transform Infrared (FTIR)**

Fourier transform infrared (FTIR) to identify chemical bonds in a molecule by the infrared absorption spectrum produced by the bonds. FTIR were performed at room temperature using NICOLET AVATAR 370 DTGS. The characteristic absorption bands of the composite films were detected at wavenumbers ranging from 500 to 4500  $\text{cm}^{-1}$ .

### 3.3.3 Thermo Gravimetric Analyzer (TGA)

Thermo gravimetric analysis was performed by a thermo gravimetric analyzer (TGA Q500) under a steady flow of nitrogen atmosphere at a heating rate 10°C/min in the range of 20-600°C.

### 3.3.4 Differential Scanning Calorimeter (DSC)

The thermal properties of composite films were characterized by a differential scanning calorimeter (DSC Q 1000). The purified and dried samples were used for DSC test. Sample was first heated from 20°C to 200°C under nitrogen atmosphere at a heating rate of 10°C/min and reheated to 200°C at the same heating rate after quenched to 20°C, at last sample was cooled to 20°C again at the cooling rate of 10°C/min.

## 3.4 Film Antimicrobial Analysis

### 3.4.1 Zone Inhibition Assay

Antimicrobial activity test was carried out using agar diffusion method. Indicator cultures were *Escherichia coli* and *Bacillus subtilis*, representing gram-positive and gram-negative bacteria. One hundred microlitres of the inoculums solution was added to appropriate soft agar, which was overlaid onto hard agar plates. Each sample was cut into squares (2cm x 2 cm) and was placed on the bacterial lawns. The plates were incubated for 24 h at 37°C in the appropriate incubation chamber. The plates were visually examined for zones of inhibition around the film and the size of the zone diameter was measured at two cross sectional points and the average was taken as the inhibition zone.

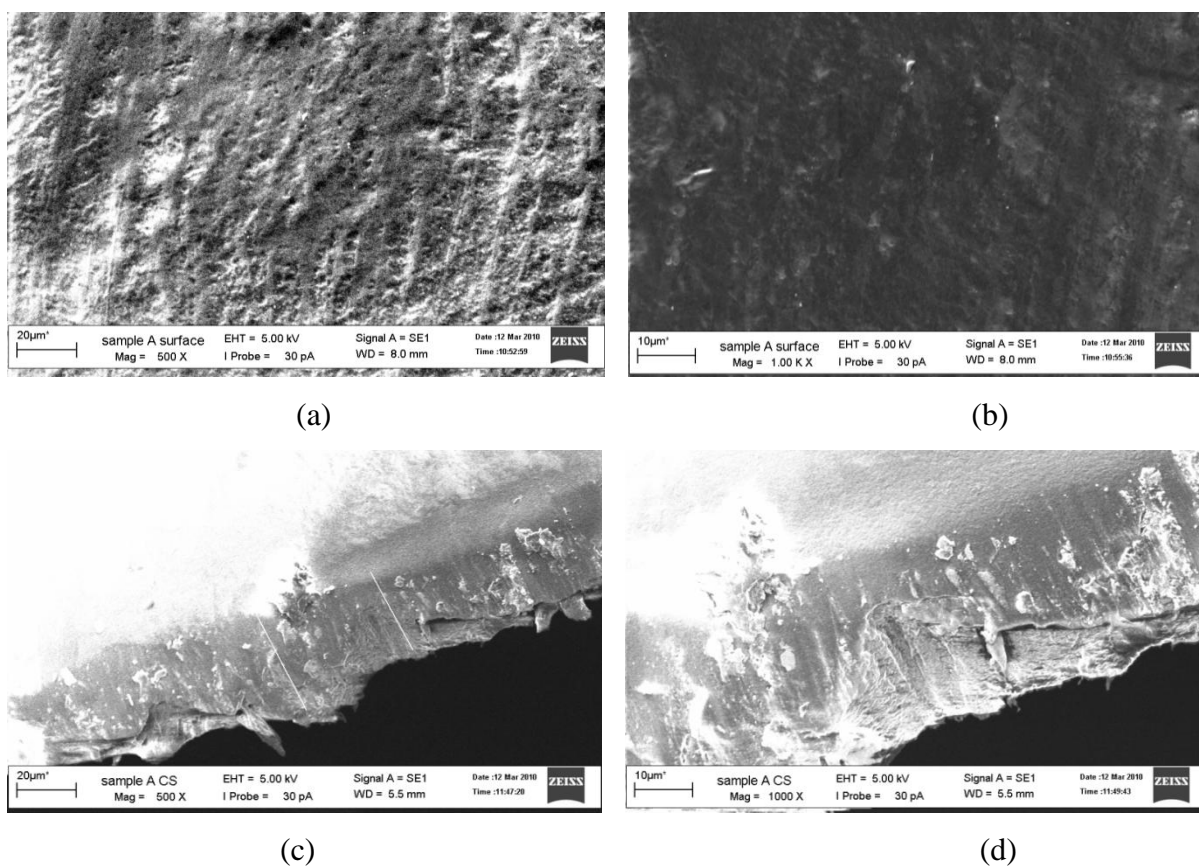
### 3.4.2 Liquid Culture Test

For the liquid culture test, each film was cut into squares (1cm x 1cm). Three sample squares were immersed in 20 ml nutrient broth in universal bottle. The medium was inoculated with 200 $\mu$ l of *Escherichia coli* and *Bacillus subtilis* in its late exponential phase and then transferred to an orbital shaker and rotated at 37°C at 200 rpm. The culture was sampled periodically (0, 2, 4, 8, 12, 24 hours) during the incubation to obtain microbial growth profiles. The optical density was measured at  $\lambda = 600$  nm using a spectrophotometer.

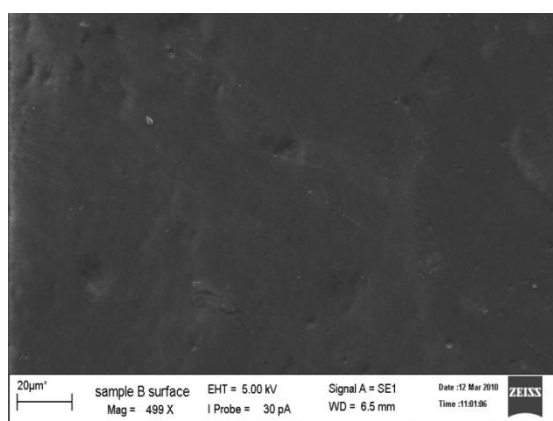
## CHAPTER 4

### RESULT AND DISCUSSION

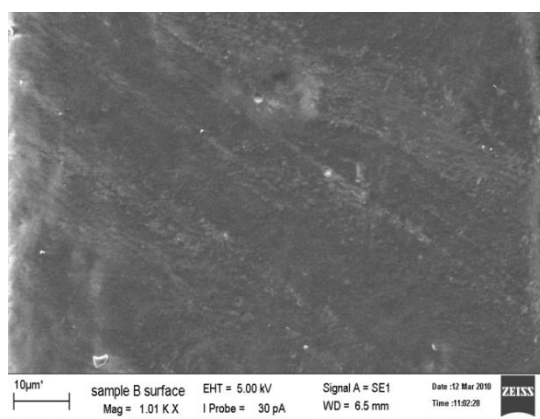
#### 4.1 Scanning Electron Microscopy (SEM)



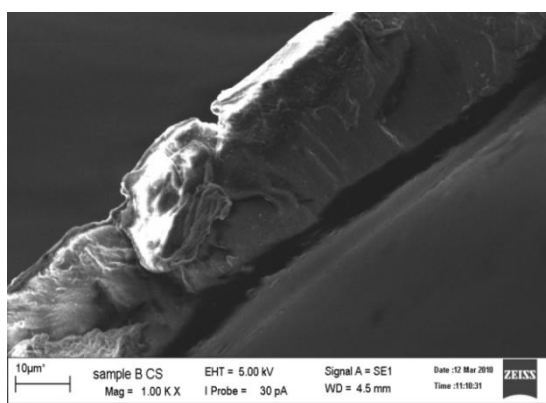
**Figure 4.1(a)-(d) : Sample A surface and cross section at 500x and 1000x**



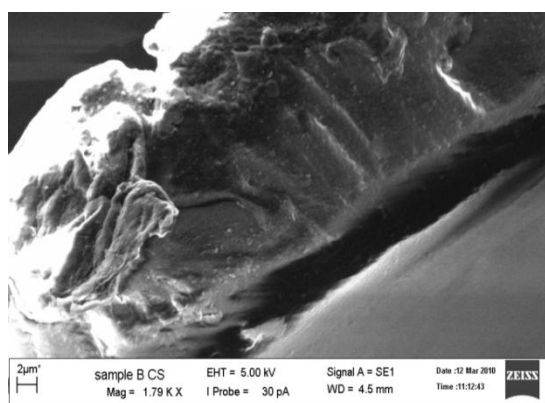
(a)



(b)

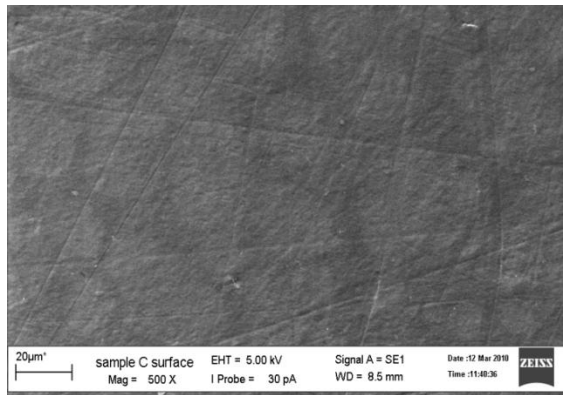


(c)

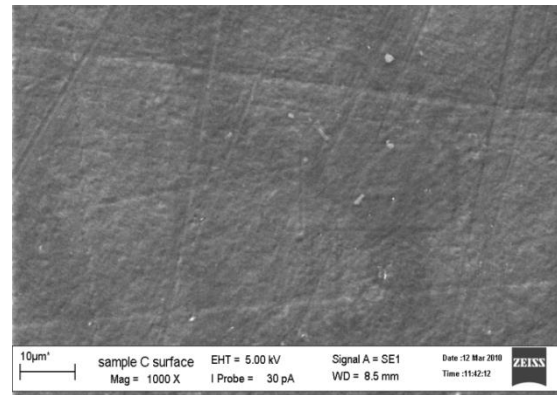


(d)

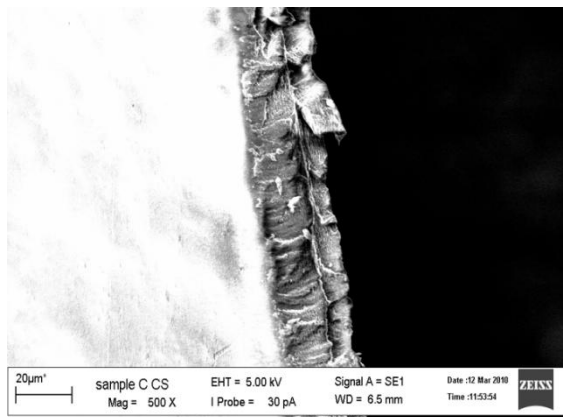
**Figure 4.2 (a)-(d):** Sample B surface and cross section at 500x and 1000x



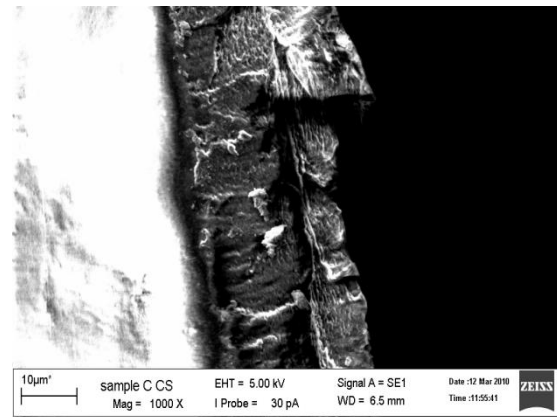
(a)



(b)



(c)



(d)

**Figure 4.3 (a)-(d):** Sample C surface and cross section at 500x and 1000x

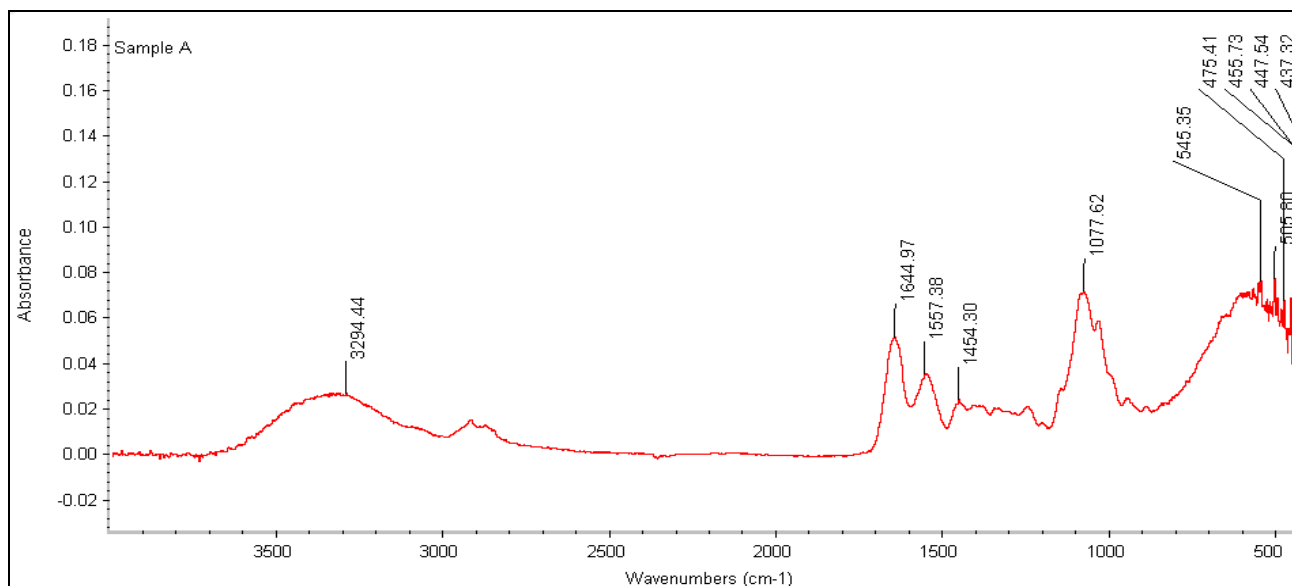
As presented in SEM images, three typical scanning electron microscopy of surfaces and cross sections of composite films were respectively presented in Figure 4.1, Figure 4.2 and Figure 4.3 respectively SEM observations were performed using the following magnification; 500 and 1000. From the results for cross sections we could find that the average thickness of the composite films is approximately 5.5 mm. For this composite film with gelatin and chitosan were characterized by a compact, uniform, dense structure and homogenous appearance.

The surfaces of sample A were rough, whereas sample B and sample C had smooth surface. This could be due to a slight interaction between the components in sample A. Meanwhile with addition of clove essential oils in sample B and sample C, the surface of samples become smooth. This may be due to the FTIR result shows the strong hydrogen bonding between hydroxyl group of the chitosan, gelatin and clove essential oils. The smoothness of surface of samples was increased as the ratios of clove essential oils increased. This may also showed smooth and compact structure indicating a good compability between the components.

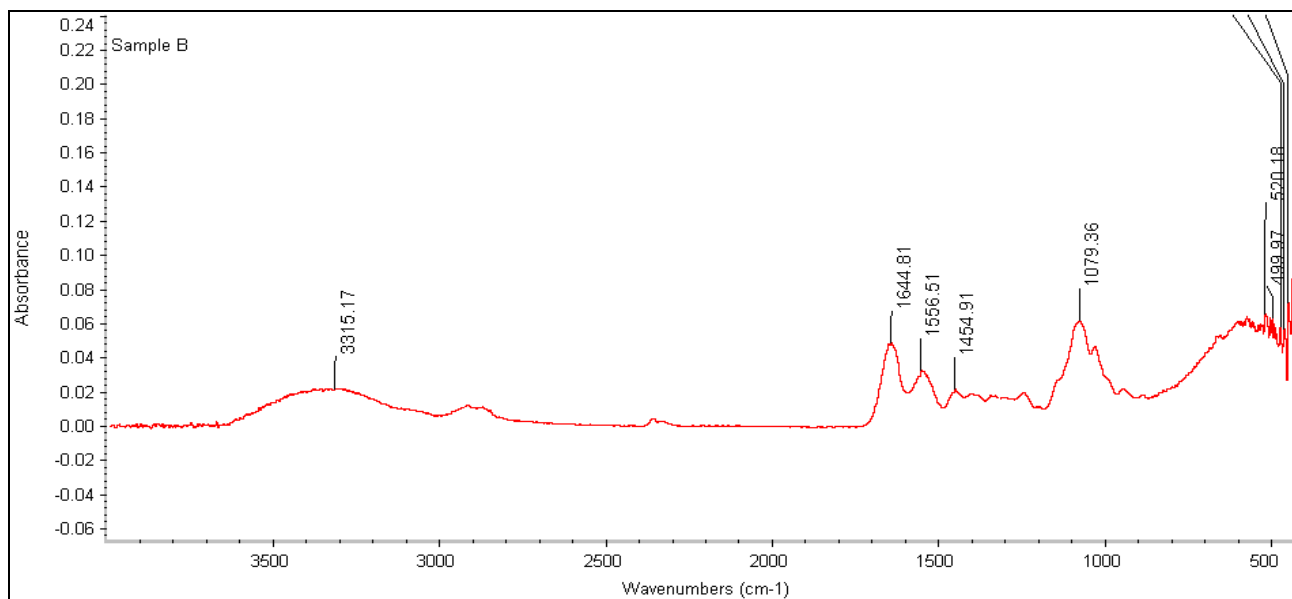
The cross section of sample A and sample C presented an absence of hollows. But the appearance of these two cross sections have some distinction where cross section of sample A was more rough that the sample C which might attribute to the addition of clove essential oils in sample C resulting in the strong hydrogen bonding in composite films. This blending ratio of clove essential oils also influenced greatly cross sections of films since sample C has compact cross section surface. However, the cross section of sample B showed presence of hollow and rough which could be observed more clearly in magnification. The reason for this phenomenon is still unclear which calls for further study. Gelation and aggregation play an important role in the structuring of all biological materials and depend on the ability of biopolymers to interact and to form organized structures (Wang *et al*, 2010).



## 4.2 Fourier Transform Infrared (FTIR)



**Figure 4.4:** The results from FTIR for Sample A



**Figure 4.5:** The results from FTIR for Sample B

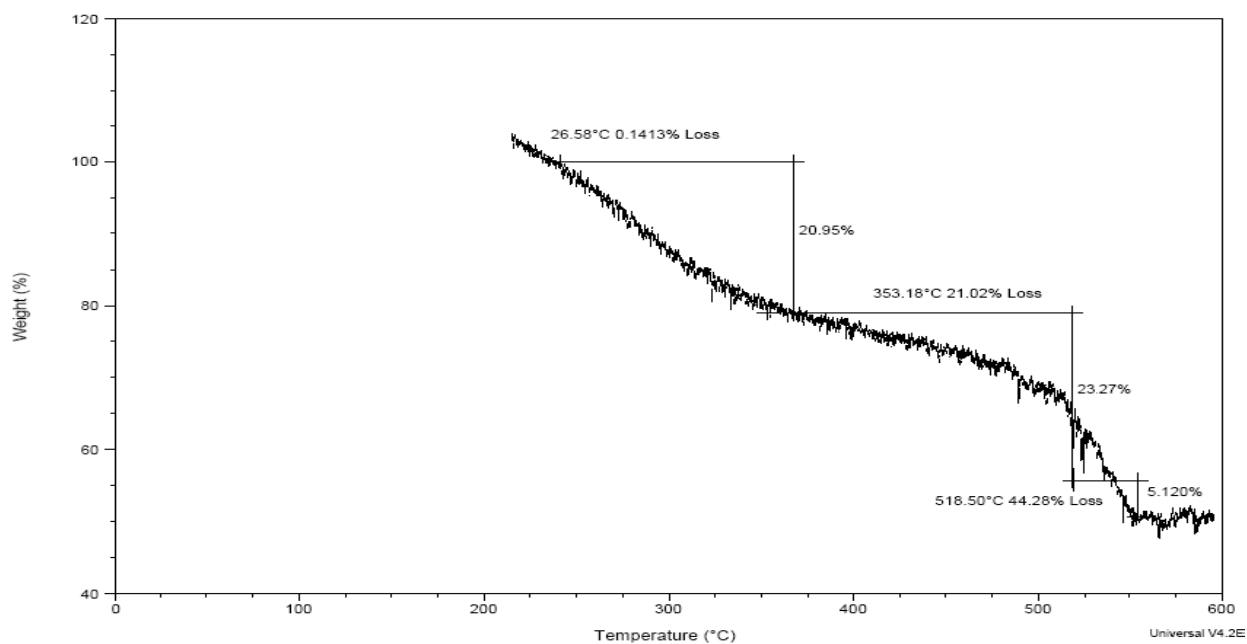
**Figure 4.7:** The results from FTIR for Sample A, B, C

Figure 4.3 shows FTIR of sample A, B and C respectively. Value of wavenumbers for sample A, B and C are similarly. This is because of the presence of similar interactions between these three samples. The wavenumber at the range of 500 to 600  $\text{cm}^{-1}$  suggest that there are aromatic rings in the film. The characteristic absorption bands of the samples around 1030 to 1155  $\text{cm}^{-1}$  which confirmed the presence of C-O in biodegradable films. This C-O interaction is in phenols and ether in clove, chitosan and polyethylene glycol. However, the peak around 1450  $\text{cm}^{-1}$  was stronger and sharper, and can be attributed to accumulation of carboxylate group ( $\text{COO}^-$ ).

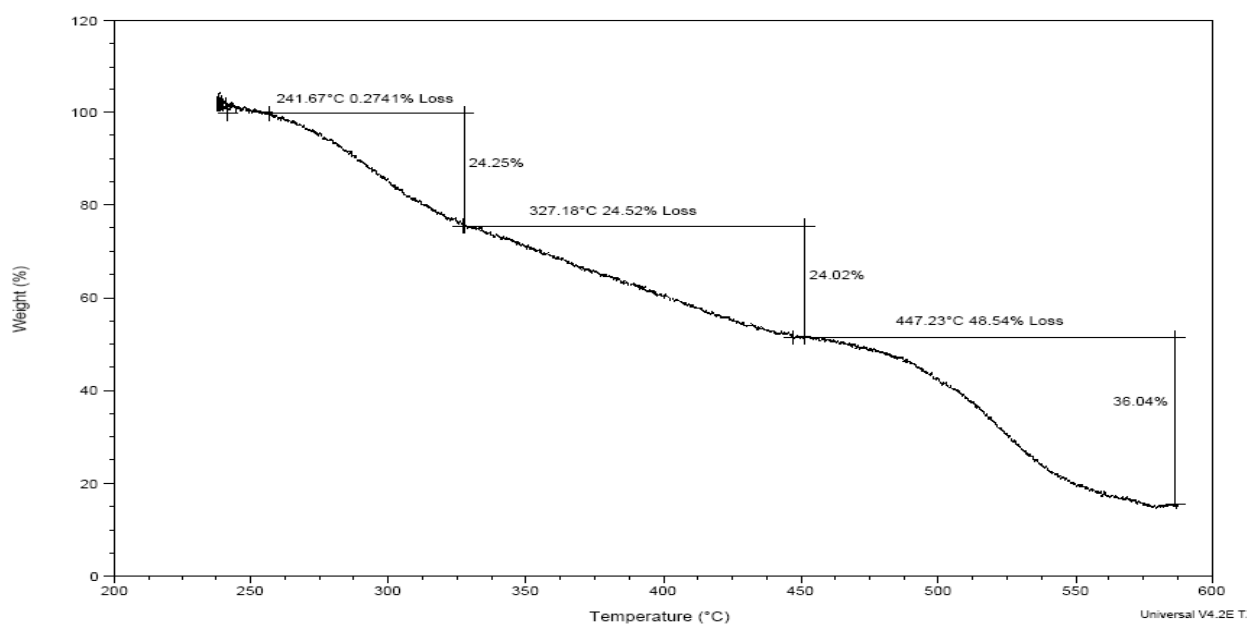
As for figure 4.3, there is  $\text{NH}_2$  interaction at 1550 to 1650  $\text{cm}^{-1}$  that shows presence of amine in gelatin and chitosan. From SEM analysis that have been described previously, surface for the film is smooth because of high compability between chitosan and gelatin. The peak of the graph is at 1660-1850  $\text{cm}^{-1}$  which shows that the carbonyl group which is in aldehyde in clove essential oils. Besides, there is O-H absorption occurs at 2500- 3500  $\text{cm}^{-1}$ . This hydroxyl group presence in chitosan, clove essential oils and polyethylene glycol. Bands due to C-H stretching vibrations overlapped in the absorption peaks at 2280  $\text{cm}^{-1}$ .

There are one or two peaks of C-H stretching shows presence of aldehyde in clove essential oils. The broad band area of 3300-3500  $\text{cm}^{-1}$  show that chitosan and gelatin shared a high composition in this film as the absorbance in that range is quite high. This N-H stretching band shows up secondary amine in gelatin and chitosan. In other words, there are only some slight differences between these two samples. When chitosan composite biodegradable films are incorporated with clove essential oils there is no modification on functional groups. There is thus no significant change on the mechanical and physical properties. This shows that the addition of clove essential oils do not affect much the absorbance and wavenumbers of these three samples.

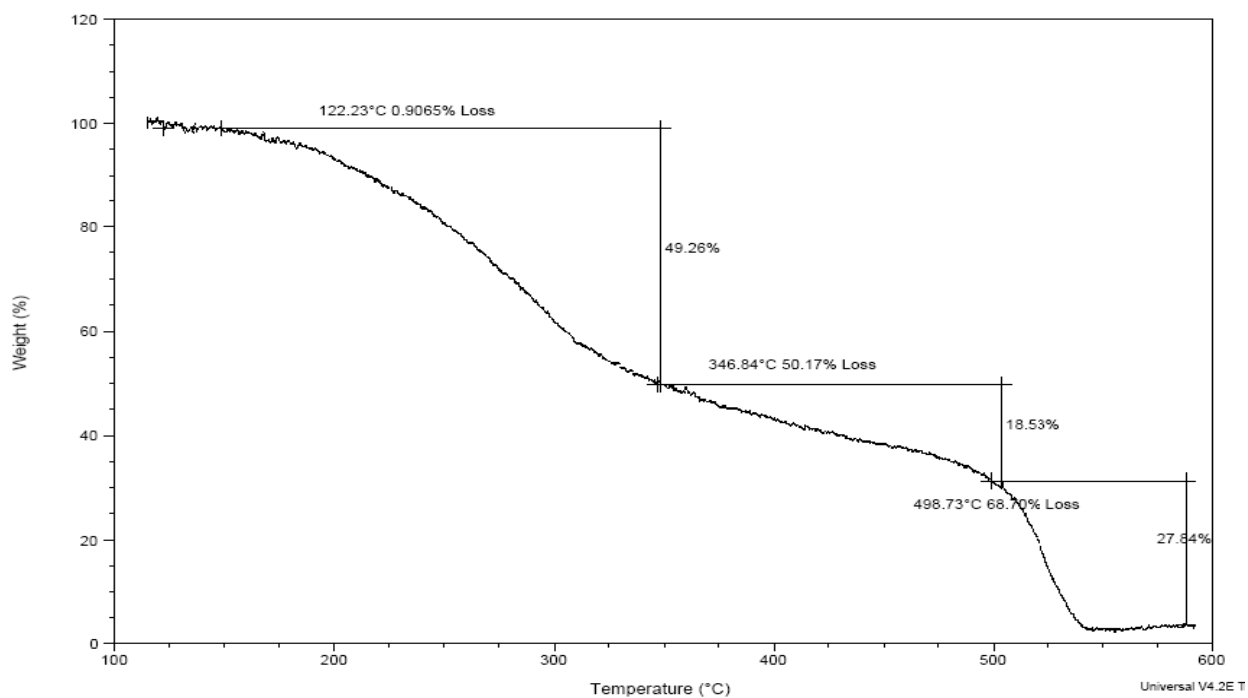
### 4.3 Thermo Gravimetric Analyzer (TGA)



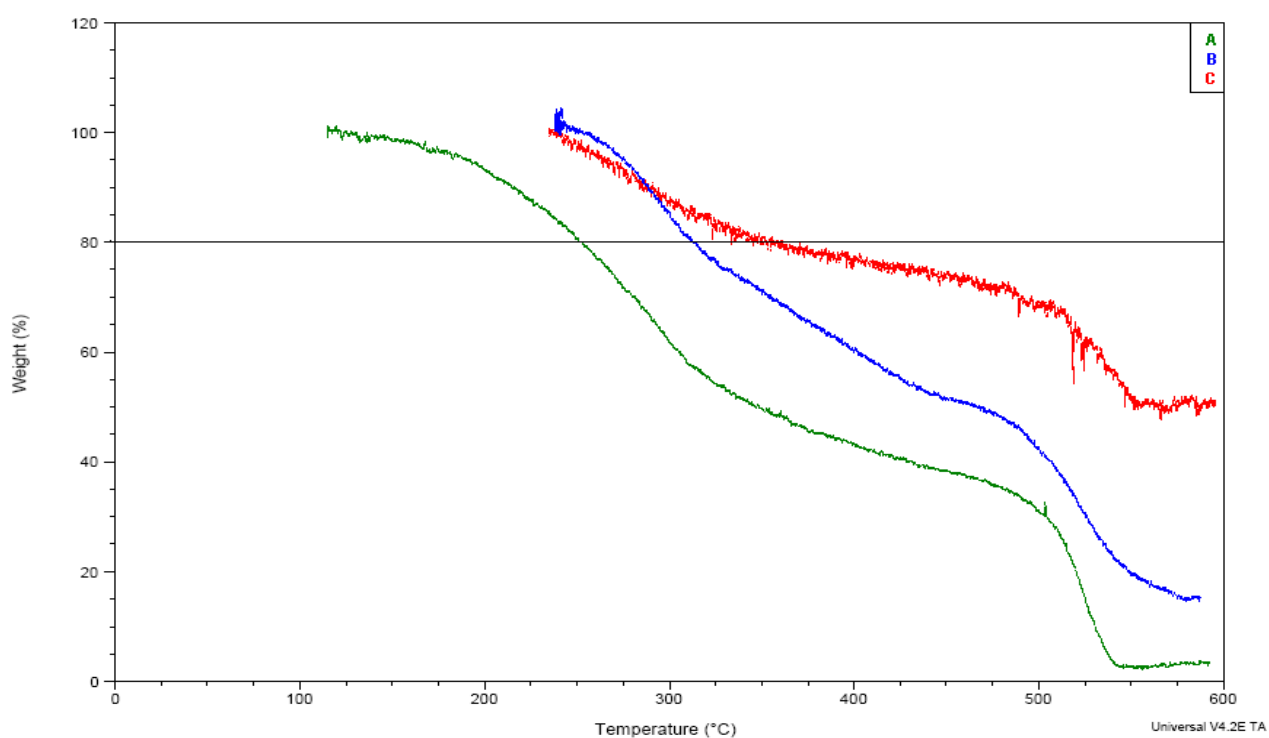
**Figure 4.8:** The result from TGA for Sample A



**Figure 4.9:** The result from TGA for Sample B



**Figure 4.10:** The result from TGA for Sample C



**Figure 4.11:** The result from TGA for Sample A, B, C

Thermal decomposition behavior of sample A, B and C were performed by thermo gravimetric analyzer. According to the thermo gram presented in Figure 4.5, at about 26 °C, the film starts to evolve the small amount of moisture, which is found to be 0.14 % by TGA. A high performance TGA instrument is required to detect this small level of moisture content. Knowing this moisture content is important as it has a major bearing on the end use properties and processing performance of films. The film undergoes thermal degradation beginning at 353 °C with mass loss of 21%. At 518°C the purge gas flowing over the sample was automatically switched to oxygen and the carbon residue was burned off and at 540°C with a mass loss of 5.1%. The sample is fully degradable only small portions of weight loss can be detected further on. The residue percentage of sample A is at 50.62 %.

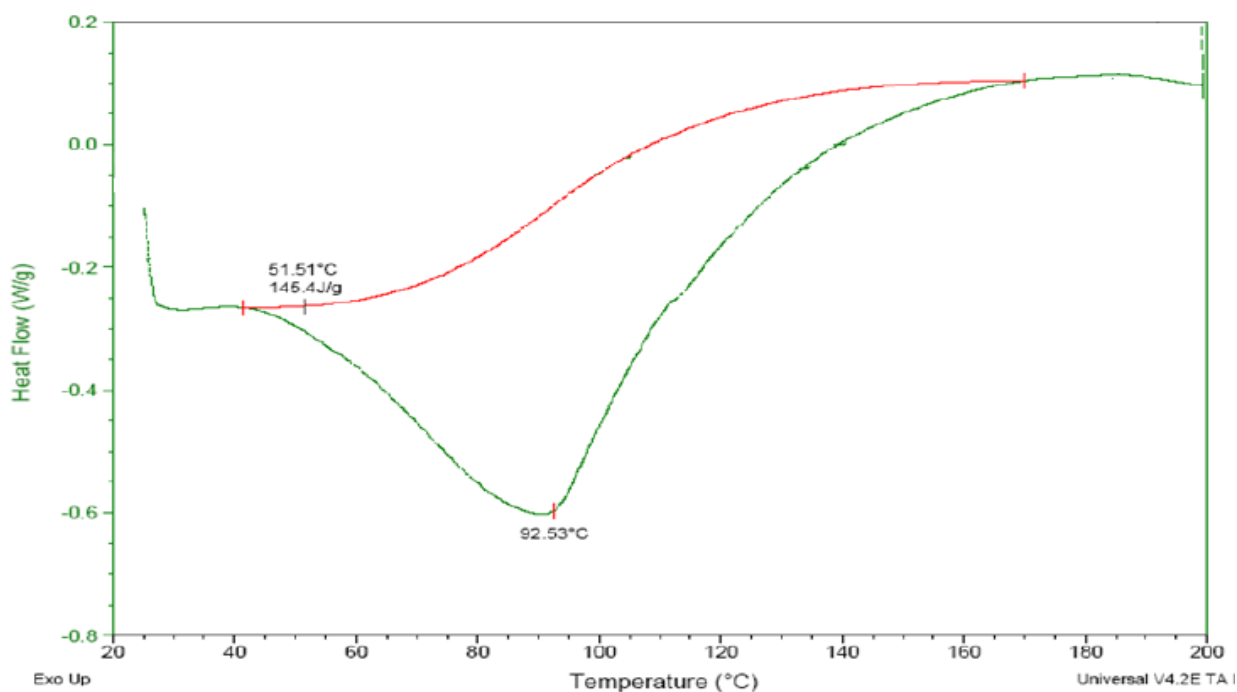
Figure 4.6 shows a quite same pattern with Figure 4.5 but there are some major differences that might be overlooked. From figure 4.6 the weight loss for water component of sample B is 0.27%. Temperature of degradation of sample B starts from 327°C. Compare to sample A, the weight loss for sample B is 24.52 %. This difference may be due to the presence of clove essential oils. After this temperature, the weight decreases in a big range, at 447.23°C the loss weight is the 96.8% and almost 100%. The residue percentage of sample B is at 15.37%.

Figure 4.7 shows the TGA result for sample C. Three weight losses are observed in TGA curve. The 0.9% weight loss at 122.23°C is due to the moisture vaporation. The second weight loss which is 50% at 346.84 °C is due to the thermal degradation of chitosan composite. Lastly, the third 68.70 % weight loss at 498.73°C is due to the by-product generated by chitosan composite during the TGA thermal degradation process. The residue percentage of sample C is at 3.47%.

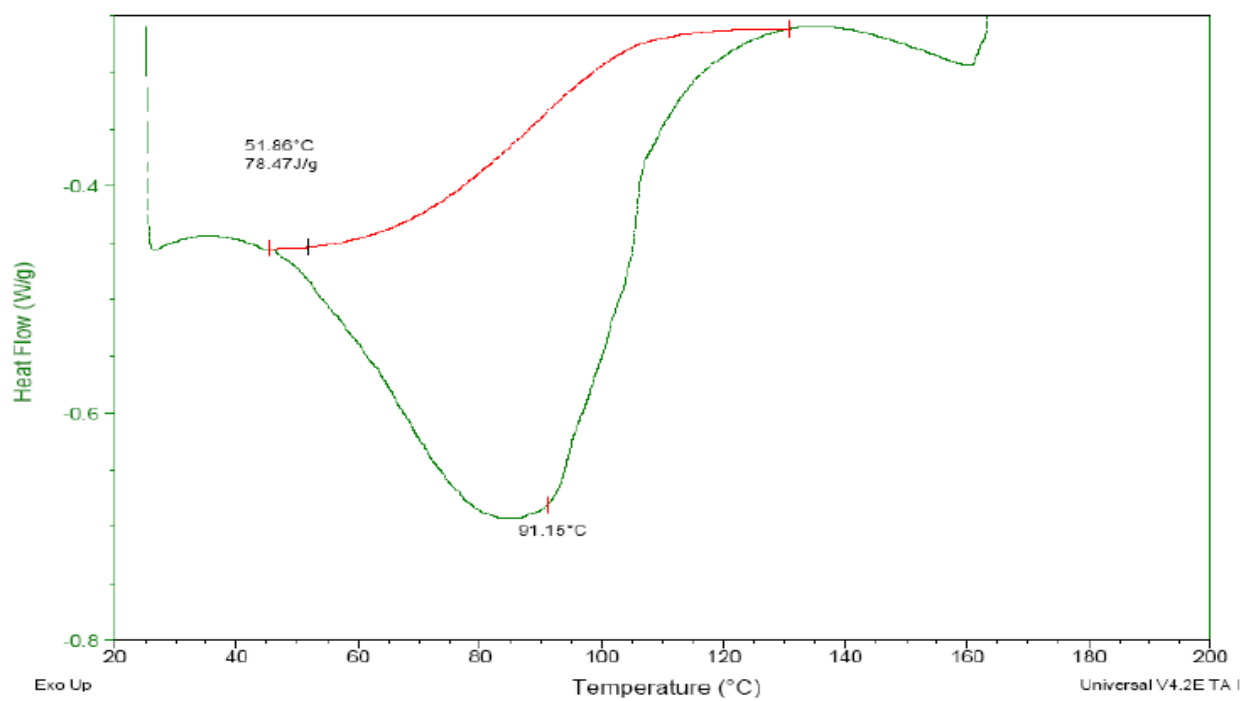
The combination of sample A, B and C is in figure 4.8. From figure 4.8, there is comparison between sample A, B and C at the same percent of weight loss which is 20% weight loss or we can say 80% weight of samples. From the graph, the sample A reached 80% weight sample at 250°C. While for sample B is at 310°C and followed by sample C which is

at 350°C. Thus this shows that sample C has high heat resistance compare to other films. From figure 4.8, sample C do not completely degrade, so it can proceed running the process at the high temperature until completely degrade. From this result show that sample C is the best because has high heat resistance.

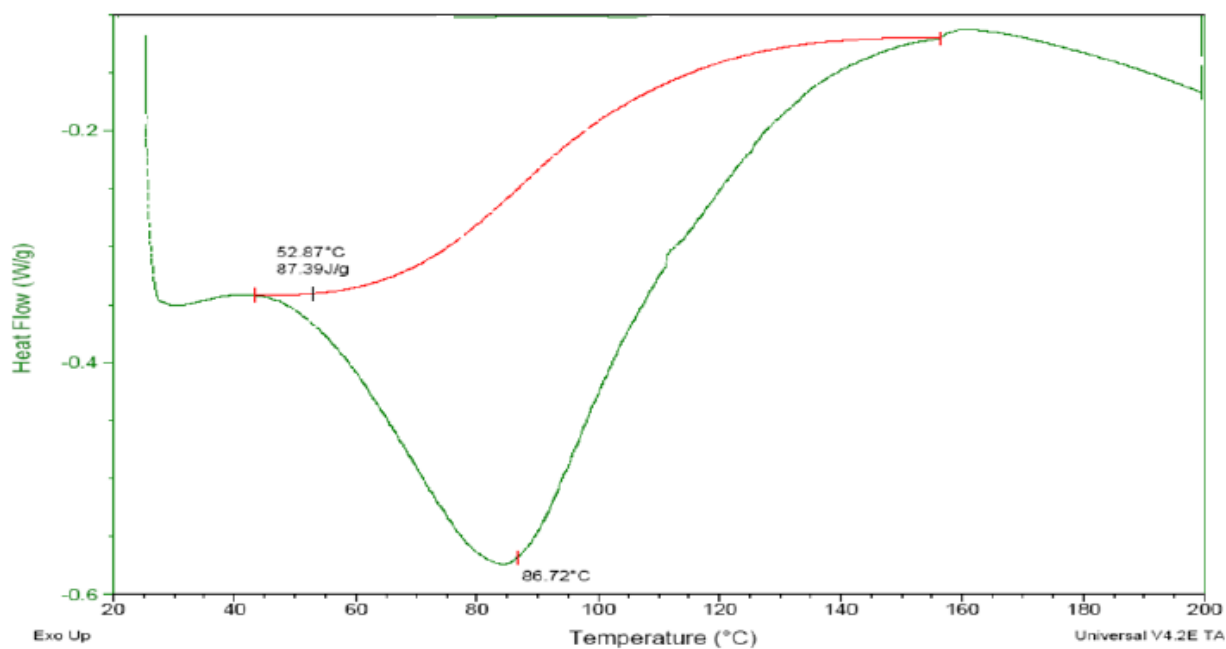
#### 4.4 Differential Scanning Calorimeter (DSC)



**Figure 4.12:** The result from DSC for Sample A

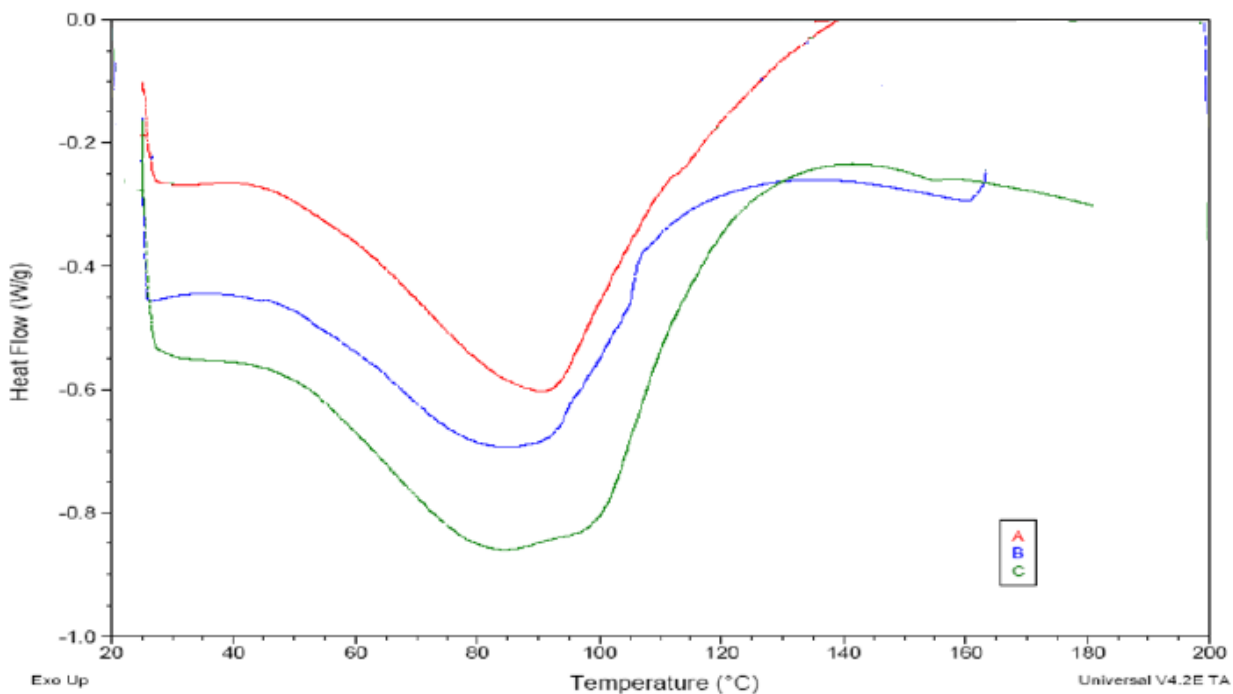


**Figure 4.13:** The result from DSC for Sample B



**Figure 4.14:** The result from DSC for Sample C





**Figure 4.15:** The result from DSC for Sample A, B, C

Differential scanning calorimeter (DSC) was selected to characterize the thermal properties of composite films. Two stage heating process was conducted for the DSC analysis. The first stage heating is used to decrease the water content in the blend films and release the stress of blend films. Since chitosan contains  $\text{NH}_2$  and  $\text{OH}$  functional groups, the hydrogen bonding force is strongly formed among molecular. It is difficult to identify the glass transition temperature ( $T_g$ ) of chitosan since this compound is semicrystalline (Chen *et al*, 2008).

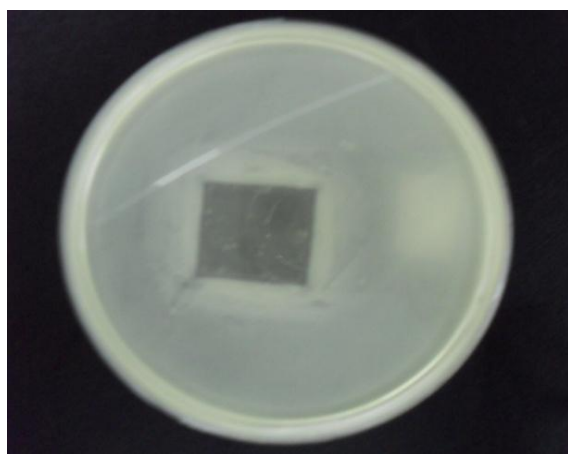
The endothermic peak in figure 4.9 is the melting point of the sample A. As presented in figure 4.9, sample A had a melting temperature ( $T_m$ ) at 51.51°C and 145.41 J/g which also confirmed the linkage of gelatin onto chitosan chain. Meanwhile, the melting temperature of sample B was 51.86°C and 78.47 J/g. This show these composite films were thermal stable and the degradation did not occur ranging from 20°C-200°C (Li *et al*, 2010). This probably due to strong hydrogen bonding between hydroxyl groups of the chitosan and polar functions

of some amino acid residues of the gelatin within the fraction composed of a homogeneous blend of gelatin and chitosan. For sample C, the melting temperature was 52.87°C and 87.39 J/g.

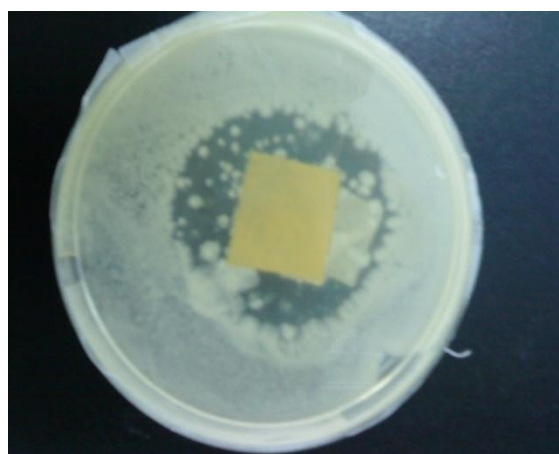
From the result, we could find that the melting temperature ( $T_m$ ) increased which is 51.51°C to 52.87°C whereas the latent heat ( $\Delta H_m$ ) of the composite films decreased which is 145.41 J/g to 87.39 J/g. This showed that the little heater under the sample pan is going to have to put a lot of heat into the films in order to melt the crystals and keep the temperature rising at the same rate as that of the reference pan.

## 4.5 Antimicrobial Analysis

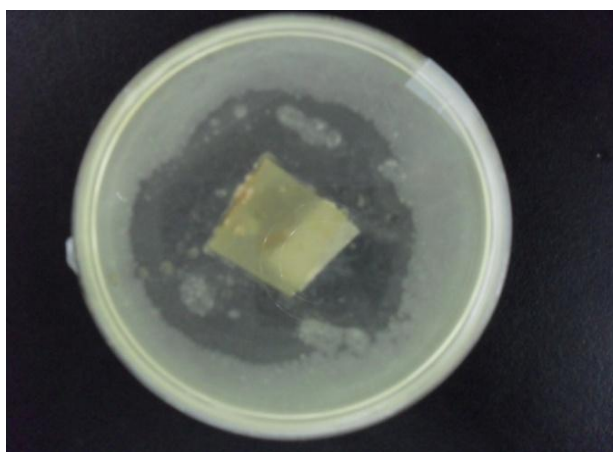
### 4.5.1 Zone Inhibition Assay



(a)

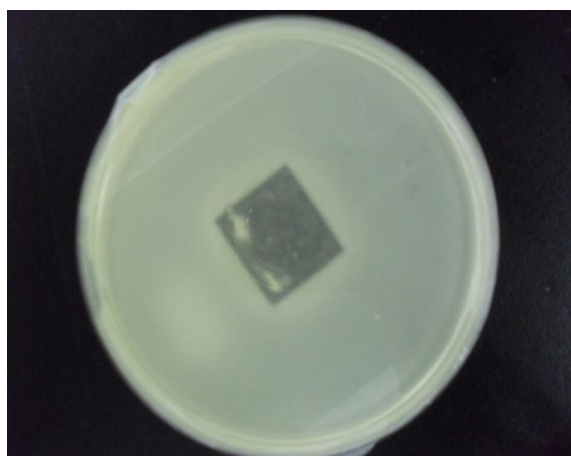


(b)

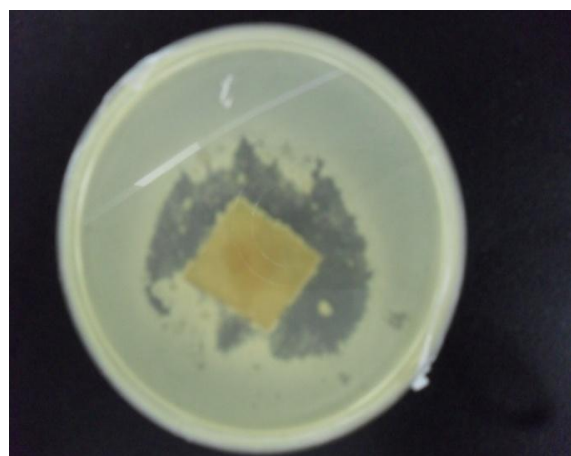


(c)

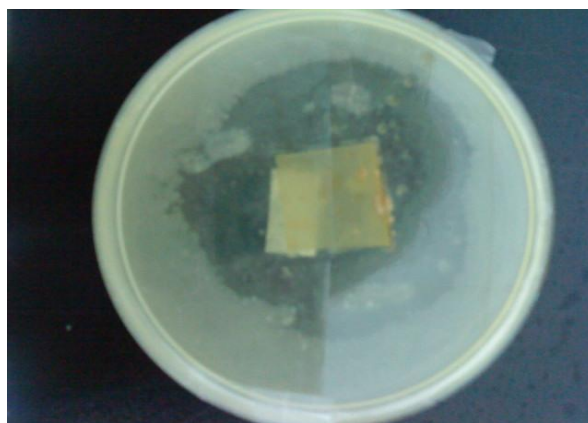
**Figure 4.16(a)-(c) :** Inhibition zone of sample against *E. coli*



(a)

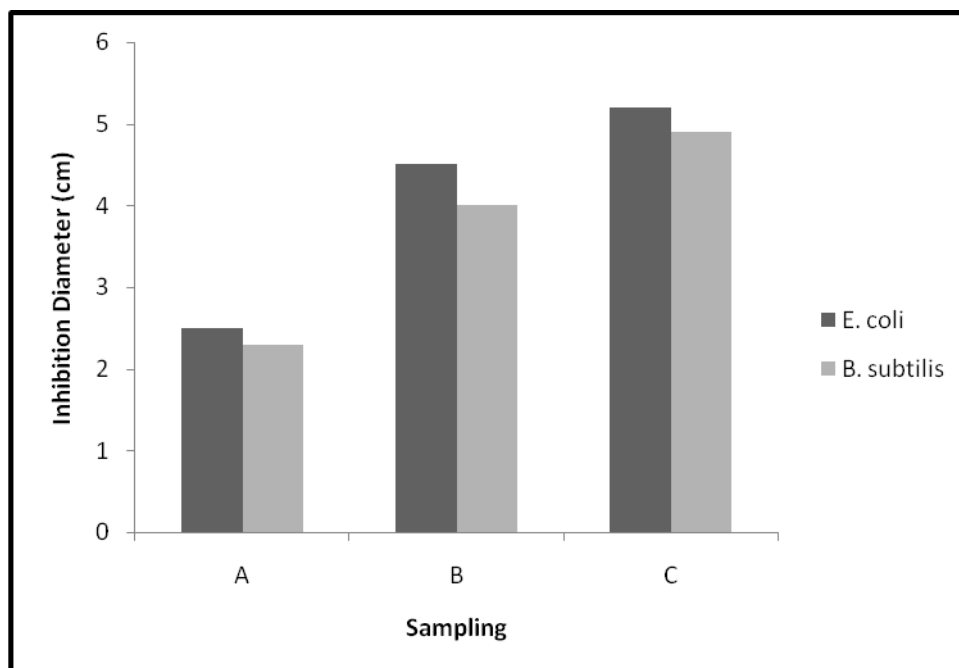


(b)



(c)

**Figure 4.16(a)-(c) :** Inhibition zone of sample against *B. subtilis*



**Figure 4.18:** Inhibition of *E. coli* and *B. subtilis* on agar plates

The details of antimicrobial activity of chitosan composite film incorporated with gelatin and clove essential oils against *E. coli* and *B. subtilis* are shown in figure 4.5. The inhibitory activity was measured based on clear zone surrounding squares film. If there was no clear zone surrounding, it was assumed that there was no inhibitory effect and furthermore, the diameter was valued as zero.

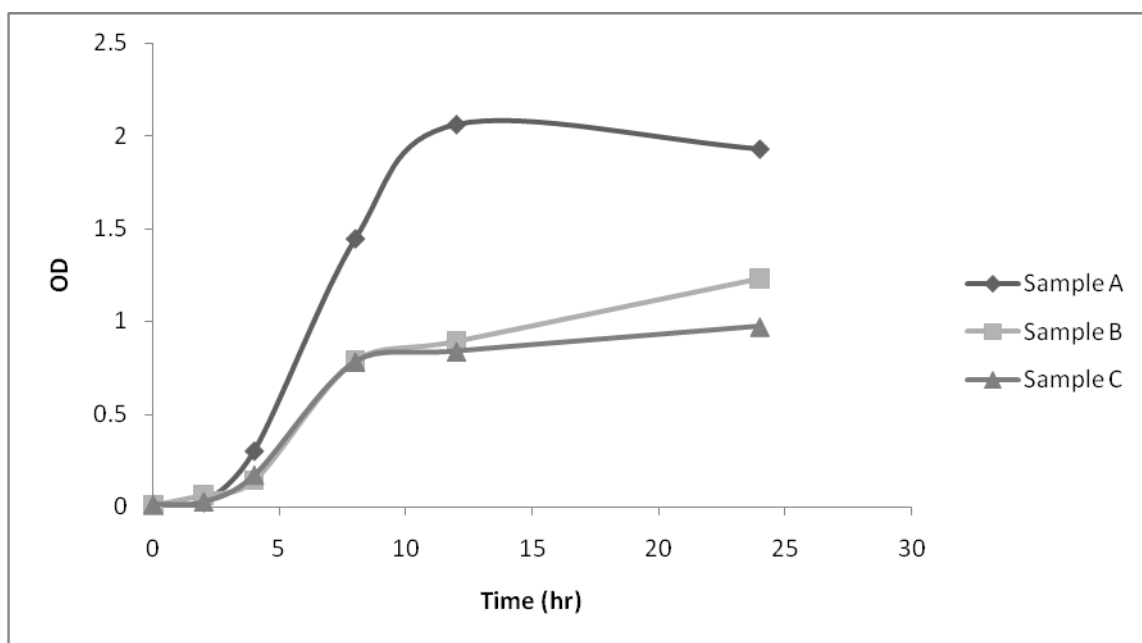
In terms of surrounding clearing zone, the sample A which is control film did not show inhibitory effect against all tested microorganisms. However, there was an inhibition occurred at the top of sample A for both *E. coli* and *B. subtilis*. This is because of an antimicrobial activity of chitosan against those microorganisms but poor.

Thus, incorporating clove essential oils into control films revealed the antimicrobial effect. The inhibitory zones were markedly high for *E. coli* and *B. subtilis*. It also reduced bacterial growth underneath films. On the contrary, sample B and sample C showed a better

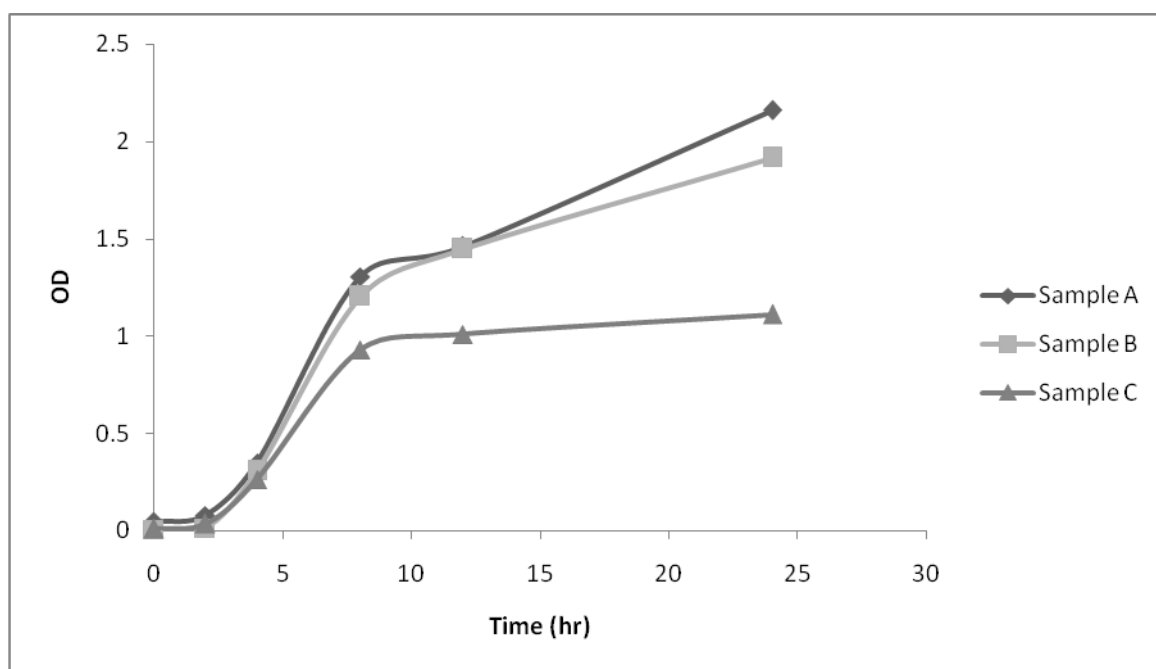
inhibition than sample A. From the figure showed that sample C has high inhibitory zone diameter. The inhibitory zone increased by the increase of clove essential oils incorporated. *E. coli* which is Gram-negative bacteria was the most sensitive against clove essential oils followed by *B. subtilis* (Gram-positive bacteria). This is because the cell wall structures of these categories of bacteria are different.

In general, chitosan film itself showed some antimicrobial effect even though it did not reveal inhibitory zone in any microorganisms tested. This is reasonable as chitosan has the innate characteristic of antimicrobial activity itself. The antimicrobial effect of chitosan occurred without migration of active agents. As chitosan is in a solid form, therefore, only organisms in direct contact with the active sites of chitosan is inhibited. Chitosan is incapable to diffuse through the adjacent agar media. The agar diffusion test is a method commonly used to examine antimicrobial activity regarding the diffusion of the compound tested through water-containing agar plate. The diffusion itself is dependent on the size, shape and polarity of the diffusing material. The chemical structure and the crosslinking level of the films also affect this phenomenon. When antimicrobial agents are incorporated, there will be diffusing materials through agar gel, and furthermore, resulting clearing zone on the bacterial growth (Pranoto et al, 2005)

#### 4.5.2 Liquid Culture Test (OD measurement)



(a)



(b)

**Figure 4.19(a) – (b):** Inhibition of samples against *E. coli* and *B. subtilis*

This result is similar to the result obtained in agar plate test. There was inhibition for both *E. coli* and *B. subtilis*. From figure 4.6, the antimicrobial film incorporated with clove essential oils was more effective against Gram-negative bacteria which is *E. coli* than the Gram-positive bacteria which is *B. subtilis* although the cell wall of Gram-negative bacteria has an outer membrane, which constitutes the outer surface of the wall.

As in figure 4.6, showed that clove essential oils have strong antimicrobial activity. This is because the high levels of eugenol contained in clove essential oils inhibit production of an essential enzyme by the bacteria or cause damage to the cell wall of bacteria. From the result, presence of clove essential oils makes the cell membrane of Gram-negative bacteria and Gram-positive bacteria weakened or even broken. This study indicated that the mechanisms of the antimicrobial activity of clove essential oils were different between those different types of bacteria.

In fact, one of the reasons for the antimicrobial character of chitosan is its positively charged amino group which interacts with negatively charged microbial cell membranes, leading to the leakage of proteinaceous and other intracellular constituents of the microorganisms. In the Gram-positive bacteria, the major constituent of its cell wall is peptidoglycan and there is very little protein. The cell wall of Gram-negative bacteria also has an outer membrane, which constitutes the outer surface of the wall. Previous study, observed that from electron micrographs for Gram-positive and Gram-negative bacteria in the presence of chitosan show the cell membrane of Gram-positive bacteria was weakened or even broken, while the cytoplasm of Gram-negative bacteria was concentrated and the interstice of the cell were clearly enlarged (Salleh and Muhamad, 2001)



## CHAPTER 5

### CONCLUSION AND RECOMMENDATION

#### 5.1 Conclusion

From the experiment, the chitosan composite biodegradable film with combination of clove essential oils were developed by casting method. The results showed that the properties of the biodegradable film were improved by combining chitosan and gelatin. The presence of molecular interaction between chitosan and gelatin was identified by FTIR, TGA and DSC. Meanwhile, the smoothness surface of composite films increased as clove essential oils ratio increased. For thermal properties, result indicates that the melting point of the films is increased when the amount of clove essential oils in films is increased. Three weight losses are observed in TGA curve. Thus, from the results showed that the biodegradable film has smoother surface, higher thermal stability and slightly higher melting point.

For antimicrobial analysis, incorporating clove essential oils into the films showed obvious effects towards inhibition of *E. coli* and *B. subtilis*. However, chitosan also has its antimicrobial activity since there is result showed inhibition of bacteria on top of the film but do not effective. Thus, with incorporating chitosan and clove essential oils demonstrates more effective antimicrobial activity against *E. coli* and *B. subtilis*. Increasing ratio of clove

essential oils make the film has greater inhibition. Thus, this antimicrobial film has potential to inhibit bacterial growth and extended the shelf life foods.

## **5.2 Recommendation**

There are still improvements and modifications that can be taken in this project for the better in the quality and quantity for the future. In this experiment gelatin was used as an additive into this composite film. However, other additive can be added to enhance the properties of the film such as starch. With addition of other additive we can identify the best method to improve the quality of composite biodegradable film. Besides, more tests should be done to prove the mechanical properties and tensile strength of the films.

For antimicrobial analysis, tests should be done on other antimicrobial ingredient in order to get strong reasons to prove the effectiveness of clove essential oil as an antimicrobial agent. The research should study on other essential oils antimicrobial activity other than clove essential oils. Rosemary essential oils and tea tree essential oils seem to be the better antimicrobial agent.

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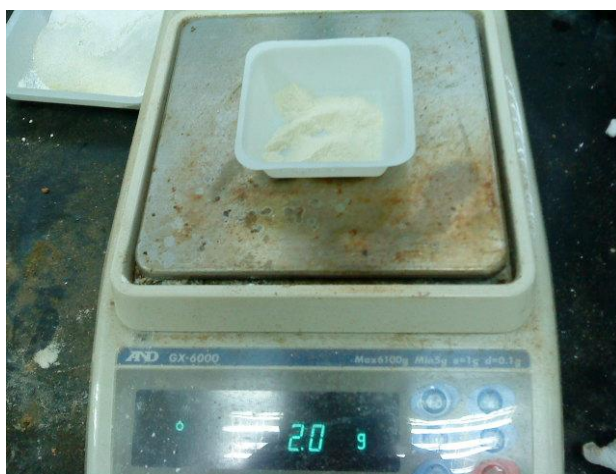
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## APPENDIX A

### MATERIALS AND METHODOLOGY



**Figure 1:** The gelatin powder used for this research



**Figure 2:** The chitosan powder used



**Figure 3:** Composite solution during the stirring process



**Figure 4:** The composite biodegradable film after it has dried on the glass plate





**Figure 5:** The casted film is peeled from the glass plate



**Figure 6:** The biodegradable film



**Figure 7:** Nutrient broth preparation for liquid culture test



**Figure 8:** Agar plate preparation for zone inhibition assay method