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

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DECLARATION

I declare that this thesis entitles "Characterization and Antimicrobial Analysis of Chitosan Composite Biodegradable Films with Addition of Lemon Grass 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.

Signature : ……..

Name : Maa Ajmalaisyatu Illani bt Mohd Nawi

Date : 30 APRIL 2010

DEDICATION

To my beloved mother and father,
My family members that always love me,
My beloved supervisor,
My research project teammate that always supporting each other,
My fellow colleagues,

For all your kindness, support and believing in me.

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 throughout this project until the research is successfully complete. With the Mercifulness from Allah therefore I can produce a complete thesis without having so much trouble along the way.

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ABSTRACT

The increasing demand in food safety, quality, convenience and environmental concerns associated with the handling of plastic waste has emphasized the importance in developing biodegradable and edible films from natural polymers, such as chitosan. Starch-based film is considered an economical material for antimicrobial packaging. This study aimed at the development of packaging based on chitosan-tapioca starch incorporated with lemon grass essential oil as antimicrobial agents. In this research, there are three samples were prepared. Sample A containing starch-chitosan as control film, sample B containing starch-chitosan with the addition of gelatin and sample C containing starch-chitosan based film incorporated with lemon grass essential oil. For the antimicrobial analysis, all the samples were tested on *B. substilis* and *E. coli*. Inhibition of bacterial growth was examined using two methods which are zone inhibition assay and liquid culture test. From the observations, sample C exhibited a wide clear inhibitory zone rather than samples A and B. From the liquid culture test, the sample C clearly demonstrated a better inhibition against *B. substilis* than *E. coli*. Incorporation of lemon grass essential oils also led to an improvement in several films properties in terms of morphology, chemical composition and thermal properties. The result from TGA and DSC has shown the higher thermal and melting temperature possesses by the sample C and slightly miscible and compatible with the starch-chitosan composition. The addition of lemon grass essential oil proves that the film is better to film based on starch-chitosan only. Lemon grass essential oil will actually improve the performance of the chitosan composite biodegradable film as tested in this research. As a conclusion, starch-chitosan composite biodegradable film has the potential to be use for food packaging.

ABSTRAK

Permintaan yang tinggi terhadap keselamatan makanan, kualiti dan masalah persekitaran alam yang berkaitan dengan pengurusan pembuangan plastik telah menekankan tentang kepentingan mencipta filem yang boleh dibiodegradasikan daripada polimer semulajadi seperti chitosan. Filem yang diperbuat daripada kanji dan chitosan ini sesuai sebagai sumber ekonomi untuk pembungkusan antimikrob. Kajian ini menekankan tentang pembungkusan yang diperbuat daripada kanji dan chitosan dan dicampurkan dengan minyak pati serai wangi. Dalam kajian ini, tiga sampel telah disediakan. Sampel A mengandungi kanji dan chitosan sahaja yang bertindak sebagai kontrol filem, sampel B mengandungi kanji dan chitosan ditambah dengan gelatin dan sampel C mengandungi kanji dan chitosan ditambah dengan minyak pati serai wangi. Untuk analisis antimikrob, kesemua sampel telah diuji terhadap *B. substilis* dan *E. coli*. Perencatan terhadap pertumbuhan bacteria telah di uji melalui dua cara iaitu *zone inhibition assay* dan *liquid culture test.* Sampel C menunjukkan kawasan zon halangan yang terbesar berbanding dengan sampel A dan B. Daripada analisis pembiakan bakteria, sampel C telah menunjukkan halangan yang tinggi terhadap *B. substilis* berbanding dengan *E. coli*. Penambahan minyak pati serai wangi telah memberi pembaharuan dari segi morfologi, komposisi kimia dan sifat terma. Keputusan dari TGA dan DSC telah menunjukkan ketahanan haba dan suhu peleburan haba yang tinggi yang tinggi dan sedikit *miscible* dan serasi dengan komposisi kanji dan chitosan. Minyak pati serai wangi akan meningkatkan kualiti chitosan komposit biodegradasi filem sebagaimana yang telah diuji dalam kajian ini. Kesimpulannya, filem komposit boleh biodegradasi yang diperbuat daripada kanji dan chitosan mempunyai potensi untuk digunakan sebagai pembungkus makanan.

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LIST OF SYMBOLS

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

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

Edible films and coatings have been particularly considered in food preservation, because of their capability in improving global food quality (Franssen and Krochta, 2003; Franssen *et al*., 2002; Guilbert and Biquet, 1996; Greener Donhowe and Fennema, 1994). The films can be used to cover food surfaces, separate incompatible zones and ingredients, form a barrier against oxygen, aroma, oil and moisture or perform as pouches or wraps. Among other important features, they can be used as carriers of functional agents, as antioxidants or antimicrobials, and to improve appearance and handling. Film production by natural and abundant biodegradable polymeric materials as cellulose, gums, starches or proteins, is also convenient due to the lower environmental consequences compared with common synthetic plastic materials (Cutter, 2006).

Edible and biodegradable films are always not meant to totally replace the synthetic packaging films (Krochta and Johnston, 1997) though it is one of the most effective methods of maintaining food quality. Usually film-forming substances are based on proteins, polysaccharides lipids and resins or a combination of these (Greener-Donhowe and Fennema, 1994). Chitosan has been found to be nontoxic, biodegradable, bio-functional, bio-compatible in addition to having antimicrobial characteristics

(Darmadji and Izumimoto, 1994; Jayakumar *et al*., 2007; Jayakumar *et al*., 2005; Jayakumar *et al*., 2006; Jongrittiporn *et al*., 2001; Wang, 1992). As compared with other bio-based food packaging materials, 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; Moller *et al*., 2004). In view of these qualities, chitosan films have been used as a packaging material for the quality preservation of a variety of food (Park and Zhao, 2004; Suyatma *et al*., 2005; Tsai and Su, 1999; Wu *et al*., 2005). Recently, a chitosan-starch film has been prepared using microwave treatment which may find potential application in food packaging. A wide variety of chitosan based antimicrobial films have recently been well documented (Tripathi *et al*., 2008).

Antimicrobial packaging has been touted as a major focus in the next generation of active packaging (Brody, 2001). Antimicrobial packaging is the packaging system that is able to kill or inhibit spoilage and pathogenic microorganisms that are contaminating foods. When the packaging system acquires antimicrobial activity, the packaging system limits or prevents microbial growth by extending the lag period and reducing the growth rate or decreases live counts of microorganisms (Han, 2000). Antimicrobial packaging can extend the food shelf-life, thus improving the quality of the food. Interest in antimicrobial packaging films has increased in recent years due to a concern over the risk of food borne illness, desire for extended food shelf life, and advances in the technology of film production. Slowing the growth of spoilage bacteria will reduce the losses of product to spoilage and extend shelf life. Reduction of pathogen growth will reduce the risk of food borne illness caused by those products (Dawson *et al.*, 2002). It can effectively control the microbial contamination of various solid and semisolid foodstuffs by inhibiting the growth of microorganisms on the surface of the food, which normally comes into direct contact with the packaging material. Antimicrobial function of the packaging system can be achieved by incorporating active substances into the packaging system by various ways (Han, 2003). The antimicrobial packaging is conducted by (1) the addition of antimicrobial containing sachets or pads into food packages; (2) the coating, immobilization or direct

incorporation of antimicrobials into food packaging materials or (3) the use of packaging materials that are inherently antimicrobial (Appendini and Hotchkiss, 2002). One of the examples of antimicrobial agents is lemon grass, besides Onawunmi *et al.* (1984) reported that the antimicrobial activity of lemongrass oil is related to high amounts of 1,8-cincole (>30%), geranial (>30%), and neral (>20%). However, citral isomers (neral, 32.2%, geranial, 41.28%) are the most abundant compounds in lemongrass oil as reported by (Choi *et al.*, 2000). These components individually showed antibacterial action on gram negative and gram-positive organisms (Onawunmi *et al.*, 1984).

1.2 Problem Statement

The rise in environmental consciousness in recent decades has included a focus on household waste. Food packaging has come to symbolize the issue of waste which formerly known as non degradable. It has expanded rapidly in recent times and perhaps most important of all, food packaging feels wasteful: used once and then promptly discarded, it seems like only a temporary presence in our lives as it rushes from factory to landfill. 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 undegraded 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, 2000). Because of the environmental concerns and technological problems such as denaturing effects of thermal polymer processing methods, extrusion and injection molding, the incorporation of bio-preservatives into biodegradable films is more suitable than incorporation into plastic films (Appendini and Hotchkiss, 2002; Han, 2000; Suppakul *et al.*, 2003)

Over the last few years, considerable research has been conducted to develop and apply bio-based polymers made from a variety of agricultural commodities and or wastes of food product industrialization (Cutter, 2006; Guilbert and Biquet, 1996). Among various degradable membrane materials, chitosan has attracted considerable attention for its unique properties, the most important one of which is abundant commercial supplies and antibacterial properties. Although chitosan membranes are highly impermeable to oxygen, they have relatively poor water vapor barrier characteristics due to its excellent hydrophilicity, which is not favorable for the usage as artificial skins (Willfor *et al*., 2008). But adding plasticizers such as glycerol had negative effects on barrier properties in spite of positive effects on mechanical properties (Srinivasa *et al*., 2007). Among efforts have been done to improve water barrier capability of chitosan (weakening its hydrophilic property) was blending chitosan with some hydrophobic materials had attached most interest. In order to improve the physical and functional properties of chitosan films, blending with other biopolymers such as starch. Starch based films have been particularly considered for the reason that they exhibit physical characteristics similar to synthetic polymers: transparent, odorless, tasteless, semi-permeable to CO_2 and resistant to O_2 passage (Nísperos and Carriedo, 1994).

Throughout the years, many researchers- have been done to improve the performance of biodegradable film in the food packaging area. A widespread trend worldwide is the movement towards natural food products. In effort to meet this demand, there has been increased interest in the food industry in using antimicrobial preservatives that are perceived as more natural. Future work will focus on the use of biologically active derived antimicrobial compounds bound to biopolymers. However many natural antimicrobials have a limited spectrum of activity and are effective only at very high concentrations. The need for new antimicrobials with wide spectrum activity and low toxicity will increase. A possible solution may be using combinations of antimicrobials (Sofos *et al.*, 1998). Instead of concentrating on development of new antimicrobial, it could be more practical to combine the antimicrobial agents that already being researched From all the points above, this research are emphasize on

make a biodegradable film from chitosan blend with tapioca starch to enhances its mechanical and functional properties with additional of lemon grass essential oil as antimicrobial agent.

1.3 Research Objectives

The objectives of this study are listed as following:

a. To fabricate a chitosan composite biodegradable film with combination of gelatin and lemon grass essential oils.

b. To analyze the fabricated films in terms of morphology, physical and chemical properties.

c. To perform the antimicrobial analysis against bacteria strain.

1.4 Scope of Study

The scopes of this study are listed as following:

a. To fabricate a chitosan composite biodegradable film with addition of tapioca starch, plasticizer, gelatin and lemon grass essential oils.

b. To analyze the fabricated films in term of antimicrobial activity using zone inhibition assay and liquid culture test against *Bacillus Subtilis* and *Escherichia coli.*

c. To characterize the fabricated films in terms of morphology, physical and chemical properties using various analysis method:

i. Scanning Electron Microscopy (SEM)

ii. Fourier Transform Infrared Spectroscopy (FTIR)

iii. Thermo Gravimetric Analysis (TGA)

iv. Differential Scanning Calorimeter (DSC)

CHAPTER 2

LITERATURE REVIEW

2.1 Composite Biodegradable Film

Bio-composites (biodegradable composites) consist of biodegradable polymers as the matrix material and biodegradable fillers, usually bio-fibers. Since both components are biodegradable, the composite as the integral part is also expected to be biodegradable (Mohant *et al*., 2000c).

Another important bio-composite category is based on agro-polymers matrixes, mainly focused on starchy materials. Plasticized starch, called thermoplastic starch (TPS) is obtained after disruption and plasticization of native starch, with water and plasticizer by applying thermo mechanical energy in a continuous extrusion process. Unfortunately, TPS shows some drawbacks such as a strong hydrophilic character, rather poor mechanical properties compared to conventional polymers and an important post-processing variation of the properties. TPS properties reach equilibrium only after several weeks. To improve these material weaknesses, TPS is usually associated with others compounds (Dufresne and Vignon, 1998; Dufresne *et al*., 2000), bleached leaf wood fibers (Funke *et al*., 1998; Ave´rous *et al*., 2001). Most of these authors have shown that between both polysaccharides, a high compatibility occurs. They have found high improvements of the performances in terms of and impact tests results, which are

in part linked to usual matrix reinforcement (Bledzki and Gassan, 1999). Another part of the mechanical properties increase is brought by the inter-relations fibre-matrix. The main attributes are higher moduli (Dufresne and Vignon, 1998; Dufresne *et al*., 2000; Funke *et al*., 1998; Ave´rous *et al*., 2001; Curvelo *et al*., 2001), reduced water sensitivity due to fibre–matrix interactions and to the higher hydrophobic character of the cellulose, which is linked to its high cristallinity (Funke *et al*., 1998; Ave´rous *et al*., 2001; Curvelo *et al*., 2001). Fibres addition induces variation of properties, due to the formation of a 3D network between the different carbohydrates, through hydrogen bonds.

2.1.2 Biodegradable Film from Starch and Chitosan Blend

Previous study showed that cassava starch can readily be cast into films. However, the cassava starch film is brittle and weak leading to inadequate mechanical properties. Overcoming the brittleness of the film can be accomplished by adding plasticizers. Common plasticizers used for starch films preparation are water, glycerol, sorbitol, and other low-molecular weight polyhydroxy compounds (Rindlav *et al*., 1998). Water is an excellent plasticizer; however, it has some disadvantages since water content varies with humidity. At low humidity there are problems with brittleness and at high humidity with softness. Glycerol and sorbitol are widely used as plasticizers because of their stability and edibility. Addition of plasticizers makes the brittle films more flexible, but also less strong. This problem has led to the development of mechanical properties of cassava starch film. Blending (Chandra and Rustgi, 1998) or laminating (Coffin and Fishman, 1993) with other materials could improve the disadvantages. The scope of films made with starch combined with other polysaccharides was widened to include chitosan for several reasons. First, chitosan is a biopolymer, obtained by N-deacetylation of chitin, which is the second most abundant polysaccharide on the earth after cellulose (Arvanitoyannis *et al*., 1998). It is commercially available from a stable renewable source, that is, shellfish waste (shrimp and crab shells) of the sea-food industry. Second, chitosan forms good films and

membranes. Chitosan films that were clean, tough, flexible and good oxygen barriers were formed by solution casting (Jeon *et al*., 2002). Composited films from chitosan and cellulose have been made by casting dispersions on the steel or chrome plates at elevated temperatures from 70 to 100˚C (Nishiyama, 1993). Some of these films contained glycerol and had good tensile strength. They were readily biodegradable either in sea water or in soil. Third, the cationic properties of chitosan offer the film-maker an opportunity to take advantage of electrostatic interactions with other anionic polysaccharides. In addition, chitosan possesses useful properties such as biodegradability, biocompatibility (Sashiwa *et al*., 2003), and non-toxicity leading to extensively use over a wide range of applications. Chitosan film has a potential to be employed as packaging, particularly as an edible packaging. This is due to its excellent oxygen and carbon dioxide barrier properties and interesting antimicrobial properties.

2.2 Chitosan

2.2.1 History of Chitosan

The history of chitosan can be traced back to 1811 when chitin was first discovered by Braconnot, a professor of the natural history in France. According to some researches, while Braconnot was conducting research on mushrooms, he isolated what was later to be called chitin (Dutta, 2009).

Twenty years later, there was a man who wrote an article on insects in which he noted that similar substance was present in the structure of insects as well as the structure of plants. He then called this astounding substance as chitin (Dutta, 2009).

Basically, the name chitin is derived from Greek, meaning tunic or envelope. The concept was further known in 1843 when Lassaigne demonstrated the presence of nitrogen in chitin (Franciele, 2009).

Following the discovery of chitin, the name chitosan emerged in the scene. It was first discovered by Rouget while experimenting with chitin. Rouget observed that the compound of chitin could be manipulated through chemical and temperature treatments for it to become soluble. Then, it was in 1878 when Ledderhose identified chitin to be made of glucosamine and acetic acid. It was not actually until 1894 that Hoppe-Seyler named the tailored chitin, chitosan (Franciele, 2009).

During the early 20th century, several researches took chitosan as their subject of study. They then involved sources of chitin, including crab shells and fungai. It was the work of Rammelberg in the 1930s that led to the confirmation on the identity of chitosan from these sources. It was also noted that by hydrolyzing chitin in several ways, it was determined by experts that chitin is a polysaccharide of glucosamine (Cholwasa, 2006).

During the 1950s, the use of x-ray analysis had advanced the study of the incidence of chitin or chitosan in fungi. However, it is only the most advanced technologies that proved the most reliable in accepting the existence of chitin as well as cellulose in the cell walls (Fujun, 2009).

2.2.2 Properties of Chitosan

Chitosan, b-1, 4 linked glucosamine and N-acetyl glucosamine, is prepared by deacetylation of chitin. Chitosan has been proved to be nontoxic, biodegradable, biofunctional, biocompatible and have antimicrobial characteristics (Wang, 1992; Darmadji and Izumimoto, 1994; Jongrittiporn *et al*., 2001).The reasons for chitosan

addition in edible films are the good film forming and mechanical properties, no toxicity, biodegradability, relative more hydrophobic nature that could provide higher moisture barrier and water resistance (Bangyekan *et al*., 2006; Mathew and Abraham, 2008).

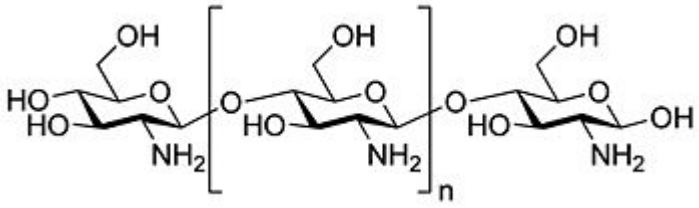


Figure 2.1: Structural molecule of chitosan

2.3 Tapioca/Cassava Starch

Cassava or tapioca is one of the economically important crops in Thailand and the cheapest raw material of starch production. Structurally, cassava starch consists of two types of molecules: amylose, a substantially linear polymer with a molecular weight of about 105; and amylopectin, a highly branched polymer with very high molecular weight of about 107. The approximate 17% of amylose content is responsible for strong film forming characteristics (Rindlav *et al*., 1998).

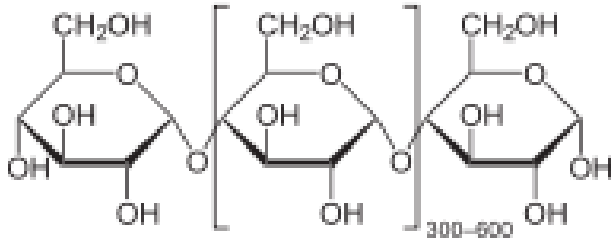


Figure 2.2: Amylose molecule

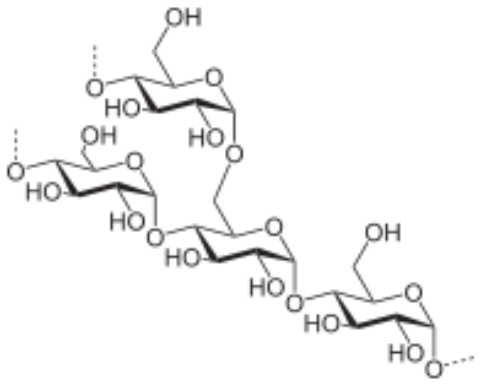


Figure 2.3: Amylopectin molecule

Tapioca starch, naturally or modified, is increasing its utility in food industry because it has some inherent properties that are demanded. The preferred properties of cassava starch include: high transparency, determining suitability for developing sauces for ready-to-eat foods; high resistance to acidity, allowing its use for acid-based sauces and jams. It is also applicable for desserts, puddings, soups, fillings and gums due to its high viscosity. As an alternative starch it could replace traditional starches because it is also a lower cost option. Unfortunately it is not easy to replace starches traditionally used because it is difficult to overcome the strong links that exist between producers, starch manufacturers and food industries that utilize this polysaccharide in main importing countries of Europe and North America (Fao, 2004). For these reasons, it is interesting to explore the possibility of developing new products based on tapioca starch, to research about alternative applications and to solve the lack of information about its role as alternative source of starch tending to help to increase its added value (Silvia, 2006)

The formation of starch edible film involves gelatinization of starch granules by heating in excess water. This procedure results in granule swelling and disruption as well leaching of soluble components (amylose) from the granule. A viscous mass is obtained and it consists of a continuous phase constituted basically by solubilized amylase and a discontinuous phase of remnant granules, mainly based on amylopectin

(Zobel, 1994). Cooling of the hot paste, results in a visco-elastic gel. The formation of the junction zones (polymer molecules joined by covalent bonds, hydrogen bonding and/or Van der Waal forces) of a gel can be considered to be the first stage of an attempt by starch molecules to crystallize. The collective processes that take part in the reduction of the solubility of dissolved starch are called retrogradation and involve the two constituent polymers, amylose and amylopectin, with amylase undergoing retrogradation at a much more rapid rate than does amylopectin. The rate of retrogradation depends on several variables, including the molecular ratio of amylase to amylopectin, structures of the amylose and amylopectin molecules (botanic source of the starch), starch concentration, presence and concentration of other ingredients, such as surfactants, lipids and salts, processing conditions like temperature and shear (Miller and Whistler, 1996). Gelation and retrogradation can be interpreted as the result of double helices forming a network of physically cross-linked molecules. As initial juncture points grow into helical segments and then aggregate into A–B-type crystallites, gels or retrograded materials become more rigid and difficult to disperse (Zobel, 1994).

2.4 Polyethylene Glycol 400 (PEG 400)

Polyethylene Glycol 400 (PEG 400) is a low molecular weight grade of polyethylene glycol. It is a clear, colorless and viscous liquid. Due in part to its low toxicity, PEG 400 is widely used in a variety of pharmaceutical formulations.

PEG 400 is strongly hydrophilic, the partition coefficient of polyethylene glycol 414 between hexane and water is 0.000015 (log$P$ = - 4.8), indicating that when polyethylene glycol 414 is mixed with water and hexane, there are only 1.5 parts of polyethylene glycol 414 in the hexane layer per 100,000 parts of polyethylene glycol 414 in the water layer. PEG 400 is soluble in water, acetone, alcohols, benzene,

glycerin, glycols, and aromatic hydrocarbons and is slightly soluble in aliphatic hydrocarbons (Renoulf *et al*., 2005).

The higher concentration of polyethylene glycol in the composite films, the more higher the water vapour 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 PEG, thus decrease the material cohesion by creating intermolecular spaces and increasing water molecule diffusion coefficient or the easier separation of PEG with the amorphous phase of polylactic acid (Renoulf *et al*., 2005).

The content of PEG will also reduce the rigidity and the brittleness of materials, thus improving their mechanical properties and their recovery. It is due to hydrolytic reactions by water absorption (Renoulf *et al*., 2005).

2.5 Food-borne Bacteria

Preservation of foods has, since the beginning of mankind, been necessary for our survival. The preservation techniques used in early days relied without any understanding of the microbiology on inactivation of the spoiling microorganisms through drying, salting, heating or fermentation. These methods are still used today, albeit using less and less preservation and combining various lightly preservation procedures to inhibit growth of microorganisms. Spoilage is characterized by any change in a food product that renders it unacceptable to the consumer from a sensory point of view. This may be physical damage, chemical changes (oxidation, color changes) or appearance of off-flavors and off-odors resulting from microbial growth and metabolism in the product. Microbial spoilage is by far the most common cause of spoilage and may manifest itself as visible growth (slime, colonies), as text-ural changes (degradation of polymers) or as off-odors and off-flavors. Despite chill chains, chemical

preservatives and a much better understanding of microbial food spoilage, it has been estimated that 25% of all foods produced globally is lost post harvest or post slaughter due to microbial spoilage (Hormazabal, 2007).

Food-borne diseases are still a major concern in some developing countries. Due to the world awareness on chemical preservatives the food industry is now reflected by the consumer opinions for safer additives and thus focusing on natural preservatives (Dillon and Board, 1994). Spices are herbal products which have been safely used by people around the world to impart desirable flavors and aromas to the local foods. It looks that there has been a natural selection for spices as these products are mainly originated from plants grown in the tropical regions with wide distribution of food-borne bacteria. Several of these spices and their essential oil extracts have been reported to possessed antimicrobial activities including garlic, savory, basil, laurel, mint, cumin, onion, sumac, thyme and lemon grass (Arora and Kaur, 1999; Delgado *et al.,* 2004; Nasar *et al.,* 2004; Ozcan and Erkmen, 2001).

2.5.1 Gram Positive Bacteria

Identification of the Gram-positive bacteria has traditionally relied on the thick peptidoglycan cell wall found in most members of this group (Bone and Balkwill, 1998; Buck, 1992; Doetsch, 1991). However, some species those are Gram-positive by phylogenetic criteria (Woese, 1997) which are lack typical cell walls and have variable or negative Gram stain reactions (Stackebrandt *et al.,* 1995). Branched-chain fatty acids have been considered as a marker for actinomycetes and Gram-positive bacteria in natural samples (Zelles and Bai, 1994), but branched-chain fatty acids are also produced by several other bacterial groups (Zelles *et al.,* 1995) in gram positive bacteria.

2.5.1.1 *Bacillus Subtilis (B. Subtilis)*

Bacillus subtilis, known as grass bacillus, is a Gram-positive, catalase-positive bacterium commonly found in soil (Madigan, 2005). A member of the genus *Bacillus*, *B. subtilis* is rod-shaped, and has the ability to form a tough, protective endospore, allowing the organism to tolerate extreme environmental conditions. Unlike several other well-known species, *B. subtilis* has historically been classified as an obligate aerobe, though recent research has demonstrated that this is not strictly correct (Nakano, 1998).

In 1835, the bacterium was originally named *Vibrio subtilis* by Christian Gottfried Ehrenberg (Ehrenberg, 1835), and renamed *Bacillus subtilis* by Ferdinand Cohn in 1872 (Nakamura, 1997). Cultures of *B. subtilis* were used throughout the 1950s as an alternative medicine due to the immune stimulatory effects of its cell matter, which upon digestion has been found to significantly stimulate broad spectrum immune activity including activation of specific antibody IgM, IgG and IgA secretion (Cohn, 1992) and release of CpG dinucleotides inducing INF A/Y producing activity of Leukocytes and Cytokines important in the development of cytotoxicity towards tumor cells (Shylakhovenko, 2003).

B. subtilis is not considered a human pathogen; it may contaminate food but rarely causes food poisoning (Ryan, 2004). *B. subtilis* produces the proteolytic enzyme subtilisin. *B. subtilis* spores can survive the extreme heating that is often used to cook food, and it is responsible for causing ropiness which is a sticky, stringy consistency caused by bacterial production of long-chain polysaccharides in spoiled bread dough.

The significance of the *Bacillus subtilis* group in food borne disease remains uncertain (Kramer and Gilbert, 1999). The identification of an enteric pathogen is dependent on demonstration of known enterotoxin genes and the functional activity of the toxins and direct proof of enterotoxigenic activity relies on animal models of

various kinds (Sears and Kaper, 1996). Given the drive to minimize the amount of animal testing, a number of in vitro assays have been employed as surrogate markers of enterotoxigenic activity. In vitro cytotoxicity assays using cell lines or erythrocytes are widely used tests. Surfactin represents a family of structurally similar heat stable cyclic lipopeptides which possess potent surfactant activity (Peypoux *et al.,* 1999). Surfactin-like compounds have been shown to be produced by *Bacillus* species other than *B. cereus* which have been isolated from food poisoning incidents (Mikkola *et al.,* 2000; From *et al.,* 2005).

2.5.2 Gram Negative Bacteria

Gram-negative bacteria are those bacteria that do not retain crystal violet dye in the Gram staining protocol (Salton, 1996). In a Gram stain test, a counterstain (commonly safranin) is added after the crystal violet, coloring all Gram-negative bacteria with a red or pink color. The test itself is useful in classifying two distinct types of bacteria based on the structural differences of their cell walls. On the other hand, Gram-positive bacteria will retain the crystal violet dye when washed in a decolorizing solution (Reid, 2001).

The pathogenic capability of Gram-negative bacteria is often associated with certain components of Gram-negative cell walls, in particular the lipopolysaccharide (also known as LPS or endotoxin) layer (Salton, 1996). In humans, LPS triggers an innate immune response characterized by cytokine production and immune system activation. Inflammation is a common result of cytokine production, which can also produce host toxicity (Reid, 2001).

2.5.2.1 *Escherichia Coli (E. coli)*

Escherichia coli (E. coli) is a Gram negative rod-shaped bacterium that is commonly found in the lower intestine of warm-blooded organisms (endotherms). Most *E. coli* strains are harmless, but some, such as serotype O157:H7, can cause serious food poisoning in humans, and are occasionally responsible for product recalls (Vogt, 2005). The harmless strains are part of the normal flora of the gut, and can benefit their hosts by producing vitamin K_2, and by preventing the establishment of pathogenic bacteria within the intestine (Reid, 2001; Feng. 2002)

E. coli are not always confined to the intestine, and their ability to survive for brief periods outside the body makes them an ideal indicator organism to test environmental samples for fecal contamination (Feng, 2002; Thompson, 2007). The bacteria can also be grown easily and its genetics are comparatively simple and easily-manipulated or duplicated through a process of mutagenic, making it one of the best-studied prokaryotic model, and an important species in biotechnology and microbiology. *E. coli* was discovered by German pediatrician and bacteriologist Theodor Escherich in 1885 (Feng, 2002), and is now classified as part of the Enterobacteriaceae family of gamma-proteobacteria (Lawrence, 2003).

2.6 Antimicrobial Agent

2.6.1 Lemon Grass Essential Oils

Lemon grasses (*Cymbopogon* spp., Poaceae) are tufted perennial C% grasses with numerous stiff stems arising from a short, rhizomatous rootstock (Weiss, 1997). Lemongrasses are indigenous in tropical and semi-tropical areas of Asia, and are

cultivated in South and Central America, Africa and other tropical countries (Weiss, 1997). Both *Cymbopogon exuosus* also known as East Indian or Cochin and lemon grass as a *Cymbopogon citrates* (DC.). Lemon grass (*Cympopogon citratus L*.) is a plant in the grass family that contains 1 to 2% essential oil on a dry basis with widely variation of the chemical composition as a function of genetic diversity, habitat and agronomic treatment of the culture (Carlson *et al.,* 2001). Lemon grass essential oil is characterized by a high content of citral (composed of neral and geranial isomers (c. 69%)), which is used as a raw material for the production of ionone, vitamin A and beta carotene (Paviani *et al.,* 2006). Several studies reported antimicrobial activities (even for human pathogenic fungi) by lemon grass oil (Appendini and Hotchkiss, 2002; Daferer *et al.,* 2003; Hammer *et al.,* 1999; Plotto *et al.,* 2003; Saikia *et al.,* 2001; Serrano *et al.,* 2005). Indeed, the lemon grass oil exhibited a broad spectrum of fungi toxicity by inhibiting completely growth of 35, 45, and 47 fungal species at 500, 1000, and 1500 ppm, respectively, and its fungi toxic potency remained unaltered for 210 days of storage, after which it started to decline, with considerable interests in the application of lemon grass oil for the preservation of stored food crops (Mishra and Dubey, 1994). Moreover, the essential oil of C. citratus was superior to synthetic fungicides like Agrosan GN, Dithane M-43 and copper oxychloride (Mishra and Dubey, 1994; Adegoke and Odesola, 1996). Lemon grass as well as oregano and bay oil inhibited all microorganisms examined at ≤2% (v/v) (Adegoke and Odesola, 1996; Hammer et al., 1999). Moreover, lemon grass oil was non-phyto toxic in nature, since it did not exhibit any adverse effects on germination and seedling growth of wheat and rice (Mishra and Dubey 1994). Interestingly, lemon grass oil showed higher activity than pure isolate (citral) as reported by Saikia *et al*. (2001).

2.6.1.1 Antifungal Activity

Lemon grass essential oil may possess antifungal activity and can be exploited as an ideal treatment for future plant disease management programs eliminating fungal spread. Recently, there has been a considerable interest in extracts and essential oils

from aromatic plants with antimicrobial activities for controlling pathogens and/or toxin producing microorganisms in foods (Reddy *et al.*, 1998; Soliman and Badeaa, 2002; Valero and Salmeron, 2003). Essential oils are natural products extracted from vegetal materials, which because of their antibacterial, antifungal, antioxidant and anti-carcinogenic properties can be used as natural additives in many foods (Teissedre and Waterhouse, 2000). In general, the levels of essential oils and their compounds necessary to inhibit microbial growth are higher in foods than in culture media. This is due to interactions between phenolic compounds and the food matrix (Nuchas and Tassou, 2000) and should be considered for commercial applications. Treatment with basil oil controlled crown rot and anthracnose prolonging storage of bananas (Anthony *et al.*, 2003) as well as cinnamon and eucalyptus oil-enrichment reduced fruit decay and improved fruit quality of tomato and strawberries. However, phyto toxicity on the fresh commodity is also to be considered while lemon grass emulsions were more damaging to the tomato tissue than thyme or oregano essential oils (Plotto *et al.*, 2003). Suppression on spore production by oil treatment could make a major contribution to limiting the spread of the pathogen by lowering the spore load in the storage atmosphere and on surfaces. The mechanism underlying the action of essential oil-enrichment on the switch between vegetative and reproductive phases of fungal development remains to be understood. The impacts of oils on sporulation may reflect effects of the volatiles emitted by oils on surface mycelia development (and thus the 'platform' to support spore production) and/or the perception/ transduction of signals involved in the switch from vegetative to reproductive development (Nikos, 2007).

2.6.1.2 Antimicrobial Activity

Lemon grass produces up to 75 to 85% citral in their essential oils (Formacek and Kubeczka, 1992). Citral is the name given to a natural mixture of two isomeric acyclic monoterpene aldehydes: geranial (*trans*citral, citral A) and neral (*cis*-citral, citral B). Normally, one isomer does not occur without the other. In addition to citral, the essential oil of *Cymbopogon* spp. consists of small quantities of geraniol,

geranylacetate and monoterpene olens, such as limonene (in *C. exuosus*) and myrcene (in *C. citratus*) (Formacek and Kubeczka, 1992; Weiss, 1997). Because of its characteristic lemon aroma, citral is of considerable importance in the food and flavour industry. Citral is also an important raw material used in the pharmaceutical, perfumery and cosmetics industries, especially for the synthesis of Vitamin A and ionones; synthetic citral, derived from conifer turpentine is normally used for those purposes (Dawson, 1994). Citral possesses antifungal activity against plant and human pathogen (Yousef *et al.,* 1997; Rodov *et al.,* 1995), inhibits seed germination (Dudai *et al*., 1994), and has bactericidal (Asthana *et al*., 1992; Kim *et al*., 1995) and insecticidal properties (Rice and Coats, 1994).

2.7 Characterization/Film Analysis

2.7.1 Scanning Electron Microscopy (SEM)

Scanning electron Microscopy (SEM) is a type of electron microscope that can take images of a sample surface by scanning it with a high-energy beam of electron 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 the information about the sample's surface topography, composition and other properties such as electrical conductivity (Umi *et al*., 2003).

The types of signals produced by an SEM include secondary electrons, back-scattered electrons (BSE), characteristic X-rays, light (cathodoluminescence), specimen current and transmitted electrons. Secondary electron detectors are common in all SEMs, but it is rare that a single machine would have detectors for all possible signals. The signals result from interactions of the electron beam with atoms at or near the surface of the sample. In the most common or standard detection mode, secondary

electron imaging or SEI, the SEM can produce very high-resolution images of a sample surface, revealing details about less than 1 to 5 nm in size. Due to the very narrow electron beam, SEM micrographs have a large depth of field yielding a characteristic three-dimensional appearance useful for understanding the surface structure of a sample. This is exemplified by the micrograph of pollen shown to the right. A wide range of magnifications is possible, from about 10 times (about equivalent to that of a powerful hand-lens) to more than 500,000 times, about 250 times the magnification limit of the best light microscopes (Qiangxian, 2008).

2.7.2 Differential Scanning Calorimetry (DSC)

Differential scanning calorimetry (DSC) is a thermoanalytical technique in which the difference in the amount of heat required to increase the temperature of a 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. The reference sample should have a well-defined heat capacity over the range of temperatures to be scanned (Wright, 2009).

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 (Preeti, 2005).

2.7.3 Thermo Gravimetric Analysis (TGA)

Thermo gravimetric analysis or thermal gravimetric analysis (TGA) is a type of testing that is performed on samples to determine changes in weight in relation to

change in temperature. Such analysis relies on a high degree of precision in three measurements: weight, temperature, and temperature change. As many weight loss curves look similar, the weight loss curve may require transformation before results may be interpreted. A derivative weight loss curve can be used to tell the point at which weight loss is most apparent. Again, interpretation is limited without further modifications and deconvolution of the overlapping peaks may be required (Preeti, 2005).

TGA is commonly employed in research and testing to determine characteristics of materials such as polymers, to determine degradation temperatures, absorbed moisture content of materials, the level of inorganic and organic components in materials, decomposition points of explosives, and solvent residues. It is also often used to estimate the corrosion kinetics in high temperature oxidation (Preeti, 2005).

2.7.4 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR is most useful for identifying chemicals that are either organic or inorganic. It can be utilized to quantitate some components of an unknown mixture. It can be applied to the analysis of solids, liquids, and gasses. The term Fourier Transform Infrared Spectroscopy (FTIR) refers to a fairly recent development in the manner in which the data is collected and converted from an interference pattern to a spectrum. Today's FTIR instruments are computerized which makes them faster and more sensitive than the older dispersive instruments (Svetlana, 2007)

Molecular bonds vibrate at various frequencies depending on the elements and the type of bonds. For any given bond, there are several specific frequencies at which it can vibrate. According to quantum mechanics, these frequencies correspond to the ground state (lowest frequency) and several excited states (higher frequencies). One way to cause the frequency of a molecular vibration to increase is to excite the bond by having it absorb light energy. For any given transition between two states the light

energy (determined by the wavelength) must exactly equal the difference in the energy between the two states (Svetlana, 2007).

Samples for FTIR can be prepared in a number of ways. For liquid samples, the easiest is to place one drop of sample between two plates of sodium chloride (salt). Salt is transparent to infrared light. The drop forms a thin film between the plates. Solid samples can be milled with potassium bromide (KBr) to form a very fine powder. This powder is then compressed into a thin pellet which can be analyzed. KBr is also transparent in the IR. Alternatively, solid samples can be dissolved in a solvent such as methylene chloride, and the solution placed onto a single salt plate. The solvent is then evaporated off, leaving a thin film of the original material on the plate. This is called a cast film, and is frequently used for polymer identification (Christort, 2002).

CHAPTER 3

METHODOLOGY

3.1 Preparation of Samples

3.1.1 Raw Materials and Equipment

In this research, there are three samples were produced with different composition of materials. In general, the materials used for this project are chitosan as the matrix material and tapioca starch as biodegradable filler. The chitosan used was chitosan, from crab shell in fine powder form, practical grade from Aldrich. For the preparation of composite solution, polyethylene glycol (PEG) with the molecular weight of 400 was used as the plasticizer. The solvent used was acetic acid with 1% v/v and distilled water. Gelatin also used as the additive and lemon grass essential oil to enhance the antimicrobial activity of the fabricated film. Mixing the solution requires the aid of several equipments such as magnetic stirrer and, plate heater. For the casting of the film, a large glass plate was used as a plane to pour over the well-mixed composite solution. A casting knife also will be needed to adjust the thickness of the sample when poured into the glass plate.

Three solutions were prepared for this research. The composition of each sample is as followed:

Table 3.1: The amount of each material.

	Sample A	Sample B	Sample C
Chitosan	2g	1g	2g
Tapioca starch	2g	2g	2g
Acetic Acid 1% v/v	100ml	100ml	100ml
Distilled water	100ml	100ml	100ml
PEG 400	2ml	2ml	2ml
Gelatin	-	1g	-
Lemon grass essential oil	-	-	0.25ml

3.1.2 Preparation of Chitosan Solutions

For preparation of sample A and C, 2g of chitosan of low molecular weight is dispersed in acetic acid (1% v/v). For sample B, 1g of chitosan is mixed with 1g of gelatin and dispersed in acetic acid (1% v/v). The dispersions are heated on slow heat (40ºC) on a hotplate for 4 hours under continuous stirring to dissolve chitosan completely.

3.1.3 Preparation of Tapioca Starch Solutions

For all samples, 2g Tapioca starch is dispersed in 100ml of distilled water. All dispersions are heated (80ºC) on a hotplate for 2 hours under continuous stirring to gelatinize starch completely.

3.1.4 Preparation of Blend Solutions (Sample A and B)

For the preparation of sample A and B, film-forming solutions are obtained by mixing chitosan and starch solution that prepared earlier. Then, 2ml of PEG 400 was added into the mixed solution and stirred for 4 hours under continuous stirring until it is completely mixed.

3.1.5 Preparation of Antimicrobial Films (Sample C)

For the preparation of sample C, film-forming solutions are obtained by mixing chitosan and starch solution that prepared earlier. Then, 2ml of PEG 400 and 0.25ml of lemon grass essential oil were added into the mixed solution and stirred for 4 hours under continuous stirring until it is completely mixed.

3.1.6 Film Casting and Peeling

After the solutions are well mixed, the solutions were degassed for 24 hours until no bubbles are visible. The prepared composite solutions were casted on a square large plate thoroughly in order to produce a film with smooth surface. The thickness of the poured degassed solutions can be adjusted using casting knife during casting process. Under ambient temperature, the casted film was left for 1 day to completely dry. Later, the film was carefully peeled from the glass plate when they are completely dry.

3.2 Film Characterization

There are six in total of the film characterization which two of them are antimicrobial analysis using Agar Diffusion Test (Zone Inhibition Assay) and Liquid

Culture Test (OD Measurement). Other four is characterization on the morphology of the films using Scanning Electron Microscopy (SEM), Fourier Transform Infrared Spectroscopy (FTIR) to identify the chemical bonds in the films, Differential Scanning Calorimeter (DSC) to identify the melting temperature of the films and Thermo Gravimetric Analysis (TGA) to measure the thermal degradation of the samples prepared.

3.2.1 Antimicrobial Analysis

3.2.1.1 Agar Diffusion Test (Zone Inhibition Assay)

Antimicrobial activity test is carried out using agar diffusion method. Indicator cultures are *Bacillus Subtilis* and *Escherichia coli*, representing Gram-positive and Gram-negative bacteria. Two hundred microliters of the inoculum solution is added to 5 ml of the appropriate soft agar, which is overlaid onto hard agar plates. Each film is cut into squares (2cm x 2cm) and placed on the bacterial lawns. The plates are incubated for 48 h at 37ºC in the appropriate incubation chamber. The plates are visually examined for zones of inhibition around the film disc, and the size of the zone diameter is measured at two cross sectional points and the average is taken as the inhibition zone.

3.2.1.2 Liquid Culture Test (Optical Density Measurement)

For the liquid culture test, each film is cut into squares (1cm x 1cm). Three sample squares are immersed in 20 ml nutrient broth in a 25 ml universal bottle. The medium is inoculated with 200µl of *Escherichia coli* and *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 is sampled periodically (0, 2, 4, 8, 12, 24 hours) during the incubation to obtain microbial growth profiles. The optical density is measured at $\lambda = 600$nm using a spectrophotometer.

3.2.2 Scanning Electron Microscopy (SEM)

Film morphology was analyzed using Scanning Electron Microscopy (SEM EDX Spectrometer EVO 50) to observe the surface and cross section of the films. The dried film samples were mounted on a metal stub with double-sided adhesive tape. The images were taken at accelerating voltage 5.00 kV with magnification of 500 and 1000 times of origin specimen size.

3.2.3 Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR analysis was based on the identification of absorption bands concerned with the vibrations of functional groups present in the sample of the films. All spectra were recorded at ambient temperature using FTIR NICOLET AVATAR 370 DTGS. The characteristics absorption bands of the composite films were detected at wave numbers ranging from 500 to 4500 cm^{-1}.

3.2.4 Thermo Gravimetric Analysis (TGA)

Thermo gravimetric analysis was performed using a Thermal Gravimetric Analyzer (TGA). For this purpose a TGA (TGA Q500) was used to carry out measurements under steady flow of nitrogen atmosphere at constant heating rate 10°C/min in the range of temperature from 20°C to 600°C.

3.2.5 Differential Scanning Calorimeter (DSC)

DSC was used to measure the thermodynamic properties or to find the melting point of the materials. All the measurements were carried out with a model DSC Q1000 series (TA Instruments) operating under Nitrogen atmosphere. 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 again at the cooling rate of 10°C/min.

CHAPTER 4

RESULT & DISCUSSION

4.1 Antimicrobial Analysis

4.1.1 Agar Diffusion Test (Zone Inhibition Assay)

The details of antimicrobial effectiveness of fabricated films of sample A, B and C are shown in Figure 4.1. The inhibitory activity was measured based on the average diameter of the clear inhibition zone as shown in Table 4.1. Figure 4.2 summarizes the results of agar diffusion test using zone inhibition assay. From Figure 4.1, all prepared samples exhibited a positive reaction towards antimicrobial activity against *E. coli* and *B. Subtilis.* Sample C exhibited significantly a greater inhibition zone diameter (a: 4.5, b: 5.9) than sample A and B. The large microbial inhibition indicates that film incorporated with lemon grass essential oil exhibited significantly higher antimicrobial activity. This could be attributed by incorporated the lemon grass essential oil and makes the film exhibited antimicrobial activity against *E. coli* and *B. Subtilis* as was also reported by Onawunmi *et al.* (1984).

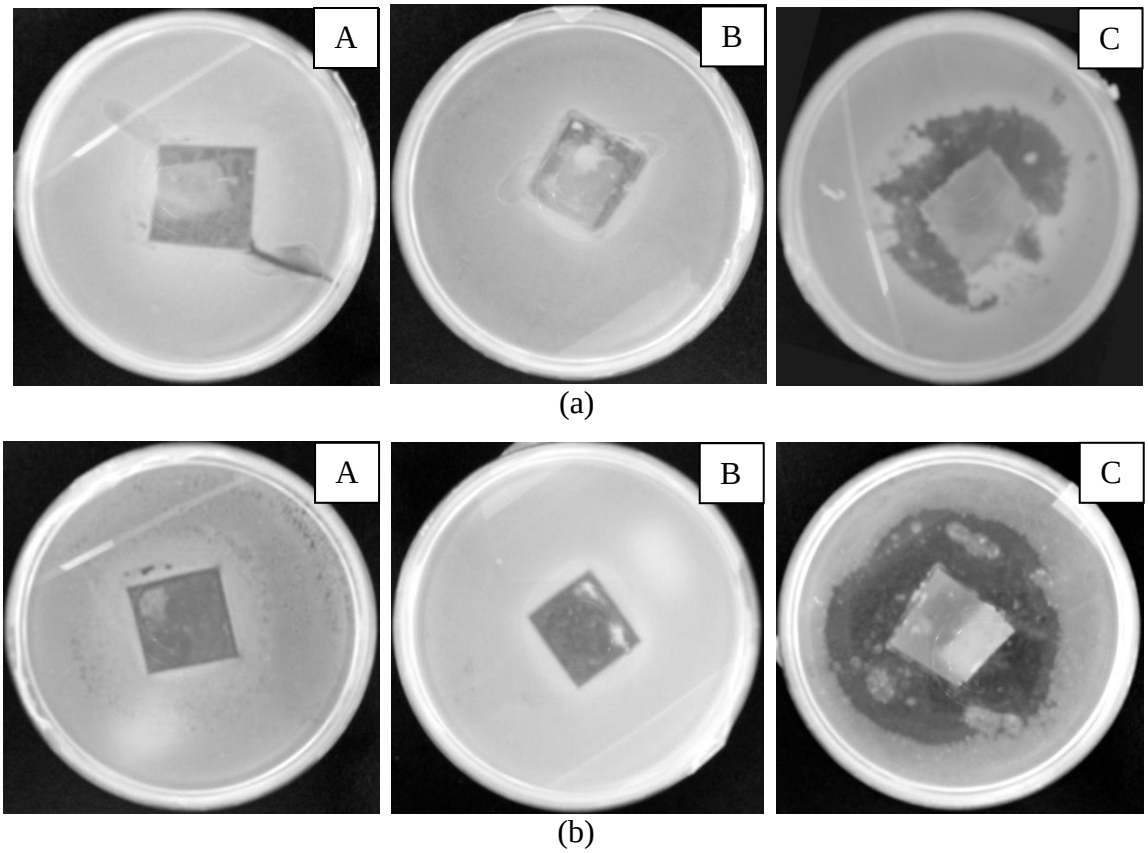


Figure 4.1: Comparison of inhibition area towards (a) *Escherichia coli* and (b) *Bacillus Subtilis* respectively.

On the other hand, samples A and B exert inhibitory action only at the film area. From several studies about biopolymer based films revealed the existence of hydrogen bonding type interactions involving chitosan and hydrophilic starch groups (Mathew and Abraham, 2008; Park *et al.*, 2004; Xu *et al.*, 2005). In summary, it is suggested that the antimicrobial action of chitosan was diminished by chitosan–tapioca starch which could reduce the availability of chitosan NH3 + position to interact with cell membrane (Coma *et al.*, 2003; No *et al.*, 2007).

Table 4.1: Inhibition of *B. subtilis* and *E. coli* on agar plates based on average zone diameter (cm) of inhibition zone.

	Sample A	Sample B	Sample C
E. Coli	2.0	2.0	4.5
B. Subtilis	2.0	2.0	5.9

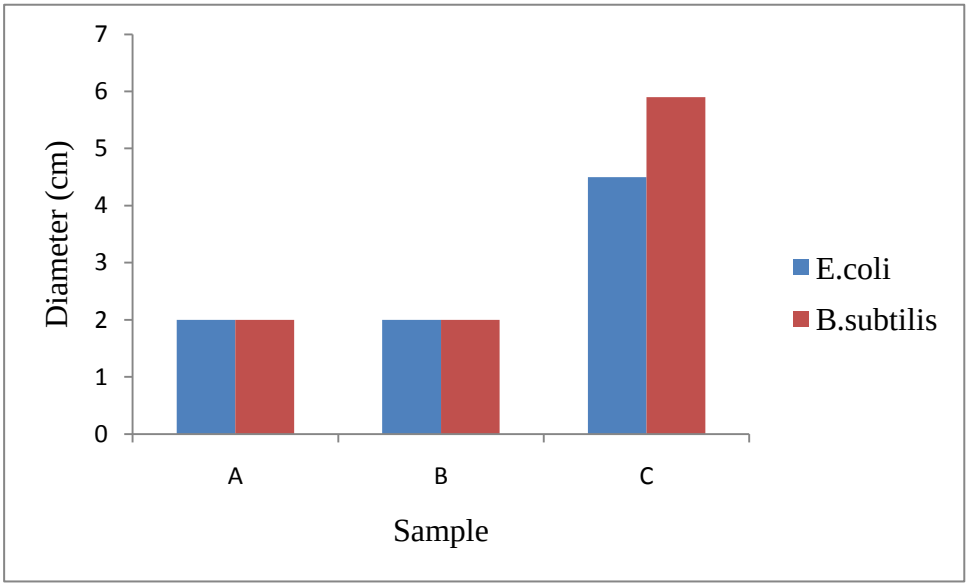
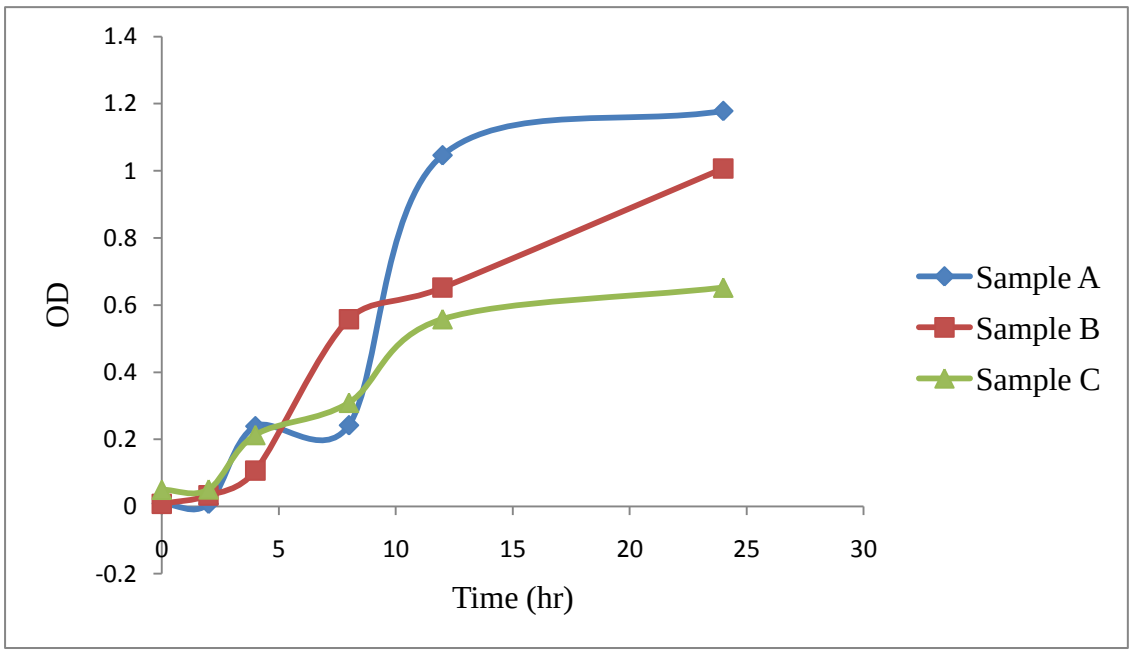


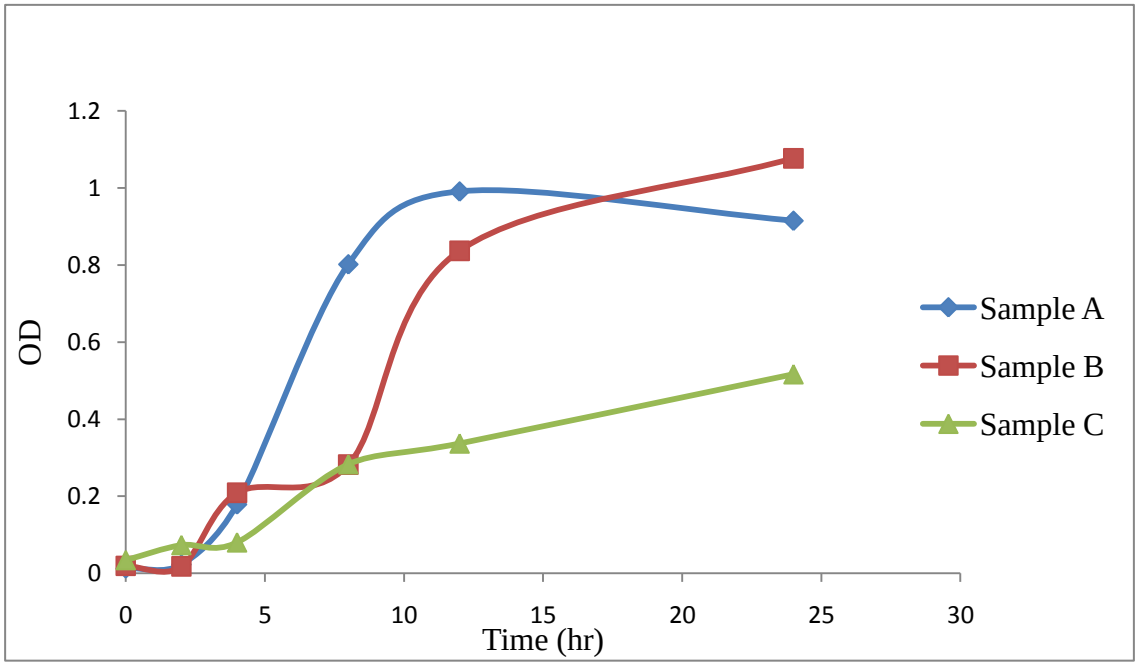
Figure 4.2: Graph of inhibition of *B. subtilis* and *E. coli* on agar plates figure based on average zone diameter (cm) of inhibition zone.

4.1.2 Liquid Culture Test (Optical Density Measurement)

In liquid cultures test, all samples are exhibited inhibition for both *B. subtilis* and *E. coli*. From Figure 4.3, sample C has shown higher inhibition because it has the lowest OD measurement in the growth profile towards both bacteria. Therefore, the incorporation of lemon grass helps to enhance the antimicrobial starch-chitosan based film strength and inhibited the growth of both bacteria.



(a)



(b)

Figure 4.3: Inhibition of sample A, B and C towards (a) *E .coli* and (b) *B. subtilis* in Liquid Culture Test (OD Measurement)

Sample A and B, also inhibited the growth of both bacteria since both of the films were antimicrobial starch-chitosan based film (Ban *et. al.,* 2005). In fact, one of the reasons for the antimicrobial character of chitosan it's 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 (Shahidi *et al.,* 1999). Sample A and B were not shown a higher inhibition maybe because low antimicrobial activity of chitosan films produced by casting is due to the previous dissolution of chitosan in acetic acid when filmogenic solution is prepared. This action protonates the NH_2 groups of chitosan and enhancing solubility. As in the extrusion process chitosan dissolution is unnecessary, the NH_2 groups are not protonated, which explains the low of the antimicrobial activity (Helander, 2001; Devlieghere, 2004).

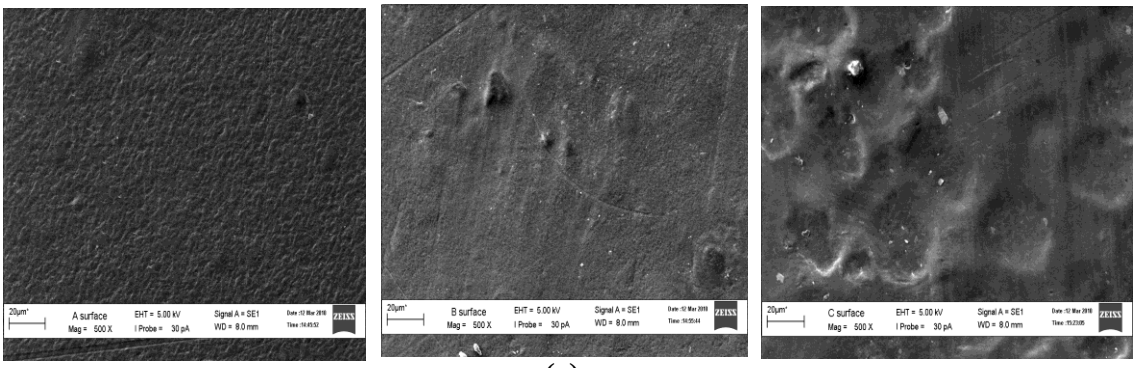
From Figure 4.3, the largest inhibition was observed for the Gram positive bacteria (b), while the smallest ones were observed for the Gram-negative bacteria (a). In the Gram-positive bacteria, the major constituent of its cell wall is peptidoglycan and there is very little protein. The cell wall of Gram-negative bacteria also has an outer membrane, which constitutes the outer surface of the wall (Zheng and Zhu, 2003). Study from Jiang *et al* (1997), 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. This study indicated that the mechanisms of the antimicrobial activity of chitosan were different between Gram-positive and Gram-negative bacteria. Additionally, the antimicrobial mechanism of chitosan might differ from that of other polysaccharides because there are positives charges on the surface of chitosan (Jiang *et al.,* 1997).

For sample C that is incorporated with lemon grass essential oil, most studies has shown the action of whole essentials oils (EOs) against food spoilage organisms and

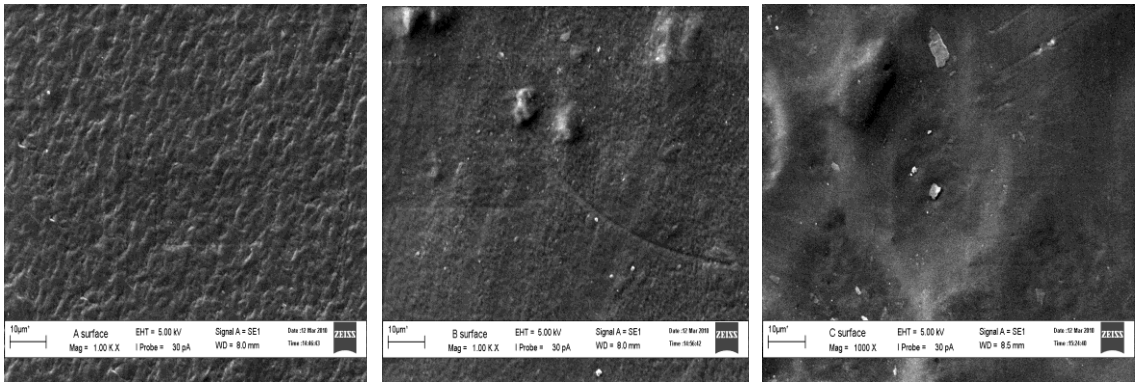
food-borne pathogens generally EOs are slightly more active against Gram-positive than Gram negative bacteria (Burt; 2004; Zivanovic, 2005).This result may be related to the presence of an additional external membrane surrounding the cell wall in Gram negative bacteria, which restricts diffusion of hydrophobic compounds through its lipo-polysaccharide covering (Burt, 2004). The proposed mechanism of antimicrobial activity of phenolic compounds of EOs is in their attack on the phospholipids 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 lipo-polysaccharides or outer membrane proteins (Zivanovic, 2005).

4.2 Scanning Electron Microscopy (SEM)

SEMs of the surfaces of the samples A, B and C are shown in Figure 4.4 (a) and (b). From the figure, sample A shows the surface to be relatively smooth, to be homogeneous and to be a continuous matrix without any pores or cracks with good structural integrity. It was flat and compact with very sparsely distributed small particles without any phase separation. Chitosan micro domains were dispersed within the starch matrix in the blend films with relatively good interfacial adhesion between the two components and were similar to the surface cellulose/ carboxymethylated-chitosan blends (Li, 2002). As the amylose molecules are preferentially dissolved by water and are easily released from the starch granules, it can be supposed that the continuous region (matrix) correspond to a network structure consisting mostly of amylose and chitosan (Jenkins, 1998)



(a)



(b)

Figure 4.4: SEM photographs of sample A, B and C respectively for (a) magnification 500X, (b) magnification 1000X for sample surface.

Sample B as compared to sample A and C, has residual granular structure present in the surface of the film. This is maybe ascribed to weak interaction between chitosan-starch with the additional gelatin. The blend films of starch-chitosan also exhibit such pattern because the increasing concentration of starch ratio to the chitosan since formulation for to produce sample B is 1 g chitosan and 2g starch. It is also maybe due to the blending film at low heat whereas water and plasticizer were not able to break up starch granules and disrupt intermolecular and intramolecular hydrogen bonds (Yu *et al.*, 2009). As shown in the Figure 4.4, sample C films had several unequalized holes, suggesting that the miscibility and compatibility in each component is lowest due to the incorporation of lemon grass essential oil.

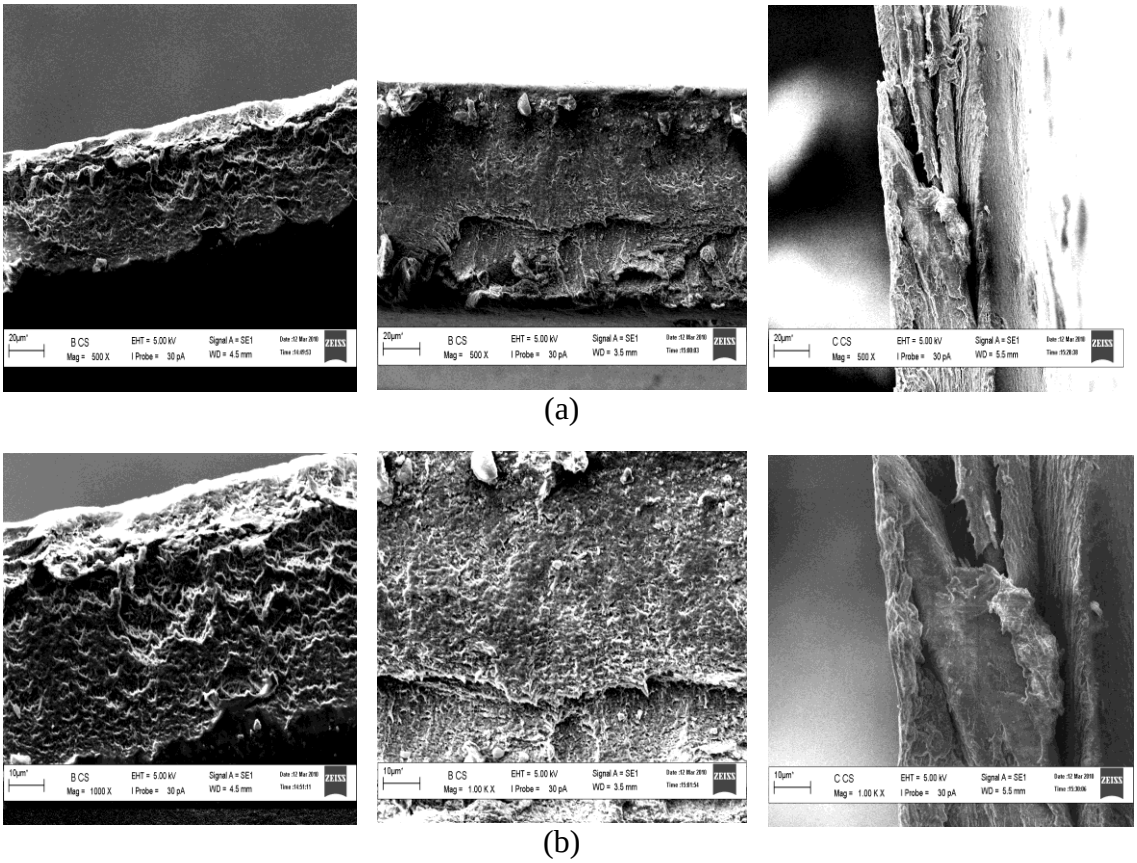


Figure 4.5: SEM photographs of sample A, B and C respectively for magnification (a) 500X, (b)1000X for sample cross section.

The SEM photographs of the cross sections for all samples are shown in Figure 4.5. Sample A and B exhibit smooth and compact cross section because of its good film forming properties and flexibility. For sample C, there is obvious phase separation was observed from SEM images. With additional of lemon grass essential has caused a layer structure as shown in the Figure 4.5. In another word, the composition of this film is poor due to low miscibility and weak interaction between chitosan-starch and lemon grass essential oil.

4.3 Fourier Transform Infrared (FTIR)

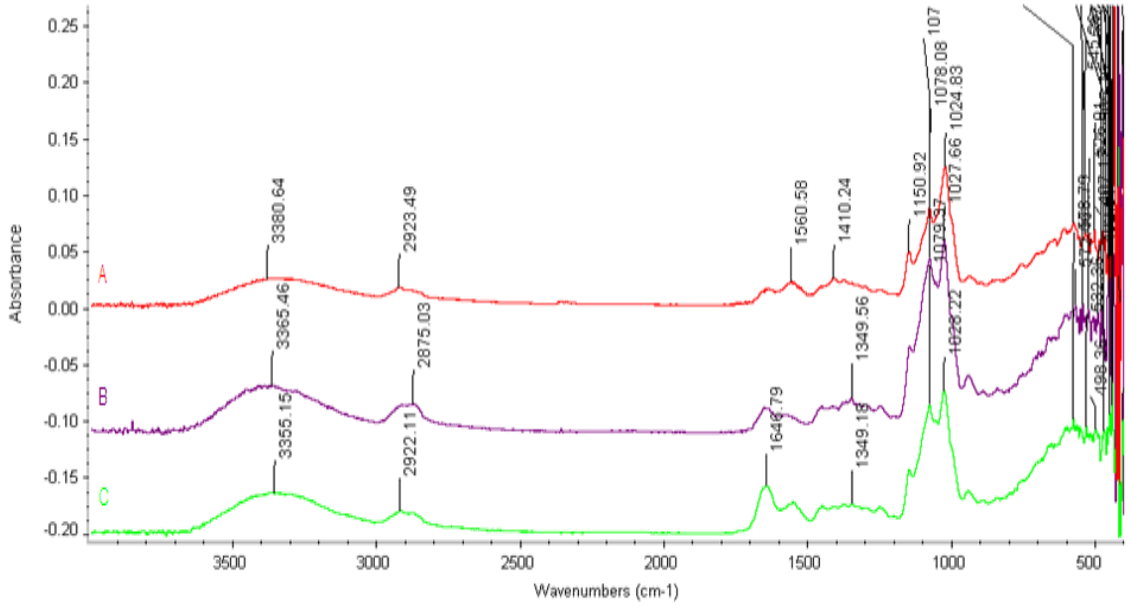


Figure 4.6: FTIR spectra of the samples in absorbance mode.

FTIR spectroscopy was used on the film samples to investigate the starch, chitosan, gelatin and lemon grass essential oil interactions. The infrared spectra of the sample A, B and C are given in Figure 4.6. The broad band at 3500 - 3000 cm-1 in the all films spectrum were ascribed to the O–H and N–H stretch vibrations as well as intermolecular hydrogen bonding of chitosan molecules, respectively (Hu *et al.*, 2007). Xu *et al*. reported that the chitosan film spectrum has abroad band at 3500 - 3000 cm-1 attributed to the OH stretching which overlaps with the stretching of NH in the same region. The band at 3000 - 2850 cm-1 corresponds to the C-H stretching, 1650 - 1350 cm-1 represent the deformation of the NH stretching plane, and the bands at 1160 – 1023 cm-1 result from the stretching of C-O in C-O-H and C-O in C-O-C bonds, respectively (Ning *et al.*, 2007).

The physical combinations as compared to the chemical interactions of two or more mixed substances are reflected by characteristic changes of the spectrum bands. The spectrum of the starch-chitosan film shows that the band relative to the amino group shifted to a high wavenumber from 1650 - 1350 cm-1, with the addition of starch. This result indicates the interaction of the hydroxyl groups of starch with the amino groups of chitosan (Xu *et al.,* 2007). The data obtained in this work suggest the compatibility of the two components, as well as their interaction.

The addition of lemon grass essential oil resulted in the higher absorbance between the range 1600 and 1400 cm-1 (Figure 4.6). This are attributed to the benzene ring insaturations and one to the actual ring. These bands are from the aromatic hydrocarbons in the composition of the lemon grass essential oil. Also, the abnormal stability resulting from the benzene ring resonance explains the preservation of this functional group in the extrusion process (Solomons *et al.*, 2001).

4.4 Thermo Gravimetric Analysis (TGA)

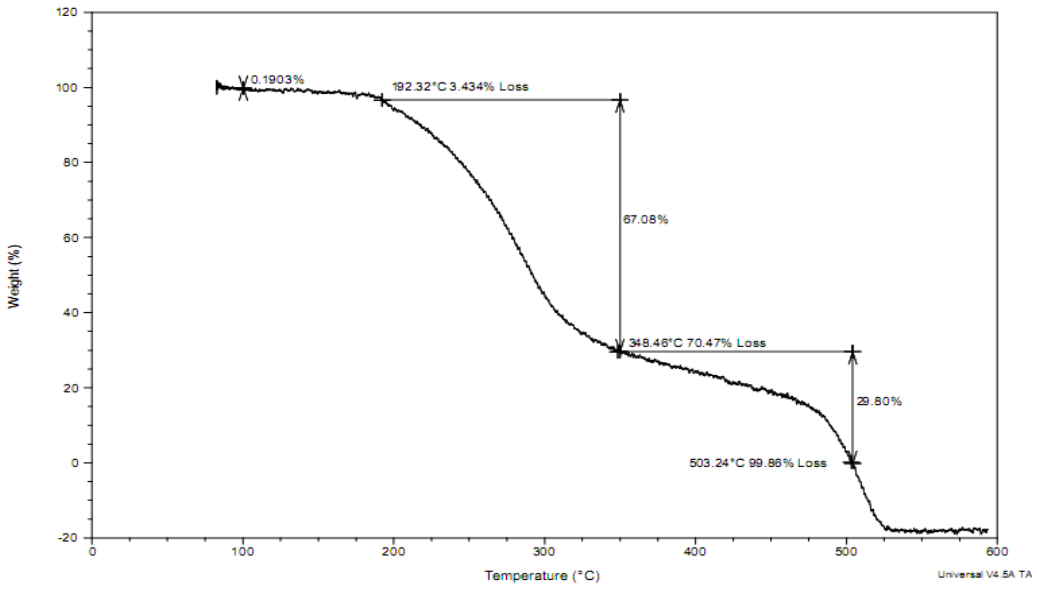


Figure 4.7: The result of TGA for sample A

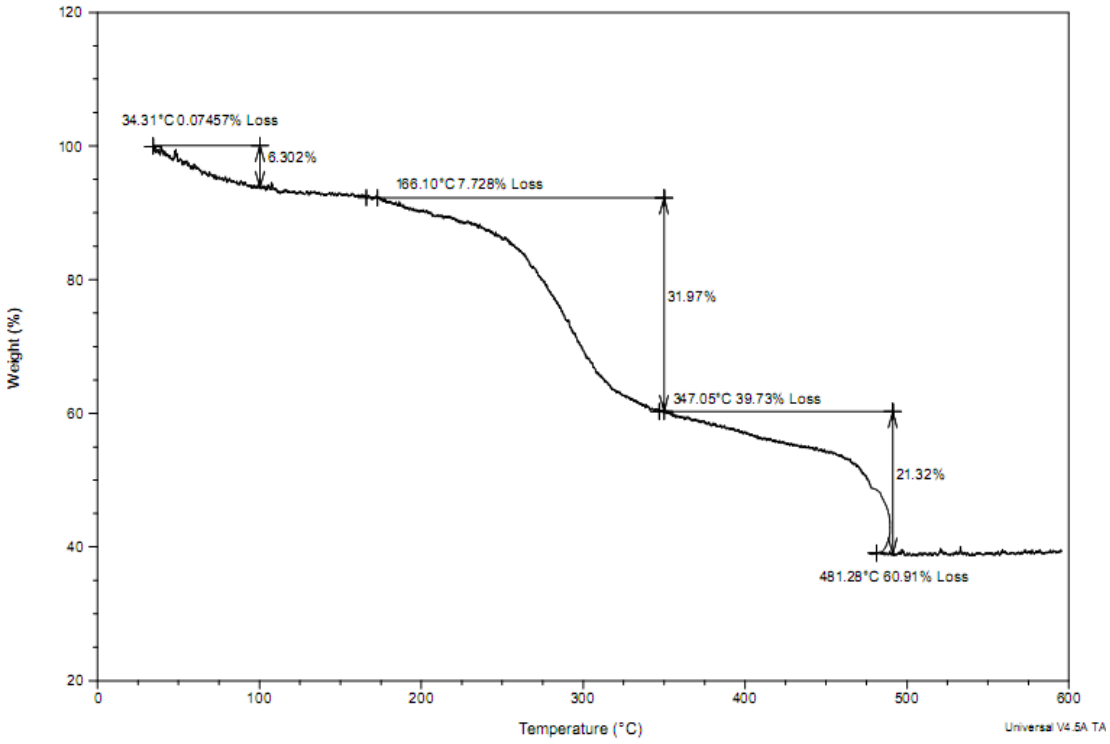


Figure 4.8: The result of TGA for sample B

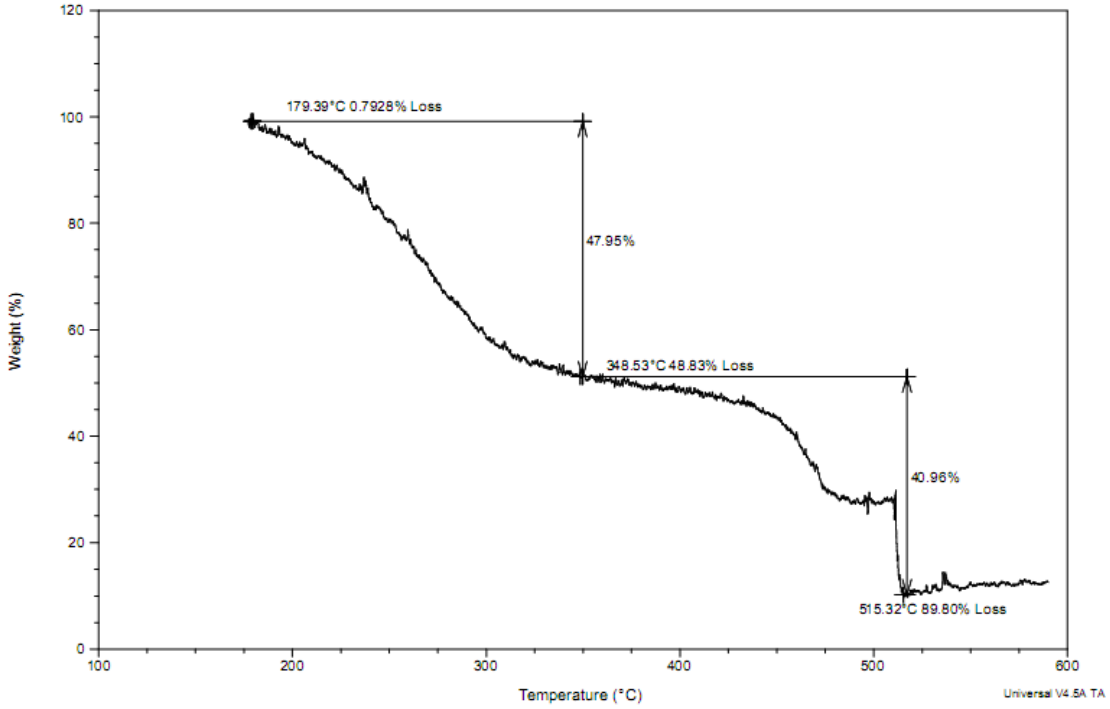


Figure 4.9: The result of TGA for sample C

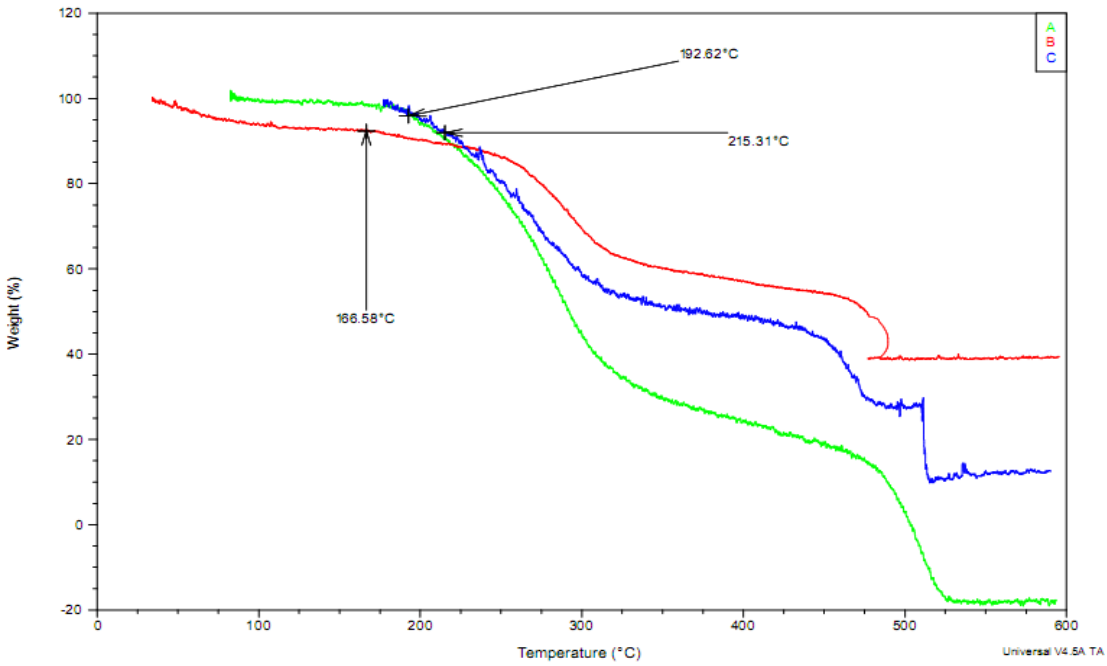


Figure 4.10: TGA curves for sample A, B and C.

TGA was performed to evaluate the thermal stability of the starch-chitosan (sample A), starch-chitosan with addition of gelatin (sample B), and starch-chitosan-lemon grass essential oil films (sample C). The TGA curves are shown in Figures 4.7, 4.8, 4.9 and 4.10. The TGA curves also are used to determine the weight loss of the material as it is heated.

The TGA curves of starch-chitosan film (sample A) showed an initial 0.1903% weight loss probably due to the moisture loss (Figure 4.7). Weight loss due to thermal degradation began at about 192.62˚C and accelerated quickly with higher temperature until about 0.14% of the original weight remained.

Weight loss for starch-chitosan with addition of gelatin (sample B) due to thermal degradation began at temperature 166.58˚C (Figure 4.10). There was an initial weight loss of about 6.302% (Figure 4.8) followed by the thermal degradation to start.

Sample A and B (Figure 4.7 and 4.8) presented three mass loss steps while sample C presented only two steps (Figure 4.9). The aromatic structures present in the lemon grass essential oil are highly stable due to the resonance of the benzene ring, which results in the decomposition of these compounds at higher temperatures (215.31˚C).

The first step, between temperature of 10 and 99˚C, is attributed to the evaporation of water absorbed by starch, chitosan, and PEG 400, along with the evaporation of the low molecular weight compounds. The weight loss in the second range (250–450˚C) corresponded to a complex process including the dehydration of the saccharide rings and depolymerization (Mathew and Dufresne, 2002). In Figure 4.10, decomposition of starch and chitosan occurs at approximately 250-350˚C. Similar results have been reported for these materials (Chen *et al.*, 2008; Cyras *et al.*, 2008; Zhang *et al.*, 2007). There is an increase in percent residue after the incorporation of the lemon grass essential oil was also observed.

4.5 Differential Scanning Calorimeter (DSC)

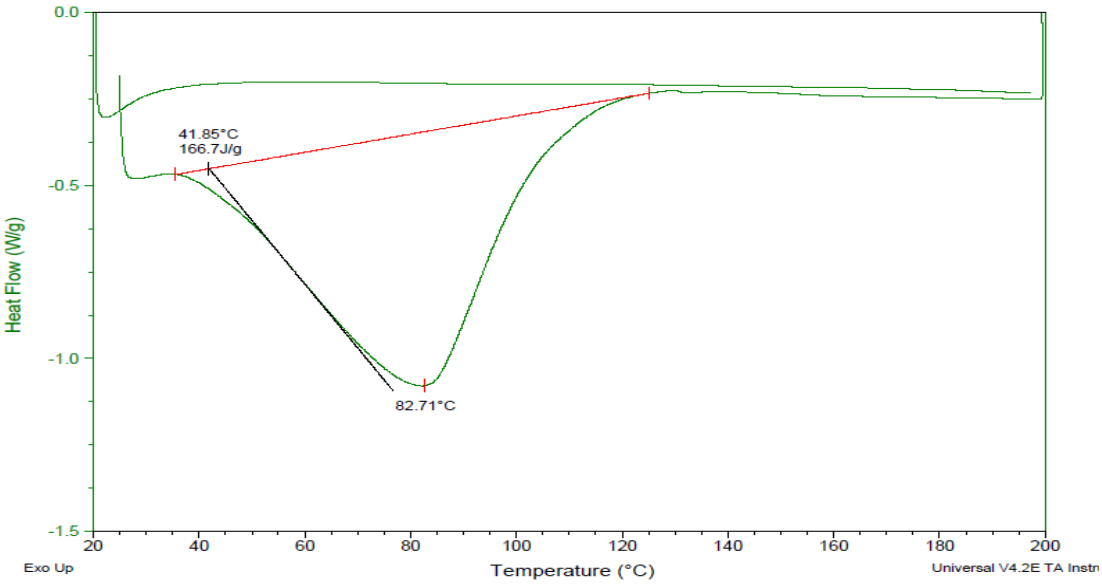


Figure 4.11: DSC thermogram for sample A.

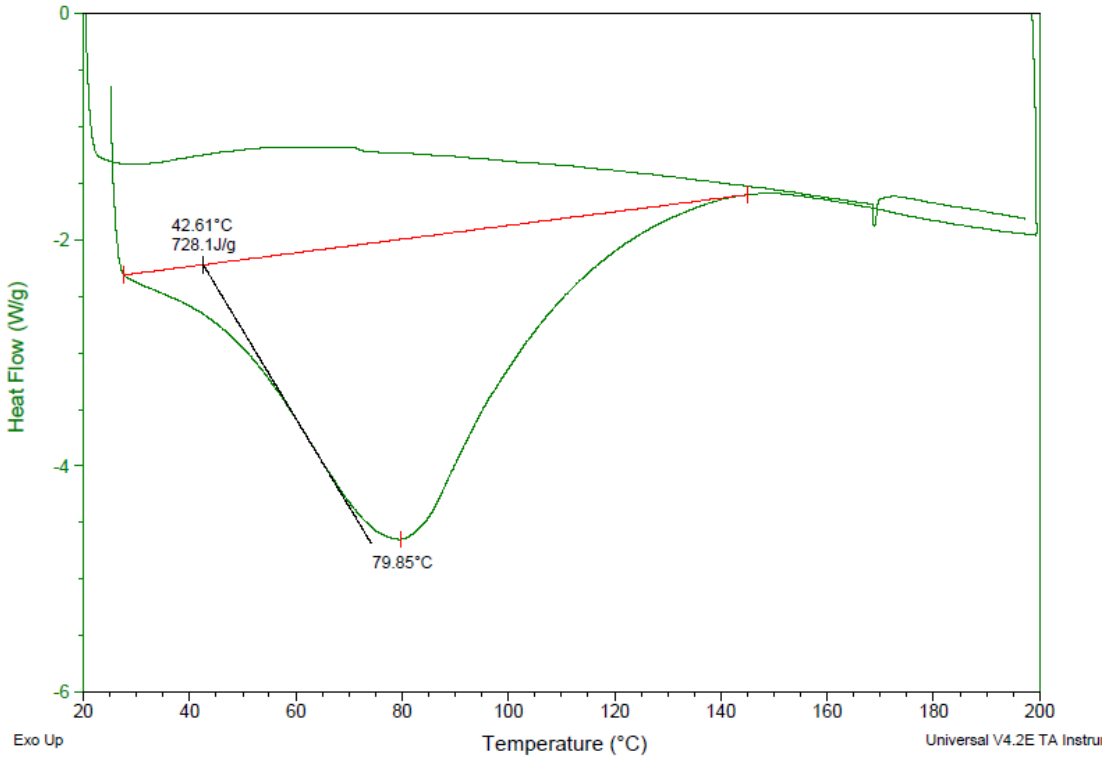


Figure 4.12: DSC thermogram for sample B.

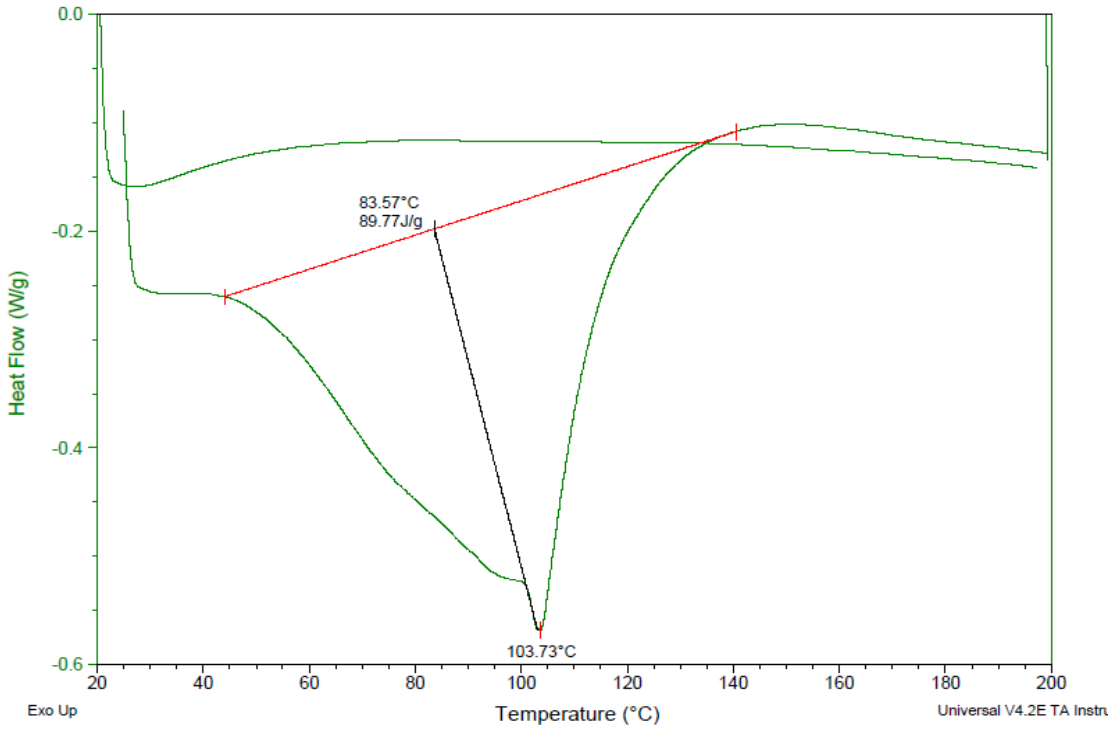


Figure 4.13: DSC thermogram for sample C.

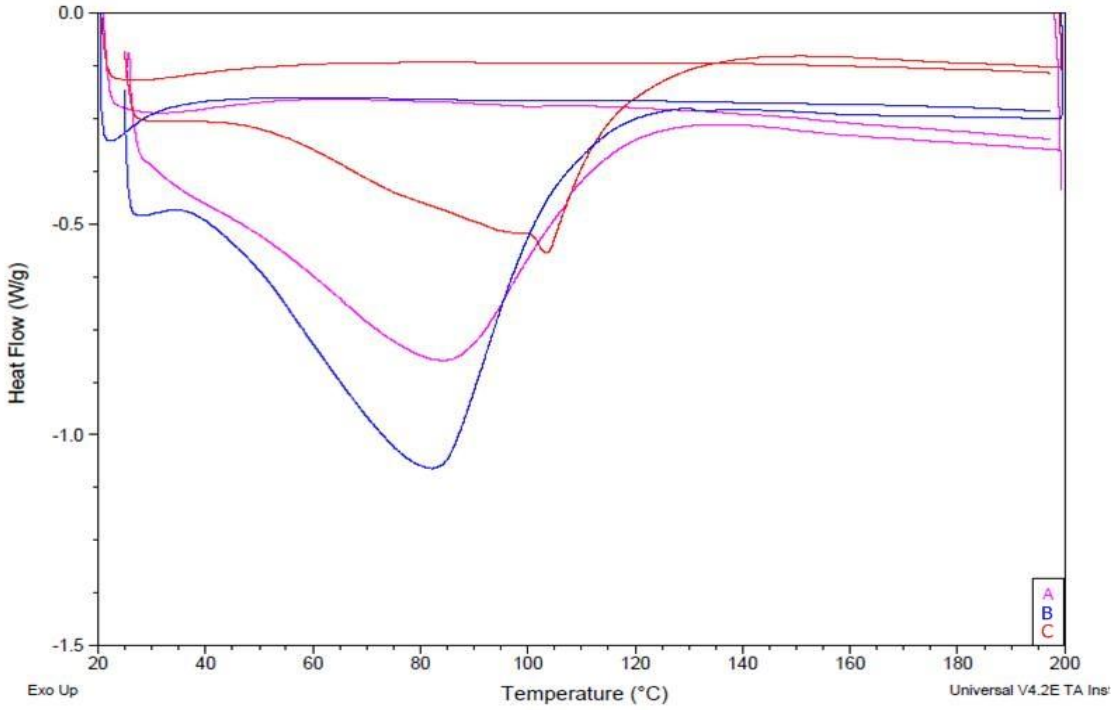


Figure 4.14: DSC thermogram for sample A, B and C.

To further understand the thermal properties of chitosan composite biodegradable films, DSC studies of all the samples containing are performed and shown in Figure 4.11, 4.12, 4.13, 4.14. This compound is semi crystalline and difficult to identify the glass transition temperature (T_g) of chitosan (Chen *et al.*, 2008). Water content is decreased in the blend films and stress of the blends film is released in the first stage of heating.

Table 4.2: Melting temperature of sample A, B and C.

Sample	**Melting Temperature, T_m (˚C)**
A	41.85
B	42.61
C	83.57

The endothermic peak in Figure 4.11, 4.12, 4.13, 4.14 is the melting temperature of the samples. The melting temperature (T_m) is shown in Table 4.2. It is observed that sample C has higher melting temperature (83.57˚C) compared to other two samples. As summarized in table 4.2, the melting temperature for sample A is 42.61˚C and for sample B is 41.85˚C. Melting temperature of sample B is quite low maybe due to that the incorporation of gelatin into starch matrix decreases the inter-molecular force of starch, and partly decreases the crystallinity of starch, resulting in a decrease in the degree of crystallinity in the blends. These results shown that these films were thermal stable and the degradation did not occur ranging from 20˚C to 200˚C (Li *et al.*, 2010).

Generally, the retrogradation of starch-chitosan biodegradable films is greatly dependent on the hydrogen bond-forming abilities of the additives with starch molecules. The stronger the hydrogen bond between starch and the additives, the more difficult for starch to re-crystallize during the storage period of (Cao *et al.*, 2007). It demonstrated that the incorporation of lemon grass essential oil formed relative strong hydrogen bonding between starch-chitosan and lemon grass essential oil to suppress starch retrogradation.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

This research was done to study the characteristic of the films produced with different composition of materials. Morphology, chemical composition and thermal properties of the composite biodegradable films were characterized to investigate their usability in bio-composite applications. From the experimental results, the film incorporated with the lemon grass essential oil shown a higher antimicrobial activity towards gram positive and gram negative bacteria compared to starch-chitosan based films. For thermal properties, the melting and degradation temperature between all samples are very close but sample C exhibit high thermal stability compared to sample A and B. From the SEM images, samples A and B shown the good miscibility and compatibility in each component for the surface and cross section images. But for sample C, it has poor miscibility and weak interaction between chitosan-starch and lemon grass essential oil.

From the experimental analysis, the film incorporated with lemon grass essential oil possesses higher antimicrobial activity, higher thermal stability, higher melting point and slightly miscible and compatible with the starch-chitosan composition. The addition of lemon grass essential oil proves that the film is better to film based on starch-

chitosan only. Lemon grass essential oil will actually improve the performance of the chitosan composite biodegradable film as tested in this research.

5.2 Recommendation

To enhance the results of the chitosan composite biodegradable films, there are several recommendations listed here. First, the incorporation of lemon grass essential oil has shown poor properties in terms of miscibility of compatibility in the starch-chitosan. It is maybe due to the higher ratios of lemon grass to the starch-chitosan solution. More experimental data will need to find the suitable ratios for this blend solution.

Second, use different plasticizer other than PEG 400 such as polylactic acid (PLA) or glycerol to improve the strength of the films. Others biodegradable fillers instead of tapioca starch also can be used as long as it will give rise to the strength of the films. This way, the research can study the effect of different variable to the properties of the composite film in order to produce a high quality film.

Third, there are other factors that must be tested before the lemon grass essential oil can be justified as the best additive for chitosan-starch composite biodegradable film. The mechanical properties, the tensile strength of the fiber, solubility in water and other more tests should be done in order to get more accurate and strong reasons to prove the effectiveness of incorporation of lemon grass essential oil.

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APPENDICES

MATERIALS AND METHODOLOGY

Figure 1: The tapioca starch powder used in this research

Figure 2: The chitosan powder used in this research

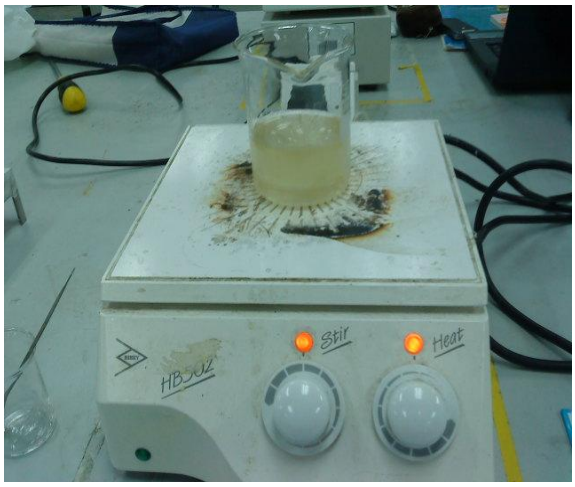

Figure 3: Preparation of chitosan solution

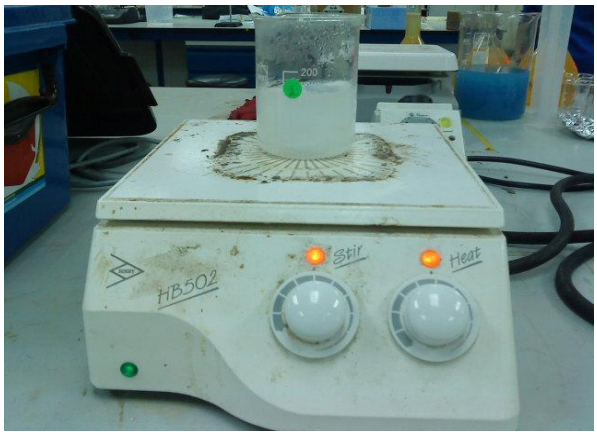

Figure 4: Preparation of tapioca starch solution

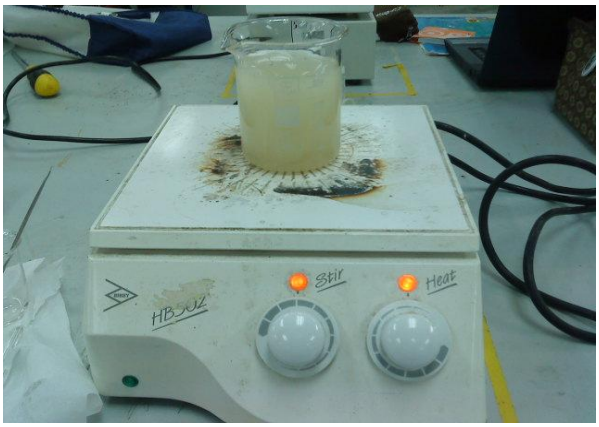

Figure 5: Preparation of blend solutions.

Figure 6: Film casting on the square glass plate

Figure 7: Film peeling

Figure 8: DSC instrument in action

Figure 9: TGA instrument in action

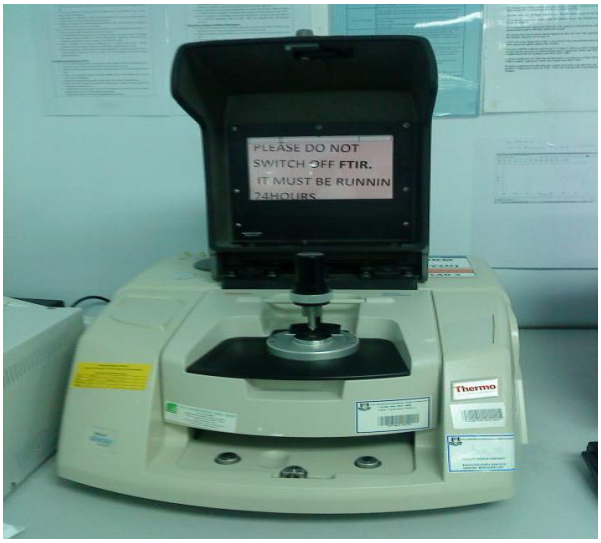

Figure 10: FTIR instrument in action