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STUDY OF AN ACTIVE ANTIMICROBIAL SYSTEM USING A BIO-SWITCH CONCEPT

Prepared by:

DR. IDA IDAYU BINTI MUHAMAD

BIOPROCESS ENGINEERING DEPARTMENT
FKKKSA UTM

ABSTRACT

In response to the dynamic changes in current consumer demand and market trends, the area of Active Packaging (AP) is becoming increasingly significant. An Antimicrobial Active Packaging can be made by incorporating and immobilizing antimicrobial (AM) agents into food packages and applying a bio-switch concept. By that, the mechanism of antimicrobial release between the developed bio-switch particles and the stimulus of a microbial contamination can be studied. The objective of this research is to develop a starch-based film incorporated with antimicrobial agents consisting of lysozymes, lauric acid, nisin and EDTA (chelating agent). The effectiveness of the AM film towards inhibition of microbial activity was evaluated. Clear zones formed on the film appearance showed the combination of both AM agents gives good inhibition to the growth of *E. coli* and *B. subtilis* with satisfying inhibition rate. With the advent of new polymer materials and antimicrobials, the development of AP could prolong the shelf life of food and reduce the risk of foodborne illness caused by microbial contamination.

ABSTRAK

Kesan terhadap perubahan dinamik dalam pasaran dan permintaan pengguna telah mengalakkan pertumbuhan dalam bidang Pembungkusan Aktif. Pembungkusan Aktif Anti-bakteria mengaplikasikan konsep 'bio-switch' yang mana ia dihasilkan dengan menggabung dan menyekat gerak agen anti-bakteria ke dalam pembungkus. Mekanisma pembebasan anti-bakteria di antara partikel 'bio-switch' dan 'stimulus' terhadap kontaminasi mikrob telah di kaji. Objektif kajian ini adalah untuk menghasilkan filem berasaskan kanji yang digabungkan dengan agen anti-bakteria iaitu lysozyme, asid laurik, nisin dan EDTA (agen penchelat). Keberkesanan filem tersebut terhadap perencatan aktiviti mikrob telah dikaji. Zon terang yang terbentuk di atas piring petri menunjukkan bahawa kombinasi gabungan anti-bakteria – anti bakteria tersebut telah memberi perencatan yang baik terhadap pertumbuhan bakteria *E. coli* dan *B. subtilis.* Pengenalan bahan polimer dan anti-bakteria yang terbaru dalam penghasilan pembungkusan aktif boleh memanjangkan jangka hayat makanan dan mengurangkan risiko keracunan makanan akibat kontaminasi dari bakteria.

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INTRODUCTION

1.1 General Introduction

Active packaging is defined and classified by the extra functions added to the packaging systems which used to have only barrier and protective functions. Packaging is termed as “active” when it performs some desired role other than to provide an inert barrier to the external environment (Hotchkiss, 1994). The extra functions include oxygen scavenging, antimicrobial (AM) activity, moisture scavenging, ethylene scavenging, ethanol emitting and so on.

Active packaging is one of the innovative food packaging concepts that have been introduced as a response to the changes in current consumer demands and market trends. It also has been defined as “a type of packaging that changes the condition of the packaging to extend shelf-life or improve safety or sensory properties while maintaining the quality of the food” by European FAIR-project. In general, active food packaging can provide several functions that do not exist in conventional packaging systems.

The demand for minimally processed, easily prepared and ready-to-eat ‘fresh’ food products, globalization of food trade, and distribution from centralized processing pose major challenges for food safety and quality. Recent food-borne microbial outbreaks are driving a search for innovative ways to inhibit microbial growth in the foods while maintaining quality, freshness and safety. Active packaging interacts with the product or the headspace between the package and the food system, to obtain a desired outcome. Likewise, AM food packaging acts to reduce inhibit or retard the growth of microorganisms that may be present in the packed food or packaging materials itself (Appendini and Hotchkiss, 2002).

Food packaging materials used to provide only barrier and protective functions. However, various kinds of active substance can now be incorporated into the packaging material to improve its functionality and give it new or extra functions. Incorporation of AM agents into a polymer can limit or prevents the microbial growth. Such active packaging technologies are designed to extend the shelf life of foods, while maintaining their nutritional quality and safety. This application could be used for foods effectively not only in the form of films but also as containers and utensil.

AM food packaging is one of the special applications of active food packaging that controls inside food and atmospheric conditions actively and responsively. Active packaging technologies are designed to extend the shelf-life, while maintaining the nutritional quality and safety of food and involve interactions between the food, the packaging material and the internal gaseous atmosphere.

1.2 Objectives of Study

The study of this research contains four main objectives to be achieve which are:

1. To formulate the AM starch-based film.
2. To evaluate the effect of AM agents towards the inhibition of microbial activity.

1.3 Scopes of Study

The scopes of this research include:

1. Formulating the AM starch-based film.
2. Evaluation of the AM starch-based film effectiveness towards the inhibition of microbial activity.

CHAPTER II

LITERATURE REVIEW

2.1 AM Starch-based Film/Food Packaging

Recently, the demand for Malaysian traditional food product pose their major problem which, their less marketing over world wide and this is due to the shelf life of the food itself (e.g. keropok lekor, tempoyak ect). The introduction of antimicrobial agents into food packaging material helps to prolong the shelf life of food products by inhibiting the growth of microorganism. AM polymers can be used in several food related applications including packaging (Hotchkiss, 1997). It can extend the shelf life and promote safety by reducing the rate of growth of specific microorganism by direct contact of the package with the surface of solid foods (e.g. meats, cheese, etc.) or in the bulk of liquids (e.g. milk or meat exudates).

Besides, AM packaging materials could also be self sterilizing. If the packaging materials have self-sterilizing ability because of their own antimicrobial activity, they may eliminate chemical sterilization of packages using peroxide and simplify the aseptic packaging process (Hotchkiss, 1997). The self-sterilizing materials could be widely applied for clinical uses in hospitals, biological labware, biotechnology equipment and biomedical devices, as well as food packaging. Such AM packaging materials greatly reduce the potential for recontamination of processed products and simplify the treatment of materials in order to eliminate product contamination. AM polymers might also be used to cover surfaces of food

processing equipment so that they self-sanitize during use (e.g. filter gaskets, conveyers, gloves, garments and other personal hygiene equipment) (Appendini and Hotchkiss, 2002).

The effectiveness of AM film to inhibit the microbial growth had been proved by a lot of researchers. The application of AM agent into food packaging could effectively used in not only in the form of film but also as containers and utensil (Han, 2000).

Active packaging technologies involve interactions between the food, the packaging material and the internal gaseous atmosphere (Labuza and Breene, 1988). Food packaging materials used to provide only barrier and protective functions. However, various kinds of active substances can now be incorporated into the packaging material to improve its functionality and give it new or extra functions. Such active packaging technologies are designed to extend the shelf life of foods, while maintaining their nutritional quality and safety (Han, 2000). The most promising active packaging systems are oxygen scavenging system (Rooney, 1981) and antimicrobial system (Rooney, 1996).

AM packaging materials have to extend the lag period and reduce the growth rate of microorganisms to prolong the shelf life and maintain food safety. They have to reduce microbial growth of non-sterile foods or maintain the stability of pasteurized foods without post-contamination.

2.2 Types of Antimicrobial Packaging

Appendini and Hotchkiss, (2002) had characterized the form of AM packaging. Below are several forms of AM packaging which are:

- Addition of sachets/pads containing volatile antimicrobial agents into packages.
- Incorporation of volatile and non-volatile AM agents directly into polymers.
- Coating or adsorbing AMs into polymer surfaces.
- Immobilization of AMs to polymers by ion or covalent linkages.
- Use of polymers that are inherently antimicrobial.

However this research will focus on incorporation of volatile and non-volatile AM agents directly into polymers. Incorporation of bioactive agents including AM into polymers has been commercially applied in drug and pesticide delivery, household goods, textiles, surgical implants and other biomedical devices (Appendini and Hotchkiss, 2002). Table 2.1 shows a few food related applications that have been commercialized.

Table 2.1: Selected commercial AM packaging available for food applications (Appendini and Hotchkiss, 2002)

Antimicrobial compound	Tradename	Producer Company	Packaging forms for food applications
Silver substituted zeolite	Aglon™	Aglon Technologies LLC	• Bulk food storage containers • Paperboard carton, plastic or paper food wraps and milk containers
	Novaron	Toagosei, Co. LTD	Many (Japan)
Triclosan	Microban	Microban Products	Deliwrap, reheatable food containers (UK)
Allylisothio-cyanate	WasaOuro	Lintec Cooperation	Pressure sensitive labels, sheets (Japan)
		Dry Company LTD	Sachets
Chlorine dioxide	Microsphere™	Bernard Technologies Inc.	Storage bag for produce, paperboard coating, rigid containers, pressure sensitive labels.
Carbon dioxide	Freshpax™	Multisorb Technologies	Sachets
	Verifrais	SARL codimer	Sachets (France)
Ethanol vapor	Ethicap	Freund	Sachets
	Negamold		
	Fretek		Sachets
	Oitech™	Nippon Kakyaku	Sachets (Japan)
Glucose oxidase	Bioka	Bioka LTD	Sachets (Finland)

2.3 AM Agents

Common AM chemicals for food products are preservatives such as organic acid and their slats, sulfites, nitrites, antibiotics and alcohols (Han, 2000). They were mixed into a wax layer for natural cheese (Melnick and Luckmann, 1954; Melnick et al., 1954; Smith and Rollin, 1954). However the release rate and migration profile of AM into food should be controlled. The AM mechanism/kinetics and the controlled-release profile of potassium sorbate form low density polyethylene (LDPE) film into cheeses were examined and mathematically simulated by Han (1996).

Biodegradable polymers are currently being studied as edible coatings or film materials (Krochta and Johnston, 1997). Pedgett et al. (1998) demonstrated the AM activity of lysozyme and nisin in soy protein isolate films and corn zein films.

The study of developing of AM packaging materials has been a great interest during the past decade. Table 2.2 shows a few of AM agents that have been used in food packaging. The inhibition of microbial growth can be observed by the clear zones around the film on the plate agar test (Figure 2.1).

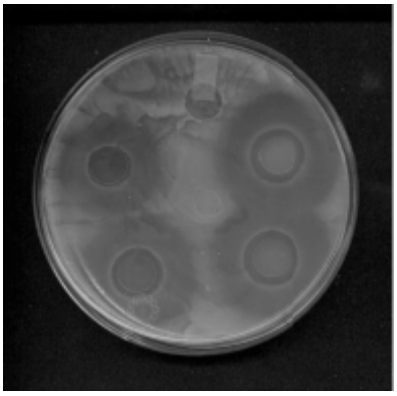


Figure 2.1. Clear zones of inhibition of the spoilage bacteria *Brochothrix thermosphacta* around discs of whey protein film containing lysozyme after 48 hr at room temperature (Han, 2000).

Table 2.2: Application of AM Food Packaging (Han J. H., 2000)

Antimicrobial Agent	Packaging Material	Food	References
Organic Acid			
Potassium Sorbate	LDPE	Cheese	Han (1996)
	LDPE	Culture Media	Han and Floros (1997)
	MC/Palmitic Acid	Culture Media	Rico-Pena and Torres (1991)
	MC/HPMC/Fatty Acid	Culture Media	Vojdani and Torres (1990)
	MC/Chitosan	Culture Media	Chen et al. (1996)
	Starch/Glycerol	Chicken Breast	Baron and Sumner (1993)
Calcium Sorbate	CMC/Paper	Bread	Ghoshh et al. (1973,1977)
Propionic Acid	Chitosan	Water	Quattara et al. (1999)
Acetic acid	Chitosan	Water	Quattara et al. (1999)
Benzoic acid	PE-co-MA	Culture Media	Weng et al. (1997)
Sodium Benzoate	MC/Chitosan	Culture Media	Chen et al. (1996)
Sorbic acid anhydride	PE	Culture Media	Weng and Chen (1997); Weng and Hotchkiss (1993)
Benzoic acid anhydride	PE	Fish fillet	Huang et al. (1997)
Fungicide/Bacteriocin			
Benomyl	Ionomer	Culture Media	Halek and Garg (1989)
Imazalil	LDPE	Bell paper	Miller et al. (1984)
	LDPE	Cheese	Weng and Hotchkiss (1992)
Nisin (peptide)	Silicon coating	Culture Media	Daeschel et al. (1992)
	SPI, corn zein film, SS	Culture Media	Padgett et al (1998)
Peptide/Protein/Enzyme			
Lysozyme	PVOH, Nylon, Cellulose Acetate	Culture Media	Appendini and Hotchkiss (1996)
	SPI film, corn zein film	Culture Media	Padgett et al. (1998)
Glucose oxidase	Alginate	Fish	Field et al. (1986)

Alcohol oxidase	-	-	Broody and Budny (1995)
Alcohol/thiol			
Ethanol	Silica gel sachet	Culture Media	Shapero et al. (1978)
	Silicon oxide sachet (Ethicap™)	Bakery	Smith et al. (1987)
Hinokithiol	Cyclodextrin/Plastic (Seiwa™)	-	Gontard (1997)
Oxygen absorber/antioxidant			
Reduced iron complex	Sachet (Ageless™)	Bread	Smith et al. (1996)
BHT	HDPE	Breakfast cereal	Hoojjat et al. (1997)
Gas			
CO_2	Calcium hydroxide sachet	Coffee	Labuza (1990)
	-	Fruit/Vegetable	Saccharow (1998)
SO_2	Sodium metabisulfite	Grape	Gontard (1997)
Other			
UV Irridiation	Nylons	Culture Media	Paik and Kelly (1995); Hagelstein et al. (1995)
Silver zeolite	LDPE	Culture Media	Ishitani (1995)
Grape fruit seed extract	LDPE	Lettuce, Soybean sprouts	Lee et al. (1998)

2.3.1 Lysozyme

Lysozyme is an enzyme (EC 3.2.1.17), commonly referred to as the "body's own antibiotic" since it kills bacteria. It is abundantly present in a number of secretions, such as tears (except bovine tears). This protein is present in cytoplasmic granules of the polymorphonuclear neutrophils (PMN) (except for bovine neutrophils) and released through the mucosal secretions (such as tears and saliva). They can also be found in high concentration in egg white. Figure 2.2 presented the 3D structure of lysozyme.

Lysozyme serves as an unspecific innate opsonin by binding to the bacterial surface to reduce the negative charge and facilitate phagocytosis of this bacterium before opsonins from the acquired immune systems enter the scene.

The enzyme functions by attacking peptidoglycans and hydrolyzing the bond that connects N-acetyl muramic acid with the fourth carbon atom of N-acetylglucosamine. It does this by binding to the peptidoglycan molecules in the binding site within the prominent cleft between its two domains. This causes the substrate molecule to adopt a strained conformation similar to that of the transition state. The amino acid side chains Glutamic acid 35 and Aspartate 52 have been found to be critical to the activity of this enzyme. Glu 35 acts as a proton donor to the glycosidic bond cleaving the C-O bond in the substrate, whilst Asp52 stabilizes the carbonium ion intermediate until it reacts with a free water molecule, extracting a hydroxl group and freeing a proton that bonds to Glu35, leaving the enzyme unchanged it.

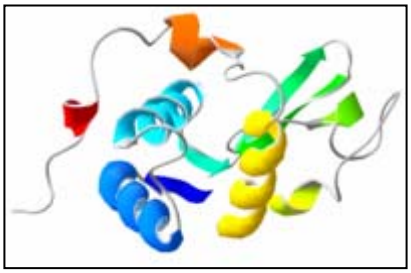


Figure 2.2: Lysozyme molecular 3D structure

2.3.2 Lauric Acid

Lauric acid is colorless, needle-like crystal and slight odor of Bay Oil. Lauric acid or dodecanoic acid is part of the class of organic compounds known as lipids, which are vital in the construction of cellular membranes and act as a source of food under starvation conditions. The molecular weight of the lauric acid are $C_{12}H_{24}O_2$ Contrary to popular beliefs, natural coconut and coconut milk are good for the health, mostly because of their high lauric acid content. Lauric acid is potential to use as the antimicrobial agent because it sources are from local sources, coconut. . Besides, it is inexpensive, has a long shelf-life, and is non-toxic and safe to handle and suitable used for incorporate in packaging. Figure 2.3 shows the 3D structure of lauric acid.

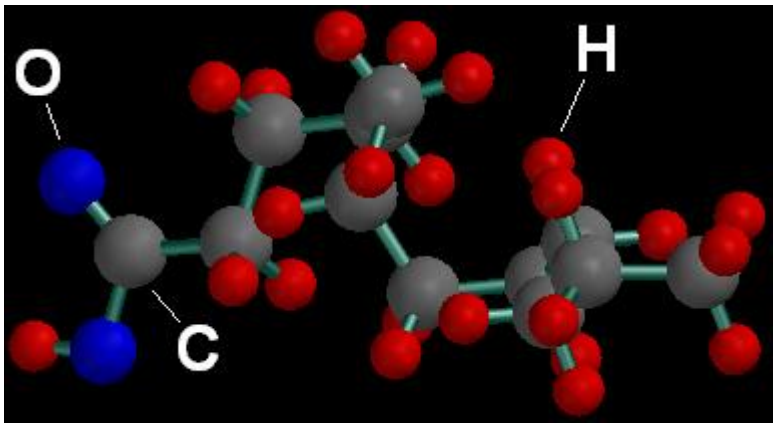


Figure 2.3: Lauric acid molecular 3D structure

It is widely used as a lubricant and as an additive in industrial preparations. It is used in the manufacture of metallic stearates, pharmaceuticals, soaps, cosmetics, and food packaging. It is also used as a softener, accelerator activator and dispersing agent in rubbers. Because of Lauric acid is insoluble in water, ethanol can be useful to solute the Lauric acid.

2.3.3 Nisin

Nisin is an inhibitory polycyclic peptide with 34 amino acid residues used as a food preservative. It contains the uncommon amino acids lanthionine, methyllanthionine, dehydroalanine and dehydro-amino-butyric acid. These special amino acids are synthesized by posttranslational modifications. In these reactions a ribosomally synthesized 57-mer is converted to the final peptide. The unsaturated amino acids originate from serine and threonine. Nisin is produced by fermentation using the bacterium *Lactococcus lactis*. Commercially it is obtained from natural substrates including milk and is not chemically synthesized. It is used in processed cheese production to extend shelf life by suppressing gram-positive spoilage and pathogenic bacteria.

There are many other applications of this preservative in food and beverage production. Due to its highly selective spectrum of activity it is also employed as a selective agent in microbiological media for the isolation of gram-negative bacteria, yeast and moulds. Subtilin and Epidermin are related to Nisin. As a food additive, nisin has E number E234.

2.3.4 Ethylenediminetetraacetic Acid (EDTA)

In this research study EDTA play the main role as the chelating agent for lysozyme to inhibit the growth of gram negative bacteria. EDTA is the chemical compound ethylenediaminetetraacetic acid, otherwise known as edetate, versene, or diaminoethanetetraacetic acid disodium salt. EDTA is a chelating agent, forming coordination compounds with most monovalent, divalent, trivalent and tetravalent metal ions, such as silver (Ag^{+}), calcium (Ca^{2+}), manganese (Mn^{2+}), copper (Cu^{2+}), iron (Fe^{3+}) or zirconium (Zr^{4+}). EDTA contains 4 carboxylic acid and 2 tertiary amine groups that can participate in acid-base reactions. EDTA forms especially

strong complexes with Mn, Cu, Fe (III), and Co(III). EDTA has a molecular mass of 292.28 g/mol. The chemical structure of EDTA is presented in figure 2.4.

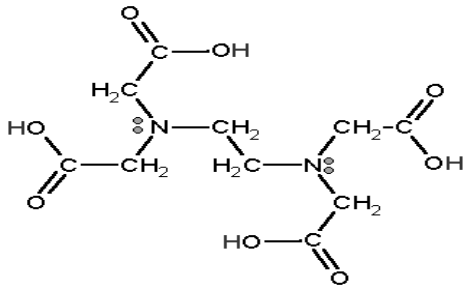


Figure 2.4: Chemical structure of EDTA

Recalcitrant chelating agents such as EDTA are an environmental concern predominantly because of their persistence and strong metal chelating properties. The presence of chelating agents in high concentrations in wastewaters and surface waters has the potential to remobilize heavy metals from river sediments and treated sludge, although low and environmentally relevant concentrations seem to have only a very minor influence on metal solubility. Elevated concentrations of chelating agents enhance the transport of metals (e.g. Zn, Cd, Ni, Cr, Cu, Pb, and Fe) in soils, and enhance the undesired transport of radioactive metals away from disposal sites.

Low concentrations of chelating agents may either stimulate or decrease plankton or algae growth, while high concentrations always inhibit activity. Chelating agents are nontoxic to many forms of life on acute exposure; the effects of longer-term low-level exposure are unknown. EDTA at elevated concentrations is toxic to bacteria due to chelation of metals in the outer membrane. EDTA ingestion at high concentrations by mammals changes excretion of metals and can affect cell membrane permeability.

2.3.5 Bunga Telang

Bunga telang is plants of the genera *Clitoria* in the pea family, having blue or lavender flowers and flat pods. It is known as butterfly pea or clitoria flower in English. The scientific name of bunga telang is *Clitoria ternatea*. Butterfly pea is most likely originated in tropical; Asia, though its true origin is obscured by extensive cultivation and neutralization around the globe. Butterfly pea has been widely distributed to many tropical and subtropical countries where it has become neutralized; South and Central America, East and West Indies, China and India. It is grown as a persistent perennial. In these areas, the flowers (figure 2.5) are used to give a blue tinge to rice cakes and boiled rice. The young pods may be consumed like string beans. Leaves are also used to dye or are eaten as a pot herb.

Figure 2.5: Bunga telang or also known as butterfly pea

The butterfly pea (*C. ternatea*) is a deep-rooted, tall slender, climbing legume with five leaflets and a deep blue flower. It is well adapted to a variety of soil types (pH 5.5-8.9) including calcareous soils. It is surviving in both the extended rainfall regions and prolonged periods of drought. Propagation is through seed; the plants may be grown with support crops (or) staked with bamboo to facilitate hand picking of the pods.

The seed of butterfly pea is very high in protein (15-25%) hence increasing the nitrogen levels in run-down cultivated paddocks, the soil fertility returns to its original level. Grass growing along the side of butterfly pea will be higher in protein due to the higher soil nitrogen levels. This had led to a higher carrying crude fiber capacity as well as better gains by cattle. The levels of crude protein and crude fiber in the leaves were 21.5% and 21.5-29% respectively (Kalamani and Michael Gomez, 2001). Total plant protein ranges from 14-20%. Seed contains 25-38% protein, 5& total sugar and 10% oil. Nitrogen concentration of whole tops range from 1.7-4.0%.

2.3.6 Release of AM Agent

Most food packaging systems consist of the packaging material, the food and the headspace in the package. Diffusion between the packaging material and the food and portioning at the interface are the main migration phenomena involved in this system. AM agents may be incorporated into the packaging materials initially and release into the food through diffusion and partitioning. Han (2000) described the migration phenomena in figure 2.6.

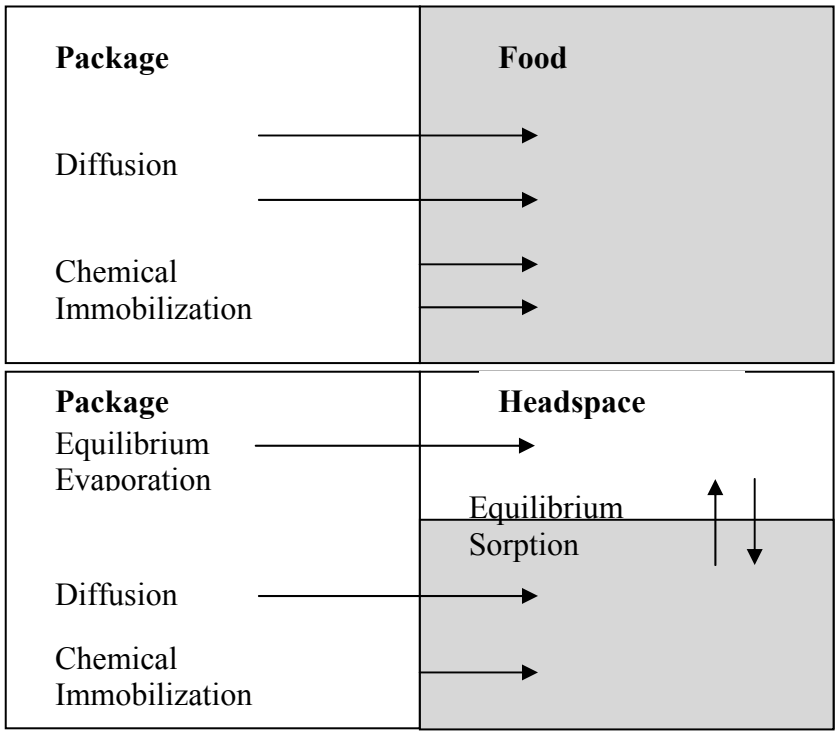


Figure 2.6: Food packaging systems and migration phenomena

Control of the release rates and migration amounts of AM substance from food packaging is very important. In principle, the extractability of a compound from a plastic by a foodstuff can be determined by placing the plastic (of known surface area) in contact with the food under defined conditions of temperature and time. In practice, the test faces difficulties for the following reasons:

a) The compounds present in the plastic under test may be unknown and may have been degraded during processing.
b) Many such compounds are difficult to determine analytically in a matrix as complex as food, particularly where only small amounts are present in the extract.
c) Compounds other than the one of interest may also be extracted and subsequently interfere with the analytically determination.
d) Most foodstuffs are stable for short periods of time only, whereas extractability data may be required from long-term studies.

e) Appropriate test conditions are not easy to define as a result of the wide variation in possible contact conditions likely to be encountered in practice in warehouses, supermarkets, corner shops and household larders.

Whilst, it is always desirable to use food themselves for extractability testing, in practice it is seldom possible, and so food simulant have to be used instead.

2.4 Mechanism of Antimicrobial film

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

Table 2.3: Concept of active packaging

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

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

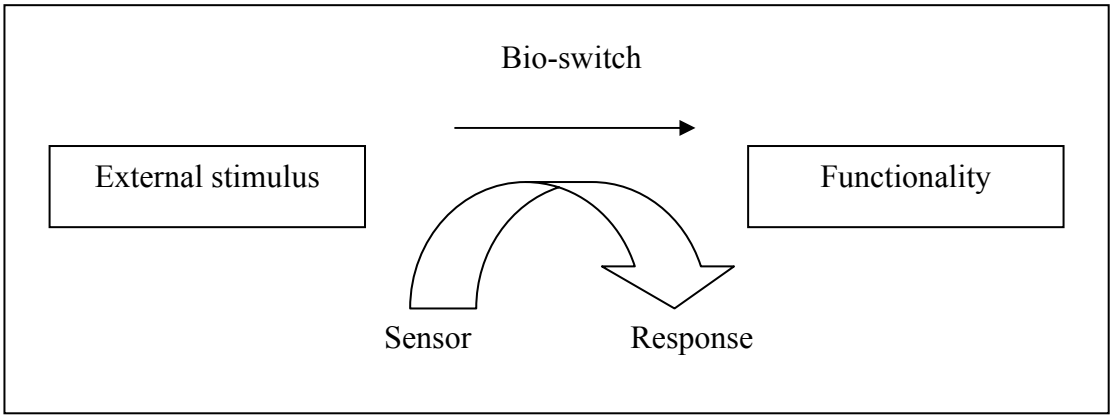


Figure 2.7: General concept of bio-switch

Figure 2.8 represent the mechanism of how the AM Active Packaging inhibit the microbial growth. Microorganism will first try to hydrolyze the starch-based particles causing the release of the AM compound which finally resulting inhibition of the microbial growth.

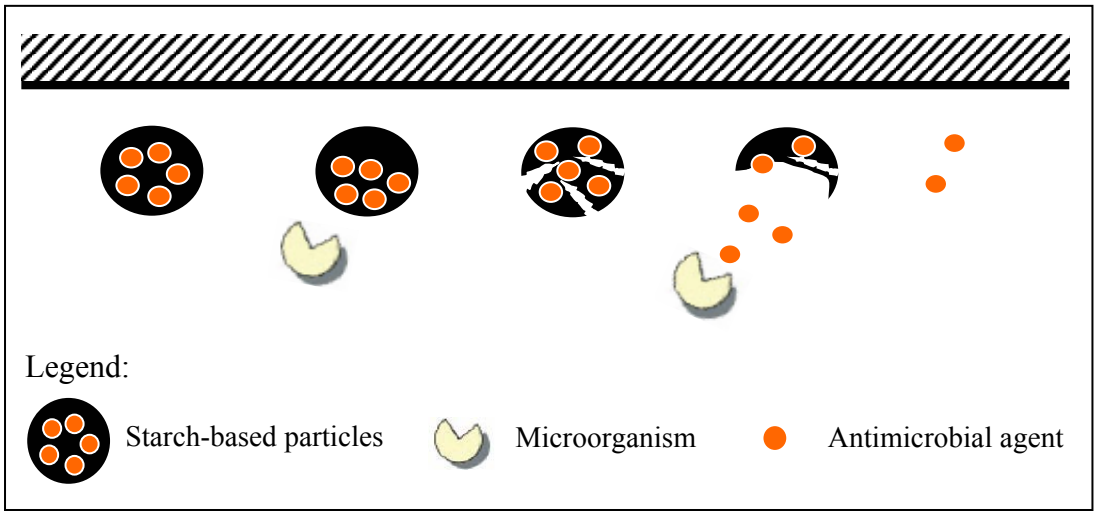


Figure 2.8: The anti-microbial active packaging action applying bio-switch concept.

2.5 Packaging Materials

2.5.1 Starch-based

Starch is a complex carbohydrate which is insoluble in water; it is used by plants as a way to store excess glucose. Starch (in particular cornstarch) is used in cooking for thickening sauces. In industry, it is used in the manufacture of adhesives, paper, and textiles. It is a white powder, and is tasteless and odorless. Chemical structure of simple starch is presented in figure 2.9 below:

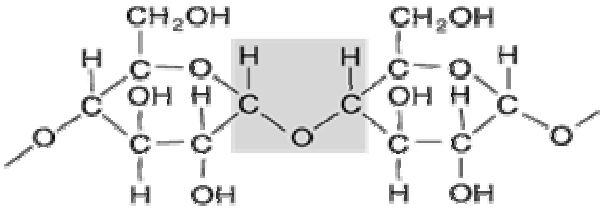


Figure 2.9: Chemical structure of starch

Van Volkenburgh W.R and White M.A indicate possible avenues to overcome some solid waste issues with special emphasis on degradable plastics that can be composted. In particular, they discuss the starch-based polymeric systems and non-starch polymeric systems as the two broad classes of microbe degradable plastics with future impact on the waste mass. The use of starch in polymer matrices to promote biodgradation, or at least biofragmentation, was popular in the late 1980's. The uses of starch as alternative binders and in starch co-polymers provide the possibility of 10-20% increase in industrial capacity. The possibilities for use of starch as a part of a polymer system using polycaprolactones, polyurethanes, polyvinyl alcohols, cellulose acetates, soybean and so forth have been investigated. The usefulness of starch as a film former is also well understood and the need for dissemination of this information is addressed by Sommerfield and Blume. Such knowledge plays a role in education and understanding of the fundamental uses of a material such as starch in environmental applications.

2.5.2 Starch (κ-carrageenan)-based

Kappa-carrageenan (κ-carrageenan) is produced by alkaline elimination from κ-carrageenan isolated mostly from the tropical seaweed *Kappaphycus alvarezii* (also known as *Eucheuma cottonii*). The experimental charge/dimer is 1.03 rather than 1.0 with 0.82 molecules of anhydrogalactose rather than one. It is classified as polysaccharides. Chemical structure of κ-carrageenans is presented below:

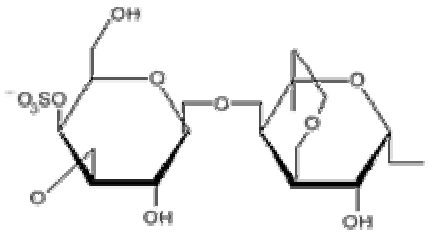


Figure 2.10: Structure of κ-carrageenans

κ-carrageenans are used mainly for thickening, suspending and gelling. κ-carrageenan form thermoreversible gels on cooling in the presence of appropriate counterions. κ-carrageenan forms a firm clear, if brittle, gel with poor freeze-thaw stability; the coil-double helix transition being followed by a K^+-induced aggregation of the helices.

κ-carrageenan gels may be softened (and is generally regarded to be synergistically strengthened[a]) with locust bean gum. κ-carrageenan stabilizes milk casein products due to its charge interaction with the casein micelles (~200 nm diameter); their incorporation into the network preventing whey separation. Such complexes are soluble when both have same charge and are held together by counter ions or oppositely charged patches. κ-carrageenans are also used as a binder in cooked meats, to firm sausages and as a thickener in toothpaste and puddings.

It may be noted that a cancer health scare concerning degraded carrageenan has recently been examined by the European Commission Scientific Committee on Food

which found no evidence in support and states that κ-carrageenan is safe to use in foods. Park (1996) reported that κ-carrageenan has excellent film forming properties with water vapor permeability of 1.87x 10-10ng m/m2s Pa and tensile strength of 22-32 MPa, which was higher that that of polyethylene film (13- 28 MPa).

2.5.3 Starch (wheat)-based

Wheat (*Triticum* spp.) is a grass that is cultivated worldwide. Globally, it is the most important human food grain and ranks second in total production as a cereal crop behind maize; the third being rice. Wheat grain is a staple food used to make flour for leavened, flat and steamed breads; cookies, cakes, pasta, noodles and couscous; and for fermentation to make beer, alcohol, vodka or bio-fuel. The husk of the grain, separated when milling white flour, is bran. Wheat is planted to a limited extent as a forage crop for livestock and the straw can be used as a ruminant fodder component or as a construction material for roofing thatch.

Wheat is widely cultivated as a cash crop because it produces a good yield per unit area, grows well in a temperate climate even with a moderately short growing season, and yields a versatile, high-quality flour that is widely used in baking. Most bread are made with wheat flour, even many breads named for the other grains they contain, including most rye and oat breads. Many other popular foods are made from wheat flour as well, resulting in a large demand for the grain even in economies with a significant food surplus.

Australian scientists have developed revolutionary packaging materials that are fully biodegradable based on wheat starch. Wheat starch-based, the new materials could be used for shopping bags, to pack vegetables, in place of polystyrene trays for baked goods, and other purposes such as mulch film for farming and gardening.

CHAPTER 111

MATERIALS AND METHODS

3.1 Materials

Chemical	Function
Lysozyme	AM Agent
Nisin	
Lauric Acid	
EDTA (Ethylenediaminetetraacetic acid)	Chelating Agent
Starch-wheat	Film Based
Starch-κ-carrageenan	
Glycerol	Plasticizer
PEG (polyethylene glycol)	

3.2 Development of AM Starch-based Film

3.2.1 Preparation of AM Starch (κ-carrageenan)-Based Film

AM starch (κ-carrageenan)-based film was prepared by dissolving a various concentration of lauric acid in DMSO and then κ-carrageenan were added to solution (2 g/ 100 mL). (50:50) w/w glycerin and polyethylene glycol (PEG) were added into the solution (0.75 g/ g κ-carrageenan). The solution was then heat and stirred at 70^{o}C for 40 minutes until a homogeny solution obtained. 20 mL of prepared film solution was then poured into casting (glass plate) and was dried constantly at room temperature for 3 days.

3.2.2 Preparation of AM Starch (wheat)-Based Film

The films were prepared by slightly modifying the method described by Gennadios et al. (1993). Starch (wheat)-based films were prepared by dissolving 8.35 g starch (wheat) in 80 mL of 20% ethanol with stirring. After the solution was completely dissolved, 3.8 mL glycerin (HmbG Chemicals) was added as plasticizer and the mixture was heated slowly to a mild boiling. Films were also formed by the combination of 15mM EDTA as chelating agent at concentration of 0.2 v/v. For AM incorporated films, AM agents were mixed with 10 mL of the film solution in a separated beaker just before casting. Five milliliters of the film mixture was pipetted into petri dishes (100 mm diameter by 15 mm depth). The petri dishes were placed for 24 hour in an oven (Memmert) set at 50^{o}C.

3.3 Testing the Effectiveness of AM Starch Based Film

3.3.1 Agar Diffusion Method (Zone Inhibition Assay)

The agar diffusion test was carried out using the method described by Dawson et al (1995). The strain selection represented typical spoilage organism groups commonly occurring in various kinds of food products. The strains were as

follows: (1) *Escherichia coli*, a conventional hygiene indicator organism, a Gram-negative rod belonging to the same family of *Enterobacteriaceae* as for example *Salmonella*. (2) *Bacillus subtilis*, a Gram-positive rod capable of forming heat-resistant spores. Spores and vegetative cells of *Bacillus* species are widely distributed in nature and are common for example in cereals. For the agar plate test, the AM film was cut into six squares (0.5 cm x 0.5 cm). Six sample squares were then placed onto the plate spreaded with bacteria (0.1 mL per plate. Duplicate agar plates were prepared for each type of film and control film. The agar plates were incubated at 37^{o}C for 48 hours.

3.3.2 Liquid Culture Test ($O.D_{600nm}$ Measurement)

For the liquid culture test (Chung, Papadakis and Yam, 2002), each film was cut into squares (1 cm x 1 cm). Three sample squares were immersed in 20 mL of specific broth in a 25 mL universal bottle. The medium was inoculated with 200μL of *Escherichia coli* in its late exponential phase, and then transferred to an orbital shaker and rotated at 37^{o}C at 200 r.p.m. The culture was sampled periodically (0, 2, 4, 8, 12, 24 hours) during the incubation to obtain microbial growth profiles. The same procedure was repeated for the control starch-based film. The optical density ($O.D._{.600}$) was measured at $\lambda = 600_{nm}$ using a spectrophotometer (Model UV-160, Shimadzu, Japan). For comparison purposes, the specific cell growth rates during the exponential growth phase were calculated as follows;

$$dX\ (t)\ dt = \mu X(t)$$

whereas; X(t) is the cell concentration of microorganism in the medium $O.D._{.600}$, μ is the specific growth rate of *E.Coli* (h^{-1}) and t is the time (h).

For AM starch (κ-carrageenan)-based film, the liquid culture test was carried out for gram positive bacteria; *B. subtitlis.*

CHAPTER IV

FINDINGS AND DISCUSSION

4.1 Development of AM Starch-based Film

4.1.1 Film formation

For formation of a good AM starch (κ-carrageenan)-based film, it is important to make sure the entire Lauric acid component is miscible and incorporate well in film. Lauric acid is insoluble in water whereas the κ-carrageenan based film is soluble in water. This incompatibility of these two different material becomes a challenge for the reaching the most suitable formulation.

A trial solvent, the ethanol is used. Ethanol (CH_3CH_2OH) is an alcohol that is a dilute aqueous solution, in which a group of chemical compounds whose molecules contain a hydroxyl group, –OH, bonded to a carbon atom. Ethanol used to dissolve Lauric acid and the solution mixes well in water based of κ -carrageenan solution. Even though it is known as disinfectant, it does not show any effect toward antimicrobial agent.

From observation, the volume of ethanol is negligible because it of immediately volatile during experiment. Besides, the drying process becomes

shortest by the present of ethanol. In other advantages, the film produce is very stable. However the using of ethanol faced casting problem. As the evaporation process is too fast and the solution is form to solid rapidly. As the way to reduce it, the quick and fast handling during casting and suitable volume is needed. Skill handling it is important by the redo the experimental for times. Besides, the volume of casting it is the critical part. If the volume too little, the solution cannot spread well at the surface of Petri dish. But if the solution is too much, the film obtained is less thick.

Furthermore the formulation is containing the plasticizers. Plasticizers give plastics properties such as color, resistance to fire, strength and flexibility and durability. Two substances that take a role for plasticizer for this film are Polyethylene Glycol (PEG) and Glycerin. PEG will be increase the biodegradability of the biopolymers and improve the water solubility of the film. Glycerin take an action to enhance the film produce follow the plastics characteristic like strengthens.

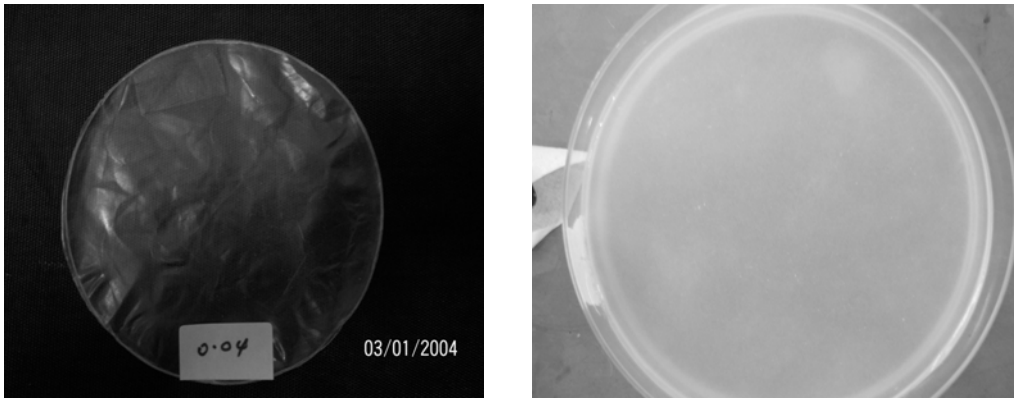
Film with wheat starch based consist of 7.75 g of pure starch,100 mL of water as solvent and 2.9 mL of glycerin shows the best film with high strength , elasticity and translucent. In contrast, potato starch film base with a same formulation have lower strength, elasticity and slightly opaque. During the development of AM starch (wheat)-based film, a translucent film with less brittleness and flexible had been successfully developed.

4.1.2 Film Appearance

Previous study claim that the addition of AM compounds affect the film thickness in compared to films with no added compounds. If there is an AM compound in a film, the film become thinner than before. Besides, the texture becomes softer and less elasticity. Figure 4.1 shows the appearance of starch (κ-carrageenan)-based AM film and starch (wheat)-based film.

Film appearance achieve for starch (κ-carrageenan)-based film is a transparent, flexible and homogeneity film. In other word, the film obtained is nearly to plastics characteristics. This film appearance is using visual observation. The volume used during casting is effect the appearance of the film. 20 mL film solution is the best volume to slow down a solid formation during pouring process. Ethanol is powerful tool for forming a thick, smooth and transparent antimicrobial film. It can be due that the concentration increase, the quality of film obtain will decrease. At high concentration of the Lauric acid added, the film become harder and the Lauric acid begin to appear at top surface of film. But at lowest concentration, film is smooth and Lauric acid well incorporated. As the solution, the selection the lower concentration is the best formulation to get good film.

The perfect film obtained is influence of the presenting ethanol and plasticizer. The study before show that film that does not contain the plasticizer, film is harder, brittle with pale yellow colour and long casting time process. However the volume of two this substances much at the specified level, even too high or low, it induces the film appearance. From this study, the best quantity of ethanol is 8%v/v and 75%w/w for plasticizer.



A **B**

Figure 4.1: Film appearance. A: starch (κ-carrageenan)-based film and B: starch (wheat)-based film.

For a Lysozyme-incorporated wheat film, as the concentration of Lysozyme increased films become thinner. Furthermore, higher amount of Lysozyme cause the film brittle and tend stick to the Petri dish. The film is translucent, while the texture is slightly stiff.

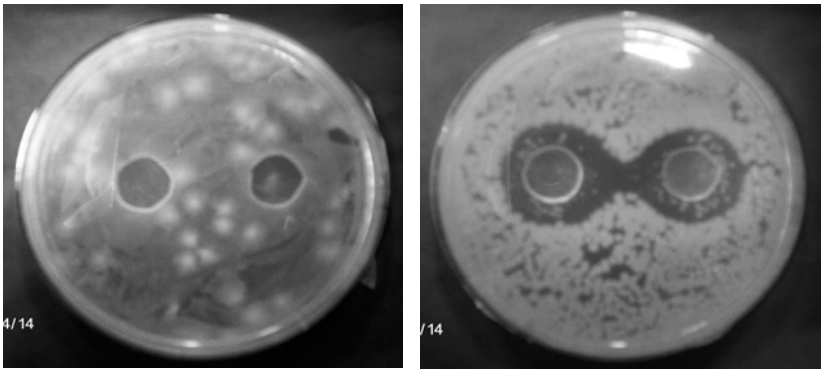
In contrast for a film incorporated with Lauric acid, the wheat films become more cohesive when the concentration of Lauric acid increased. Besides, at a concentration of Lauric acid 0.04, 0.07 and 0.2 g/mL the film started to crack and tear into pieces.

4.2 Effect of AM film Towards Microbial Activity

4.2.1 Inhibition of *Escherichia coli* and *Bacillus subtilis* on Agar Plate Test

4.2.1.1 Inhibition of *Escherichia coli* and *Bacillus subtilis* on Agar Plate Test containing AM Starch (κ-carrageenan) Film

The agar diffusion test provides the rapid assessment for microbial activity. Figure 4.2 shows the clear zone formed around the disc film after in contact with microbe colonies. It's indicated that lauric acid works better to inhibit the growth of *B. Subtilis*.



A **B**

Figure 4.2 Inhibition zone for A: *E. coli* and B: *B. subtilis* for agar plate test

Figure 4.3 shows the comparison of inhibition zones between *E coli* and *B. subtilis.* It is shown that lauric acid gives a very small impact on E. coli growth. From the 10mm diameter as initial diameter, it expended only 1mm to 2mm diameter. However, lauric acid gives a good inhibitory effect against *B.Subtilis*. It is shown that as the concentration of lauric acid increased the inhibition zone increased.

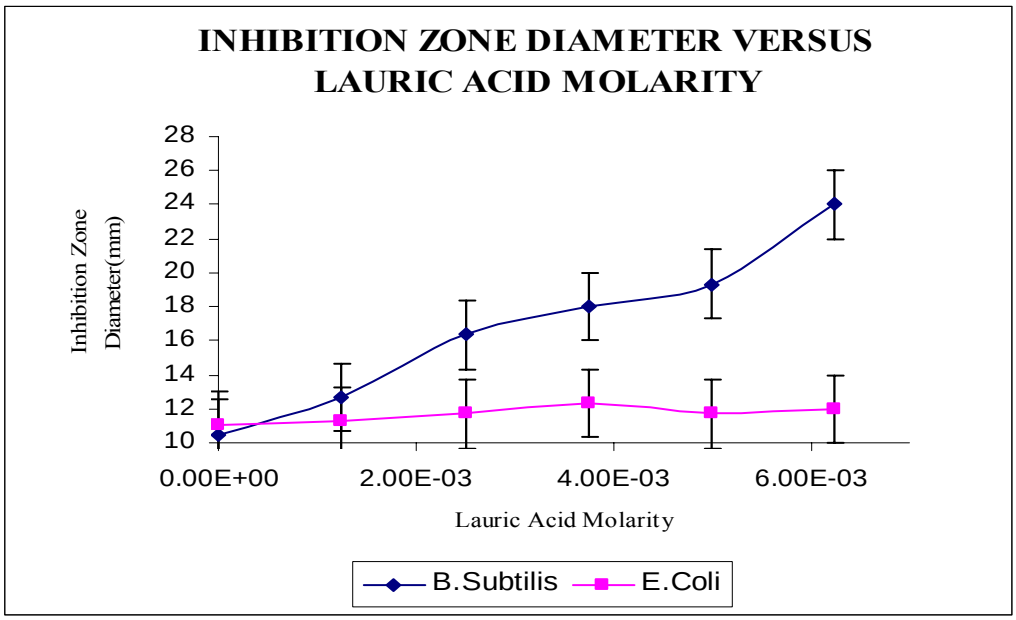


Figure 4.3: The comparison of inhibition zone between *B. subtilis* and *E. coli*

A statistical analysis had been carried out as the function to identify the method and parameter based on data collected. One very common procedure dealing

with comparison of multiple population means which compared more than two population means, is ANOVA (analysis of variance).

The F value is greater than F critical, therefore from the initial assumption Ho which is value of two types are same are rejected. That means, as the approach to get the two data are different is significant, the F must be bigger than the F critical. Otherwise is not significant. Table 4.1 shows the statistical data for the inhibition diameter for both *B.subtilis* and *E.coli*.

Table 4.1: The significant comparison of types of bacteria at different concentration

Concentration Lauric acid (10^{-2}M)	F value	F critical Value	Significant
0.000	0.968	7.709	F< F crit No
0.125	12.800	7.709	F> F crit Yes
0.250	98.000	7.709	F>F crit Yes
0.375	289.000	7.709	F> F crit Yes
0.500	264.500	7.709	F> F crit Yes
0.625	432.000	7.709	F> F crit Yes

F crit = F critical

It is shown that the comparison data were accepted for inhibition diameter for both bacteria at different concentration. That mean the method and parameter used for this experiment are suitable.

4.2.1.2 Inhibition of *Escherichia coli* and *Bacillus subtilis* on Agar Plate Test containing AM Starch (wheat) Film

All samples were examined for possible inhibition zones after incubation at 37°C for 48 hours. Figure 4.4 shows the plotted graph of inhibition area versus concentration of lysozyme. It showed that, as concentration of Lysozyme increased, the amount of inhibition also increased for both methods. An overall difference of grid method to graph method is only about 8.7 %. As it was only a small differences, either grid or graph method can be used in order to determine the inhibition area.

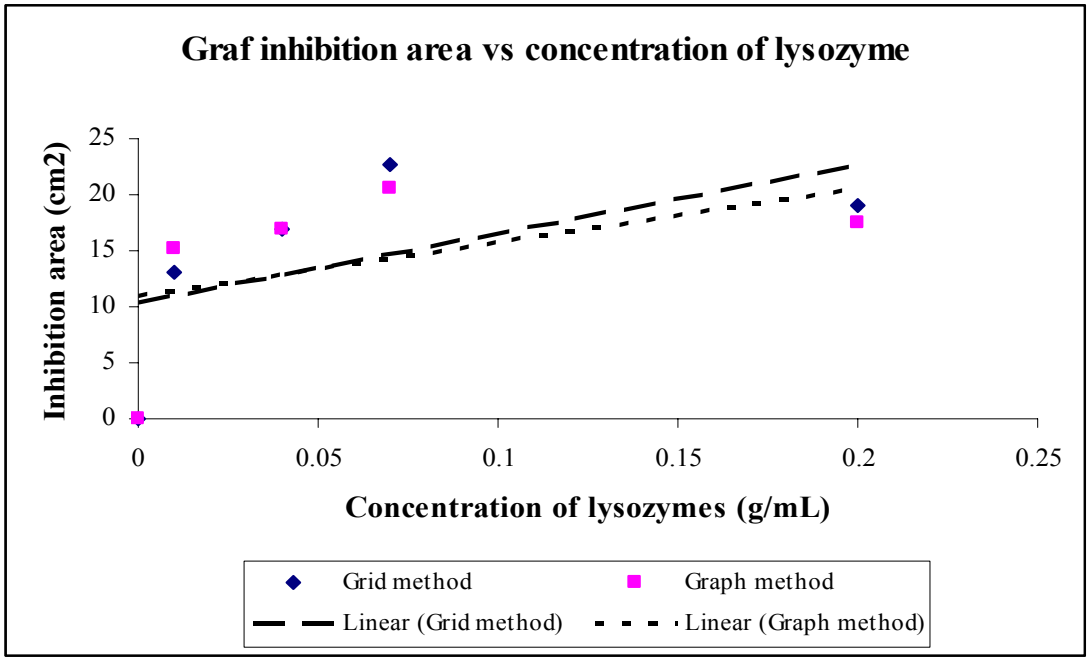


Figure 4.4: Graph inhibition area of *E. coli* growth versus concentration of lysozymes for grid and graph method.

Figure 4.5 shows the effect of various concentration of lauric acid towards the growth of *E. coli*. It is shown that as the concentration of lauric acid increased, the amount of inhibition area also increased. An overall difference of grid method to graph method is only about 19.2 %. As it was only a small differences, either grid or graph method can be used in order to determine the inhibition area.

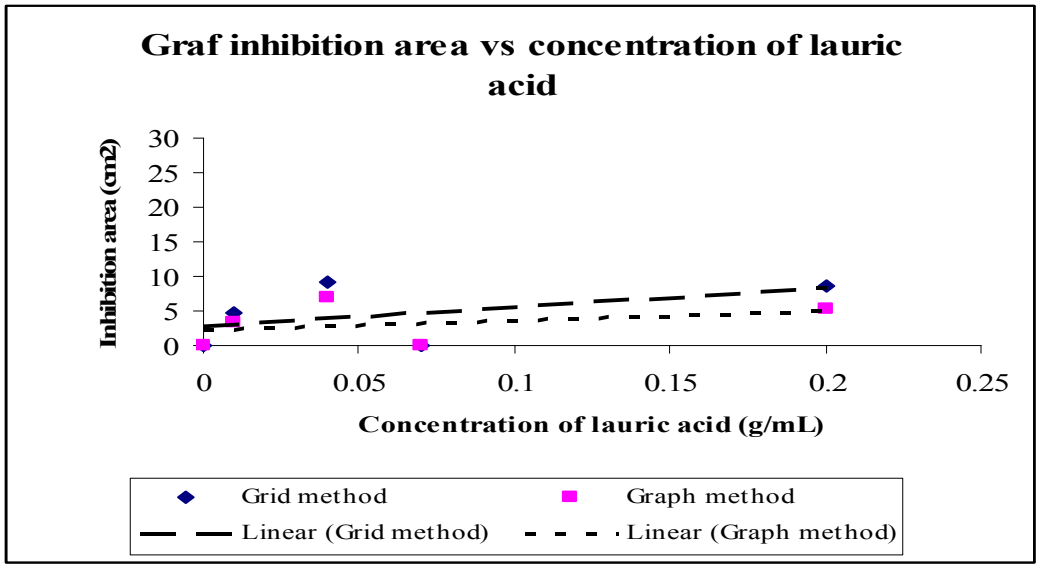
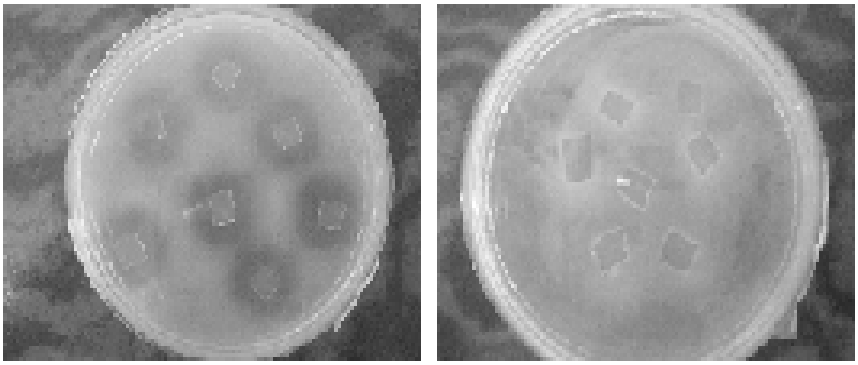


Figure 4.5: Graph inhibition area versus concentration of Lauric acid for grid and graph method.

Besides lysozyme and lauric acid, nisin had also being choose to be an AM agent. All samples were examined for possible inhibition towards the growth of *E. coli* and *B. subtilis* after incubation at 37^{o}C for 48 hours. Figure 4.6 shows the agar plate contained AM incorporated film in comparison to control film that contain no AM compound at all. From the observations, the AM-incorporated films showed clear zone formed on the agar plate after in contact with the microbe colonies. For this test a measurement of inhibition zones on/around film squares on inoculated bacteria was determined.



AM film Control film

Figure 4.6: Inhibition area of AM incorporated film in comparison to control film.

Table 4.2 lists calculated inhibition area for each plate test. The control films (Plate 1) showed no inhibition area and colonies were formed all over the plate. Film containing EDTA (Plate 3) showed inhibitory growth of both *Escherichia Coli* and *Bacillus subtilis*. Film containing nisin (Plate 2) and lysozyme (Plate 4) alone showed no inhibition towards *E. coli*. This is not surprising since nisin and lysozyme had benn regarded as being inactive against Gram-negative *(*Appendini and Hotchkiss, 1997. Nisin and lysozyme combined with EDTA (Plate 5 & 7) inhibited growth of *B. subtilis* and *E. coli.*

Table 4.2. Inhibition of Escherichia coli and Bacillus subtilis on agar plates expressed as an area (cm^2) of inhibition zone

Film	*Bacillus subtilis* (48 hours @ 37^oC)	*Escherichia Coli* (48 hours @ 37^oC)
Control	-	-
Nisin	6.60	-
EDTA	13.77	17.97
Lysozyme	5.57	-
Nisin + EDTA	20.30	15.18
Nisin + Lysozyme	3.52	1.44
EDTA + Lysozyme	15.00	20.63

4.2.2 Liquid Culture Test ($O.D_{600\ nm}$ Measurement)

Microbial growth is one of approach that can be manipulated for this experiment. In this method, the log OD versus time will be plotting to achieve the growth curve. A typical growth curve includes the following phases:

1. Lag phase
2. Logarithms or exponential phase

3. Deceleration phase
4. Stationary phase
5. Death phase

Lag phase occurs immediately after inoculation and the period of adaptation of cell to new environment. During this phase cells mass may increase a little without an increase in cell number density. Exponential growth phase, the cells can multiply rapidly, cell mass and cell number density increase exponential with time. The deceleration phase is due to either depletion of one or more essential nutrients or the accumulation of toxics by-product of growth. Then it continues with stationary phase, when the net growth rate is zero (no cell division) or when a growth rate is equal to the death rate. Even thought the net growth rate is zero during this phase, cell still metabolically active. Finally the death (decline phase) follows the stationary phase.

4.2.2.1 Liquid Culture Test ($O.D_{600\ nm}$ Measurement) for AM starch (κ-carrageenan) film

The growth curve of E.coli (figure 4.7) showed a similar pattern for all different concentration of Lauric acid. It is shown that *E.coli* is can growth in the medium incorporated with Lauric acid. It is because of the structure of *E.coli* cell which had an outer membrane supported by a thin peptidoglycan layer.

This peptidoglycan is the complex polysaccharide with acid amino and form a structure somewhat analogous to a chain link fence. A second membrane (inner or cytiplasmic membrane) exists and it separated from the outer membrane by perisplasmics space. The cell envelop serve to retain important cellular compound, preferentially exclude undesirable compound in environment and it is crucial to transport of selected material in and out of the cell.

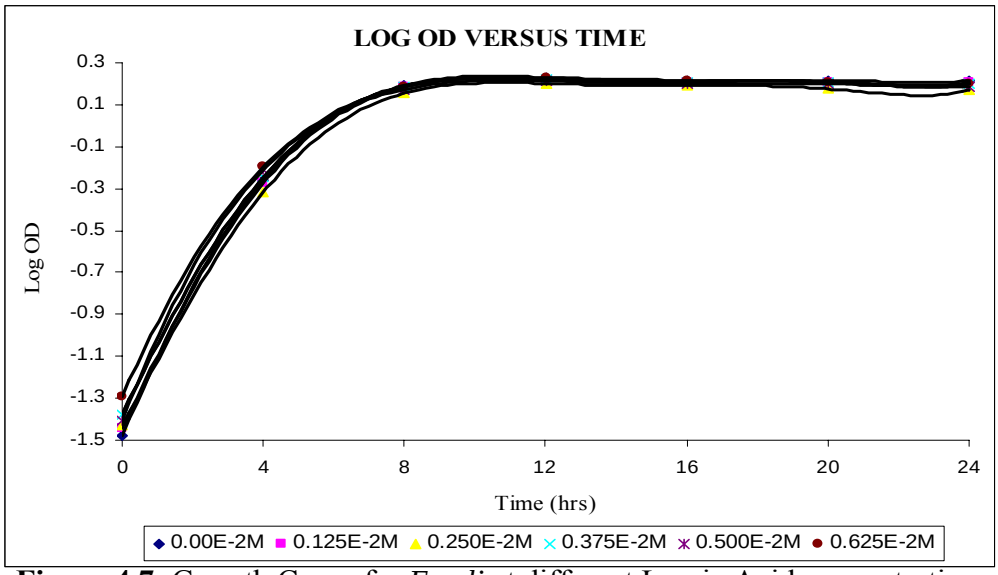


Figure 4.7: Growth Curve for *E.coli* at different Lauric Acid concentration

However Lauric acid gives a better potential as AM agent towards the inhibition of *B subtilis*. Figure 4.8 shows the growth curve for *B. subtilis*. At $0.125x10^{-2}$ M concentration of Lauric acid, it responsible the delay about 8 hours the lag phase of *B .subtilis*. The delaying due to *B .subtilis* to adapted to Laurics acid which effected the concentration of the nutrient liquid medium. However the cell forward to exponential phase after a long lag phase. For $0.250x10^{-2}$M, the curve pattern is similarly to $0.125x10^{-2}$M. It could be due to the fact that the concentration does not give obvious effect on Laurics acid.

In contrast, the $0.375x10^{-2}$M Lauric acid starts to adjust the style of *B. subtilis* growth curve. Time delaying become 12 hours before it starts to enter the exponential phase and only achieve the deceleration phase at 20 hours incubation. However it is obvious different growth curve appear for $0.500x10^{-2}$M and $0.625x10^{-2}$M compare to others. The Lauric acid totally depresses the growth of the *B. subtilis* until there are no more microbial activity involve. It can be indicated that at this concentration, the *B. subtilis* cannot be survive and no antimicrobial activity occurs.

This effectiveness of the Lauric acid to enhance the growth of *B. subtilis* as the gram positive is due of the kind of that microorganism. Gram positive cell do not have an outer membrane. Rather they have a very thick, rigid cell wall with

multilayer of peptidoglycan. This structure causes the *B. subtilis* cell easily to destroy or effected with Lauric acid.

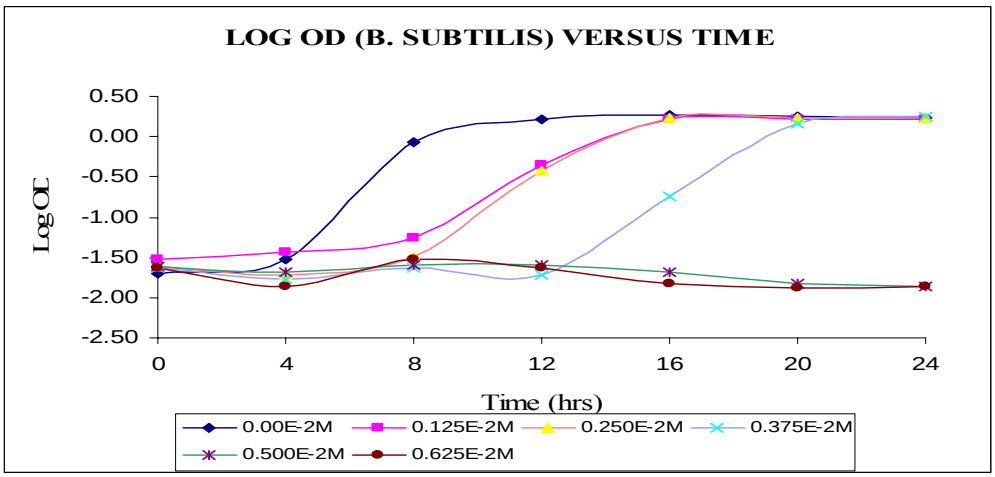


Figure 4.8: Growth Curve of *B. Subtilis* at different Lauric Acid concentration

4.2.2.2 Liquid Culture Test ($O.D_{600\ nm}$ Measurement) for AM starch (wheat) film

The effect of AM concentration on the inhibition is distinguishable much more clearer in Figure 4.9. The effects keep increase until it become almost consistent after 30 hour. It is shown that as concentration increased, the reading of OD will decreased. It means that higher concentration of Lysozyme in a film will inhibit more of *E.coli*.

Compared to control film, 0.01 g/mL lysozymes film manage to inhibit the growth but 0.2 g/mL of lysozymes film inhibit the most. It inhibits about 73% of *E.coli* growth while for 0.01 g/mL only inhibits about 14%, 0.04 g/mL was about 30 % and lastly 0.07g/mL lysozymes film inhibit 39 %.

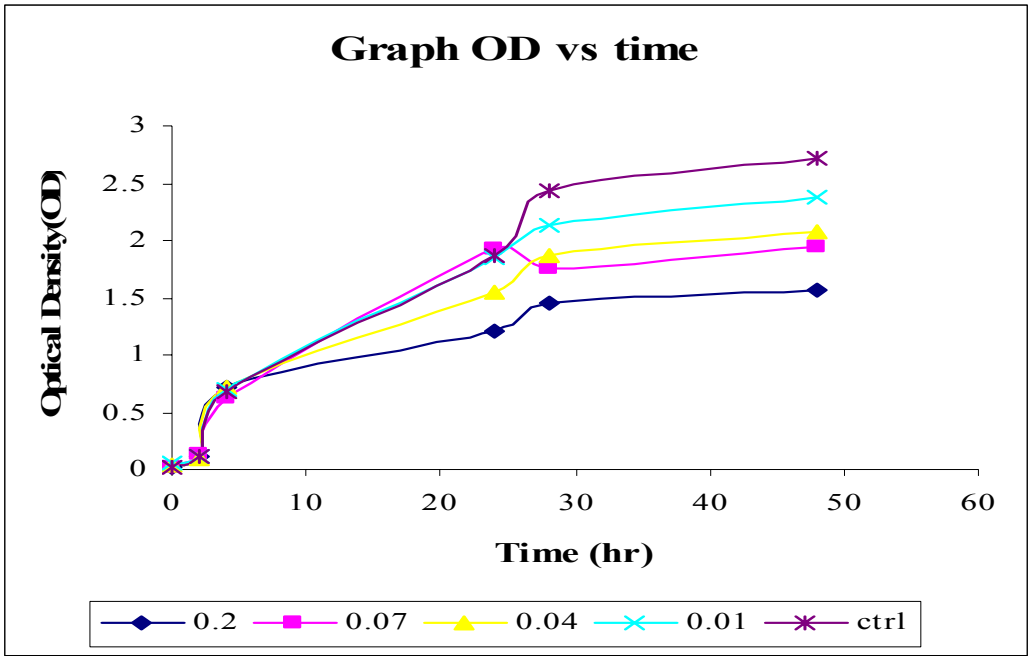


Figure 4.9: Graph OD versus time

Figure 4.10 shows the effect of E. coli growth after 48 hours incubation with Lauric acid AM film. It is shown that when the concentration of Lauric acid in a film increased, the inhibition effects towards *E.coli* growth also increased. This is also observed for 0.07 g/mL Lauric acid-incorporated film which is different with the agar diffusion test. This might be because of the differences in the degree of Lauric acid insolubility.

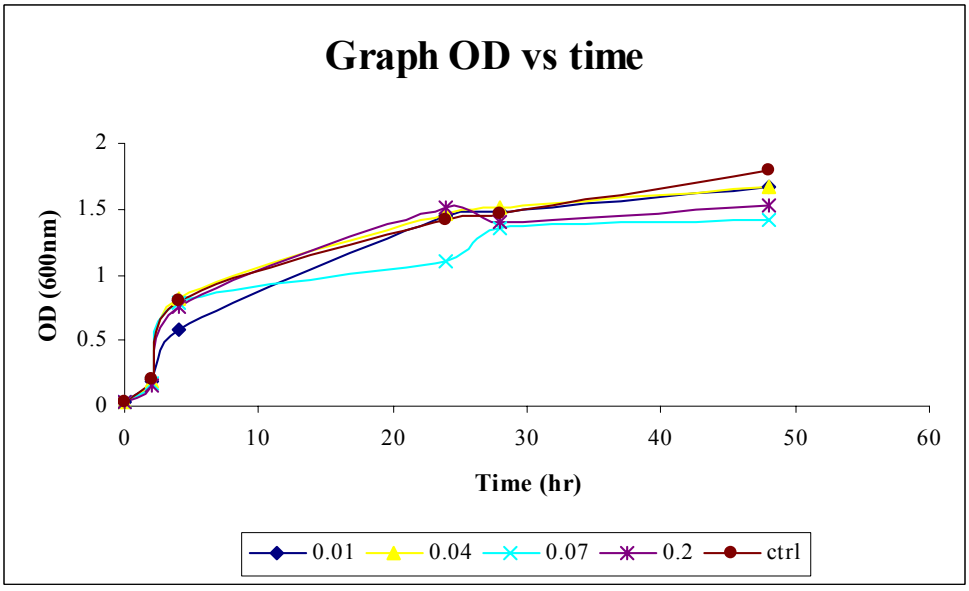


Figure 4.10: Graph OD versus time

AM starch (wheat)-based film containing nisin, nisin-EDTA, lysozyme, lysozyme-EDTA nisin-lysozyme and EDTA give a different inhibitory effect towards the growth of *E. coli.* Figure 4.11 shows the inhibition of *E. coli* by the AM films in liquid culture test. Clearly, combination of lysozyme and EDTA shows a large reduction of stationary growth phase. At the stationary growth phase, the cell concentration in the control medium ($O.D_{600nm}$ = 1.175) was 3 times higher than the cell concentration in the medium containing the starch-based film incorporated with lysozyme and EDTA ($O.D_{600nm}$ = 0.412). From the above discussion, it can be concluded that lysozyme combined with EDTA enable inhibition of both bacteria growth. As a chelating agent, EDTA plays an important role for the AM to function in the film matrix.

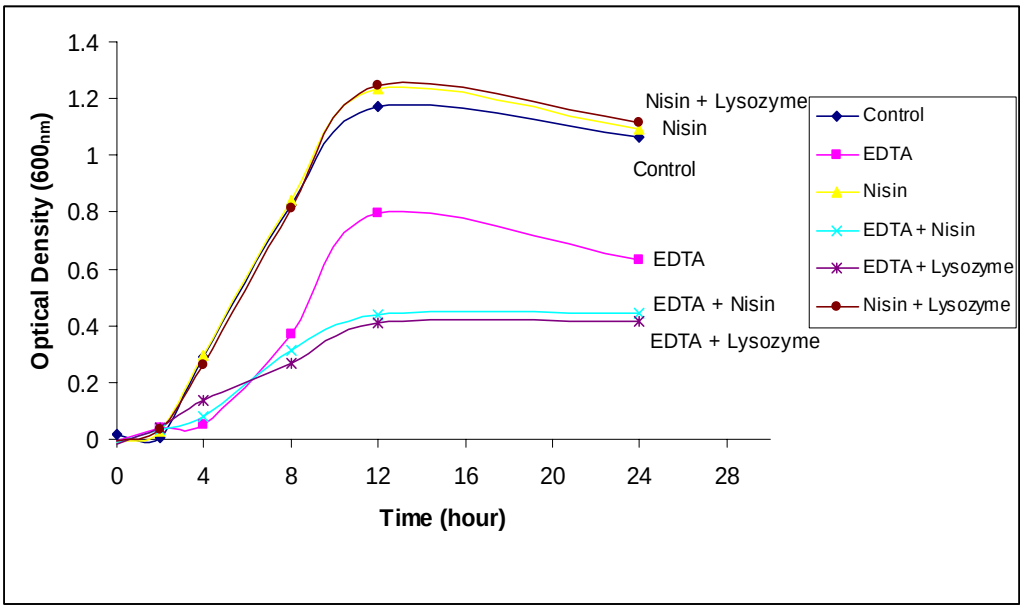


Figure 4.11. Inhibition of Escherichia Coli by the starch-based film containingantimicrobial agents in a liquid culture medium at 37°C

Figure 4.12 summarized the ability of lysozyme, nisin and Lauric acid to inhibit the growth of E. coli in a liquid culture medium at 37°C. Clearly, lysozyme shows the largest reduction of exponential growth phase. Broth culture exposed to lauric acid incorporated film had the lowest inhibition effect towards *E. Coli* growth.

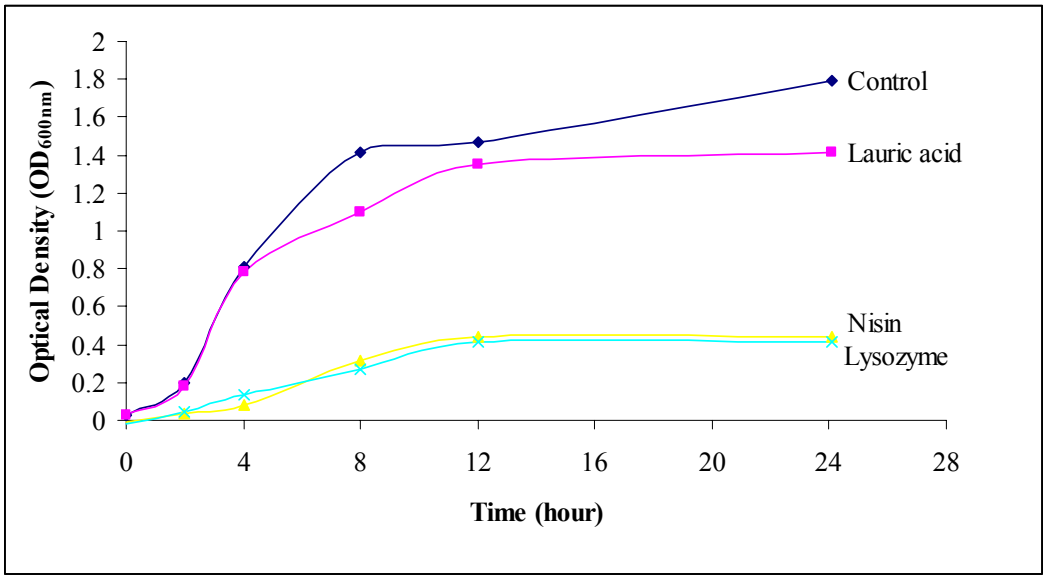


Figure 4.12: Inhibition of *E. Coli* by the starch-based film containing AM compound in a liquid culture medium at 37^{o}C.

CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

The purpose of this research is to develop an AM starch-based film. AM active packaging starch-based particles can help prolong the shelf life of food product while maintaining the quality, safety and integrity of packaged food. It was found that the film is effective towards inhibition of microbial activity. Combination of EDTA and nisin and EDTA and lysozyme help to inhibit the growth of both *E. coli* (gram positive) and *B. subtilis* (gram negative bacteria). Whilst, Lauric acid had a better inhibition effect towards the growth of *E.coli*. And lastly, a translucent AM starch-based film with less brittleness and flexible had been successfully developed.

5.2 Recommendations

It is recommended that this research will be extends to the study for new antimicrobials agent with wide spectrum of activity and low toxicity. It is also

recommended that the study will be further on the development of starch-based film from other starch sources such as tapioca, corn etc.

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