

**DEVELOPMENT OF ANTIMICROBIAL COMPOSITE  
FILMS OF CHITIN NANOFIBER REINFORCED CHITOSAN  
CONTAINING SILVER SALT**

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**FACULTY OF ENGINEERING  
UNIVERSITY OF MALAYA  
KUALA LUMPUR**

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FILMS OF CHITIN NANOFIBER REINFORCED  
CHITOSAN CONTAINING SILVER SALT**

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## ABSTRACT

Pure chitosan films have poor tensile strength and elasticity. Hence development of high strength composites that is biocompatible for biomedical applications are desired. In this work, 16.7 wt. % of chitin nanofiber, extracted using acid hydrolysis process was incorporated into chitosan polymer matrix to improve the tensile strength and elasticity of the composite films. Composite films were prepared from chitosan by solution casting after incorporating chitin nanofibers as nanofillers and different types of silver salt as antimicrobial agent. Silver sulfate,  $\text{Ag}_2\text{SO}_4$  and silver acetate,  $\text{AgC}_2\text{H}_3\text{O}_2$  with various amounts of 0.1, 0.3, 0.5 and 0.7 g were doped into the polymer matrix to improve the antimicrobial properties of the composite films. Results show that the tensile strength of the chitosan films can be increased by incorporating chitin nanofibers and silver sulfate up to a certain amount without appreciable change in the chemical structure of the chitosan films. The addition of 0.1 g of silver sulfate could improve greatly the tensile strength of neat chitin-chitosan composite while providing improved antimicrobial properties. The antimicrobial efficacy of the composite films was proven using the Kirby-Bauer disk diffusion susceptibility test with *Escherichia coli*. Silver ion release test demonstrates that the amount of silver salt incorporated into the composites could produce sufficient silver ion for enhancing antimicrobial capability. Overall results suggested that chitin nanofiber-chitosan doped with 0.1 g silver sulfate have a potential for application where antimicrobial characteristics is needed such as wound healing dressings, food packaging industries or coatings for medical devices.

## ABSTRAK

Filem kitosan tulen mempunyai kekuatan regangan dan keanjalan yang lemah. Oleh itu pembangunan komposit yang mempunyai kekuatan tinggi serta bersesuaian dari segi biologi untuk aplikasi bioperubatan adalah diperlukan. Dalam kajian ini, 16.7 % berat nanofiber kitin, diekstrak menggunakan proses hidrolisis asid telah ditambahkan ke dalam matriks polimer kitosan untuk meningkatkan kekuatan tegangan dan keanjalan filem komposit. Filem komposit disediakan daripada kitosan melalui kaedah “solution casting” selepas menggabungkan nanofiber kitin sebagai nanofillers dan pelbagai jenis garam argentum sebagai agen antimikrobial. Argentum sulfat,  $\text{Ag}_2\text{SO}_4$  dan argentum asetat,  $\text{AgC}_2\text{H}_3\text{O}_2$  dengan pelbagai amaun 0.1, 0.3, 0.5 dan 0.7 g telah didopkan ke dalam matriks polimer untuk meningkatkan ciri-ciri antimikrob filem komposit. Keputusan menunjukkan bahawa kekuatan regangan filem kitosan boleh ditingkatkan dengan menggabungkan nanofiber kitin dan garam argentum sulfat sehingga jumlah tertentu tanpa perubahan ketara dalam struktur kimia filem kitosan. Penambahan argentum sulfat sehingga 0.1 g dapat menambah kekuatan regangan filem kitosan disamping meningkatkan sifat anti-mikrob. Keberkesanan antimikrob filem komposit telah terbukti dengan ujian Kirby-Bauer resapan cakera kerentanan dengan menggunakan *Escherichia coli*. Ujian pelepasan ion aregantum menunjukkan bahawa jumlah garam argentum yang ditambahkan ke dalam komposit dapat menghasilkan ion argentum yang mencukupi untuk meningkatkan keupayaan anti-mikrob. Keputusan ini mencadangkan bahawa filem komposit nanofiber kitin - kitosan bersama 0.1 g garam argentum sulfat mempunyai potensi untuk aplikasi di mana ciri-ciri anti-mikrob diperlukan seperti pembalut penyembuhan luka, industri pembungkusan makanan atau sebagai salutan untuk alat alat perubatan.

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## LIST OF SYMBOLS AND ABBREVIATIONS

MRSA	:	<i>methicillin-resistant Staphylococcus aureus</i>
mRNA	:	Messenger Ribonucleic Acid
DNA	:	Deoxyribonucleic Acid
FESEM	:	Field Emission Scanning Electron Microscope
PBS	:	Phosphate Buffer Saline solution
RPM	:	Revolutions per minute
XRD	:	X-Ray Diffraction
FTIR	:	Fourier Transform Infrared Spectroscopy
TEM	:	Transmission Electron Microscopy
HCl	:	Hydrochloric acid
PVA	:	Polyvinyl alcohol

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## CHAPTER 1: INTRODUCTION

### 1.1 Introduction

Infection due to surface contamination by bacteria has raised a lot of concern. As the second highest cause of death after cardio vascular diseases, 1 out of 4 deaths recorded in year 2010 was caused by infectious disease (Dye, C., 2014). Studies show that *methicillin-resistant Staphylococcus aureus* (MRSA) can survive on walls for more than 2 months, or 2 days if it dries on a plastic laminated surface. It can withstand temperature and humidity variations and exposure to sunlight (Smith J et al., 1999). Under these conditions, bacteria can spread by direct hand contact and other methods.

Food and medical industries are the largest users of antimicrobial coatings where it is crucial to prevent any contamination to the surface. Contamination is very likely to occur at all stages of food preparation from the producer to the personnel that handles the food (A. Bridier et al., 2015). Therefore, the development of antimicrobial coatings is critical in fight against infections.

The requirement for practical antimicrobial coatings are low cost, suitable for long term usage and storage at designed operating temperature, not soluble in water or common solvents, does not decompose to emit toxic products, should not be toxic or irritating to human and environment, can be regenerated to restore its antimicrobial capability, and biocidal to a broad spectrum of pathogenic microorganisms in brief times of contact (El-Refaie Kenawy et al., 2007).

Amongst potential surface coatings, chitosan coating is a very good candidate to prevent the spread of infections due to its high antimicrobial properties. Chitosan with an amino group at the C-2 site possesses a polycationic structure. This desired property enables chitosan to penetrate the bacteria cell wall and hinders the synthesis of messenger

ribonucleic acid (mRNA) and deoxyribonucleic acid (DNA) transcription. Previous research also found that chitosan can interact with the cell surface and subsequently change the cell permeability by forming a barrier around the cell (Page K., M. Wilson et al, 2009).

Chitosan with its excellent antimicrobial capability had been studied extensively. However, pure chitosan has disadvantages of poor tensile strength and elasticity. Chitosan also have high water permeability and easily dissolved in weak low concentration acid. These properties limit its long-term usage and practicality (Thakhiew, Champhahom, Devahastin, Soponronnarit, 2015). Hence development of high strength composites is necessary for antimicrobial coating applications. Various research had been carried out to improve the mechanical property of chitosan by incorporating plasticizer or high strength filler materials. High strength chitosan composite was developed by using chitin nanofibers as filler materials into chitosan polymer matrix (Shelma R. et al., 2008).

Chitin can be degraded by hydrolytic enzymes called chitinase. Despite presence of nitrogen in its polymer chain, it has very low immunogenicity making it safe to human. Chitin have comparable low chemical reactivity and insolubility to cellulose. Chitin is so similar to cellulose that it may be regarded as cellulose with hydroxyl at position C-2 replaced by an acetamino group. Chitin functions naturally as a structural polysaccharide on organism such as insects and crustacean. Pure chitin is white, hard, inelastic, nitrogenous polysaccharide and major source of surface pollution in coastal areas (Majeti, 2000).

Silver has been used as an antimicrobial agent since early 20<sup>th</sup> century. While low intake of silver will not cause major harm to human, silver ion is strong antimicrobial agent due to highly reactive nature which binds to tissue proteins and causes structural

changes in the bacterial cell wall, intracellular and nuclear membranes. This will lead to cellular distortion and loss of activity of the bacteria. Silver ion also exhibits its bacteriostatic properties by binding to and thus denaturing bacterial DNA and RNA, and ultimately inhibiting bacterial replication (Castellano et al., 2007). A study involving impregnated silver nanoparticles onto the surface of cotton and other materials as carrier has shown great capability to reduce gram negative bacteria like *Escherichia coli* and gram-positive bacteria like *Staphylococcus aureus* (M.H. El-Rafie et al., 2010).

Researchers have long been trying to incorporate the antimicrobial capability of silver with other materials to bring out its full potential. The downside of these research is that it does not study the full application of the antimicrobial coating. The prospect of higher mechanical properties of chitin-chitosan composite coupled with strong antimicrobial capability of silver has paved the direction for finding the most suitable antimicrobial coatings with characteristics of high strength, high antimicrobial capabilities while at the same time remain biodegradable and safe to the environment.

## **1.2 Problem Statement**

As one of the highest cause of fatality, infections is a threat to human life. Infectious disease can spread through many different mediums such as air, water and direct contact. Infectious disease need to be controlled at the root where prevention of its occurrence is a better solution. Thus, a strong and long lasting antimicrobial materials need to be developed to prevent spreading of infectious disease by eliminating chances of surface contamination.

Chitin nanofiber reinforced chitosan composite doped with silver salts has great potential as an antimicrobial composite. The strength and biocompatibility of chitin-

chitosan composite coupled with slow release of silver sulfate and silver acetate salts could pave a way for strong and long lasting antimicrobial composite.

### **1.3 Objectives**

1. To develop chitin-chitosan composite incorporating antimicrobial properties by doping with silver salts using solution cast method.
2. To study physical properties of the composite such as tensile strength, crystallinity indexes, and surface morphology.
3. To determine antimicrobial efficacy of the composite and its relationship to silver ion release.
4. To study degradation of the antimicrobial composite in in-vitro environment.

### **1.4 Scope of Thesis**

The thesis will start with brief introduction in Chapter 1 where the problem statement and brief background of the research was discussed. Objective and scope of thesis will also be presented in this chapter.

Chapter 2 is literature review where detail research background and previous research was presented. The information in literature review leads to the development of this research title.

Research methodology developed based on information from Chapter 2 was presented in Chapter 3. This chapter outline the materials and procedure to require to carry out the research.

The results were presented in Chapter 4 with discussion on the results based on our research objective and literature review.

The thesis will be concluded in Chapter 5 where application and implication of this research as well as possible future development recommendation will be discussed.

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## **CHAPTER 2: LITERATURE REVIEW**

### **2.1 Antimicrobial Coating**

#### **2.1.1 Biofilm Formation and Infections**

The major cause of fatalities includes infectious disease which accounted for 20% of the world-wide fatality figure (Dye, C., 2014). Infections can occur by contact with contaminated surface where 80% of infections are caused by surface contaminated with bio-film (Mario. S, 2014).

Bacteria and fungi could colonize both biotic and abiotic surfaces like surface of medical devices, food preparation tools, and even ordinary surfaces like table and tiles. This could lead to serious infections which can't be prevented even with the help of highly dosed antibiotics. Antibiotic could not mitigate the risk of re-infection while also aid in the evolution of bacteria by increasing its antibiotic resistance. Thus, the prevention of formation of bio-films are crucial in the fight with infectious disease (A. Bridier et al., 2015).

Great interest was observed recently in field of antimicrobial to use of interesting composite that can provide non-adhesive and antimicrobial properties imparted by its functional elements.

#### **2.1.2 Desired Properties for Antimicrobial Coatings**

Low-fouling coatings are coatings with surfaces that doesn't interacts with the biological environment. Polymers are used extensively in this application due to its nature of being hydrophobic which reduces chances of bacteria or fungus sticking to the surfaces. Common polymers used include poly-acryl-amide, poly saccharide, and polyethylene glycol. Their ability to impede bio-molecule adsorption could effectively prevent formations of bio-films (Page K., M. Wilson, et al., 2009).

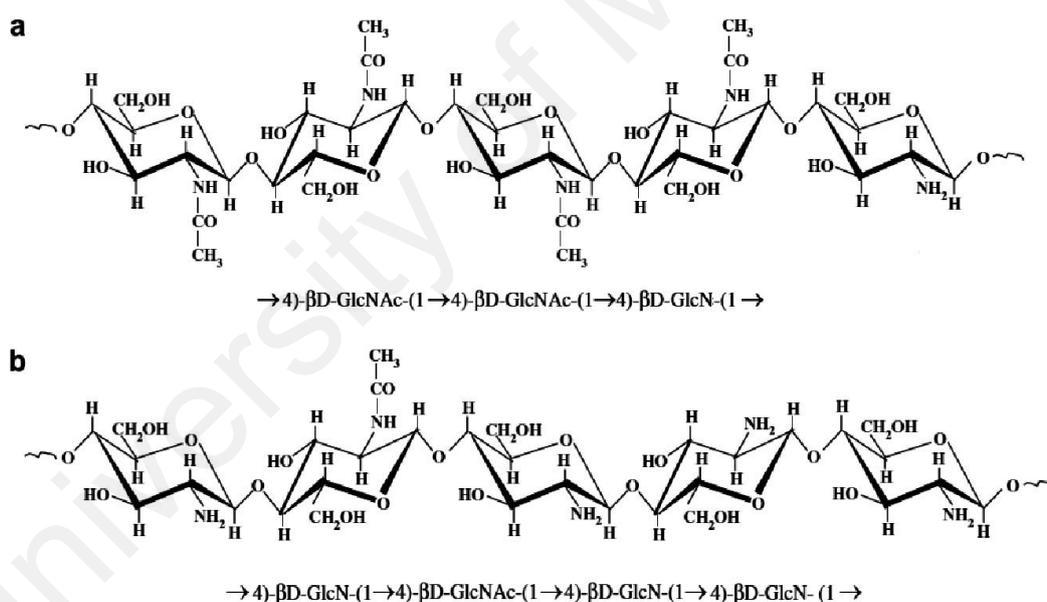
Previous research shows that surfaces with high contact angle could prevent bio-molecule adsorption and thus inhibit the colonization of bacterial on substrate surfaces. However, the formation of bio-films could not be prevented completely if the surface property degrades over time or damaged site due to handling. Thus, an effective antimicrobial would require more than one antimicrobial mechanism. This can be achieved by introduction of additional elements to aid in inhibiting or dispersing bio-films formation. Common doping including silver, guanidine salts, peptides and proteins. Peptide and proteins based doping is only suitable for short term applications as peptides are released or delaminated after prolonged period. Thus, it is important to develop new materials that can used in long term application with slow release of its antimicrobial elements and less intrusion to its environment. Embedded silver nano-particles are common these days for antimicrobial and long-term usability due to slow release rate. The down side is nano-materials are in its early stage of development and there are currently no known side effects of using these new materials (El-Refaie Kenawy, S. D. W., and Roy Broughton, 2007).

## **2.2 Chitin**

### **2.2.1 Chitin in Nature**

Chitin was discovered in 1811 by French botanist H. Braconnot who isolated it from mushroom which was known as *Fungine* after its origin. The difference of it from other polysaccharides is the presence of nitrogen in its polymer chain. In year 1823, another French scientist by the name of A. Odier identified chitin in demineralized crab carapace and suggested that it is the basic building block of the exoskeleton of all insects. The term chitin is derived from Greek word meaning "tunic" or "covering" indicating its structural function in living things. It is known that both chitin and chitosan are linear co-polymers of D-GlcN and D-GlcNAc monomers distributed randomly and not blocked together, the

monomers are linked entirely in the  $\beta$ -1,4-configuration (Figure 2.1); the various depolymerization products and structural and spectroscopic studies provide ample proof for this. The  $\beta$ -1,4-configuration results in a rigid and unbranched structure. The abundance of hydroxyl groups (1 primary hydroxyl at C-6 and 1 secondary hydroxyl at C-3) and highly reactive amino group (at C-2) or its N-acetyl counterpart (wholly in chitin) with concomitant tendency for intra- and intermolecular hydrogen bonds results in the formation of linear aggregates with extensive crystallinity. The latter contributes to the strength shown by chitinous structures, and also to the insolubility of chitin in common solvents, particularly water at neutral pH (K.V. Harish Prashanth and R.N. Tharanathan, 2007).



**Figure 2.1: Primary structure of a) Chitin and b) Chitosan (K.V. Harish Prashanth and R.N. Tharanathan, 2007).**

### 2.2.2 Chitin Source and Extractions

Chitin is considered to be second most abundant biopolymer on earth after cellulose. Each year an estimated of 10 gigatons of chitin is biosynthesized and degraded. Thus, it poses great challenge to our environment as most of the chitin waste came from food industry. While being one of the most abundant natural polymer, chitin was for a long time considered as an untreatable polymer because of its inherent insoluble nature and intractable molecular structure (C. Pilai et al., 2009).

One of major source of chitin is from crustacean waste such as crab shells which is composed of chitin which form a chitinoproteic complex with proteins. The composition are proteins (15-50%), minerals (30-50%), and chitin (15-30%). Thus, to extract chitin from these bio waste, process of removing proteins and minerals are required (C. Pilai et al., 2009).

Traditionally, chitin extraction involves strong acid and alkali treatment under high temperatures for demineralization and de-proteinization respectively. The adverse condition of the process would cause chitin de-polymerization which leads to further environmental pollution. Biotechnological processes developed recently could allow us to extract chitin by using proteolytic enzymes or proteolytic microorganisms. This method can obtain higher molecular weights chitin compare with chemically prepared variants. Furthermore, fermentation process is a cheap technique which could retains the nutritional quality of the by products such as bio peptides (Rasha M. Abdel-Rahman, et al., 2015).

### 2.2.3 Mechanical Properties of Chitin

Chitin occurs as a highly-organized micro- and nano-fibril structure, whose role is that of providing support and protection to living systems, mainly to crustaceans, insects and fungi, as reinforcing and functional elements. Chitin in nature forms part of a well-organized composite, increasing from nanometer to millimeter scale. Highly crystalline nanofibrils packed in amorphous regions making it semi crystalline. Proteins enveloped these nanofibrils and assemble into nanofibers bundles. At micrometer level, network of bundles is formed that created a twisted plywood structure that gives the structure strength. These structure repeats to form endocuticle, exocuticle and epicuticle at the macroscopic level which are the exoskeleton of crustaceans. The tensile strength of chitin from different source was shown in table 2.1. The high tensile strength properties show that chitin can be used as a durable structural material while maintaining environmental friendly due to its biodegradable nature (I. Joffee, H. R. Hepburn, et al, 1975).

**Table 2.1: List of tensile strength of chitin from different source**

Chitin Source	Tensile strength in dry state
Crab shell	36N/mm <sup>2</sup>
Shrimp shell	21N/mm <sup>2</sup>
Beetle shell	80N/mm <sup>2</sup>

*Reference: I. Joffee, H. R. Hepburn, et al, 1975.*

### 2.2.4 Chitin Nanofibrils and its Properties

Chitin nanofiber can be obtained using various method both chemically and mechanically. The most common method is acid hydrolysis using hydrochloric acid. The separation process includes acid hydrolysis, mechanical treatment, ultrasonication process and chemical process. These methods can be use individually or combined to obtain the desired chitin nanomaterials (Rasha M. Abdel-Rahman, et al., 2015).

Acid hydrolysis process involve heating 3M HCl solution at 90°C for 3 hours with vigorous stirring under refluxing. HCl to chitin ratio is maintained at 30mL g<sup>-1</sup>. The main purpose of heating chitin under strong hydrochloric acid was to hydrolyze the amorphous regions of the chitin. The suspension was centrifuged at 10,000 rpm for 10 minutes and the acidic solution was removed and replaced with distilled water. This process was repeated trice to remove the hydrochloric acid. The chitin whiskers were transferred to a dialysis to remove residual acid present in the chitin. The dialysis process was conducted by soaking the dialysis bag in distilled water for 24 hours. The resulting suspension pH was adjusted to 2.5 using diluted HCl and subsequently subject to ultrasonication for 10 minutes. This method was reported to produce nanofiber with diameters of 28nm and length of 300nm which are sourced from shrimp shells (V. Rubentheren, Thomas A. Ward, C.Y. Chee, C.K. Tang 2015).

The variations in size, shape, crystallinity, aspect ratio and morphology of chitin nanofiber can be attribute to the biosynthesis process (chitin source) and isolation process of the chitin nanofiber which very much depend on the type and harshness of the separation process. Their elongated fiber shape usually with high width to length ratio of up to 1:300 allow it to self-assembled into bundles of chitin fiber with high tensile strength. The width varies from less than 10nm up to 100nm and length ranging from 500nm to 10μm depending on the source of chitin nanofiber (Phongying, Aiba, and Chirachanchai, 2007).

Its desired properties include renewable and biodegradable characteristics, small size, low density, large surface due to its nano sized dimensions, chemical reactivity, biological activity and non-cytotoxicity makes it an ideal candidate for wide range of applications in medical, nanomaterials, food packaging, adsorption treatments, among others (A.M. Salaberria, Rene H. Diaz, et al. 2015).

Chitin nanofibrils also reportedly able to activate fibroblasts and induced the regeneration of epidermal keratinocytes by increasing the expression of  $\alpha$ - and  $\beta$ -defensins in endothelial cells and that of  $\beta$ -defensins in keratinocytes (R. Izumi et al., 2015). These properties together with its bacteriostatic properties proved that chitin nanofibrils are suitable for use in wound healing applications. Chitin nanofibers also reportedly have high antifungal capability (A.M. Salaberria et al. 2015). Thus, it has been extensively used in biotechnology and medical industries.

### **2.2.5 Chitin Nanofibrils in Composite Materials**

Large number of researches currently focus on using chitin nanofibrils as filler for polymer matrices in polymer composites. Its great mechanical properties and biological activity make it a great choice for use in composites. Chitin nanofibrils was used to create composites with different polymeric matrices such as thermoplastic starch, chitosan, polyvinyl alcohol, and thermoplastic poly-urethane (A.M Salaberria, J. Labidi, et al., 2015).

Chitin nanofiber was proven to improve tensile strength and Young modulus when doped to various polymer matrices such as thermoplastic starch. The addition of chitin nanofiber also modified the composite's water vapor transmission and permeability thus improving its applications in wet and moist environment (A.M Salaberria et al., 2015). This barrier properties also could help prevent moisture transfer between the two sides of the composite making it a suitable candidate for protective coating.

Research by Qiaoyun Deng et al also shows that composite of poly(vinyl alcohol) with chitin nanofiber could reduce the coefficient of thermal expansion greatly compare with plain PVA film. Neat PVA film coefficient of thermal expansion of  $124\text{ppm K}^{-1}$  was successfully reduced to  $25\text{ppm K}^{-1}$  on PVA-chitin naofiber composite. The 80% reduction

was suggested to come from the strong atomic bonding of highly crystalline chitin nanofiber. Presence of chitin nanofiber in PVA matrices successfully restrain the expansion of PVA thus reducing the overall coefficient of thermal expansion of the composite (Qiaoyun Deng, et al., 2014).

#### **2.2.6 Chitin in Antimicrobial**

Chitin often used in composite with various materials for antimicrobial purposes. Chitin in nanofiber form had been incorporated with chitosan, silver nanoparticles, starch, and carrageenan to improve not only its mechanical and barrier properties. Chitin also act as a functional element to improve antimicrobial properties of the composites. Chitin nanofiber was reportedly having superior antifungal activity against *A. niger*. While additional of silver nanoparticle further improve the antifungal property greatly. Study had showed that chitin nanofiber coupled with silver nano particle could deter spore germination for various types of fungus such as *A. brassicae*, *C. hingginianum* while greatly reducing rate of spore germination for various other types of fungus such as *A. alternate*, *B. oryzae* and *P. digitatum*. In this case Chitin nanofiber had shown to be a great substrate to properly disperse silver nanoparticles without causing coagulations (A.M. Salaberria, Rene H. Diaz, et al. 2015).

Chitin nanofibrils was used as reinforcing materials in carrageenan nanocomposite films. While improving its mechanical properties such as improved tensile strength and higher Young's Modulus, addition of chitin nanofibrils also shows to exhibit antimicrobial property versus *L. monocytogenes* but not against *E. coli*. Proposed antimicrobial mechanism is that chitin makes bacteria flocculate and prevent growth through lack of nutrients and oxygen (mass transfer limitation). Chitin which are positively charged also said to interact with negatively charged bacteria cell membranes

and thus increased its permeability and causing cell rupture and losing its intracellular material.

### **2.2.7 Disadvantages of Chitin**

While research shown that chitin is good candidate as a reinforcing material in biodegradable products, it is less effective in the fight against bacterial infections. Chitin nanofibrils shows bacteriostatic properties rather than bactericidal. Thus it was reported to be able to prevent spreading of certain types of bacteria and fungus only. Chitin was reportedly able to inhibit growth of *A. niger*, *L. monocytogenes* and *A. alternate*. Chitin shows no inhibition to growth of *E. Coli* (A.M. Salaberria, Rene H. Diaz, et al. 2015). Pure chitin nanofiber is unable to reduce the rate of spore germination for various types of fungus such as *B. cinerea*, *C. orbiculare* and *P. oryzae* (Ifuku, et al, 2014). Thus chitin is often used in composite with one or more types of functional elements in antifungal and antimicrobial applications.

## **2.3 Chitosan**

### **2.3.1 Chitosan as Derivative of Chitin**

Chitosan is derivative from chitin where it is partially N-deacetylated. Chitosan depending on its source and derived process is categorized by different degree of deacetylation and molecular weight.

Chitosan and its derivatives have attracted considerable attention as biomedical materials, owing to their unique biological effects such as antioxidant, anti-allergic, anti-inflammatory, anticoagulant, anti-coagulant, anti-obesity, anti-diabetic, anti-HIV, anti-microbial, anti-cancer and anti-tumor activities (K.V. Harish Prashanth and R.N. Tharanathan, 2007).

### **2.3.2 Method to produce chitosan**

Chitosan was produced by deacetylation of chitin. Where chitin was treated with strong alkaline to remove the acetyl groups from the molecular chain of chitin. Chitosan with various degree of deacetylation can be obtained by using different concentrations of sodium hydroxide solution. While chitosan is characterized by its degree of deacetylation, higher degree of deacetylation can be obtained from reaction with higher concentration of sodium hydroxide.

Treatment of chitin in 30% sodium hydroxide yield chitosan with degree of deacetylation of 45% and increases to 95.5% degree of deacetylation when treated with 60% sodium hydroxide. This treatment was performed at 90°C under reflux with 1:30 w/w solid to solvent ratio for 1-5 hours (Rasha M. Abdel-Rahman, et al., 2015).

### **2.3.3 Properties of Chitosan**

Chitosan is a cationic polymer derivative of chitin with superior antimicrobial properties with good biocompatibility, biodegradability, non-toxicity and flexibility which are of great interest to researchers.

Chitosan's cationic properties allow it to inactivate a variety of bacterial and fungi. While it has great antimicrobial properties, it is pH sensitive polymer where it loses antimicrobial activity under alkaline. There are three types of reactive functional groups with one amino/acetamino group and two hydroxyl groups at C-2, C-3 and C-6 site respectively. The main factors that contributes to the differences in chitosan is the amino content which often refer to as degree of deacetylation. Amino content would affect structures and chemical properties of chitosan (Kumar, M.N., 2000).

Chitosan physical and mechanical properties vary with different molecular weight (MW). Studies shows that chitosan with higher molecular weight will have higher tensile

strength but lower elasticity. This is due to the formation of intra- and inter-molecular hydrogen bond where longer molecular chain allows for more formation of hydrogen thus increasing its strength (M. J. Bof et al. 2015).

#### **2.3.4 Chitosan in Antimicrobial**

Chitosan with its free amino groups is able to become positively charged and this cation properties plays a big role in fighting with gram positive and gram negative bacteria such as *E. coli*. The antibacterial activity of chitosan can be directly correlate to its degree of deacetylation. The higher the degree of deacetylation the higher the antimicrobial properties of chitosan. The antibacterial or antifungal activity of chitosan depends on the presence of cationic charge on chitosan chain on deacetyled step. The more cationic would present higher bacteriocidal properties (Jonathan Rhoades, Bob Rastall, 2008).

There are few mechanisms of antimicrobial properties of chitosan. Such as the cationic properties would cause the chitosan chain to attach to the surface of the cell and forming a polymeric membrane which act as a barrier for nutrients and oxygen from entering the cell and thus starving it to death. It is also suggested that chitosan could penetrate through the cell wall of *E. coli* then thus causing it to rupture and lose it cellular liquid (I. Younes, S. Hajji, V. Frachet et al. 2014).

#### **2.3.5 Disadvantages of Chitosan**

Chitosan's superior antimicrobial activity and good film forming ability due to its inter- and intra-molecular hydrogen bond makes it a good candidate for antimicrobial edible films and coatings. The major disadvantages of chitosan is its hydrophilic nature which makes it highly permeable to water vapor. It is reported that the strength of the

chitosan film would decrease greatly when water content in the environment is increased (M.A. Gámiz-González et al. 2015).

Chitosan also lose its antimicrobial activity in high pH environment. This is because lower pH values allow the presence of more  $-NH_3^+$  in the environment. These  $-NH_3^+$  binds to bacteria cell wall which destabilizing cell structure (Shun-Hsien Chang, Hong-Ting Victor Lin, Guan-James Wu, Guo Jane Tsai, 2015). High pH would make the environment less favorable for cation activity thus reducing the efficacy of chitosan.

## **2.4 Chitin-Chitosan Composite**

### **2.4.1 Chitin-Chitosan Composite Properties**

Chitin being a good structural material have poor film forming capability due to its high degree of crystallinity. The high degree of crystallinity would cause the film to become brittle and weak because there are strong intra and inter-molecular hydrogen bond inside the crystal but there is no bonding between nanofiber strands.

Chitosan with its high degree of deacetylation shows better antimicrobial activity because there is less blocking of the amino groups compare with chitin. The extra amino groups in chitosan contributes greatly to its antimicrobial activity (R.M. Abdel-Rahman et al. 2015). Chitosan have low tensile strength which makes it less useful in various applications.

Thus, there are various research to create composite film or coatings by creating composite of structural materials and chitosan as polymer matrix to improve its mechanical properties. Chitin nanofiber is the most suitable candidate due to its high crystallinity and rigidity. Chitin nanofiber can improve chitosan film's mechanical property greatly if it is used as filler material. Previous research had shown that mechanical strength can be increase with increasing amount of chitin nanofiber up to 20%

weight percentage of chitin nanofiber (Shelma R., Paul W., and Sharma C.P. 2008). Chitosan are derivative of chitin both having similar structures, this enable formation of strong electrostatic interaction and hydrogen bonding between them. Thus composite of chitin nanofiber with chitosan have excellent interfacial adhesion and compatibility. Previous study shows that tensile strength could increase from 38.5MPa on neat chitosan to 110.3MPa on chitin nanofiber reinforced chitosan which amount to almost 3 times increase in strength (B. Ma, A. Qin, X. Li, et al. 2014).

#### **2.4.2 Applications of Chitin-Chitosan Composite**

Chitin-chitosan composite with superior mechanical strength coupled with great antimicrobial properties have huge potential in biomedical applications such as antimicrobial coating and wound healing coating. Research by Shelma et al shows that chitin nanofiber reinforced chitosan could improve wound healing rate when tested on Guinea pigs and rabbits. It also prevent infections and act as a barrier to foreign particles when coupled with alginate and poly ethylene glycol on human subject with chronic non-healing ulcers (Shelma, et al., 2008).

Chitin-chitosan composite also a suitable candidate for antifungal applications where research by A.M. Salaberria et al. (2015) demonstrated that chitin-chitosan composite have strong antifungal activity against *A. niger*. B. Ma et al. also found out that chitin-chitosan composite have effectively inhibit growth of *S. aureus*, *E. coli* and *C. michiganence*. The research proposed that chitin-chitosan composite could be suitable for use in packaging and wound dressing applications (A.M. Salaberria et al., 2015).

#### **2.4.3 Development of Chitin-Chitosan Composite.**

Recently there are great interest on chitin-chitosan composite due to its antimicrobial, high strength, biocompatibility, biodegradability, and able to regenerate naturally. These

properties make it a great candidate to replace petroleum based polymer which not only can pollute our environment also does not completely compatible with human body.

The current development trend on chitin-chitosan composite focus on two major areas of improvements: 1) Improvement in mechanical properties, and 2) Improvement in antimicrobial properties. Studies had shown that incorporating cross-linking chemical such as tannic acid could improve its mechanical properties by almost two-fold from 22.02 MPa to 39.75 MPa (V. Rubentheren, T.A. Ward, C.Y. Chee, and C.K. Tang, 2015). Varying the amounts and dimensions of chitin nanofibers also could improve mechanical properties of composite by three-fold when compare with neat chitosan film (Ma et al., 2014). While improving tensile strength was ideal it often comes with lower elasticity and breaking of film occur at shorter elongation. This could reduce the flexibility of the film and limit its applications. The optimal chitin nanofiber content as found out by previous research is 17% weight percent which gives optimal tensile, elastic and water permeability property (Shelma et al. 2008).

The antimicrobial property of chitin-chitosan could be improved greatly by incorporating bacteriocidal materials such as copper (Ilnicka, Walczyk and Lukaszewicz, 2015), silver nanoparticles (T.V. Mathew and S. Kuriakose, 2013) or zinc sulfate (D. Gupta, D. Singh, N.C. Kothiyal et al., 2015). Chitin-chitosan composite act as a carrier based for controlled release of these bacteriocidal materials to improve its antimicrobial properties.

## **2.5 Silver Ions**

### **2.5.1 Silver Ions Properties**

Silver-based antimicrobials capture much attention not only because of the non-toxicity of the active  $\text{Ag}^+$  to human cells but because of their novelty being long lasting biocide with high temperature stability and low volatility. The antimicrobial activity of silver ions has been well established. Silver ions are significant antimicrobials by virtue of their antiseptic properties with only few bacteria being intrinsically resistant to this metal. Silver is well known being a significant resource for topical therapy because of its beneficial antimicrobial properties in medical devices such as catheters, cannulae etc .

The antimicrobial activity of silver is dependent on the silver cation  $\text{Ag}^+$ , which binds strongly to electron donor groups in biological molecules containing Sulphur, oxygen or nitrogen. Hence the silver-based antimicrobial polymers have to release the  $\text{Ag}^+$  to a pathogenic environment in order to be effective. The oxidation of the metallic silver to the active species  $\text{Ag}^+$  is possible through an interaction of the silver with the water molecules. A steady and prolonged release of the silver biocide in a concentration level (0.1 ppb) capable of rendering an antimicrobial efficacy is a key factor for the design of this class of materials (Radhesh Kumar, Helmut Münstedt, 2005).

### **2.5.2 Application of Silver in Antimicrobial**

Polymers are widely used as a material basis for medical and life-care products. For such applications polymers should preferably possess antimicrobial efficacy to reduce the risk of device-related infections. Instead of organic biocidal agents, elemental silver can be used for the preparation of antimicrobial polymers. In the presence of humidity and oxygen from air, elemental silver particles release small amounts of silver ions that exhibit a biocidal activity against a broad spectrum of microorganisms.

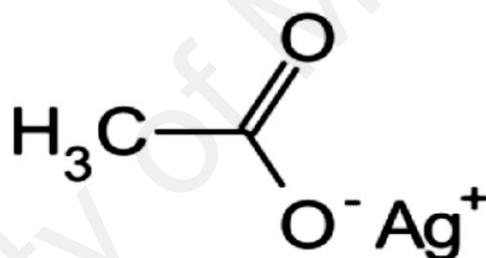
A dressing composed of freeze-dried chitosan acetate incorporating nanoparticle silver was compared with a dressing of chitosan acetate alone in an in vivo burn model infected with bioluminescent *P. aeruginosa*. The survival rates of mice treated with silver-chitosan was higher than in regular chitosan or untreated samples. Silver-chitosan dressings can effectively control the development of systemic sepsis. These studies suggest that a dressing combining chitosan acetate with silver leads to improved antimicrobial efficacy against fatal burn infections (Liyi Huang, 2011 & Jayakumar, 2011).

The chitin/chitosan samples were doped with silver salts because studies have indicated that such chitosan/silver films provide excellent antibacterial action against model bacteria such as *Escherichia coli* and *Bacillus*. This approach can be easily used in the large-scale production of such silver-nanoparticles-loaded chitosan films. These films can be used as antimicrobial packaging materials, as wound dressings and can also be grafted onto various implants (Thomas V, 2009).

### **2.5.3 Release of Silver Ions from Silver Salts**

There are two types of silver salts which are soluble in water and not soluble in water. Silver nitrate are salts that can have high solubility in water while silver chloride is non-soluble in water. The ideal properties for application in antimicrobial film is to have controlled sustainable release of silver ion into the surrounding. Thus, silver sulfate and silver acetate are a suitable candidate where their solubility in water is low. Silver sulfate solubility in water is 8.3 grams per liter while silver acetate solubility is 11.11 grams per liter of water (Radhesh Kumar, Helmut Münstedt, 2005, F. H. MacDougall, 1947). The low solubility could enable a slow release of silver ions into the environment and longer the time to depletion of silver ions greatly.

Ionic selective electrodes (ISE) can be used to detect concentration of silver ions in solution. ISE are membrane electrodes that respond selectively to specific ions in the presence of others. The principle of measurement is based on the potential generated across the membrane. The electrodes have an internal reference electrode and the potential difference between the internal and external electrodes always remain constant. Thus, any alterations in the potential are due to the changes in potential across the membrane and the aqueous solution of immersion. The potential difference measured by the electrode depends on the activity of the specific ion in solution i.e. concentration of the ion. Solid state electrodes utilize relatively insoluble inorganic salts in a membrane. The potentials are developed at the membrane surface due to the ion-exchange process.



**Figure 2.2: Silver Acetate (F. H. MacDougall and S. Peterson, 1947)**

Silver acetate is an organic compound with the empirical formula CH<sub>3</sub>COOAg. This compound is a photosensitive, white crystalline solid (F. H. MacDougall, 1947). Due to the partial solubility of silver acetate in water, silver acetate will dissolve to form silver ion and acetate ion.  $\text{CH}_3\text{COOAg} = \text{CH}_3\text{COO}^- + \text{Ag}^+$  (F. H. MacDougall, 1947). As it is mentioned above, the total electrolyte concentration in a solution will eventually affect the solubility or dissociation rate of the salts. In other words, the ionic strength increased initially to a certain value. Where equilibrium between the solution and the un-dissolved remained solute crystal will be established. The silver ion concentration will reach an

equilibrium when the solution saturates. Beyond this point, there will be no further increase in silver and acetate/sulfate ion (Nic, Jirat, & Kosata, 2006).

Ionic concentration of a solution is the basic parameter which acts as a yardstick to indicate the ionic strength of the solution. Ionic strength of a solution is defined by the concentration of ions in the solution. When ionic compounds in the solution dissolve, it will dissociate into ions. The total electrolyte concentration in a solution will affect the solubility of different salts and the dissociations rate. Solution with dissolved ions is characterized by their ionic strength. The formula used in calculating the ionic strength,  $I$  of a solution is the function of concentration of all ions present in the solution (Nic, Jirat, and Kosata, 2006).

Ionic selective electrodes can be used to detect concentration of silver ions in solution. ISE are membrane electrodes that respond selectively to specific ions in the presence of others. ISE are relatively simple to use, cost-effective as the cost of the initial set-up for analysis is quite low and not affected by interferences such as the color of the sample. The basic equipment required for measuring ionic strength using ISE include a digital meter (capable of providing measurements in millivolts), a selective solid-state ion probe for immersing in the sample to detect the specific ion concentration and other necessary consumables such as an ionic strength adjuster and filling solutions required for ionic strength adjustments and calibration procedures.

## **2.6 Literature Review Summary**

New antimicrobial composite was proposed based on literature study carried out. The new antimicrobial composite should incorporate a strong structural carrier and a strong antimicrobial agent as a doping element

The high strength of chitin nanofiber reinforced chitosan composite was combined with silver salts that dissolved partially in water. The resulting composite should have both high strength through chitin nanofiber reinforced chitosan composite and high antimicrobial properties from its silver salt doping.

However, the effect of doping with silver salts to chitin-chitosan composite need to be studied from physical properties (tensile strength, crystallinity indexes, and surface morphology) to its antimicrobial efficacy (Kirby Bauer test and silver ion release test) as well as long-term degradation of the composite was determined to ensure the robustness of the new material and its antimicrobial capability.

## CHAPTER 3: MATERIALS AND METHODS

### 3.1 Introduction

This chapter describes the production of an antimicrobial composite film which involves extraction of chitin nanofiber from chitin flakes, preparation of chitosan polymer matrix, and doping with silver salts. These composite films were subjected to various test to study its properties comparing to neat chitin/chitosan composite film. The chart of the work flow is shown in Figure 3.1: Overview of research methodology.

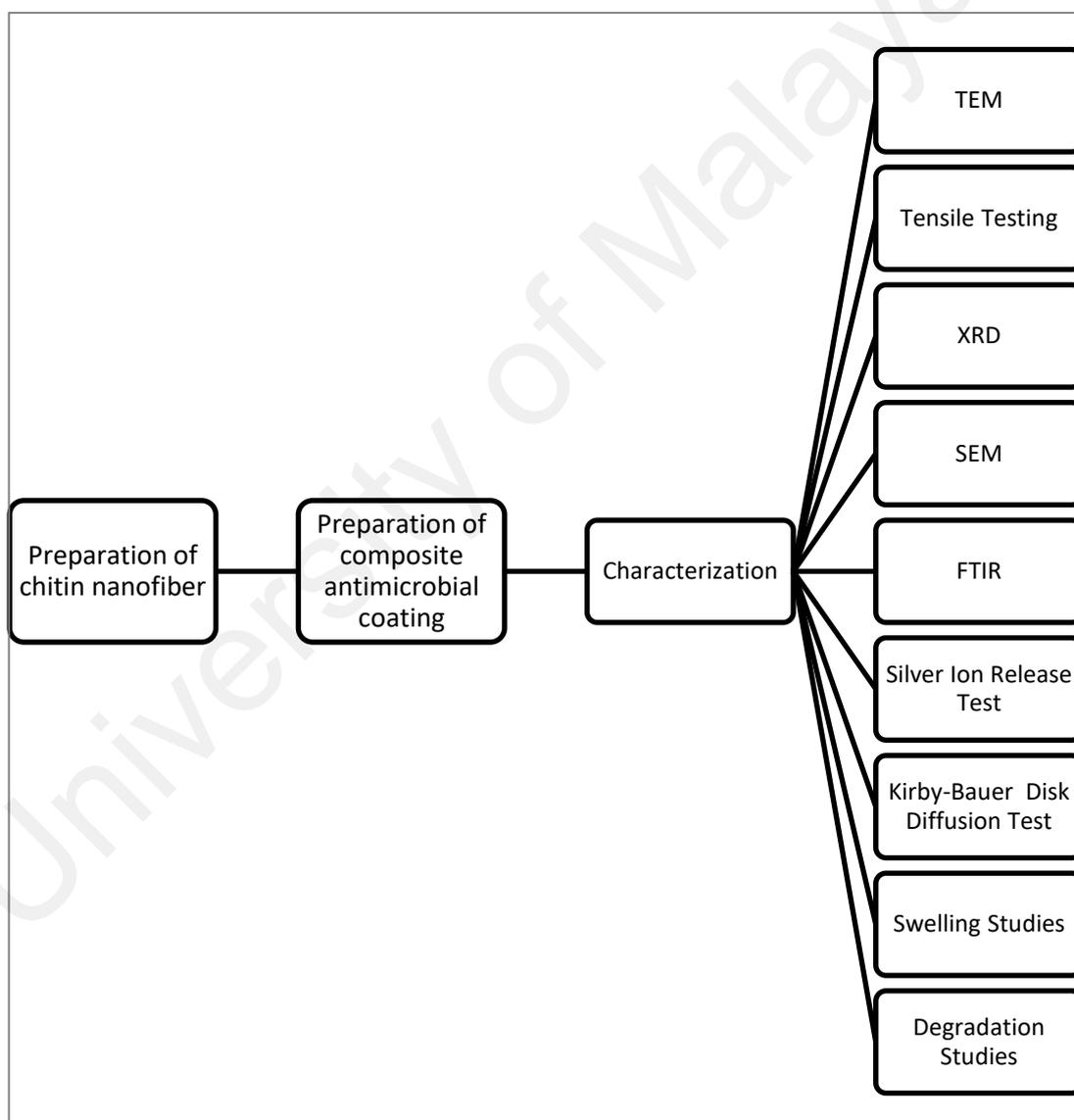


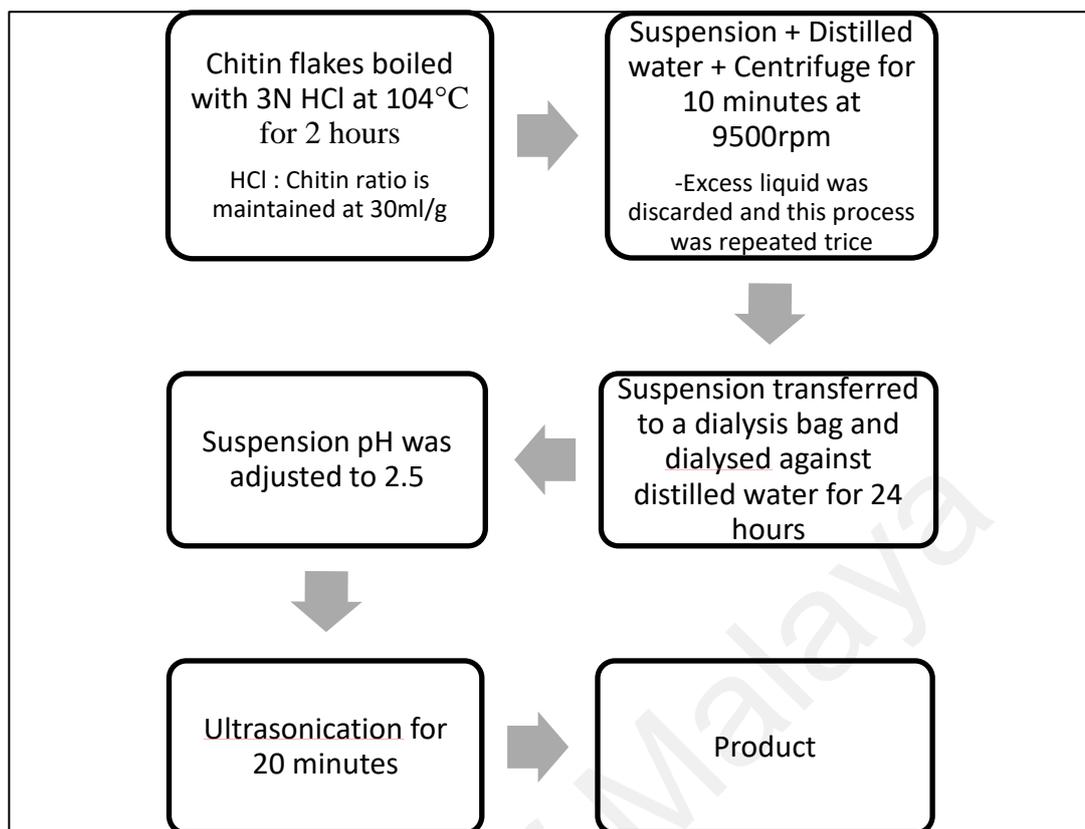
Figure 3.1: Overview of research methodology

### **3.2 Material**

Chitin coarse flakes (sourced from shrimp shell), high molecular weight chitosan (sourced from shrimp shell with molecular weight 310000-375000 Da, viscosity 800-2000 cP, 1 wt. % in 1% acetic acid(25 °C) and various chemicals such as silver sulfate salts 99% purity, silver acetate salts 99% purity, >99.7% concentration glacial acetic acid (diluted to 1% volumetric acetate acid for dissolving chitosan), 6N hydrochloric acid (diluted to 3N concentration by mixing with distilled water in ratio of 1:1 for acid hydrolysis) and sodium hydroxide (1N concentration) was obtained from Sigma-Aldrich Malaysia.

### **3.3 Preparation of Chitin Nanofiber**

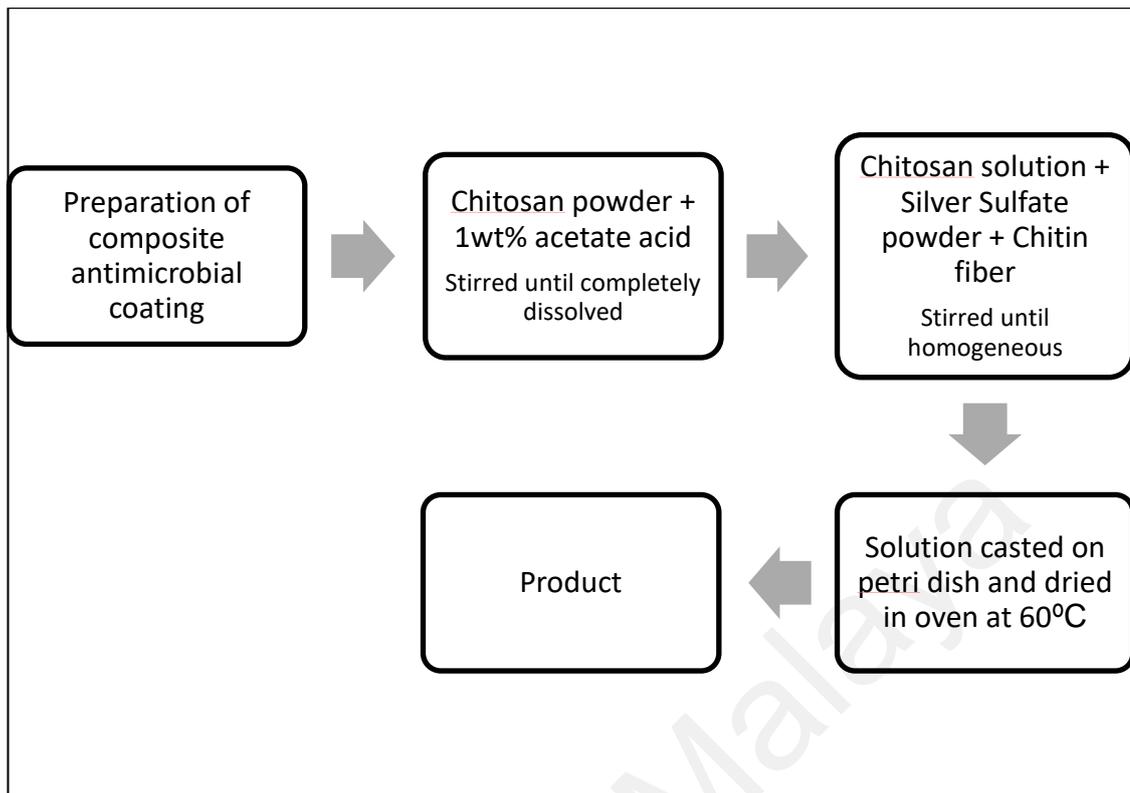
Chitin powder was hydrolyzed by adding 3N hydrochloric acid under stirring for 2 hours at 104°C. Hydrochloric acid to chitin ratio was maintained at 30ml/g. After hydrolysis, suspension was diluted with distilled water followed by centrifugation at 9500rpm for 10 minutes. This process was repeated trice. The suspension was then transferred to a dialysis bag and dialyzed against distilled water for 24 hours. The pH of the suspension was adjusted to 2.5 by adding hydrochloric acid and subsequently subjected to ultra-sonication for 20 minutes. The resulting suspension in colloidal form due to the protonation of amino group of chitin.



**Figure 3.2: Preparation procedure of chitin nanofiber**

#### **3.4 Preparation of chitin nanofiber reinforced silver salt doped chitosan membranes**

Chitosan was dissolved in 1% acetic acid solution. Solution of silver sulfate was mixed with the chitosan solution and stirred until homogeneous. Silver sulfate that was added are 0.1g, 0.3g, 0.5g and 0.7g respectively. Chitin fiber as prepared in the first part of experiment was added to the solution and stirred for 2 hours. The solution was spread on a Petri dish followed by oven drying. The dried membrane was peeled off from the plate and washed with 1N sodium hydroxide solution to neutralize the residual acid. The membrane was then washed with distilled water repeatedly and then place in oven for drying. The dried membrane was kept in desiccators for characterizations.



**Figure 3.3: Preparation procedure of chitin nanofiber reinforced silver sulfate doped chitosan membranes**

### 3.5 Characterization

#### 3.5.1 Transmission Electron Microscopy (TEM)

TEM was performed on chitin nanofiber after acid hydrolysis process to determine the dimension of individual fiber. Chitin nanofiber suspension after acid hydrolysis was diluted at 1 drop of suspension into 100ml of distilled water. The diluted suspension was subjected to ultrasonication to disperse the nanofiber evenly. Five different samples were prepared from different batch of chitin nanofiber suspension. One drop of each sample was drop onto a carbon coated grid and leave to dry in drying cabinet. Five samples from different batch of hydrolyzed chitin nanofiber was prepared. The carbon coated grid with chitin nanofiber was examined using a Zeiss EFTEM Libra 120 transmission electron

microscope at 20,000 times magnification. The dimension of individual chitin nanofiber was measured randomly using ImageJ software.

Random strain of chitin nanofiber was measured and the image was saved for analysis. Minimum of 5 measurement will be performed on each sample to obtain average diameter and length of nanofiber.

### 3.5.2 Tensile Test

This study was conducted to investigate the effect of silver sulfate and silver acetate towards the tensile strength of chitin and chitosan composite film. In this test, samples was secured on tensile testing machine and stretched by increasing the load on the samples. Initial dimension of sample was 1.4 cm in width, 2.8 cm in length and 0.015 cm thickness. All measurements of dimension were done using Vernier caliper.

### 3.5.3 X-ray Diffraction (XRD)

The crystalline/amorphous nature of the film were analyzed using XRD method with the D8 Advance X-Ray Diffractometer at Combicat, University of Malaya. The CuK $\alpha$  radiation was set at 40 kV and 20 mA. The data was collected with  $2\theta$  ranged from  $5^\circ$  to  $90^\circ$  with step size of  $0.02^\circ$  and a step time of 1s. Identification of phases was made by comparing the diffraction patterns of film with Joint Committee for Powder Diffraction Studies (JCPDS) standard of silver (893722), chitin and chitosan (401518).

Crystallinity index was calculated using Equation 3.1 as below (Ca;ärdenas, Cabrera, Taboada, & Patricia Miranda, 2004).

#### Equation 3.1:

$$CI = \frac{I_{110} - I_{am}}{I_{110}} \times 100$$

Where  $I_{110}$  is the maximum intensity (arbitrary units) of the diffraction of the (110) plane at  $2\theta = 19^\circ$  and  $I_{am}$  is the intensity of the amorphous diffraction at  $2\theta = 12.6^\circ$ .

#### **3.5.4 Field Emission Scanning Electron Microscopy (SEM)**

FESEM image was taken using Auriga-39-22 FESEM manufactured by Carl Zeiss SMT at Mechanical Department of Engineering Faculty, University of Malaya. Sample was cut into small pieces for viewing. Low electron acceleration potential of 0.80kV was used to prevent damage to surface of sample.

Image was obtained at 10,000 times magnification to have clear view of surface morphology of the composite film. Change in surface morphology due to additional of silver salts can be observed upon further inspection of the FESEM image.

EDX scanning function was used to determine the chemical composition of the composite. EDX point mode was used to pin point the exact concentration of particles found on the surface of the composite with higher doping concentration of silver salts.

#### **3.5.5 Fourier Transform Infra-Red Spectroscopy (FTIR)**

In this research, FTIR equipment brand of Thermo Scientific model Nicolet Is10 was used to identify the absorption spectrum of the samples. The infrared spectra were registered to FTIR equipment connected to computer with OMNIC software for data processing. The samples were analyzed in KBr smart scan ranging from wave length of 4000 to 650  $\text{cm}^{-1}$ .

#### **3.5.6 Silver Ion Release**

Silver ion was known to be a potent antimicrobial agent. The doping of silver salts enables the release of silver ion to its surrounding thus enabling active mode of

antimicrobial. Silver ion release test was carried out to determine the ability of developed composite to release silver ion.

Silver ion release test was carried out using Orion 4-Star pH/ISE meter with Orion Ion plus Silver/Sulfide electrode to determine the ability of the membrane to release silver ion in the presence of water. Samples were cut into 1 cm by 1 cm square. Readings were taken after 24 hours immersion to determine the silver ion concentration in respective samples.

### **3.5.7 Kirby-Bauer Disk Diffusion Susceptibility Test**

The Kirby-Bauer disk diffusion susceptibility test was used to determine the sensitivity or resistance of pathogenic aerobic and facultative anaerobic bacteria to various antimicrobial compounds.

Briefly, the Mueller-Hinton medium was inoculated with *Escherichia Coli* and antimicrobial film was placed on top of the agar. The plate was then placed in an incubator and incubated for 16 to 18 hours at 35°C. The *Escherichia Coli* was grown on Mueller-Hinton agar medium in the presence of various antimicrobial agents. The presence or absence of growth around the film is an indirect measure of the ability of that compound to inhibit that organism (S. Baar et al., 2001).

### **3.5.8 Swelling Studies**

A total of 8 vials were required for the 8 days of analysis. All 24 samples (previously prepared) were weighed individually using the digital balance to obtain the initial dry weight ( $W_i$ ). Three samples were placed in each vial with the order of placement and the initial mass values recorded. The vials and the samples were sterilized using ethanol (96%) and double distilled water before placement inside the vials. The samples were immersed in 10 mL of phosphate buffer saline (PBS) solution poured into each vial and

the pH readings for each individual vial were recorded. The vials were labeled and placed in an oven maintained at a temperature of 37°C. The samples were placed inside the PBS buffer solution for different time durations such as 1, 3 and 7 days respectively. After the predetermined time, the samples were removed in the same pre-arranged order and the surface adsorbed solution was removed by gently dabbing with a Kimwipes wiper and wet weight was recorded as  $W_w$ . The ratio of swelling was determined using Equation 3.2 below.

**Equation 3.2:**

$$\text{Percentage Swelling} = \frac{W_w - W_i}{W_i} \times 100$$

Swelling percentage was expressed as a mean. (Sowmya, et al., 2011) This entire procedure was repeated for the other compositions.

### 3.5.9 Degradation Studies

#### 3.5.9.1 Mass Loss

Degradation study was carried out to determine the degradation rate of the composite when exposed to PBS solution. Chitosan was prone to degrade due to its ionic nature and weaker polymeric chain. Thus, it was important to study the rate of degradation to investigate the effect of addition of silver salts to chitin chitosan composites.

The samples that was completed swelling study was placed on labeled Petri dishes and left to dry for a period of 5 hours after which the dry weight ( $W_d$ ) was obtained. The degradation of composite film was calculated using Equation 3.3 (Sowmya, et al., 2011).

**Equation 3.3:**

$$\text{Degradation (Rate of weight loss \%)} = \frac{W_i - W_d}{W_i} \times 100$$

Degradation rate was recorded as mean. As a precautionary measure, the vials were sealed with a plastic lid designed for the vials, to prevent evaporation of the PBS over the long period of the experiment and the readings for every time interval was taken simultaneously for all samples (Ambalangodage C. Jayasuriya & Mauch, 2011).

### 3.5.9.2 Ionic Strength Degradation Study

In this experiment, we will focus on ionic strength in PBS solution after immersion of chitin nanofiber reinforced chitosan composite doped with silver salts. The main contribution of ionic strength was silver sulfate and silver acetate doped in the samples. According to solubility rules, all silver ions are insoluble in water except silver nitrate, silver sulfate and silver acetate.

Sample was cut into small 1 cm by 1 cm size and immersed in PBS solution stored in a vial. The ionic strength was measured using Orion 4-Star pH/ISE meter with Orion Ionplus Silver/Sulfide electrode. Reading was taken 1<sup>st</sup> day, 4<sup>th</sup> days, 7<sup>th</sup> days, 11<sup>th</sup> days, and 15<sup>th</sup> days after immersion of sample in PBS solution. Silver acetate is an organic compound with the empirical formula  $\text{CH}_3\text{COOAg}$ . This compound is a photosensitive, white crystalline solid (F. H. MacDougall, & S. Peterson, 1947). Due to the partial solubility of silver acetate in water, silver acetate will dissolve to form silver ion and acetate ion.  $\text{CH}_3\text{COOAg} > \text{CH}_3\text{COO}^- + \text{Ag}^+$  (F. H. MacDougall, & S. Peterson, 1947). Silver sulfate on the other hand was ionic compound which dissolved partially in water to form silver and sulfate ions.  $\text{Ag}_2\text{SO}_4 > 2\text{Ag}^+ + \text{SO}_4^{2-}$ .

The total electrolyte concentration in a solution will eventually affect the solubility or dissociation rate of the salts. In other words, the ionic strength increased initially to a certain value. Where equilibrium between the solution and the un-dissolved remained solute crystal will be established (Nic, Jirat, & Kosata, 2006). The silver ion concentration

will reach an equilibrium when the solution saturates. Beyond this point, there will be no further increase in silver and acetate/sulfate ion (Nic, Jirat, & Kosata, 2006).

### **3.5.9.3 Change of pH Over Extended Period**

One of the important parameter that determine the antimicrobial effectiveness is the ability to maintain or change the pH of solution. Research shown that antimicrobial capability could be increased in alkaline environment while acidic environment reduced antimicrobial capability.

Study was carried out by measuring the pH of PBS solution after 1, 4, 7, 11 and 15 days of immersion of chitin nanofiber reinforced chitosan film doped with silver salts. The influence of pH could be due to scission of chitosan when expose to PBS and also dissolved silver salts.

The composite film produced was immersed in vials filled with PBS solution. The pH reading was taken using Orion pH/ION meter with pH probe after set period of 1, 4, 7, 11 and 15 days respectively. The reading was recorded for analysis.

## CHAPTER 4: RESULTS AND DISCUSSIONS

### 4.0 Introduction

Chitin chitosan composite doped with silver salts was prepared and characterized. TEM was conducted to measure the dimension by using ImageJ software to verify the chitin used is nanofiber. FTIR was done to confirm the complexation of chitin chitosan composite with silver salt and polymeric chain identification of the samples. XRD was carried out to verify the phase composition and nature of composite base on its degree of crystallinity. The surface of the composite was investigated using FESEM to study surface morphology of composite. Tensile test was conducted to study the change in mechanical property due to doping of silver salts.

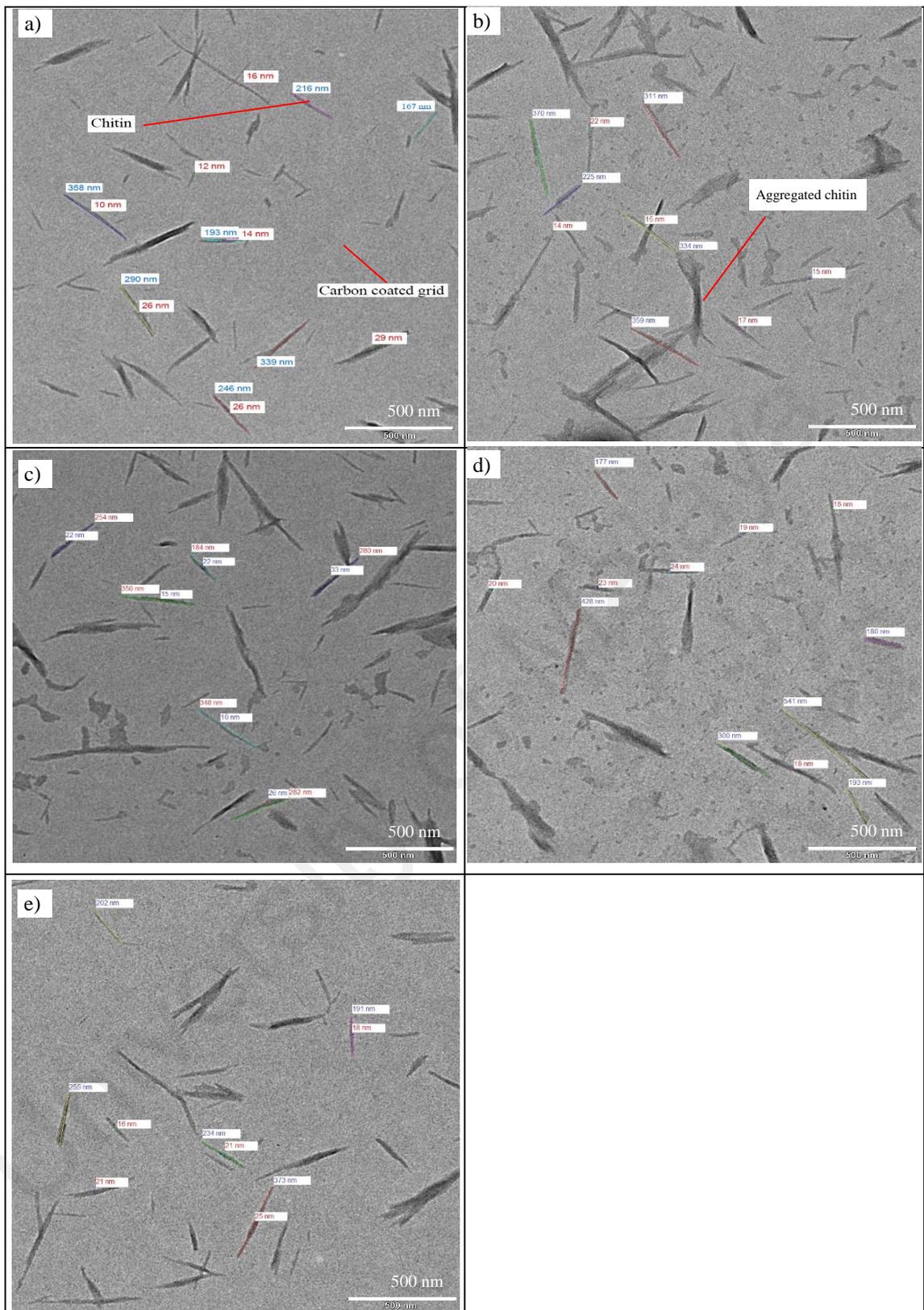
The composite developed was proposed to be an antimicrobial composite, Kirby-Bauer test was conducted to study the antimicrobial efficacy of the composite. The antimicrobial capability was further studied in silver ion release test that confirmed the release of silver ion of the composite.

The final part of the research focus on degradation of the composite to ensure the long-term application of the composite could be realized. The degradation study involve swelling, mass loss, ionic concentration, and finally pH change over extended exposure to environment.

#### 4.1 Transmission Electron Microscopy (TEM)

The dimension and morphology of the chitin fiber used to fabricate the composite with chitosan was studied using TEM. Figure 4.1 shows that some chitin exists as individual strains whilst some was not completely segregated into individual strains resulting in aggregated form. The separation of the chitin fiber is dependent on the harshness of the isolation process such as higher treatment temperature, longer treatment time, and strong mechanical force which could break the chitin nanofiber into smaller dimensions (Salaberria et al., 2015).

ImageJ software was used to measure the dimension of chitin fiber obtained from the TEM micrograph. It was evident that the diameter of chitin fiber is within nanometer range of 10 to 33nm with average of  $(20 \pm 1.05)$  nm. The measured length of chitin fiber was found to be ranged from 200 to 400 nm with average length of 282 nm ( $\pm 16.4$  nm). The dimension of chitin fiber clearly indicates that the chitin is nanofiber shape due to the high aspect ratio of length to diameter in nanometer range. These measurements correspond to the dimension of chitin nanofiber extracted from shrimp shells reported in previous research by Phongying, Aiba, and Chirachanchai which suggested where chitin nanofiber from shrimp shell had dimensions of 200-560 nm length and diameter of 18-40 nm (Phongying, Aiba, and Chirachanchai, 2007).



**Figure 4.1: Image of chitin suspension on different carbon coated grid (n=5) with scale bars of 500 nm. Blue indicates length of chitin fiber and red indicates diameter.**

## 4.2 Fourier Transform Infra-Red Spectroscopy (FTIR)

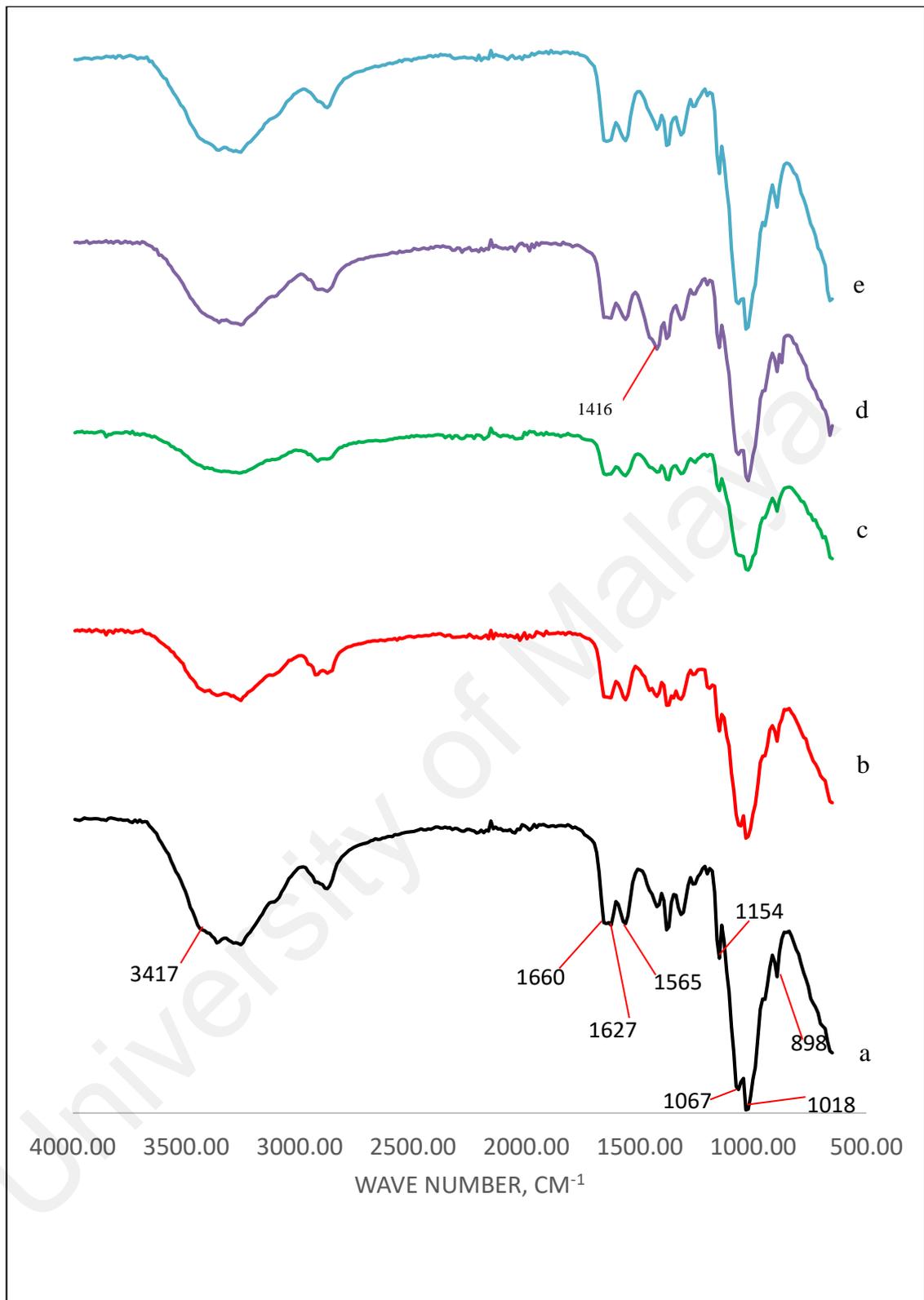
FTIR was performed to confirm the polymeric structure of chitin reinforced chitosan film doped with silver sulfate and silver acetate as shown in Figure 4.2 and Figure 4.3. All samples exhibit transmission peak at 1018 to 1067  $\text{cm}^{-1}$ , which show presence of saccharide moiety (Shankar, Reddy, Rhim, Kim, 2015). The peak around 3417  $\text{cm}^{-1}$  corresponds to the vibration of N-H and O-H bond. Peaks at 1565  $\text{cm}^{-1}$  was due to the  $\text{NH}_2$  group in both chitin and chitosan.  $\text{NH}_2$  characteristic peak was shifted from 1565  $\text{cm}^{-1}$  to 1555  $\text{cm}^{-1}$  and intensified as more silver sulfate was doped. This change in characteristic peak can be attributed to bonding between protonated amino and sulfate from silver sulfate doped. This was not observed in doping with silver acetate which suggested there are little or no interaction between acetate anion with amino functional groups of chitin and chitosan (Fajardo, Lopes, Caleare, Britta, et al, 2013).

FTIR results from composites doped with silver acetate shows increasing peaks at 1416  $\text{cm}^{-1}$  which are attributed to  $-\text{COO}$  symmetric stretching on acetate anion. Peak appears to increase in absorbance as amount of silver acetate increases indicating the increased amount of acetate presence in the matrix (Pang, Wu, Zhang, 2015).

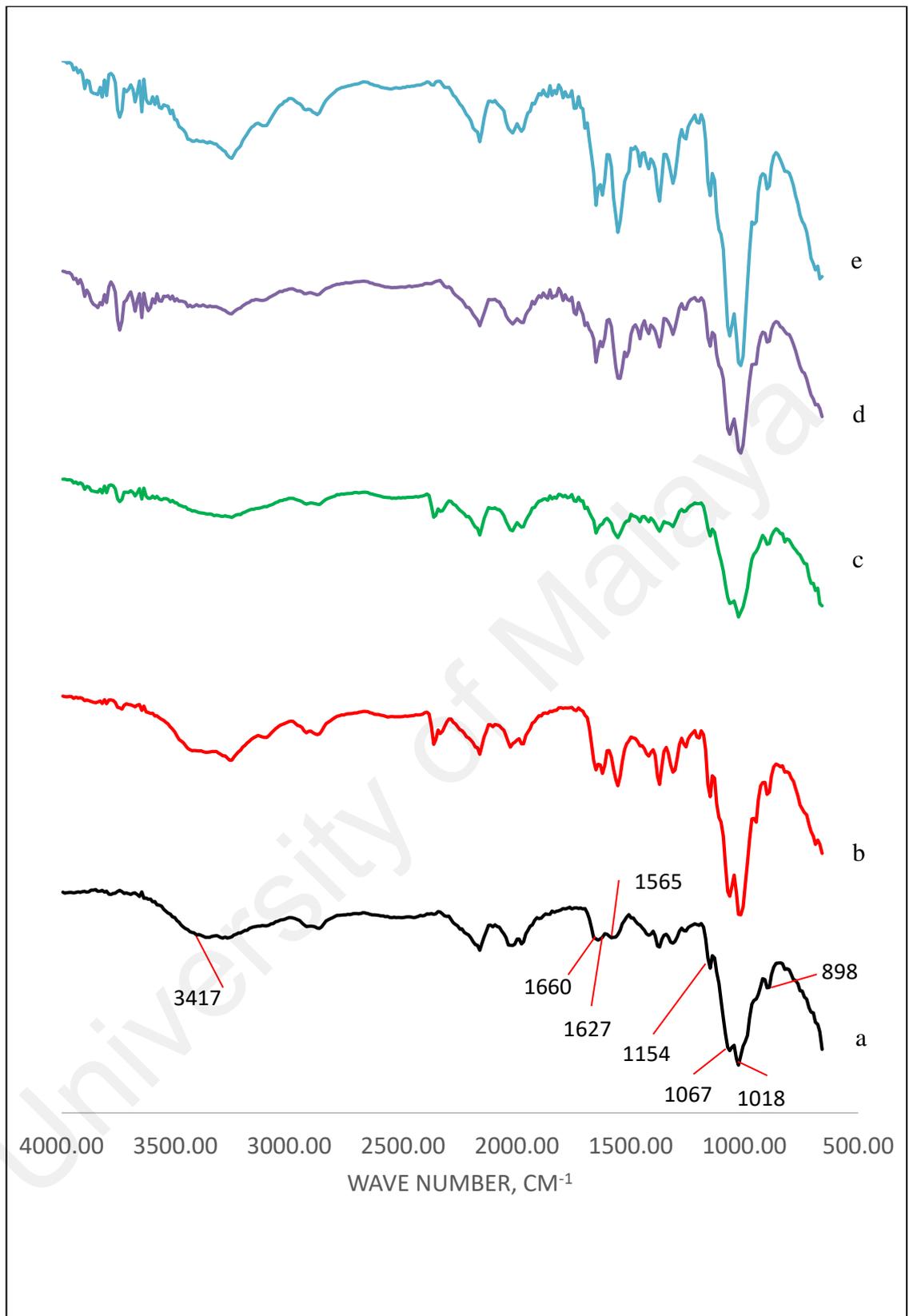
Peaks observed in the region of 1660 and 1627  $\text{cm}^{-1}$  are due to amide I band which is the characteristic of  $\alpha$ -chitin in this case, from shrimp shell. Half of the carbonyl groups are bonded through hydrogen bonds to the amino group inside the same chain (C=O-H-N) that is responsible for the vibration mode at 1660  $\text{cm}^{-1}$ . Remaining carbonyl groups produces the same bond plus another with the group  $-\text{CH}_2\text{OH}$  from the side chain. This additional bond produces a decrease in the amide I band at 1627  $\text{cm}^{-1}$ . The existence of these inter-chain bonds is responsible for the high chemical stability of the  $\alpha$ -chitin structure (Mohammad R. Kasaai, 2007). The FTIR absorption band and their assigned bonding type was summarized in Table 4.1.

**Table 4.1: FTIR absorption band position and corresponding band assignment.**

Absorption band position, $\text{cm}^{-1}$	Absorption band assignment	Reference
900-1200	Saccharide moiety	<i>Shankar, Reddy, Rhim and Kim, 2015</i>
1416	-COO symmetric stretching (Acetate)	<i>Pang, Wu, Zhang, 2015</i>
1565	-NH <sub>2</sub>	<i>Fajardo, Lopes, Caleare, Britta, et al, 2013</i>
1627 & 1660	Amide I band	<i>Shankar, Reddy, Rhim and Kim, 2015</i>
3417	N-H and O-H vibration	<i>Fajardo, Lopes, Caleare, Britta, et al, 2013</i>



**Figure 4.2: FTIR results for a) chitin-chitosan composite, chitin-chitosan composite doped with various amount of silver acetate, b) 0.1 g, c) 0.3 g, d) 0.5 g and e) 0.7 g**



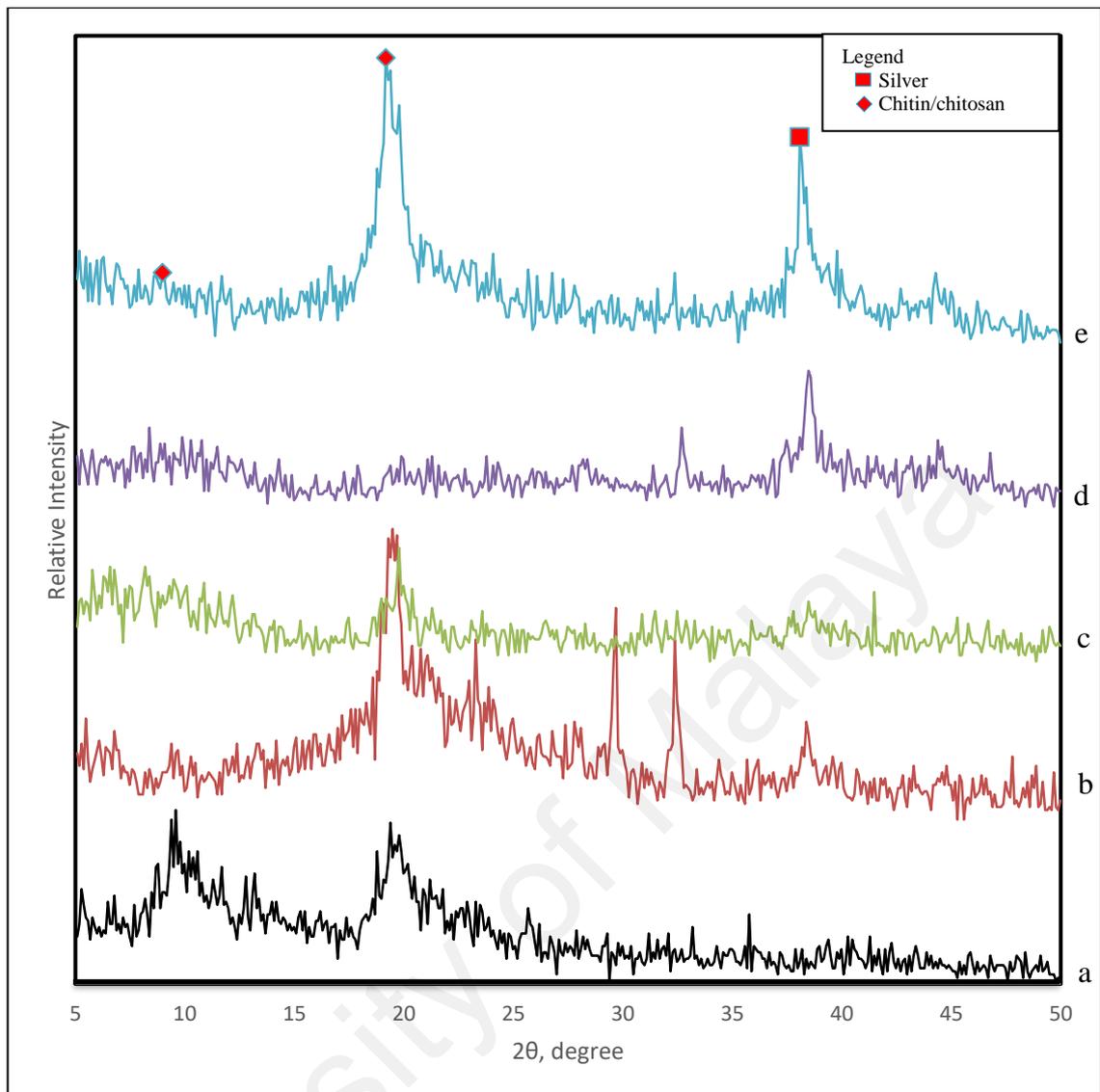
**Figure 4.3: FTIR results for a) chitin-chitosan composite, chitin-chitosan composite doped with various amount of silver sulfate, b) 0.1 g, c) 0.3 g, d) 0.5 g and e) 0.7 g**

### 4.3 X-Ray Diffraction (XRD)

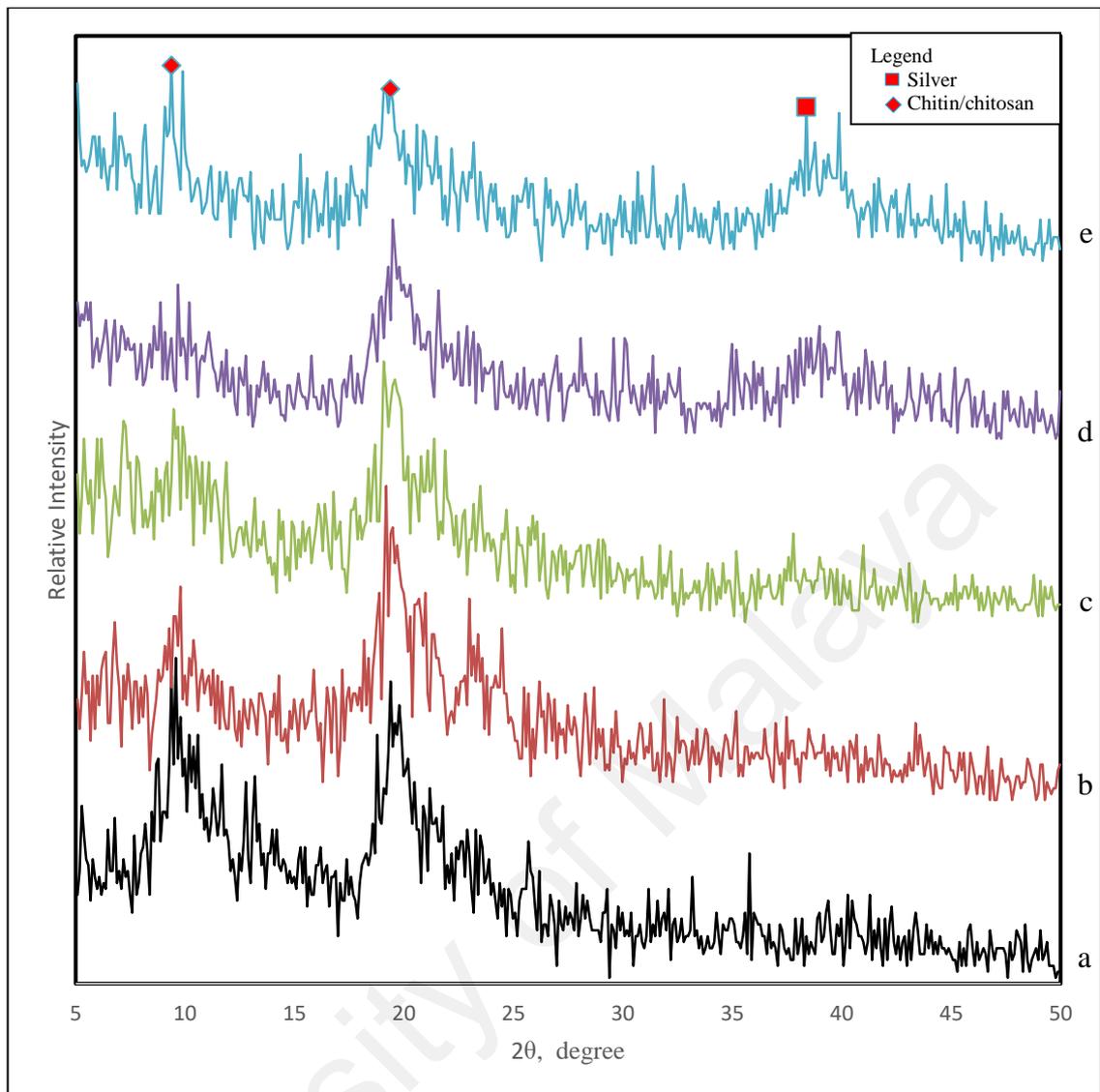
XRD results indicate that chitin nanofiber reinforced chitosan film doped with silver acetate have a much lower crystallinity index compared to film doped with silver sulfate as shown in Figure 4.4 where the peak at intensity  $2\Theta = 9^\circ$  and  $19^\circ$  was decreased as more silver acetate salt added into chitin chitosan composite. This is due to weaker anion of acetate unable to form strong electrostatic interactions with  $\text{-NH}_3^+$  on chitosan in the matrix. The larger anion size of acetate further causes steric hindrance effect that reduce the intramolecular bonding between chitosan (Wu, Wei, et al., 2007).

Figure 4.5 shows XRD pattern for chitin nanofiber reinforced chitosan film doped with various amounts of silver sulfate. Peaks at  $2\Theta = 9^\circ$  and  $19^\circ$  as well as  $2\Theta = 20^\circ$  were observed and indicated the presence of  $\alpha$ -chitin. As silver sulfate amount was increased, an increase in intensity at  $2\Theta = 38^\circ$  was observed due to the increase in amount of silver in the composite film (Yimin Fan et al., 2009).

XRD results for both chitin nanofiber reinforced chitosan doped with silver sulfate and silver acetate showed that doping amount of 0.5 grams and above will start to cause the excessive silver salts to precipitate onto the surface of the film. Excessive amount of silver salts means the steric hindrance effect was at its maximum proven in the reduced tensile strength of the film at these two concentrations of doping. The postulation was proven by the FESEM image taken where the presence of silver nano particle and rough surface morphology both pointing towards reduced strength of the film structure at higher doping concentration.



**Figure 4.4: XRD results for a) chitin-chitosan composite, chitin-chitosan composite doped with various amount of silver acetate, b) 0.1 g, c) 0.3 g, d) 0.5 g and e) 0.7 g**



**Figure 4.5: XRD results a) chitin-chitosan composite, chitin-chitosan composite doped with various amount of silver sulfate, b) 0.1 g, c) 0.3 g, d) 0.5 g and e) 0.7 g**

**Table 4.2: Crystallinity index of the antimicrobial film calculated using Equation 3.1 from results in Figure 4.4 and Figure 4.5**

Sample	Crystallinity index of Silver Sulfate (%)	Crystallinity index of Silver Acetate (%)
Chitin chitosan	32	32
0.1g silver salt doped	50	63
0.3g silver salt doped	46	35
0.5g silver salt doped	26	12
0.7g silver salt doped	39	69

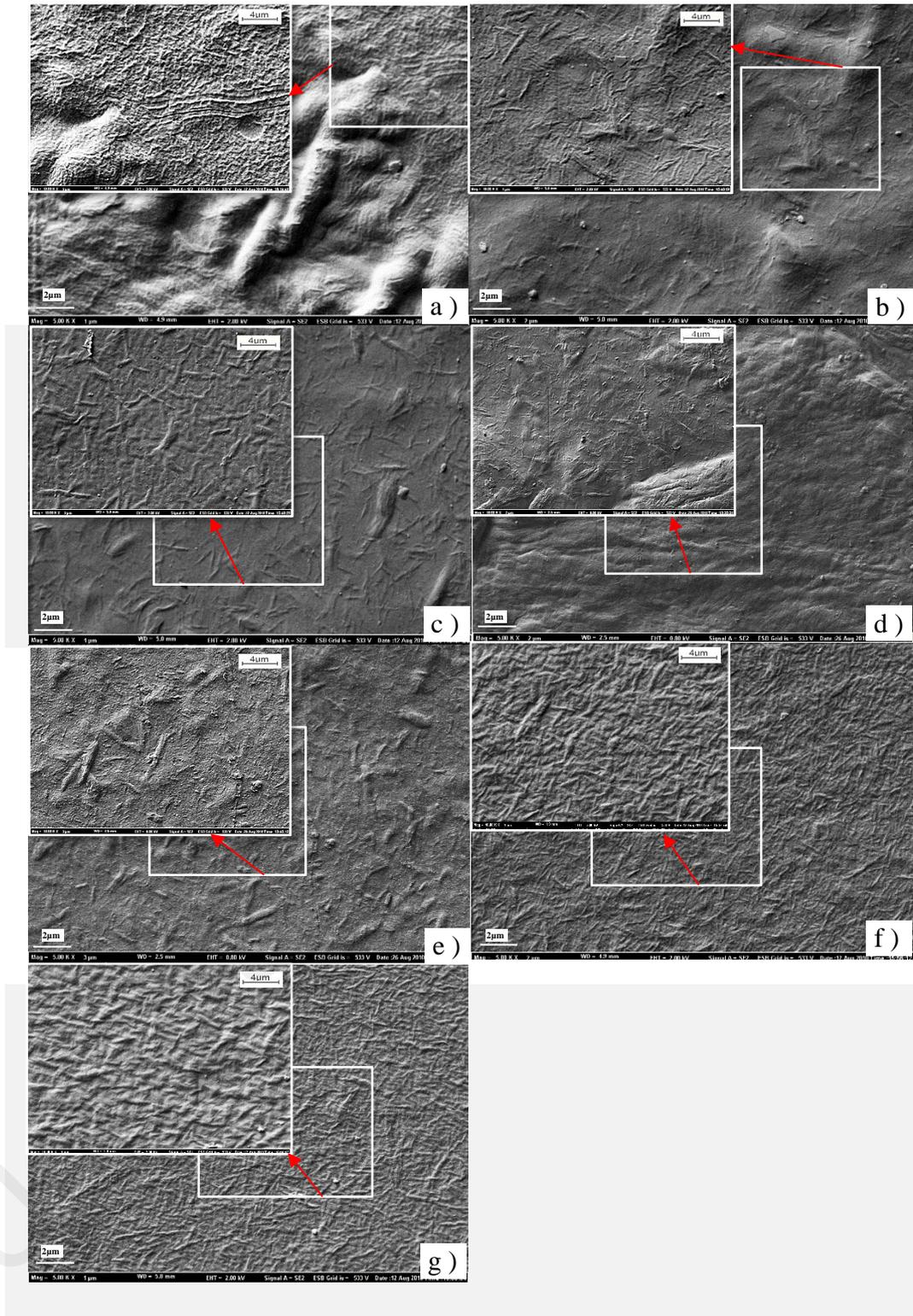
#### 4.4 Field Emission Scanning Electron Microscopy (FESEM)

FESEM image was captured to inspect the surface morphology of the coating. Chitin nanofiber appear visually to be bound in the chitosan matrix. Chitin nanofiber was found to be distributed randomly and uniformly in the chitosan matrix.

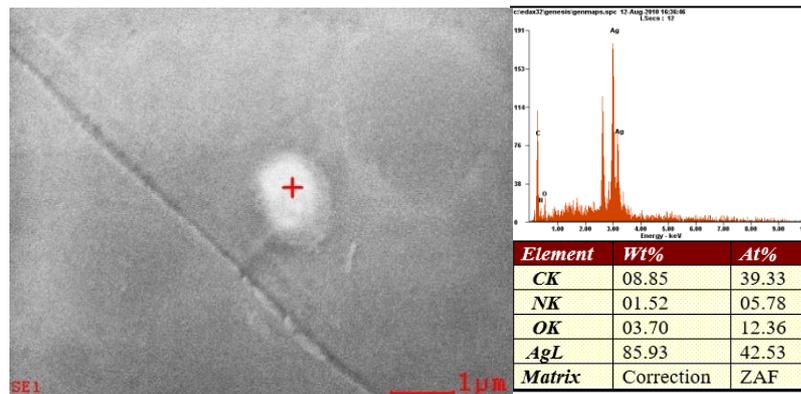
Surface of chitin nanofiber reinforced chitosan film doped with silver sulfate appears to be generally smooth with occasionally appear of small nano-sized particle. The sample with highest doping (above 0.5 grams) appears to have rough surface (Figure 4.6 d,e). This coincide with the XRD spectrograph where the extra silver salts start to precipitate onto the surface of the film where peaks of silver increases at  $2\Theta = 38^\circ$  for samples doped with 0.5 grams of silver salts and 0.7 grams of silver salts.

Surface of chitin nanofiber reinforced chitosan composite doped with silver acetate appears to be rough with a lot of surface features Figure 4.6 f and g. The rough surface coincides with the XRD spectrograph where the polymer appears to be very amorphous thus resulting in the lower tensile strength. Amorphous nature of the polymer matrix could be due to the limitation of steric hindrance between silver acetate and chitosan. FESEM image for higher silver acetate loading was not obtained as the silver acetate salt do not appear to be able to dissolve in chitin-chitosan composite for doping of 0.5g and 0.7g thus the uneven surface was not able to be observed clearly under FESEM.

EDX spot results on the particles (Figure 4.7) that appears on the surface of the composite shows that it is silver nanoparticle that precipitate out of the composite and agglomerated on the surface of the composite.



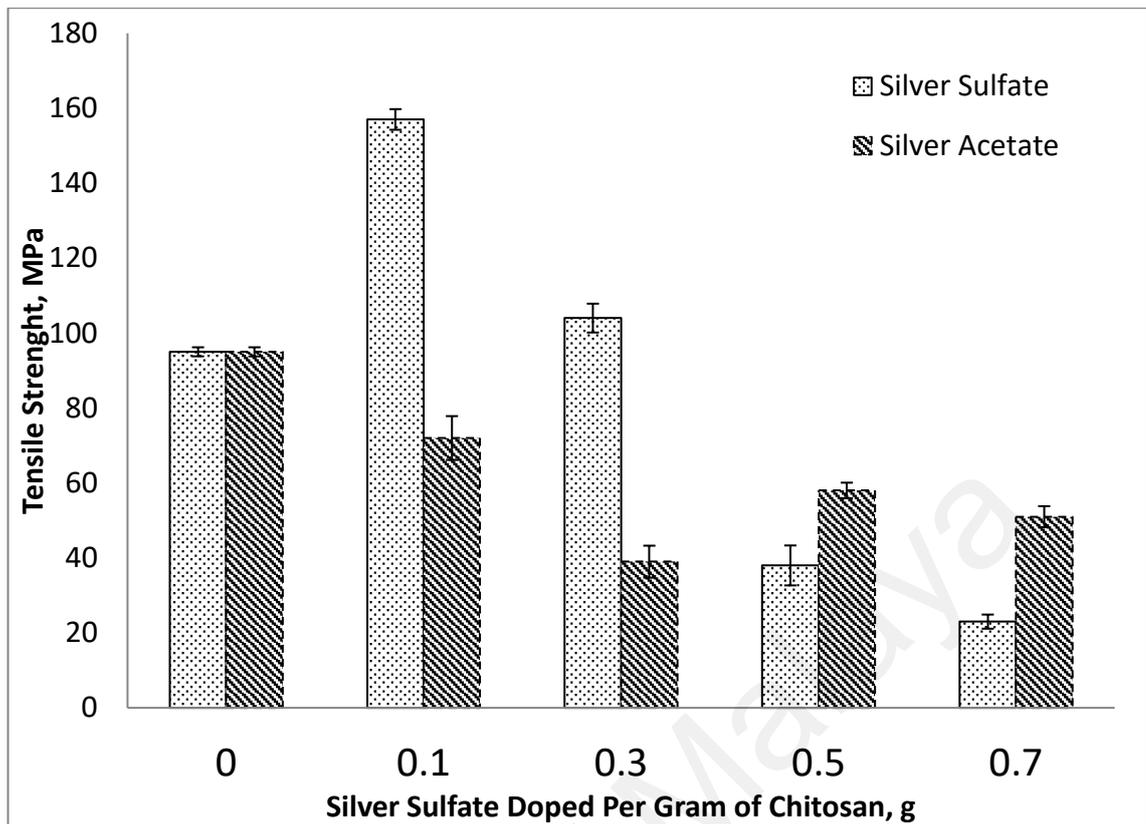
**Figure 4.6: FESEM images for a) chitin-chitosan composite, chitin-chitosan composite doped with silver sulfate of various concentration b) 0.1g, c) 0.3g, d) 0.5g, e) 0.7g, and chitin-chitosan composite doped with silver acetate of various concentration of f) 0.1g and g) 0.3g**



**Figure 4.7: EDX spot analysis of particle on surface of composite film with 0.7 g silver salt doping and its weight concentration.**

#### 4.5 Tensile Strength

Tensile testing was carried out on chitin nanofiber reinforced chitosan film doped with 0 to 0.7 grams of silver sulfate and silver acetate. Results on Figure 4.8 showed that addition of 0.1 grams of silver sulfate to the coating would produce the strongest film. The ultimate tensile stress was calculated to be 157 MPa for coating with 0.1 grams silver sulfate. As the amount of silver sulfate was increased, the strength will decrease until only 23 MPa for 0.7 grams of silver sulfate doped sample. The tensile strength of pure chitin/chitosan film decreased from 95 MPa to 39 MPa when doped with 0.3 g silver acetate.



**Figure 4.8: Ultimate tensile strength versus silver salt doped (n=3)**

Results from XRD supports the tensile test trend in Figure 4.8. Crystallinity index was directly related to the tensile strength of the samples as shown in Figure 4.9 and Figure 4.10 where crystallinity index decreases with increasing amount of silver sulfate doped. This showed that tensile strength decreased as crystallinity index in the composite film was decreased.

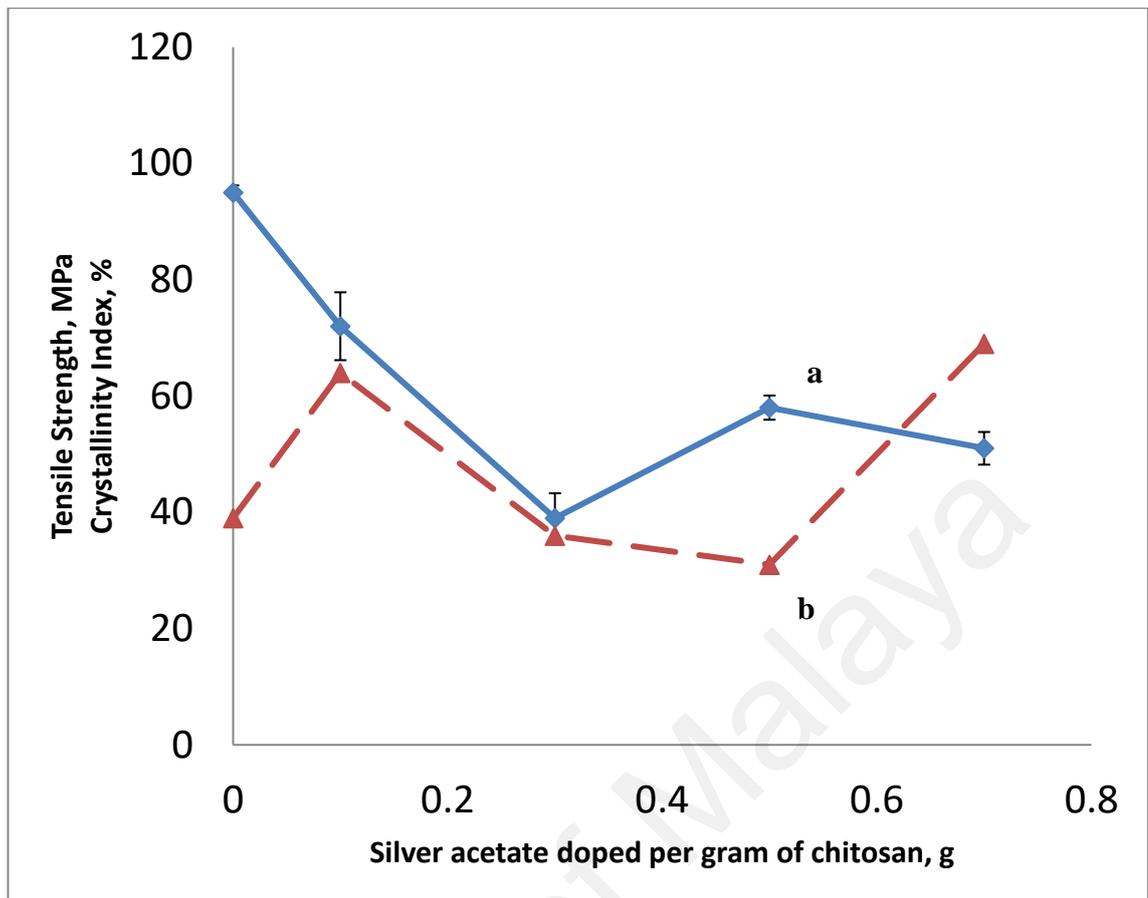
Silver sulfate with doping quantity of 0.1 grams have seen positive increment in tensile strength of chitin-chitosan composites. We can deduce that the strong anion power of sulfate ( $\text{SO}_4$ ) in silver sulfate salts aid in formation of cross linking of chitosan between its  $-\text{NH}_3^+$  and sulfate anion. Results calculated from XRD spectrograph on table 4.2 observed an increase in degree of crystallinity when chitin nanofiber reinforced chitosan

film is doped with 0.1g of silver sulfate. This shows that the molecules are packed more aligned and closely together.

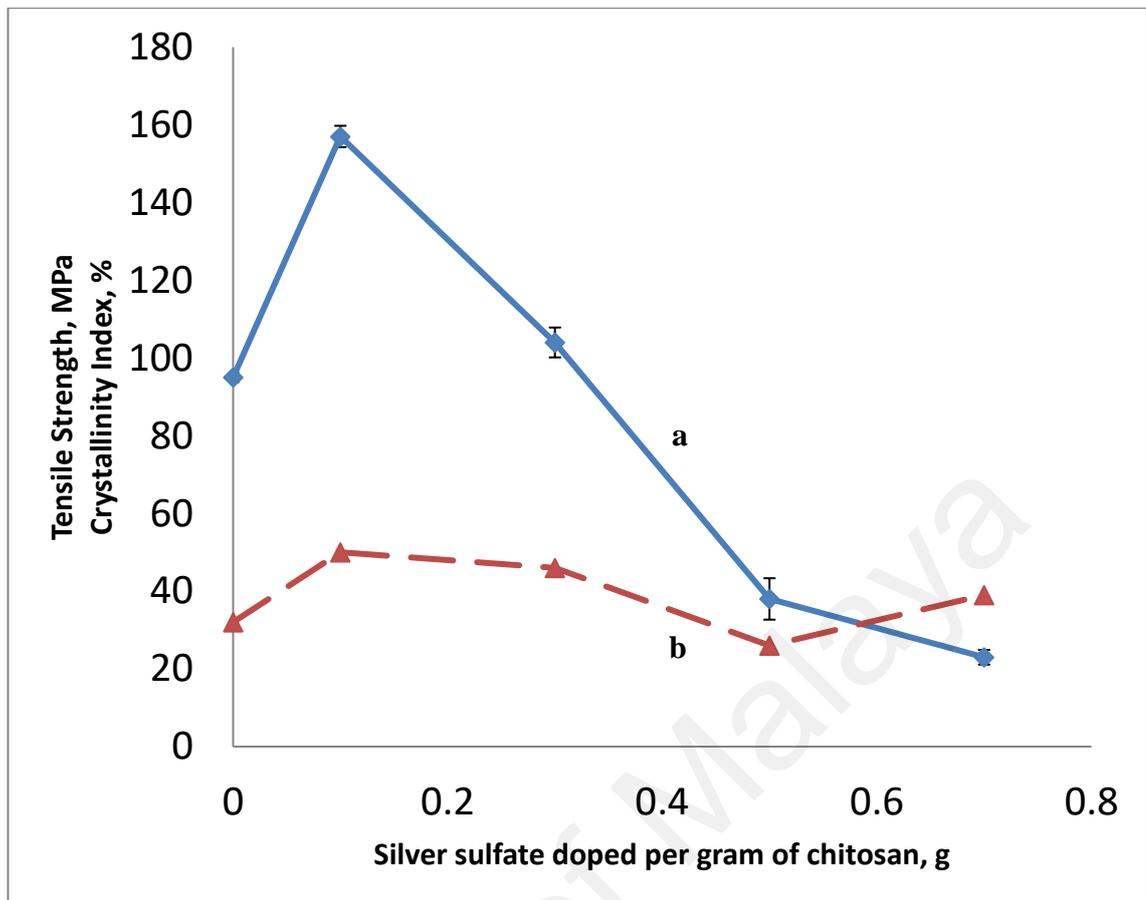
Further increase of silver sulfate salts decreases tensile strength as the steric hindrance effect of silver sulfate which not only reduce the effects of sulfate anion but also prevented the formation of hydrogen bonds as silver ions occupied the active sites in chitosan (Wu, Wei, Wang, Su, Ma, 2007).

Silver acetate was unable to form strong electrostatic interactions with  $-NH_3^+$  on chitosan due to its weaker anion of acetate. This weaker interaction with chitosan matrix directly reflected in its tensile strength where it decreases as more silver acetate was added. The presence of extra silver acetate creates steric hindrance in the matrix which disrupt the formation of intra molecular hydrogen bond of chitosan polymer chain (Wu, Wei, Wang, Su, Ma, 2007).

Additional of 0.7 grams of silver salts had showed increase in crystallinity index compared with samples doped with 0.5 grams of silver salt which is in opposite trend of reducing crystallinity index when doping increase. This increase in crystallinity index does not improve the tensile strength of the composite as shown in Figure 4.2.1. Investigation on FESEM image shown rough surface morphology where silver salt precipitate onto the surface of the film. The presence of salt particle and rough surface created stress concentration zone which resulted in lower tensile strength of samples doped with 0.7 grams of silver salts.



**Figure 4.9: Comparison of a) tensile strength with b) crystallinity index of chitin-chitosan composite doped with silver acetate.**



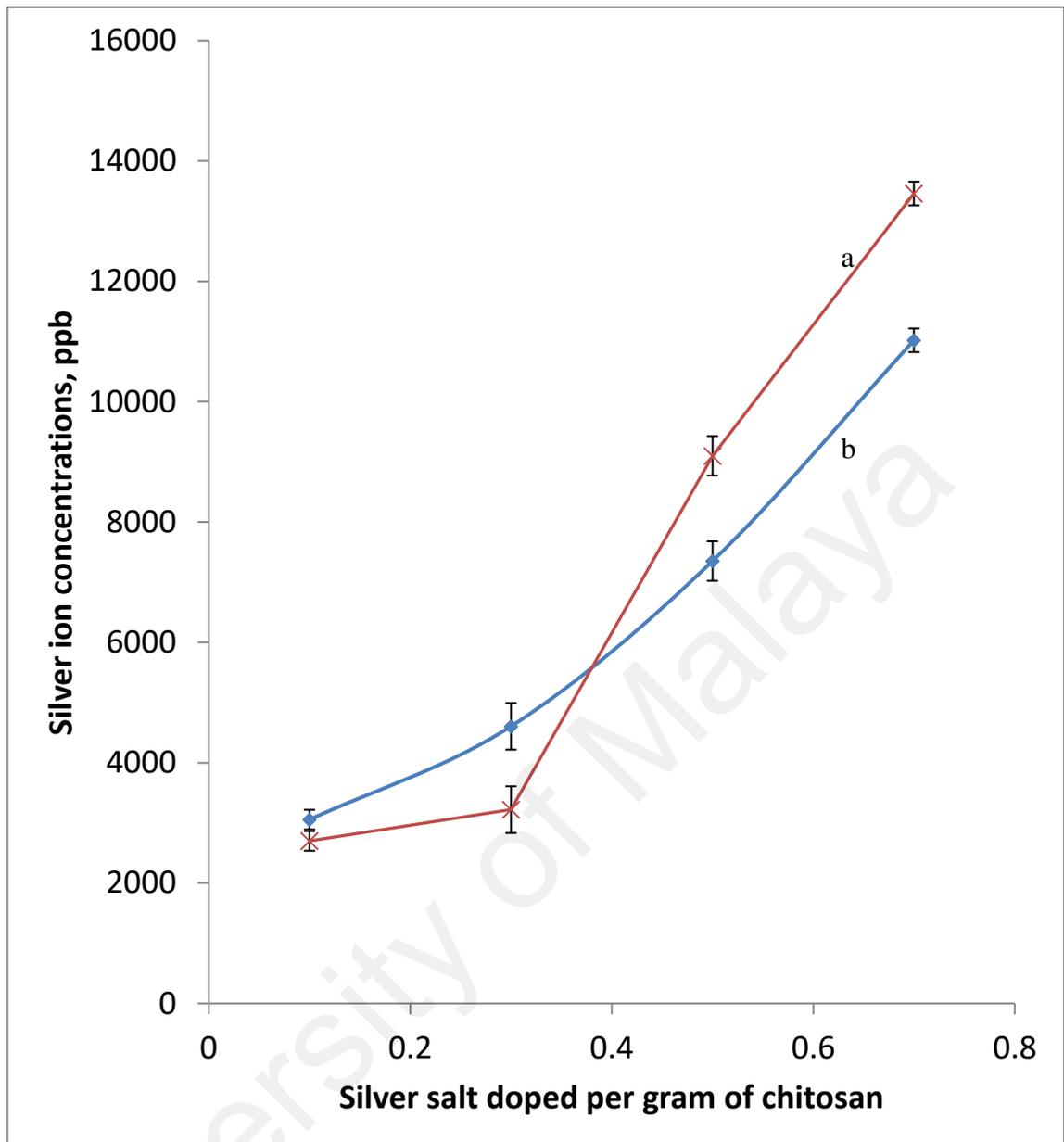
**Figure 4.10: Comparison of a) tensile strength with b) crystallinity index of chitin-chitosan composite doped with silver sulfate.**

#### 4.6 Silver ion release test

The main purposes of doping of silver salts was to improve the composite film's antimicrobial properties. Silver sulfate and silver acetate will ionize (dissociate) into silver cation and its respective anion partially in presence of water (Michael O. Hurst, Ryan C. Fortenberry, 2015). Chitosan's  $-NH_2$  and  $-OH$  groups contributes to this chelating property where silver ions are chelated to chitosan in chitosan polymer matrix (Nivethaa, Narayanan & Stephen, 2015). This chelating properties enable chitosan to act as a substrate to immobilize silver ions in the film and release it to surrounding when in contact with moist or wet environment as water penetrate the polymer matrix.

Silver ion release test showed the ability of the composite to release silver ions increasingly with doping amount increases from 0.1 to 0.7 grams of silver salts (Figure 4.11). As more silver cations are immobilized in chitosan matrixes, it can be released to the surrounding when the composite film is in moist or wet environment as silver ions dissolved in water.

Silver ion release ability measured by concentration of silver ions after 24 hours of immersion in distilled water increases from 3054 ppb to 11000 ppb for silver sulfate doped samples. Silver acetate samples showed least silver ion release of 2700 ppb at 0.1 grams doping weight. The concentration of silver ions increases from 2700 ppb to 13460 ppb at 0.7 grams doping weight. Chitin nanofiber reinforced chitosan doped with silver sulfate and acetate both have strong antimicrobial capability when compared with previous studies which concludes that minimum concentration for effective antimicrobial properties for silver ions is 900 ppb (Mikihiro Yamanaka, 2005). This shows that chitin nanofiber reinforced chitosan is a great carrier for silver cation at up to 0.1 grams of doping which have superior mechanical properties such as high tensile strength, good barrier properties against different environment, and biocompatibility.

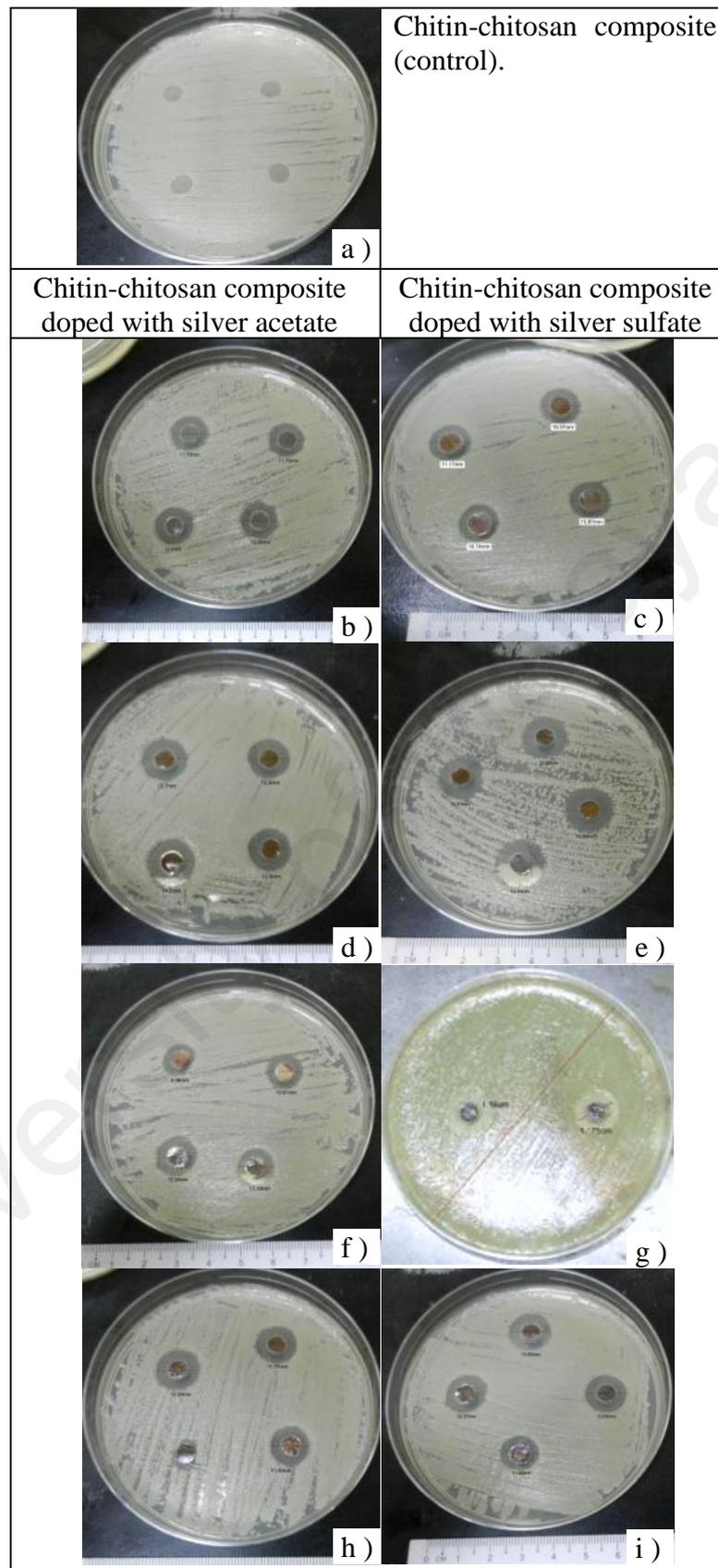


**Figure 4.11: Concentration of silver ion presence in solution after 24 hours of immersion in distilled water a) chitin-chitosan composite doped with silver acetate, b) chitin-chitosan composite doped with silver sulfate.**

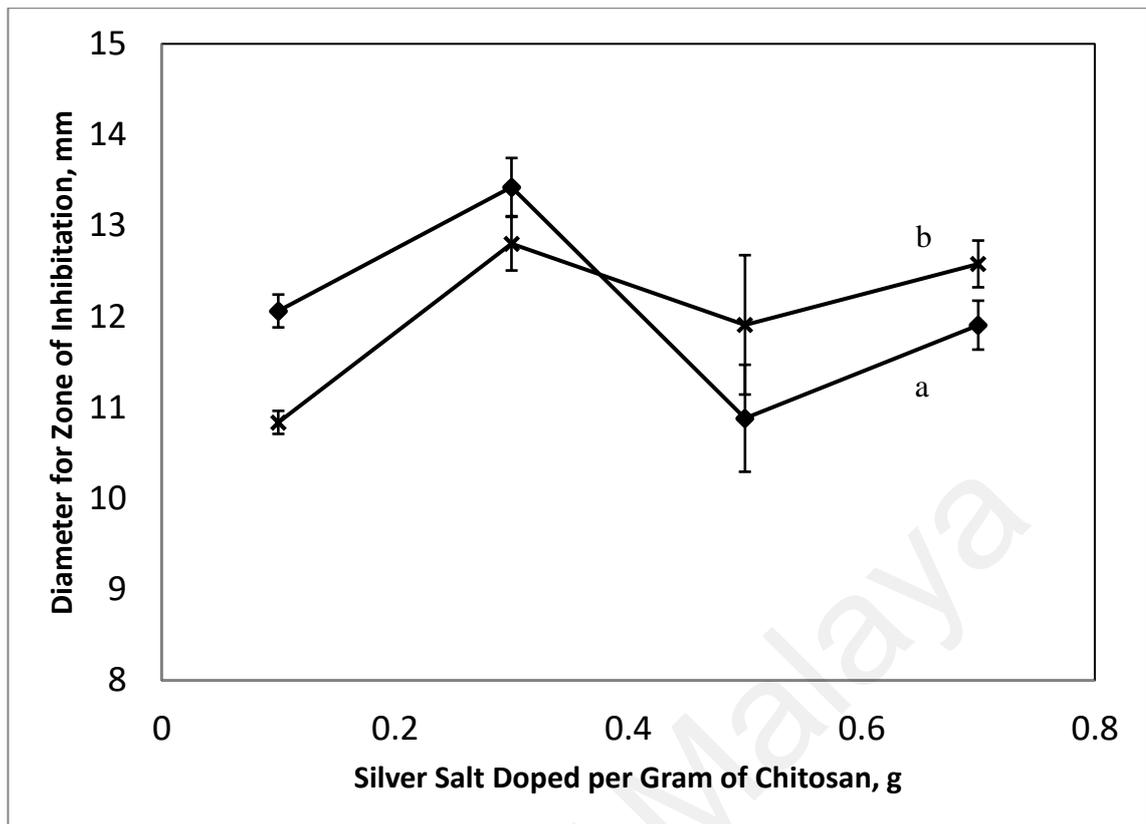
#### 4.7 Kirby-Bauer disk Diffusion Susceptibility Test

The Kirby-Bauer disk diffusion susceptibility test successfully showed antimicrobial strength of the newly developed antimicrobial coating. All samples doped with silver acetate and silver sulfate showed similar size of inhibiting zone of more than 10 mm in diameter as shown in Figure 4.13. Samples doped with 0.3 grams of silver acetate had the best antimicrobial property with zone of inhabitation of 13.42 mm diameter in average.. The region could clearly be distinguished by the clear gel compare with cloudy color on region habitat by *E. coli* (Figure 4.12 b to i). This proved that the film could be a very strong antimicrobial agent.

Neat chitin nanofiber reinforced chitosan film without any doping shows no region of inhibitory zone around the samples but it is evident that Figure 4.12(a ) show no growth of *E. coli* colony both above and below the sample. This coincide with previous studies that chitin and chitosan is bacteriostatic instead of bacteriocidal where it flocculates the bacteria and starve it off its nutrient and oxygen (M.S. Benhabiles, R. Salah, H. Lounici, et al, 2012). This mode of action is limited by the distance of antimicrobial activity can remain positive as the large molecular size of the chitosan prevented chitosan from dissolve in wet or moist environment.



**Figure 4.12: Antimicrobial results from Kirby-Bauer test. a) chitin-chitosan composite (control) and chitin-chitosan composite doped with different concentration of silver acetate and silver sulfate respectively b) 0.1g silver acetate, c) 0.1g silver sulfate, d) 0.3g silver acetate, e) 0.3g silver sulfate, f) 0.5g silver acetate, g) 0.5g silver sulfate, h) 0.7g silver acetate and i) 0.7g silver sulfate.**



**Figure 4.13: Diameter for zone of inhibition on samples with varying amount of silver doped measured from samples in Figure 4.12 (n=4). a) Chitin-chitosan composite doped with silver acetate, b) chitin-chitosan composite doped with silver sulfate.**

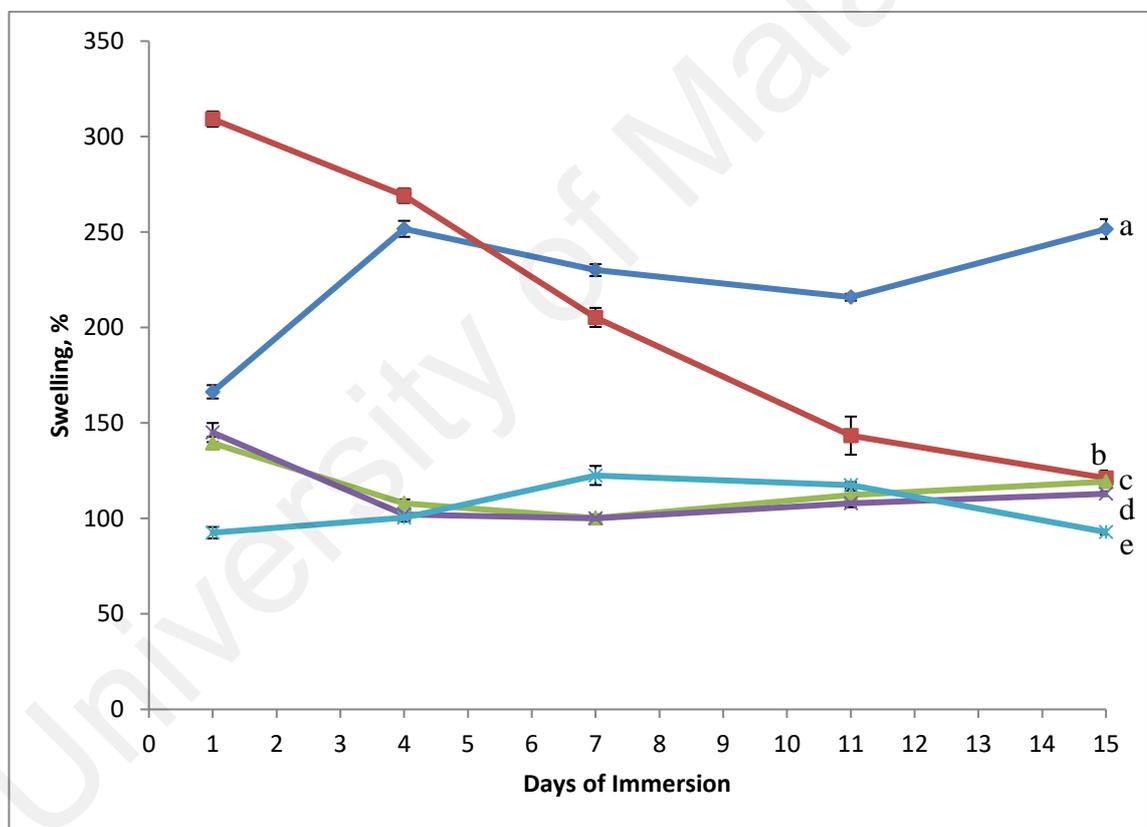
#### 4.8 Swelling study

Figure 4.14 and Figure 4.15 showed that samples doped with silver salts exhibits lower swelling percentage probably due to the interstitial bond created during the doping that prevented the degradation process. Thus, we can conclude that doping of silver salts could improve the durability and reduce the degradation rate in chitin nanofiber reinforced chitosan composite.

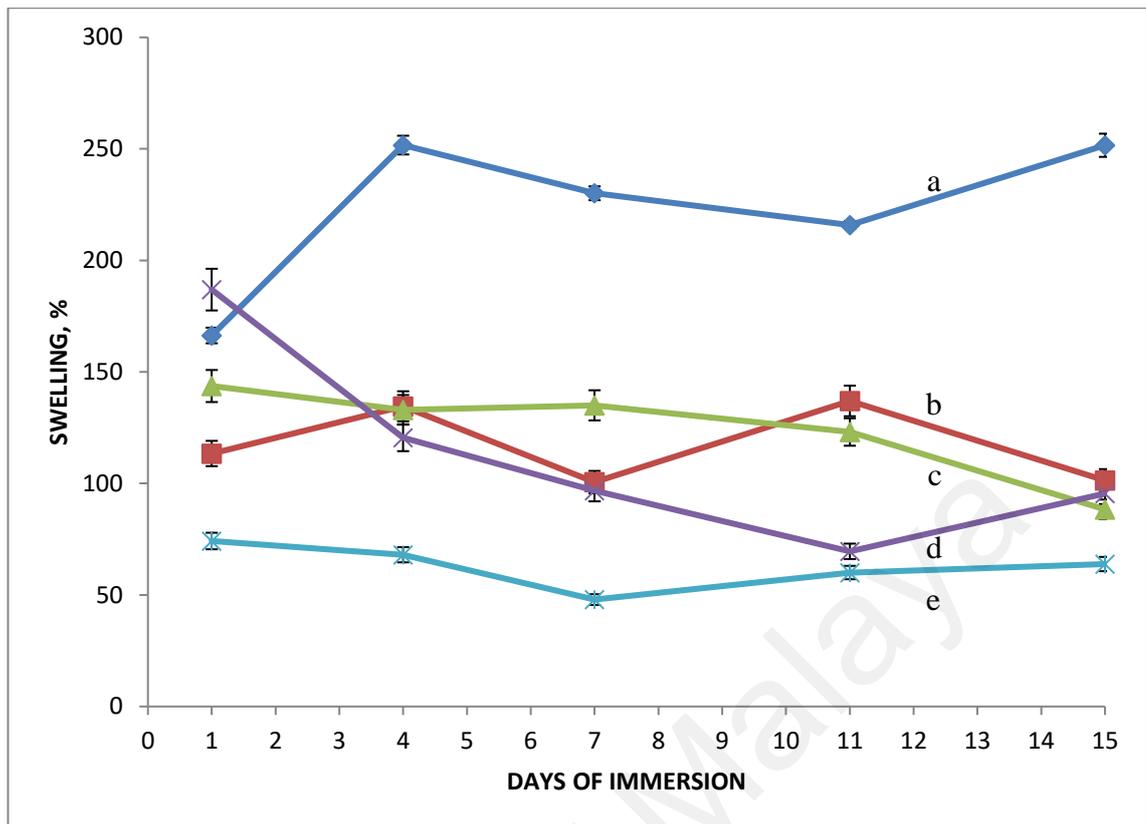
Experiment shows that neat chitin-chitosan composite film has higher degree of swelling at equilibrium followed by samples doped with 0.1 gram of silver salt. Composite film doped with 0.3 and 0.5 grams of silver salts show similar degree of

swelling. Samples with 0.7 grams doping of silver salt have lowest degree of swelling compared to the other samples.

The degree of swelling decreases as the amount of doping increases. This shows that there are interactions between silver salts and chitosan matrixes which causes the decrease in rate of degradation of chitosan in water. The decrease of degree of swelling as doping increases can be attributed to the increase in degree of crystallinity. Higher degree of crystallinity indicates more and stronger cross-linking between polymer chain. This limits the empty spaces between polymer chain for liquid to enter the composites.



**Figure 4.14: Swelling percentage for chitin nanofiber reinforced chitosan samples doped with various amount of silver acetate. a) Chitin-chitosan composite, chitin-chitosan composite doped with various amount of silver acetate, b) 0.1 g, c) 0.3 g, d) 0.5 g and e) 0.7 g**

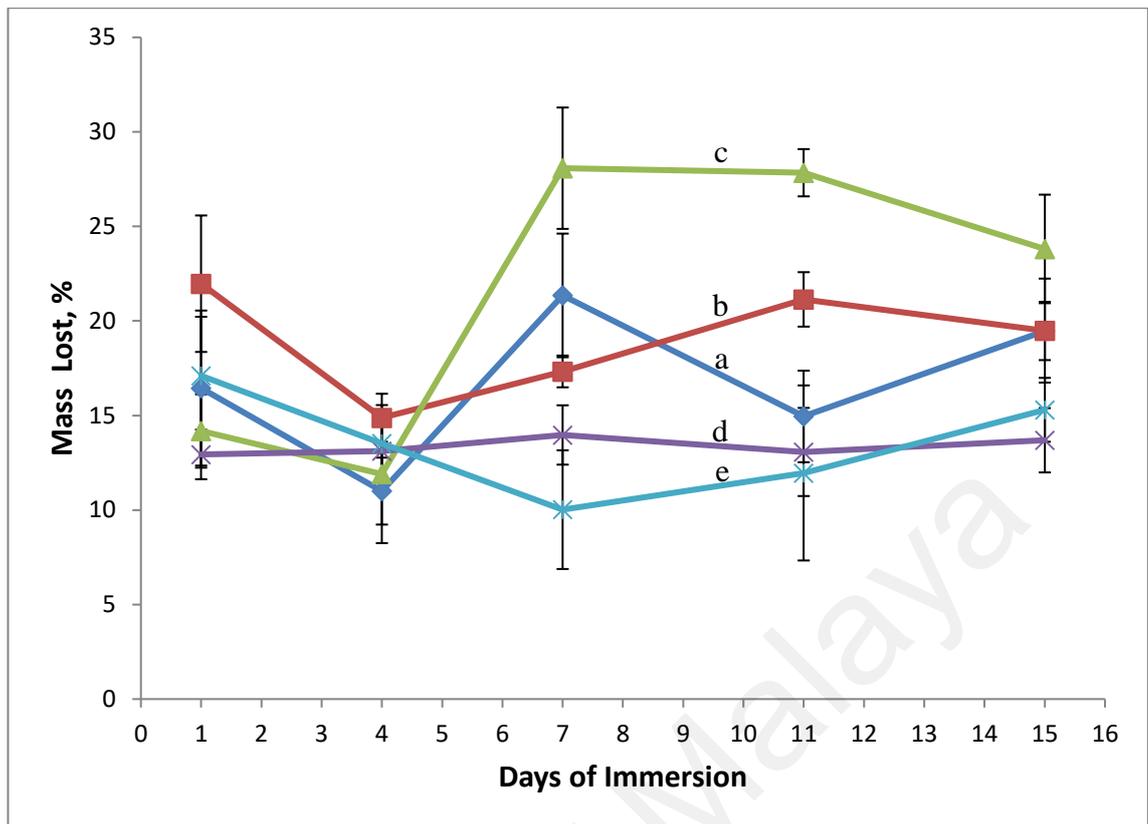


**Figure 4.15: Swelling percentage for chitin nanofiber reinforced chitosan samples doped with various amount of silver sulfate. a) Chitin-chitosan composite, chitin-chitosan composite doped with various amount of silver acetate, b) 0.1 g, c) 0.3 g, d) 0.5 g and e) 0.7 g**

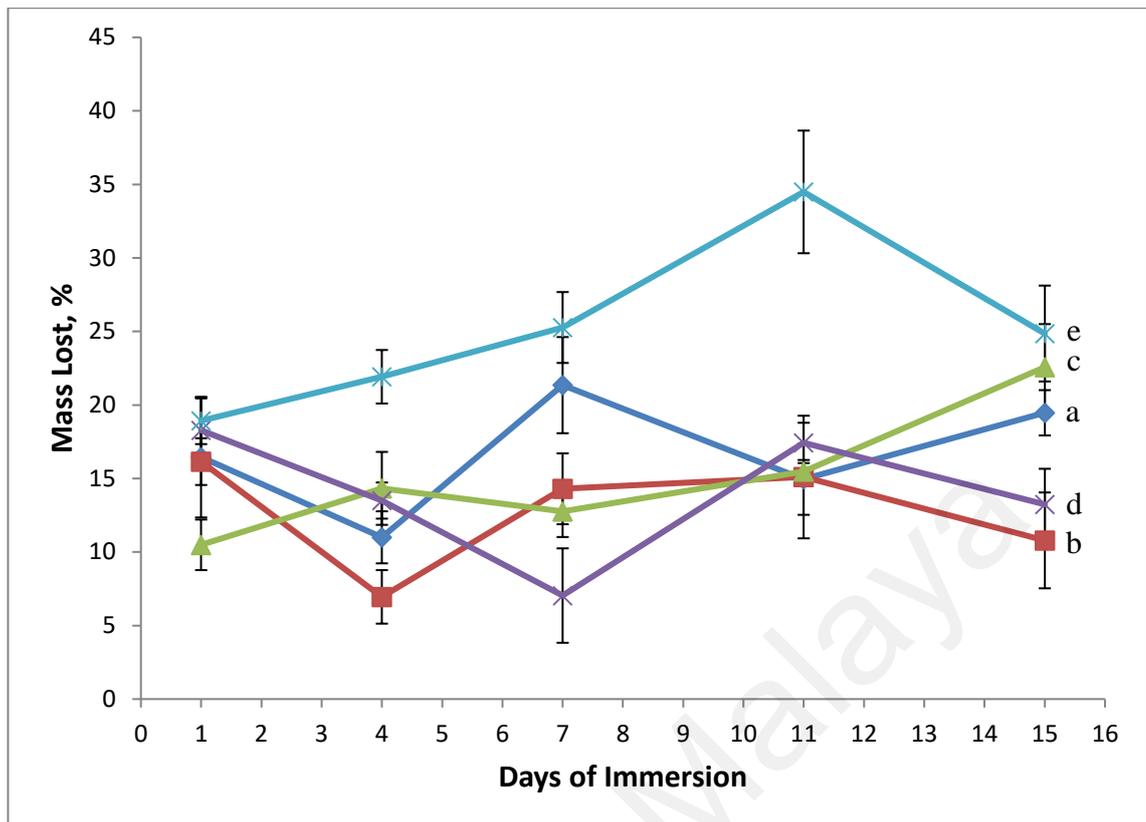
## 4.9 Degradation study

### 4.9.1 Mass Loss

From the graphs on mass loss (Figure 4.16 & Figure 4.17) films doped with 0.3g silver acetate and 0.7g silver sulfate showed higher mass loss while the remaining samples (though undergo degradation) do not display an exponentially increasing trend in mass loss. All samples show similar amount of mass loss of 10 to 22% on mass loss after one day of immersion and subsequent measurements after day 1 remain within range of 10 to 22% except for the samples doped with 0.3g silver acetate and 0.7g silver sulfate. The results are directly related to crystallinity of the samples where 0.3g silver acetate and 0.7g silver sulfate doping have similar crystallinity index of 35% and 39%.



**Figure 4.16:** Mass lost percentage over days of immersion for a) chitin-chitosan composite doped with various amount of silver acetate of b) 0.1 g, c) 0.3 g, d) 0.5 g, and e) 0.7 g



**Figure 4.17:** Mass lost percentage over days of immersion for a) chitin-chitosan composite doped with various amount of silver sulfate of b) 0.1 g, c) 0.3 g, d) 0.5 g, and e) 0.7 g

#### 4.9.2 Ionic potential in PBS

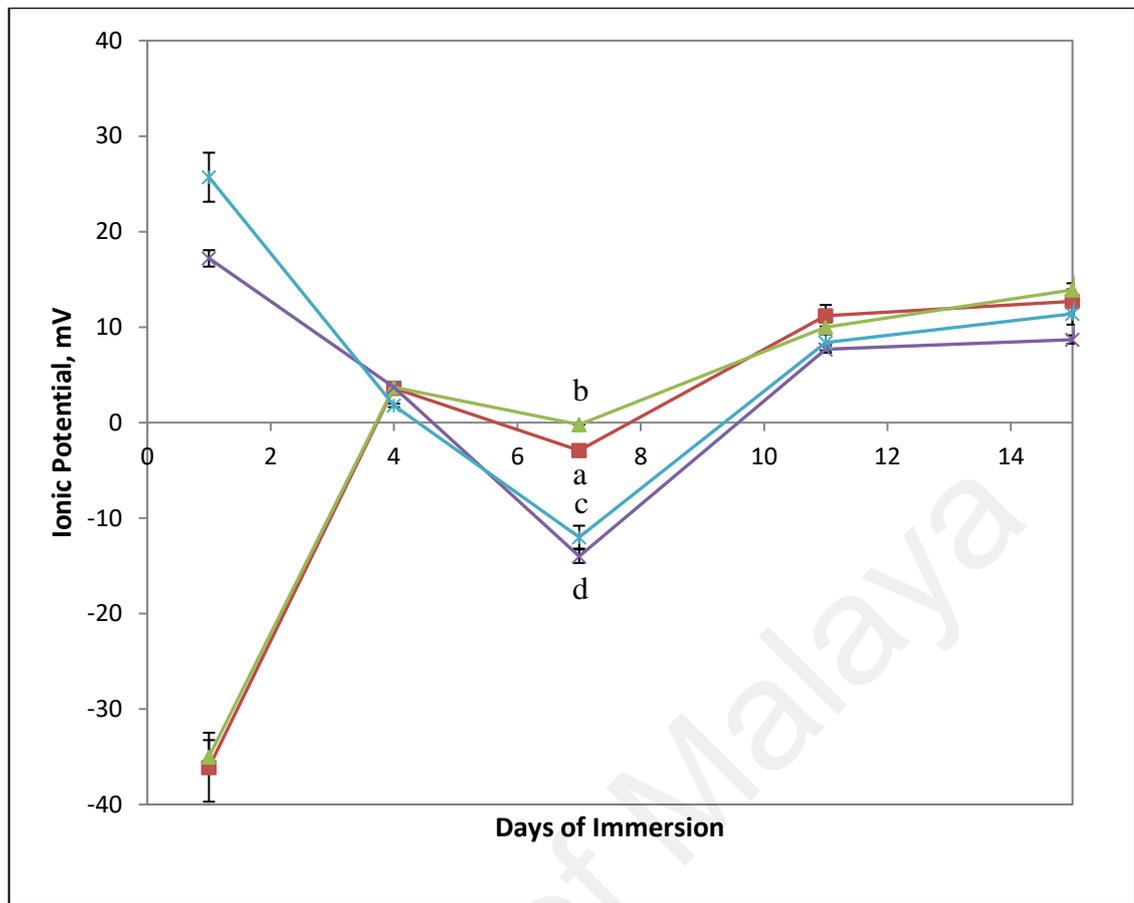
In this experiment, we will focus on ion release silver acetate and silver sulfate. According to solubility rules, all silver ions are insoluble in water except silver nitrate, silver sulfate and silver acetate. Silver nitrate dissolve completely in water while silver sulfate and silver acetate dissolve partially in water.

Previous study reported that chitosan samples in paste form released a greater amount of silver ions as compared to solid form as paste samples consist of loosely packed polymer chains with a greater polymer free volume and higher water content. Thus, upon immersion in the medium, water molecules will quickly diffuse into the samples, and so there will be higher diffusion of silver particles into the medium. The tolerance level of

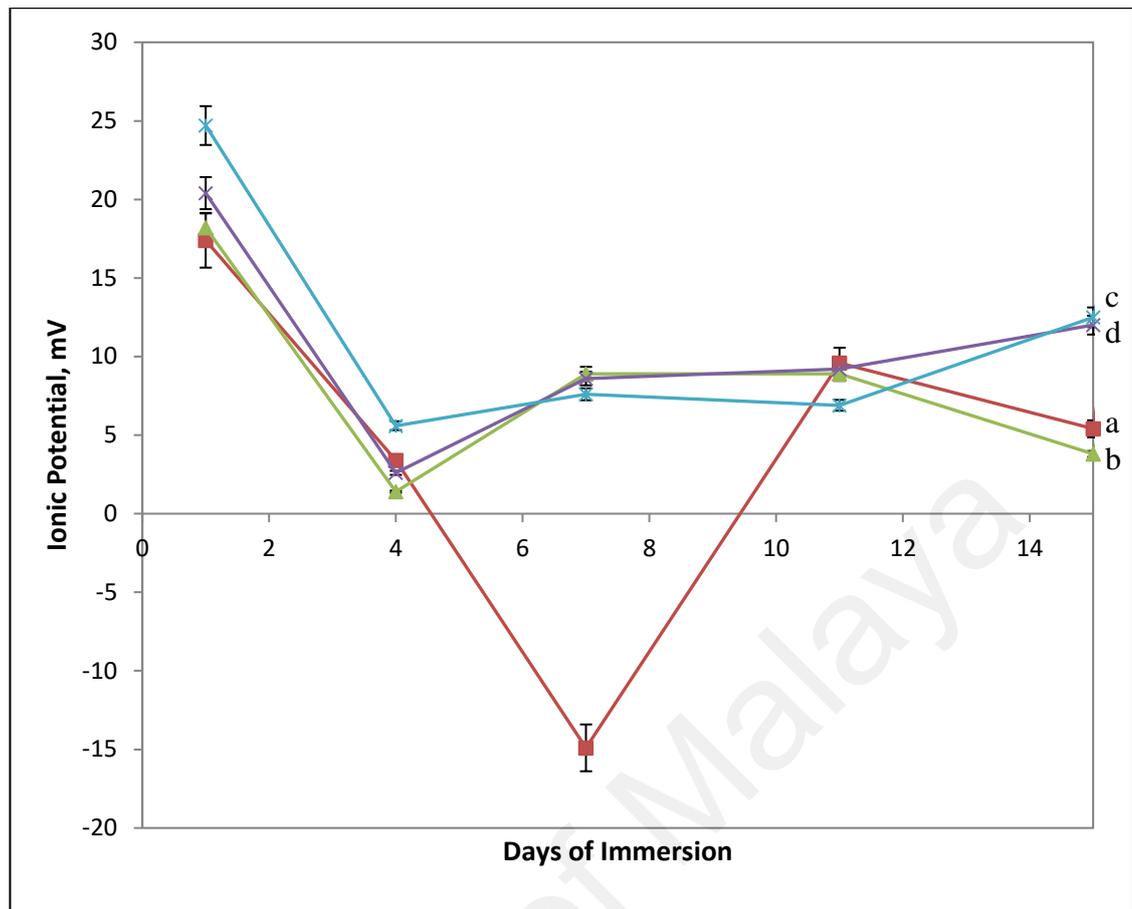
silver in human body is between 50–200 ppm and concentration of ion release decreased with time in Sabudin's study (Sabudin, Derman, Zainol, & Noorsal, 2012).

Ionic concentration after 24 hours shows a trend of increasing ion concentration with increasing doping content. However, this trend for the ion potential is not maintained for the entire duration of the study. The initial ionic potential for lower doping amount of 0.1 and 0.3 grams of silver acetate is lower than samples doped with 0.5 and 0.7 grams of silver acetate. This is due to the silver precipitated to the surface of the antimicrobial composite with higher amount of silver salt doping causing initial ionic potential after 1 day of immersion much higher. The same trend was observed on samples doped with silver sulfate where initial ionic potential is higher and only stabilized after 11 days of immersion with positive ionic potential of 5 to 10mV.

This proved our postulation of the slow release rate of silver ion is much suitable for long term usage. The antibacterial activity of chitosan can be improved by incorporating silver compounds and the slow release of the silver ions in the chitin-chitosan composite doped with silver salt is believed to be an effective method for the long-term inhibition of the growth of bacteria.



**Figure 4.18: Relative ionic potential – chitin-chitosan composite doped with various amount of silver acetate, a) 0.1 g, b) 0.3 g, c) 0.5 g and d) 0.7 g**



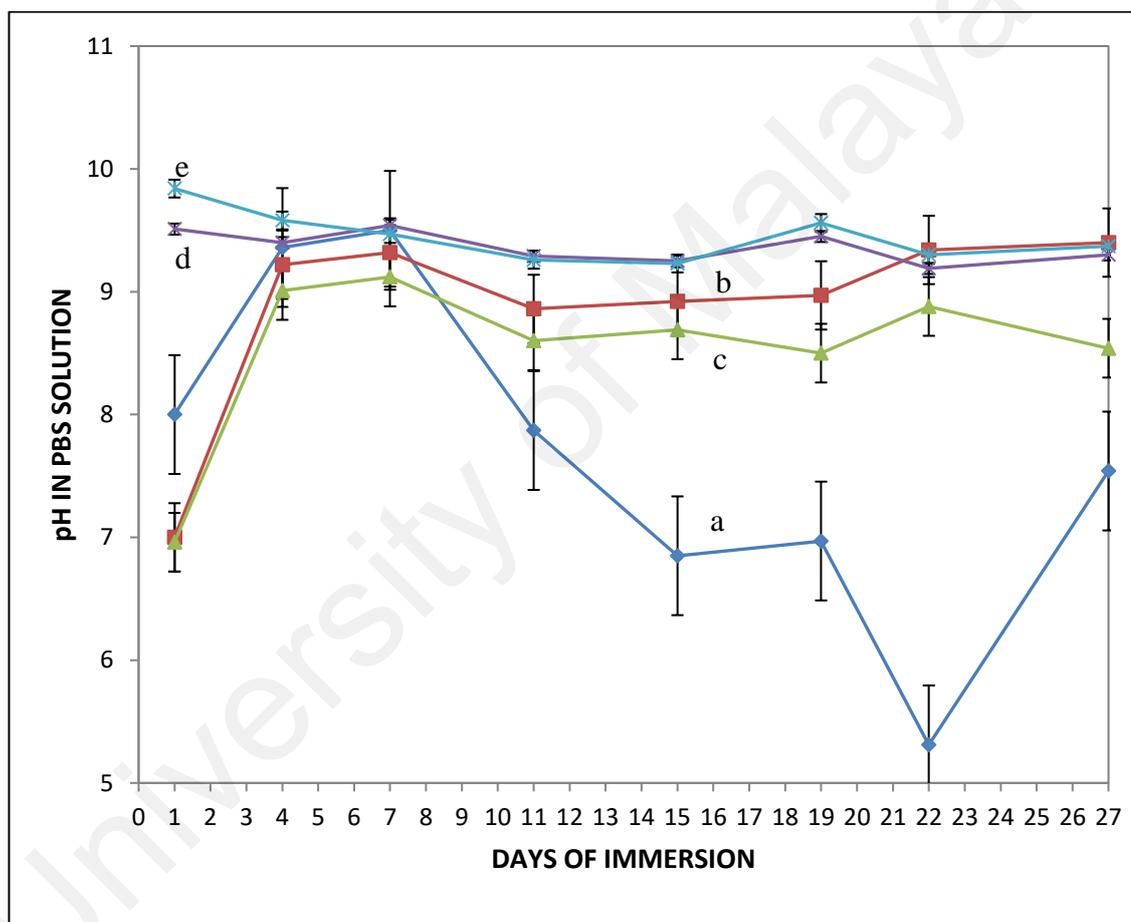
**Figure 4.19: Relative ionic potential – Chitin-chitosan composite doped with various amount of silver sulfate, a) 0.1 g, b) 0.3 g, c) 0.5 g and d) 0.7 g**

#### 4.9.3 Final pH in Phosphate Buffer Saline Solution

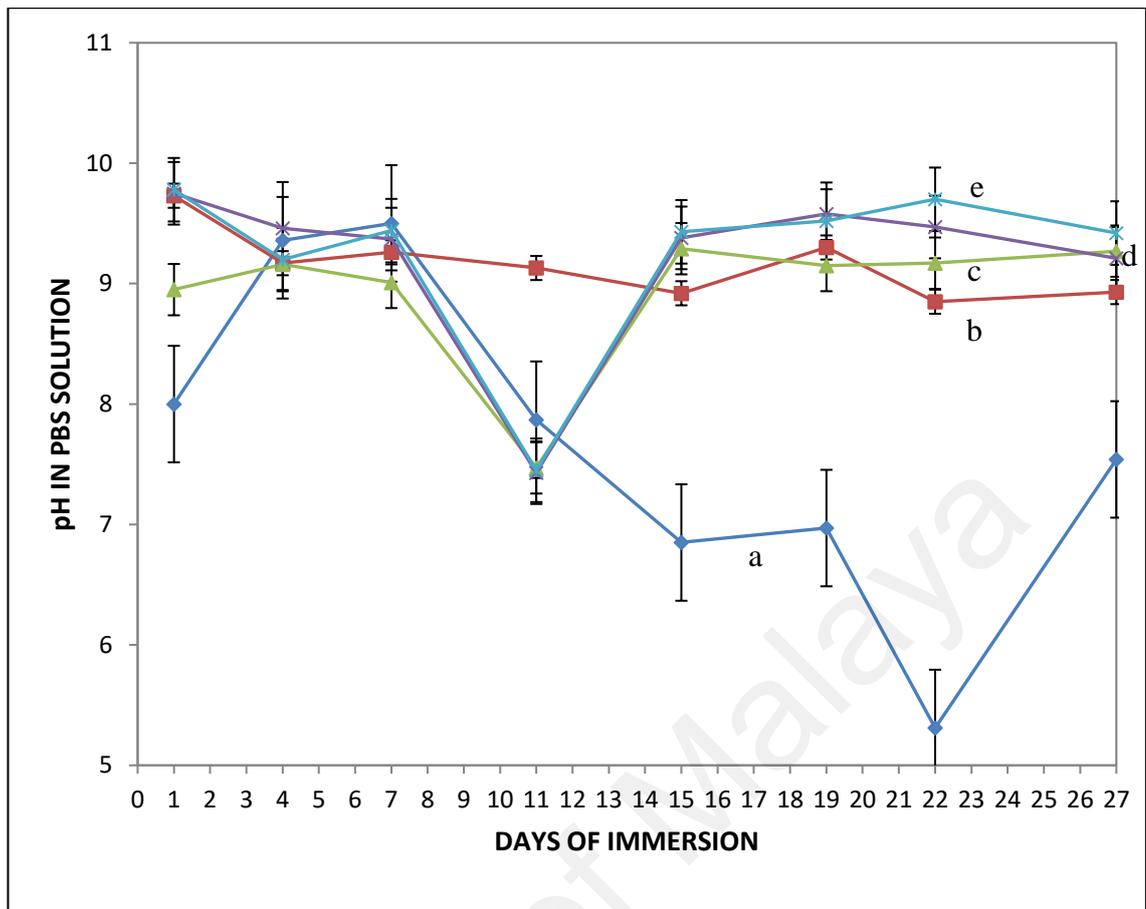
Chitin and chitosan are basic polysaccharides (Kumar, 2000) and while the nitrogen content of chitin varies from 5 to 8% (depending on extent of deacetylation), the nitrogen content of chitosan is mostly in the form of primary aliphatic amino groups. Thus, chitosan often undergoes reactions similar to amine group (Kelesoglu, 2007) and this may be a contributing factor for the PBS solutions to become more alkaline. The pH of the solution immersed with pure chitin/chitosan composite maintains at alkaline until day 7 where it decreases as immersion days increases. The pH for PBS solution immersed with chitin-chitosan composite without doping decreased to acidic on day 22 to pH 5.31 and increased to pH 7.54 on day 27 indicating the chitin-chitosan composite is unable to

maintain the pH and the solution turn into acidic. As more amine was dissolved into the solution due to degradation of the composite, the pH level raises again to slightly alkaline.

Samples doped with silver salts maintain its alkaline pH for far longer compared with pure chitin/chitosan samples Figure 4.25 and Figure 4.26. This suggested that the silver ion helps to increase the pH or the incorporation of silver salts into the composites helps to reduce the degradation rate of chitosan thus maintaining the pH in alkaline state.



**Figure 4.20: Final pH in PBS solution – a) chitin-chitosan composite and chitin-chitosan composite doped with various amount of silver acetate, b) 0.1 g, c) 0.3 g, d) 0.5 g and e) 0.7 g**



**Figure 4.21: Final pH in PBS solution – a) chitin-chitosan composite and chitin-chitosan composite doped with various amount of silver acetate, b) 0.1 g, c) 0.3 g, d) 0.5 g and e) 0.7 g**

#### 4.10 Summary of Research Findings

- Chitin nanofiber was extracted from coarse chitin flakes using acid hydrolysis. Chitin nanofiber with dimensions of 282 nm length and 20 nm diameter was obtained indicating shrimp shell origin.
- Tensile strength of chitin nanofiber reinforced chitosan film doped with 0.1 grams of silver sulfate shown 65% increases in tensile strength compared with neat chitin chitosan composite film. The tensile strength increases from 95 MPa to 157 MPa in 0.1gram silver sulfate doped sample.

- X-ray Diffraction results showed increase in degree of crystallinity when 0.1 grams of silver salts was added. The crystallinity index reduces with further addition of silver salt until it increases again when doped with 0.7g of silver salts. Characteristic peak of silver also started to show up at  $2\theta = 38^\circ$  at doping weight of more than 0.3 grams of silver salts.
- Surface morphology of neat chitin chitosan composite showed rough surface. Addition of silver sulfate of 0.1 grams to 0.5 grams produce smoother surface with clearly visible of impregnation of chitin nanofiber in chitosan matrix. Addition of 0.7 grams of silver sulfate resulted in rough surface where silver particle started to precipitate from the matrix onto the surface of the composite.
- FTIR showed characteristic peak of chitin and chitosan. Samples doped with silver sulfate showed interaction of sulfate ions with  $\text{NH}_2$  group where peak at  $1565 \text{ cm}^{-1}$  shifted to  $1555 \text{ cm}^{-1}$  and the peak intensified as more silver salt was added.
- FTIR results from composites doped with silver acetate showed increasing peaks at  $1416 \text{ cm}^{-1}$  which were attributed to  $-\text{COO}$  symmetric stretching on acetate anion. Peak appeared to increase in absorbance as amount of silver acetate increases indicating the increased amount of acetate presence in the matrix.
- Silver ion release test proved that the composite was able to release silver ion and its silver ion release capability increases with extra silver salts doping. All composite tested were able to release silver ion to concentration of more than 900 ppb which also indicates its antimicrobial efficacy.
- Kirby-Bauer disk diffusion susceptibility test successfully demonstrated the improved antimicrobial efficacy of chitin chitosan composite doped with silver

salts. All samples showed zone of inhibition diameter of more than 10mm. Neat chitin chitosan composite showed no zone of inhibition around the film but inhibited growth of bacteria on its surface.

- Swelling study showed that samples reached an equilibrium state and maintain its swelling percentage. All samples doped with silver salts exhibit lower swelling percentage compare with neat chitin chitosan composite.
- Degradation study showed that the mass loss of chitin-chitosan composite doped with 0.3g silver acetate and 0.7g silver sulfate show higher mass loss while the remaining samples do not display any increasing trend in mass loss. All samples shown similar amount of mass loss after initial mass loss of 10 to 20% mass loss except 0.3g silver acetate and 0.7g silver sulfate.
- Study of silver ion concentration over time showed that ionic potential increased to certain ionic potential and level up as the concentration reaches equilibrium.
- The study of the pH of liquid over days of immersion of samples in phosphate buffer solution showed sample doped with silver salts can maintain alkaline pH whereas neat chitin chitosan drops in pH after 7 days of immersion.

## CHAPTER 5: CONCLUSIONS

### 5.1 Conclusions

Antimicrobial composite was successfully developed by doping chitin nanofiber reinforced chitosan composite with silver sulfate and silver acetate. Chitin nanofiber was successfully extracted from chitin flakes with acid hydrolysis method. The average dimension of chitin nanofiber is 282 nm length and 20nm diameter.

Doping of silver sulfate at 0.1 grams manage to improve tensile strength by up to 65% compared with neat chitin-chitosan composite. The increase in tensile strength is due to increasing the crystallinity of the polymer composite while also give the sample a smooth surface morphology compared with neat chitin-chitosan composite.

Kirby-Bauer disk diffusion susceptibility test successfully demonstrate the improved antimicrobial efficacy of chitin chitosan composite doped with silver salts. All samples showed zone of inhibition diameter of more than 10mm. Neat chitin chitosan composite showed no zone of inhibition outside of the chitin chitosan film but inhibited growth of bacteria on its surface.

Degradation study showed that the mass loss of chitin-chitosan doped with silver salts is uniform across different doping concentration of around 10 to 22% except for 0.3g silver acetate and 0.7g silver sulfate doping which show higher mass loss of over 25% after 7 days of immersion in PBS solutions.

Study of silver ion concentration over time showed that the ionic potential increases to certain potential and remain constant as the concentration reaches equilibrium. This shows the sustained silver ion release capability of the composite.

The study of the pH of liquid over days of immersion of samples in phosphate buffer solution showed sample doped with silver salts was able to maintain alkaline pH whereas neat chitin chitosan starts to drop in pH after 7 days of immersion.

The ideal antimicrobial film from our research is chitin nanofiber reinforced chitosan film doped with 0.1 grams of silver sulfate. The advantage of extra tensile strength coupled with strong antimicrobial capability allows it to be more versatile and ensure longer application life time.

## **5.2 Future work and recommendation**

Future study to fine tune the silver salts concentration and identify other types of antimicrobial salts to be incorporated into the composite could be conducted. The high tensile strength after silver salt doping enable the composite to be used in high strength low weight requirement application.

The research had already drawn interest from University Malaya Engineering Faculty, Mechanical Department interest in their research in mechanical wing flying devices for the wing film application due to high tensile strength of our composite. Joint research study already produced one journal paper on development of the composite (V. Rubentheren, Thomas A. Ward, C.Y. Chee, C.K. Tang, 2015).

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## APPENDIX

### APPENDIX A: Dimensions of chitin nanofiber calculated using ImageJ software from TEM results.

Count	LENGTH (nm)	DIAMETER (nm)
1	310.94	13.62
2	370.09	14.79
3	359.48	17.47
4	333.89	22.43
5	224.93	14.65
6	428.18	18.72
7	300.32	18.17
8	540.74	24.37
9	177.10	18.39
10	192.97	22.71
11	179.79	20.00
12	357.92	25.85
13	216.36	10.15
14	167.21	11.84
15	339.22	26.48
16	245.91	14.22
17	290.09	16.37
18	193.04	29.99
19	350.16	14.79
20	253.55	21.59
21	347.58	10.15
22	282.48	25.69
23	280.34	33.06
24	183.91	21.59
25	372.50	18.39
26	234.44	20.91
27	202.06	24.54
28	191.08	15.86
29	255.09	20.71
Average	282.12	19.57
Standard Deviation	88.56	5.66
Standard error	16.45	1.05

### APPENDIX B: Tensile test results

	Test 1	Test 2	Test 3	Average	Standard Deviation	Standard Error
Chitin-Chitosan Composite	97	95	92.8	95	2.101	1.213
Doped with 0.1 g Silver sulfate	158.8	152	161.2	157	4.772	2.755
Doped with 0.3 g Silver sulfate	99.8	110.2	102.3	104	5.429	3.134
Doped with 0.5 g Silver sulfate	30.3	34.6	48	38	9.232	5.330
Doped with 0.7 g Silver sulfate	21.6	21.1	27	23	3.272	1.889
Doped with 0.1 g Silver acetate	83.1	63.8	68.2	72	10.115	5.840
Doped with 0.3 g Silver acetate	45.3	30.5	40.3	39	7.529	4.347
Doped with 0.5 g Silver acetate	61.4	54.3	56.8	58	3.601	2.079
Doped with 0.7 g Silver acetate	56	46.4	49.8	51	4.868	2.810