UNIVERSITI PUTRA MALAYSIA

PROPERTIES OF STARCH/NANOCELLULOSE COMPOSITE FILM CONTAINING THYMOL FOR FOOD PACKAGING APPLICATION

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ABSTRACT

Application of starch films has been limited due to poor properties of the films compared to synthetic plastic. However, this can be encountered by adding nanofiller particularly nanocellulose. Food product can be easily spoiled by microbial thus affecting shelf life of the food product. This can be overcome by addition of antimicrobial agent such as thymol.

Antimicrobial nanocomposite film particularly starch/nanocellulose films incorporated with thymol was produced using solvent casting method. The starch/nanocellulose films were incorporated with different concentrations of thymol which were 0.1%, 0.3%, and 0.5% w/v. The physical, mechanical, thermal, barrier, and antibacterial properties of the films were analysed. Physical properties particularly the colour was determined using colorimeter and the thickness of the films were determined using micrometre; mechanical properties using texture analyser; thermal properties using thermogravimetric analysis (TGA); and barrier properties particularly water vapor permeability (WVP) and oxygen permeability using modified dry cup method and oxygen analyser respectively. For antibacterial properties of the films, in vitro study was conducted by observing inhibition zone of the films against Staphylococcus aureus and Escherichia coli. For in vivo study, the films were used to wrap chicken meat and the quality of the chicken meat was observed at specified interval of time.

It was found that tensile strength (TS) and Young modulus (YM) decreased but elongation at break (EAB) increased with the addition of thymol demonstrating that the films are suitable to be used as plastic wrapping for food packaging application. Besides that, addition of thymol improved thermal stability and barrier properties of the films. Starch/nanocellulose films incorporated with thymol produced in this work exhibit high potential to be applied as antimicrobial food packaging since the films exhibit improvement in the properties and positive inhibition towards bacterial growth.

ABSTRAK

Penggunaan filem kanji telah terhad disebabkan oleh sifat-sifat buruk filem berbanding dengan plastik sintetik. Walau bagaimanapun, ini boleh ditemui dengan menambah nanofiller terutamanya nanocelulosa. Produk makanan boleh dirosak kan dengan mudah oleh mikrob yang menjejaskan jangka hayat produk makanan. Ini dapat diatasi dengan penambahan agen antimikrobial seperti thymol.

Filem nanocomposite antimikrobial terutamanya filem kanji / nanoselulosa yang digabungkan dengan thymol dihasilkan menggunakan kaedah pemutus pelarut. Filem kanji / nanoselulosa digabungkan dengan kepekatan thymol yang berbeza iaitu 0.1%, 0.3%, dan 0.5% w / v. Ciri-ciri fizikal, mekanikal, haba, halangan dan antibakteria filem-filem dianalisis. Ciri-ciri fizikal terutamanya warna ditentukan menggunakan warna dan ketebalan filem ditentukan menggunakan mikrometer; sifat mekanikal menggunakan penganalisis tekstur; sifat haba menggunakan analisis termogravimetrik (TGA); dan sifat halangan terutamanya kebolehtelapan wap air (WVP) dan kebolehtelapan oksigen yang menggunakan kaedah pai kering dan penganalisis oksigen yang diubah suai. Untuk ciri-ciri antibakteria filem, kajian in vitro dijalankan dengan memerhatikan zon inhibisi filem terhadap Staphylococcus aureus dan Escherichia coli. Bagi kajian vivo, filem-filem itu digunakan untuk membungkus daging ayam dan kualiti daging ayam diperhatikan pada selang masa tertentu.

Telah didapati bahawa kekuatan tegangan (TS) dan modulus Young (YM) berkurangan tetapi pemanjangan pada rehat (EAB) meningkat dengan penambahan thymol yang menunjukkan bahawa filem-filem yang sesuai digunakan sebagai pembungkus plastik untuk aplikasi pembungkusan makanan. Selain itu, penambahan thymol meningkatkan kestabilan haba dan ciri-ciri halangan filem. Filem kanji / nanoselulosa yang digabungkan dengan thymol yang dihasilkan dalam pameran

ini mempamerkan potensi tinggi untuk digunakan sebagai pembungkusan makanan antimikrob kerana filem memperlihatkan peningkatan sifat dan perencatan positif terhadap pertumbuhan bakteria.

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LIST OF ABBREVIATIONS

ASTM	American Society of Testing and Material
TGA	Thermogravimetric Analyser
TS	Tensile strength
WVP	Water vapor permeability
WVTR	Water vapor transmission rate
YM	Young modulus
TA	Texture analyser
OP	Oxygen permeability
OTR	Oxygen transmission rate
PBS	Polybutylene succinate
PCL	Polycaprolactone
PE	Polyethylene
PET	Polyethylene terephthalate
PGA	Polyglycolic acid
PLA	Polylactic acid
PP	Polypropylene
PS	Polystyrene
PVA	Polyvinyl alcohol

CHAPTER 1

INTRODUCTION

1.1 Background of Study

The world's production and use of plastics for packaging have been massively expanded over the past 20 years, this contribute to the waste disposal issue. According to the latest market data compiled by European Bioplastics in cooperation with the Nova Research Institute, global production capacity for bioplastics is expected to rise from around 2.11 million tons in 2018 to around 2.62 million tons in 2023 (European Bioplastics, 2018). As a result, growing environmental sustainability concerns and demand for bioeconomic and green products are increasing. This led to significant bio-based polymer development. The term biodegradable materials are used to describe materials that can be degraded by living organism enzymatic action such as bacteria, fungi, and yeast. Due to its non-toxicity, non-pollutant, biodegradable, and availability, biopolymer materials have great potential to replace the conventional plastics (Dutta et al., 2009; Pereda et al., 2012; Mendes et al., 2016).

Generally, there are 3 main categories of biodegradable polymers; 1) Natural polymers such as polysaccharide (starch, cellulose, chitosan, and agar), protein (casein, gelatine, gluten, alginate, whey protein, and collagen), and lipid that are abundant and can be naturally found on earth (Malhotra et al., 2015); 2) Bio-derived synthetic polymers such as polylactic acid (PLA), polycaprolactone (PCL), polyglycolic acid (PGA), polyvinyl alcohol (PVA) and succinate polybutylene (PBS) (Rhim et al., 2013); 3) Polymers from microbial fermentation or genetically modified bacteria such as PHB (poly hydroxybutyrate) (Anne, 2011). Considering the high cost and complicated method to synthesis synthetic biopolymer, application of synthetic biopolymer as food packaging material is limited thus packaging materials from natural biopolymer is a good alternative for food packaging application (Imre & Pukanszky, 2015).

Starch-based biopolymers such as maize, rice, corn, yam, potato, and tapioca can be used to produce thin film (Jimenez et al., 2012). An amount of 75% of all organic material on earth is present in the form of polysaccharides. A starch is a major polysaccharide. In addition, the process of making the thin film from starch does not involve any harmful chemicals, thus producing the non-toxic and edible film that is useful as food packaging material. Starch is also low cost and renewable (Xu et al., 2005). Because of its good film-forming properties, abundant availability and inexpensive (Jimenez et al. 2012; Kowalczyk et al. 2015), in this study, corn starch was used as the main biopolymer matrix to produce the films. However, starch application reveals several disadvantages due to its inherently strongly hydrophilic character (water sensitivity) and poor mechanical properties compared to conventional synthetic polymers that limit their use as food packaging material.

Incorporating an additive like plasticizer such as glycerol into a biopolymer matrix may increase elasticity of the starch film (Vieira et al., 2011). Starch film can also be mixed with nanomaterials such as nanocellulose to produce bio-nanocomposite which is a promising route to enhance mechanical and barrier properties of the starch biopolymer. In addition, additives such as antimicrobial agent can be added to the polymer matrix to improve consumer safety and extend the shelf-life of food products. Antimicrobial agent can inhibit microorganism growth or kill the microorganism. Since this agent is used for packaging, it can dramatically increase the shelf-life of food products without adding additives or preservatives in food product. Among the antimicrobial agent, thymol ($C_{10}H_{14}O$) is very promising as it exhibits excellent antimicrobial activity (Sanchez-Garcia et al., 2008).

Thymol is a phenolic compound, a component of thyme oil, a compound in the thyme plant (Thymus vulgaris). It occurs naturally in foods like lime honey, cooking herbs, cranberry and mandarin orange oils, and mandarin. It has a thyme-like smell and a pungent taste. Thymol is a volatile oil that can be used as a vermifuge for its antiseptic, antibacterial, and antifungal actions (Dorland, 28th ed).

1.2 Problem Statement

The use of biopolymers such as starch to produce food packaging film reveals several disadvantages compared to conventional synthetic polymers. Biopolymer films exhibit poor mechanical, thermal and barrier properties. This limit the application of the film (Pelissari et al, 2009). However, incorporating starch film with nanosized filler such as nanocellulose and glycerol as plasticizer to produce bio-nanocomposite may improve their properties.

In addition, food product can be easily spoiled by bacteria. Additive such as antimicrobial agent particularly thymol can be added to the food packaging film to produce active food packaging system. Thymol can help to increase the functional properties of films, especially antimicrobial properties in order to extend the shelf life of food products.

However, addition of nanocellulose and different concentrations of thymol into the films may affect the mechanical, thermal, barrier, and antimicrobial properties of the films. Thus, this work is directed towards investigating the effects of nanocellulose and different concentrations of thymol addition towards the mechanical, thermal, barrier, and antimicrobial properties of the films.

1.3 Objectives

Objectives of this study are:

1. To produce and characterize properties (physical, mechanical, thermal, and barrier) of starch/nanocellulose films incorporated with different concentrations (0.1%, 0.3%, and 0.5% w/v) of thymol.

2. To investigate the antibacterial properties of the starch/nanocellulose films incorporated with thymol via in-vivo and in-vitro analysis.

CHAPTER 2

LITERATURE REVIEW

2.1 Food packaging

Packaging plays a critical role in the food supply chain. The primary role of food packaging is to protect food from environmental contamination and other influences (such as odors, shocks, dust, temperature, physical damage, light, micro-organisms, and humidity) and is a key to ensure the quality and safety of food and at the same time extending the shelf-life and minimizing food losses and wastage (Carocho, et al, 2015). Packaging shall serve as a food container that allows efficient transport throughout the supply chain, prevents physical damage, and protects against manipulation and theft.

Figure 1 shows the general properties of food packaging material. Food packaging materials must exhibit good mechanical and thermal properties to provide physical protection and maintain physicochemical properties food products. Food packaging material also act as a barrier to control permeation of oxygen, carbon dioxide and other volatile compounds, so that the quality and aroma of food is preserved (Rhim et al., 2013)

Figure 1. General food packaging properties.

(Source: Rahim et al.,2013)

Food packaging is usually selected according to the types of food product to ensure that food packaging effectively preserves the food quality (Rhim et al., 2013). Packaging materials are usually made from plastic, paper, metal, and glass. Among them, plastic is the most popular food packaging material because of its special properties, which can be formed and moulded in various shapes and thicknesses (Shin & Selke, 2014). Petroleum-based plastics including Polyethylene (PE), Polypropylene (PP), Polyethylene terephthalate (PET), and Polystyrene (PS) are usually used to produce food packaging materials such as cling wrap, shrink film, sachet, stand-up pouches, sachet, and container (Tang et al., 2012). However, petroleum-based plastic is non-degradable, thus can lead to the abundant municipal solid waste on the landfill which creates a serious global issue on environment (Iwata, 2015). In Malaysia, petroleum-based plastic dominated the second highest in the landfill which is 24% of the total solid waste (Moh and Abd Manaf, 2014). In addition, petroleum is non-renewable, and will eventually run out.

To solve this issue, biopolymers have been introduced as an alternative material to replace petroleum-based plastics because of growing environmental concerns and fossil-source shortages (Rhim & Ng, 2007). Their natural properties offer the packaging materials to degrade in a reasonable period without causing environmental waste problems.

2.2 Biopolymers

Over the two decades, renewable resource polymers or biopolymers have attracted tremendous attention. The growing appreciation of biopolymers is mainly due to environmental concerns and the rapid depletion of petroleum resources. Generally, biopolymers can be classified into three groups: (1) natural polymers, (2) synthetic polymers from natural monomers, and (3) polymers from microbial fermentation as can be seen from Figure 2. All the three polymer groups are derived from renewable resources except the last group that is derived from petroleum products. Among the biopolymers, starch is one of the most promising natural biopolymers due to its availability, low price, biodegradable and good film-forming capability.

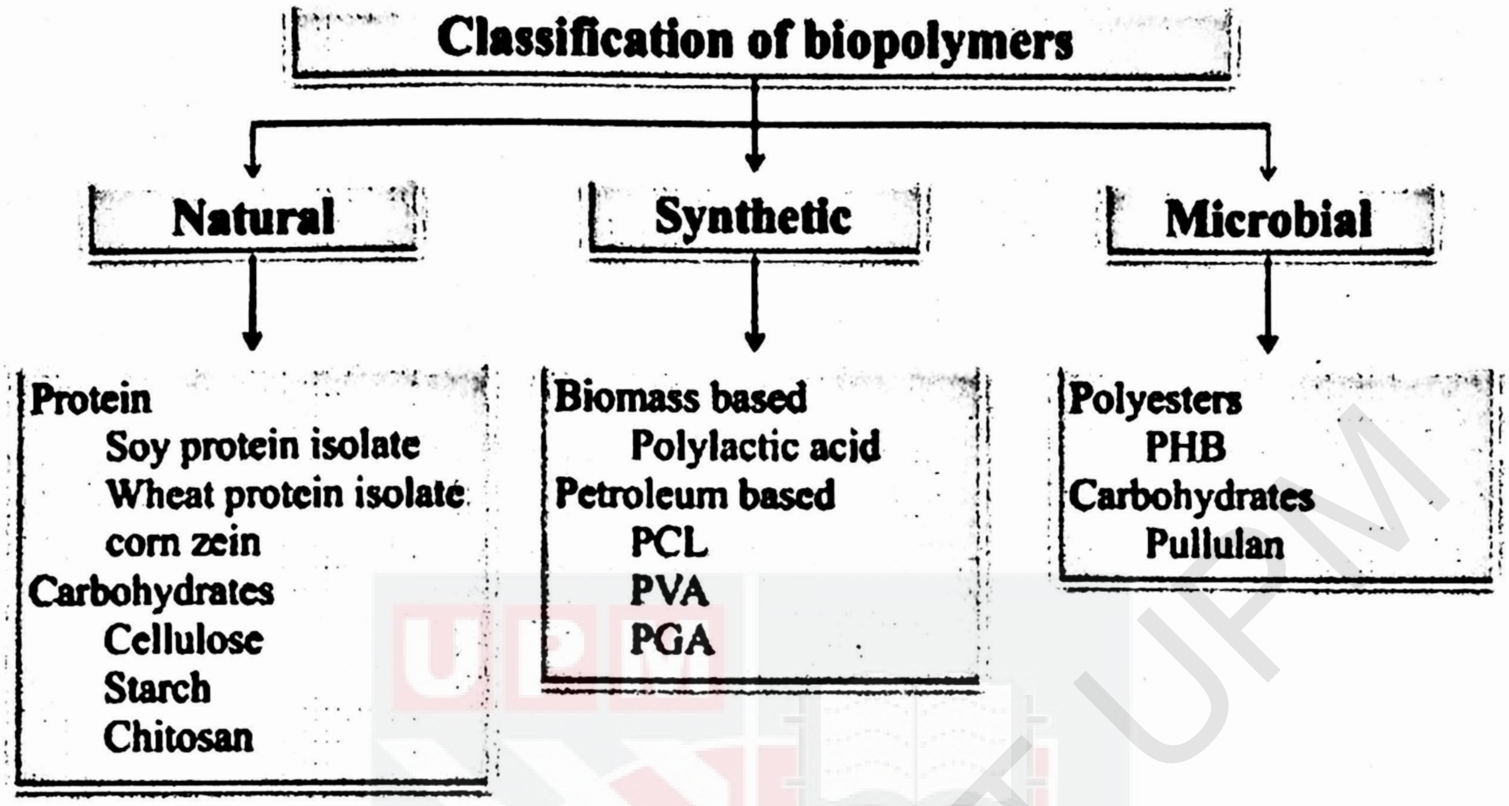


Figure 2. Type classification of biopolymers.

(Source: Talegaonkar et al., 2017)

2.2.1 Starch film

There are two main components of starch which are amylose and amylopectin. According to Mishra and Rai (2006), composition of amylose and amylopectin in starch is dependent on the origin of starch which can be categorized into cereal (maize, rice, and corn) and tuber (tapioca, yam, potato, and sweet potato) which could affect the properties of the produced films such as mechanical, barrier and thermal properties. Amylose is a straight chain sugar polymer molecule with the α-1,4-glucosidic bond linkage. When looked at a large scale, the amylose is helical, and it becomes a colloidal when disperse in hot water. The average molecular weight of the molecule is 10,000-50,000 atomic mass unit, and it averages 60-300 glucose units per molecule. Figure 3 show the chemical structure of amylose.

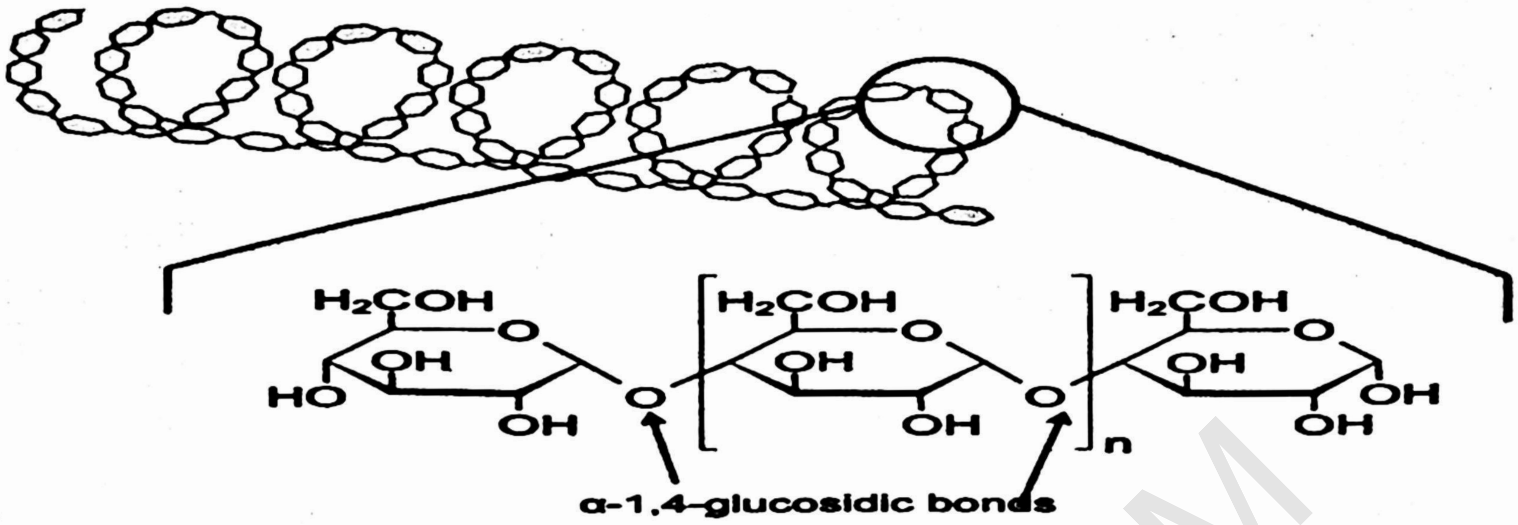


Figure 3. Chemical structure of amylose (Source: Dutton, 2018).

Meanwhile, amylopectin is branched whereby the α-1,4-glycosidic bonds are connected to each straight chain monomer and a α-1,6-glycosidic bond connects the branches. Compared to amylose, it is insoluble and the average molecular weight is 300,000 atomic mass unit, averaging 1800 units of glucose per molecule. Amylopectin is approximately 10 times the amylose size. Figure 4 shows the chemical structure of amylopectin.

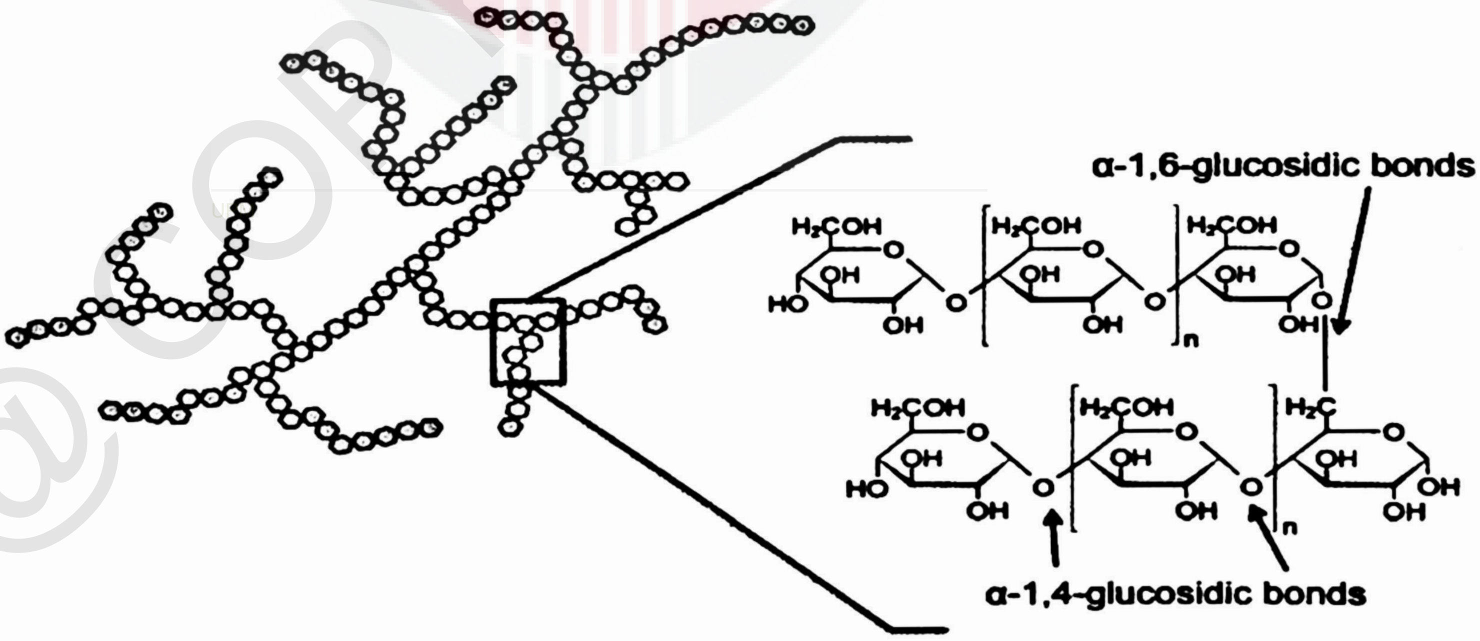


Figure 4. Chemical structure of amylopectin.

(Source: Dutton, 2018)

Table 1 shows the dry-based comparative chemical composition of tapioca and corn starch representing the tuber starch and cereal starch respectively. It shows that tapioca starch consists of 19% amylose, while corn starch consists of 25% amylose higher than tapioca starch. Due to high amylose crystallinity, high amylose composition in corn starch produces stronger and more compact film compared to tapioca starch. In addition, the amylopectin composition in tapioca starch is 81% higher than the amylopectin composition in corn starch, which is 75%. Amylopectin is the amorphous starch region that can influence the starch's crystallinity. High amylopectin shows that the starch exhibits low crystallinity thus may reduce the strength of the film produced. Different types of starch can produce different properties of films due to the different composition of amylose and amylopectin that exhibited different behaviours in terms of film-forming capacity, crystallinity and film properties (Mali et al., 2004).

Table 1. Chemical composition of corn and tapioca starches (Dry basis).

(Source: Mali et al., 2004)

Component	Corn starch	Tapioca starch
Amylose (%)	25 ± 2	19 ± 2
Amylopectin (%)	75 ± 5	81 ± 5
Protein (%)	0.27 ± 0.01	0.11 ± 0.01
Fat (%)	0.29 ± 0.01	0.28 ± 0.01
Ash (%)	0.06 ± 0.01	0.22 ± 0.01

2.2.2 Gelatinization process

Starch granules in cold water are insoluble. Granules absorb water and swell when starch is heated in water. Water absorption within the granules by amorphous region destabilizes their crystalline structure, resulting in the loss of birefringence, a definition of gelatinization (Ratnayake et al. 2007). Due to the straight forward process and low cost of production, solvent casting is among the techniques used to produce thin film (Karki et al., 2016). Solvent casting generally involves film-forming solution preparation by dissolving polymer or biopolymer in aqueous solvents such as water and ethanol. The polymer solution is then poured onto the casting mould and left to dry, allowing the solvent to evaporate and produce the independent film (Irfan et al., 2016).

Water is commonly used as a starch solvent because of the hydrophilic nature of starch to prepare film-forming solution (Suppakul et al., 2013). Starch is then heated to enable the gelatinization process. Figure 5 shows the starch gelatinization process mechanism. Due to the hydrophilic properties of starch, starch granules began to swell when starch is dissolved in water. The combination of heat and shear stress from mixing in the starch solution increases the kinetic energy, causing the granules to rupture and release the amylose. At this point, starch solution's viscosity increases due to amylose structure forming the gel matrix that has the ability to form the film. Due to different degrees of crystallinity, the temperature of starch gelatinization is strongly dependent on the starch origin. Corn starch gelatinization process occurs when the temperature is approximately 70–85 °C (Ratnayake et al., 2007)

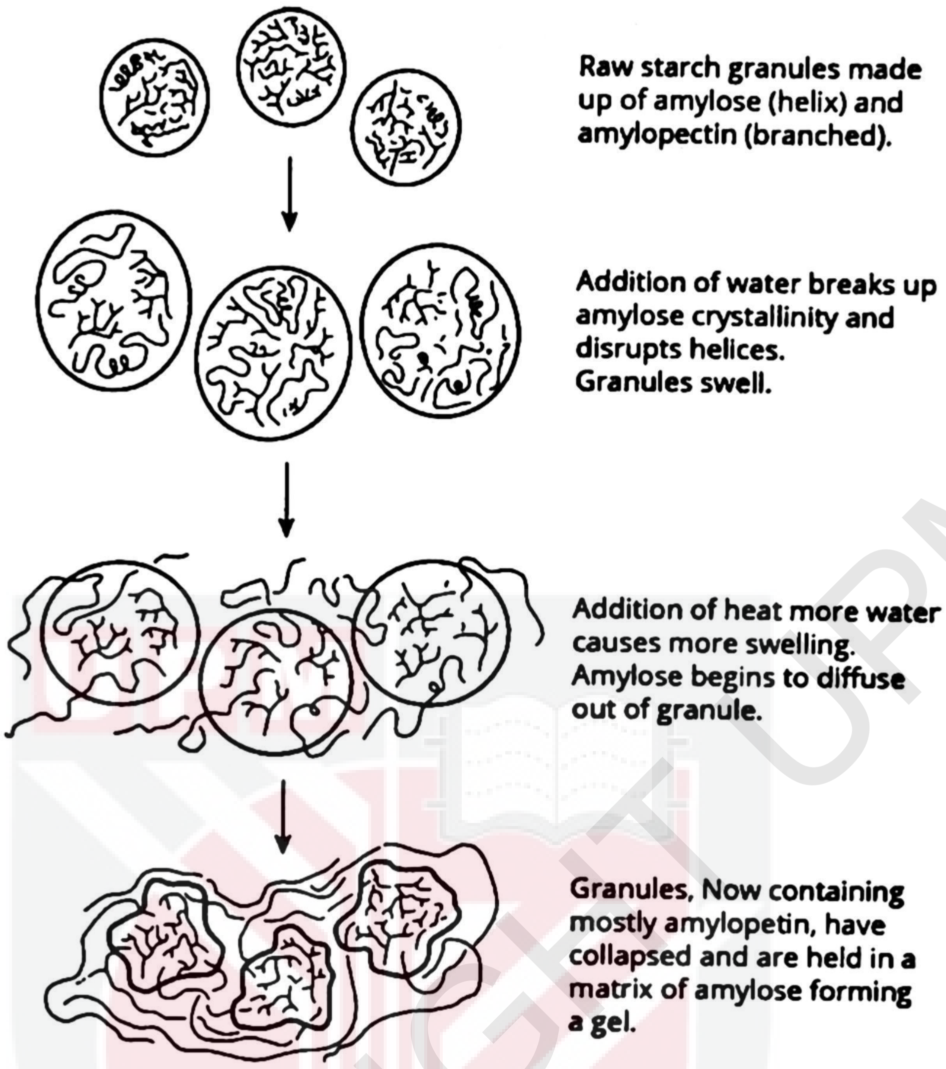


Figure 5. Illustration of gelatinization process of starch.

Source: (Forte, 2018)

2.3 Nanocellulose as reinforcing agent

As a nano reinforcement for polymer matrices, nanocellulose has received significant attention in the food packaging sector, especially for those bio-based and biodegradable material. Polymer nanocomposites usually have much better polymer/filler interactions than conventional composites (Ludueña et al., 2007). Nanocellulose are interesting materials because of low cost, lightweight, and high-strength nanocomposites (Helbert et al., 1996; Podsiadlo et al.,2005). Nanocellulose is an exciting nanofiller for the growth of bio-nanocomposites, derived from the most abundant polymeric resource in nature and with intrinsic biodegradability. Nanocellulose is simple to functionalize due to the elevated amount of hydroxyl groups on its surface, primarily to enhance its dispersion within the polymer matrix. Well-dispersed nanocellulose can improve multiple polymer characteristics, including heat, mechanical, barrier, and surface wettability characteristics, as well as control of active compound release.

The addition of nanocellulose in starch films could increase mechanical properties of starch films due to similarities in the chemical structure (both are polysaccharides), which promotes good interfacial interaction between the two components through hydrogen bonds (Salehuddin et al., 2014). Mechanical properties of nanocellulose-reinforced polymer nanocomposites usually improve with uniform dispersion and distribution of nanocellulose in the polymer matrices. This is due to the stiffing effect of cellulosic nanomaterials due to their large specific surface area in contact with polymer matrix (Mondal, 2017). The capacity to strengthen, however, depends heavily on the amount of nanocellulose added. Too high concentration may result in poor dispersion and non-uniform distribution of stress in the film matrix. This leads to a reduction in properties of tensile. Crystalline nature of nanocellulose and ability to form a good network with matrix by hydrogen bonding will subsequently increases the tortuosity path for the diffusing molecules (water vapor, oxygen) as can be observed from Figure 6 (Mondal, 2017; Sorrentino et al., 2007).

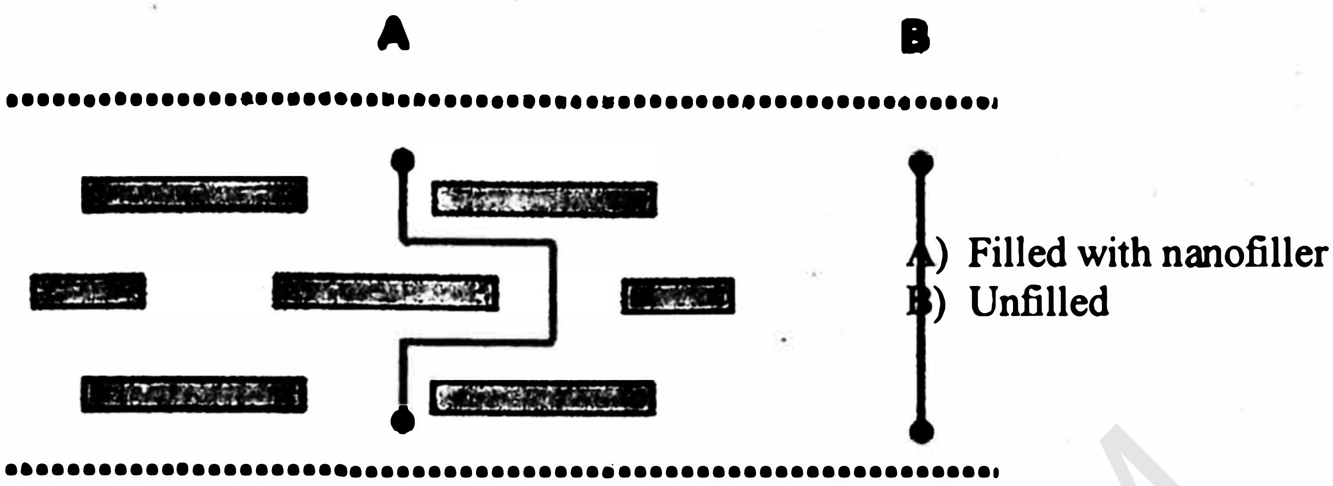


Figure 6. Schematic illustration of the tortuosity of (a) Filled polymer, (b) Unfilled polymer.

(Source: Sorrentino et al., 2007)

According to the studies by Cheng et al. (2017), incorporation of cellulose nanocrystal (CNC), cellulose nanofiber (CNF), and micro fibrillated cellulose (MFC) into starch causes a shift of the onset degradation temperature toward higher temperature (about 50°C ahead). In other words, the onset degradation temperature of starch can be significantly improved by addition of CNFs. The improvement in the thermal stability of starch composites with the presence of cellulose nanofibers (CNC, CNF, and MFC) can be ascribed to the uniform dispersion and confined network structure of CNFs in the starch nanocomposites as well as a strong interfacial interaction between matrix and the fillers.

2.4 Antimicrobial packaging

Nowadays, consumers are demanding for less use of chemicals in food, but still expect high quality of food while preserving the freshness of the food product and the quality of the sensors. Among emerging technologies, many applications such as oxygen-scavenging packaging and antimicrobial packaging are among the most promising innovations in active packaging technologies (Floros et al., 1997). Antimicrobial packaging is a packaging system that is capable of killing or inhibiting food contaminating spoilage and pathogenic microorganisms (Han, 2003). The primary objectives of an antimicrobial packaging system are (i) safety assurance, (ii) quality maintenance, and (iii) extension of shelf life. Research on antimicrobial packaging generally began with the development of antimicrobial packaging materials whereby in their macromolecular structures containing antimicrobial agent.

Due to the different antimicrobial mechanisms and different physiologies of the microorganisms, all antimicrobial agents have different activities on microorganisms. Some antimicrobial agents inhibit essential microorganism metabolic (or genetic reproductive) pathways, while others alter the structure of the cell membrane/wall (Sofi et al, 2017). Meat, dairy, and bakery as well as fruits and vegetables are the common food products which are easily attacked by microorganism and can be protected from spoilage by antimicrobial packaging (Leiter, 1994). Antimicrobial agent can generally be classified into three groups which are chemical agents, natural agents, and probiotics. Table 2 shows the example of potential antimicrobial agents:

Table 2. Example of potential antimicrobial agents for antimicrobial food packaging system.

A) Chemical antimicrobial agent. • Organic Acids - Acetic acid, benzoic acid, lactic acid, citric acid, malic acid, propionic acid, sorbic acid, succinic acid, tartaric acid, mixture of organic acids • Fungicides - Benomyl, Imazalil, sulfur dioxide
B) Natural antimicrobial agents • Polysaccharide - Chitosan • Phenolics - Catechin, cresol, hydroquinone • Plant volatiles - Allyl isothiocyanate, cinnamaldehyde, eugenol, linalool, terpineol, thymol, carvacrol, pinene • Plant/spice extract - Grape seed extract, grapefruit seed extract, hop beta acid, Brassica erucic acid oil, rosemary oil, oregano oil, basil oil, other herb/spice extracts and their oils
C) Probiotics antimicrobial agents • Lactic acid bacteria

(Source: Han, 2004)

2.5 Thymol

Thymol is a naturally occurring antimicrobial carvacrol isomer. Thymol is the main component of essential oils of oregano and thyme (Thymus and Organum sp.) Thymol is also called 2 isopropyl-5-methylphenol and is a white crystalline substance. Figure 7 shows the chemical structure of thymol.

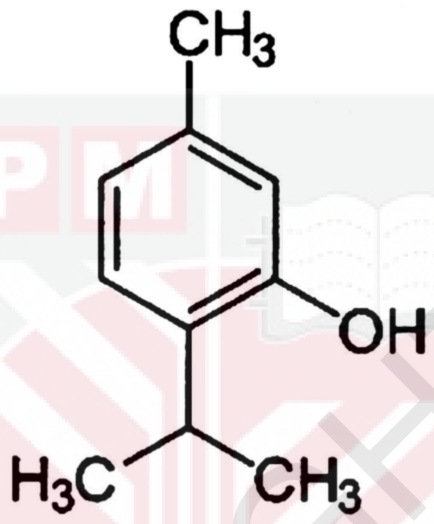


Figure 7. Chemical structure of thymol (Source: Kaur et al., 2013).

Thymol gives a pleasant smell of aroma. In relation, thymol is a hydrophobic phenolic compound in essential oils and is likely to dissolve between lipid acyl chains in the hydrophobic domain of the cytoplasm of bacterial cells. Thymol is used as antimicrobial agent and has a high potential to increase shelf life and improve the safety of food products. Antimicrobial activity of thymol against bacteria, mold, and yeast has been demonstrated (Guarda et al., 2011). Thymol also exhibits high potential for use in the food industry as it is considered by the US Food and Drug Administration as "Generally Recognized as Safe (GRAS)" (Paola et al., 2009). It is also suitable for food applications as it has migratory release properties that can provide the food in the packaging to continuous antimicrobial activity (Tawakkal et al., 2014). The activity of an essential oil as antimicrobial agent can affect both the external envelope of the cell and the cytoplasm. Thus, the hydrophobic nature of thymol allows them to penetrate microbial cells and cause alterations in its structure and functionality. This could explain why thymol are generally most effective, with some exceptions (Kim et al., 1995) against Gram-positive microorganisms. The external capsule of some Gram-negative bacteria limits or prevents the penetration of thymol into the microbial cell. The compounds present in the thymol are also capable of interfering with proteins in the wall that are often involved in the transport of essential molecules into the cell.

2.6 Characterization of the films

2.6.1 Colour properties

Colour attributes are of the utmost importance of food packaging material because they directly influence the acceptance of consumers towards the food products as well as the food packaging material. Film colour parameters range from L*: 0 (black) to 100 (white), a*: -60 (green) to +60 (red) b*: -60 (blue) to +60 (yellow) can be measured using the Easy Math Calorimeter (HunterLab Color Reader) according to ASTM D1925 standard test method (Chillo et al.,2008; Gutierrez et al., 2015). The total colour difference (ΔE), can be measured by the magnitude of the resulting vector of the three components, which are ΔL: brightness difference, Δa: red – green chromaticity difference and Δb: yellow – blue chromaticity difference.

Usually, starch film is colourless and transparent thus suitable for food packaging and food coating (Mali et al., 2004). The concentration of plasticizers added in the film may affect the film's colour. The higher concentration of film-forming plasticizers affected the yellowness (+b) of the film (Risyon, 2014).

2.6.2 Mechanical properties

Food packaging material should exhibit good mechanical strength to meet the food packaging application requirement. It is important to enable the food package to withstand stress during handling, storage, and transportation. The material used for food packaging are dependent on the types of food and usually should be strong, flexible, and unable to deform or change its shape to provide structural integrity or strengthen the structure of food (Gontard et al., 1992). There are many standards for measuring the mechanical properties of polymer provided by expert societies such as the American Society of Testing and Materials (ASTM), the British Standard, and the Japanese Industrial Standard (JIS). Each standard offers a variety of standard methods and steps to characterize the mechanical properties of the materials' mechanical properties including tensile testing, puncture testing, and peel testing. According to Aguirre et al. (2013), Antoniou et al., (2015), and Tang et al., (2008), a standard test method to determine mechanical prorperties is ASTM D882-91 and this method is commonly used to characterize thin film biopolymer film for tensile properties.

In this study, tensile strength, Young's modulus and elongation at break are among the mechanical properties that were characterized. Mechanical properties of thin films can be determined using a universal texture analyser. The tensile strength of the films can be measured through tensile stress and strain curves as shown in Figure 8. Tensile strength measures the force at specific acceleration to pull something. In addition, the maximum tensile stress is achieved before the sample test specimen starts to fail. The breaking point of the specimen is the test failure point (Risyon, 2014). As the loads continue to increase, the axial deflection of the specimen leads to a failure (break point). In the meantime, the tensile strain is the elongation of the test specimen measured when the break point was reached.

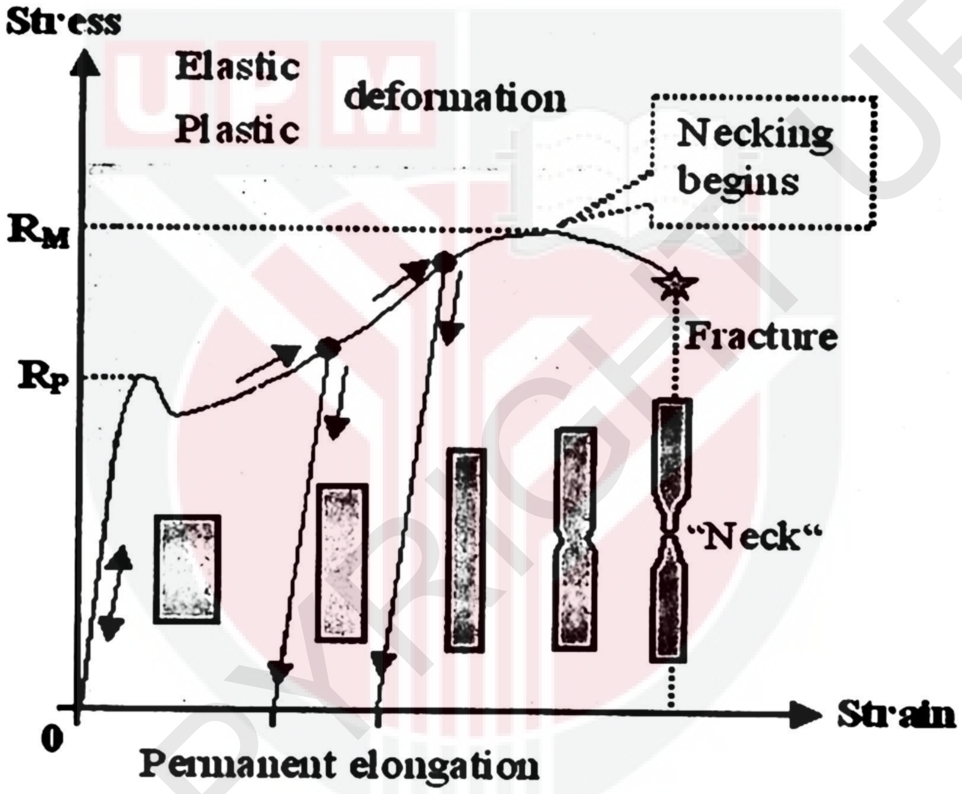


Figure 8. Typical stress strain curve.

Source: H. Föll (2019)

Figure 8 shows the typical stress-strain curve obtained from tensile test. Stress-strain curve is divided into three regions which are elastic deformation, uniform plastic deformation, and non-uniform plastic deformation (Faridmehr et al., 2014). The shape of materials will temporarily change during elastic deformation and return to the original shape after the load is removed. If the load is continuously exerted on the material, it will undergo uniform plastic deformation by permanently changing the shape of the materials after removing the load. Continuous pulling of the material will result in non-uniform deformation of the plastic by breaking the material and fracturing it. Bondenson and Oksman (2007) obtained a clear improvement in the elastic modulus (from 2600 MPa to 3,100 MPa), tensile strength (from 35 MPa to 52 MPa), and a slightly higher elongation at break (from 1.8% to 3.1%) for PLA reinforced with 5 wt.% of CNC due to the interactions by hydrogen bonding (Favier et al., 1995). The percolation theory predicts that enhancement in composite properties requires the presence of an amount of filler high enough to form a continuous structure, properly dispersed within the matrix (Ljungberg et al., 2005). Maximum modulus (2.6 GPa) and strength (58 MPa) were achieved by addition of 50 wt% BCNF, corresponding to 106- fold and 20-fold the values for pure starch/glycerol film. The incorporation of 20 wt% CNF to xylan-rich hemicellulose films plasticized with 25 wt% sorbitol (Peng et al., 2011) increased film strength and modulus from 11.9 MPa and 0.74 GPa, respectively, to 15.0 MPa and 17.2 GPa.

2.6.3 Thermal properties

When heated over time, thermal properties of food packaging material determine the behaviour of how the material reacts. These properties provide information about material thermal stability and material decomposition when reacting to heat (Hammer, 2008). Thermogravimetric analysis (TGA), differential scanning calorimetry (DSC), differential thermal analysis (DTA), and thermomechanical analysis (TMA) are usually used to characterize the thermal properties of the materials.

TGA measures decomposition of materials at the specific temperature (Liu et al., 2016). The decomposition usually occurs in a few stages which depends on the composition of the materials. The film component such as moisture, solvents, plasticizers, and monomers begin to degrade at the first stage of decomposition. Due to the low boiling point, volatile materials tend to evaporate, thus reducing the sample mass. The final stage of mass loss in the curve shows maximum temperature degradation (T_{max}). As an excess product, polymer decomposition and final residues such as ash, filler, and glass fibres are produced. (Hammer, 2008). Incorporation of nanofiller such as nanocellulose into biopolymer matrix can improve the thermal stability of the films. Similar study reported by Moreira et al. (2013) revealed that addition of magnesium hydroxide nanoplates $Mg(OH)_2$ into the pectin matrix improved the thermal properties of pectin film. The improvement of pectin/$Mg(OH)_2$ film was due to the strong ability of $Mg(OH)_2$ in hindering the decomposition of pectin.

(Cheng et al., 2017) find that incorporating each of cellulose nanofibers (CNC, CNF, and MFC) into starch causes a shift of the onset degradation temperature toward higher temperature (about 50°C ahead). In other words, the onset degradation temperature of starch can be significantly improved by addition of cellulose nanofibers. The improvement in the thermal stability of starch composites with the presence of cellulose nanofibers can be ascribed to the uniform dispersion and confined network structure of the cellulose nanofibers in the starch nanocomposites as well as a strong interfacial interaction between matrix and the fillers.

2.6.4 Barrier properties

Food packaging also act as a barrier between surrounding and food inside the packaging. A good food packaging needs to be able to prevent excess gas such as oxygen, carbon dioxide, and water vapor from entering the food. This excess gas and water can spoil food and affect food and shelf-life which usually causing food deterioration due to lipid and vitamin oxidation, resulting in changes in sensory and nutrient (Biji et al., 2015).

Food packaging also acts as a barrier to prevent the loss of food components in packaging such as moisture, minerals, and flavours. In other words, barrier properties are related to the permeability of the films and dependent on food packaging material types, thickness, and relative humidity of the surrounding (Siracusa, 2012). Water vapor permeability (WVP) can be determined as the absorption of moisture permeating through the film from the surrounding (Bertuzzi et al., 2007). Meanwhile, film barrier properties against oxygen can be quantified by the oxygen permeability (OP) by measuring oxygen permeation through film thickness.

Figure 9 shows the water vapor and gas mechanism that penetrates through a packaging film which involve adsorption, diffusion, and desorption processes. The molecules of water and gas diffuse and penetrate on one side of the film and move between the polymer matrix within the void space, then desorb on the other side of the film from the polymer surface. The chemical structure, amylose ratio to starch amylopectin, polymer packing, crystallinity, plasticizer, filler, and environmental humidity influenced the film's permeability (Chung and Lai, 2007). Generally, starch biopolymer film has poor moisture resistance due to its hydrophilic characteristic, thus exhibit high WVP (Petersson and Stading, 2005). However, reinforcement of biopolymer with nanofiller such as nanocellulose will result to improvement in the barrier properties of the films against water vapor and volatile gas by the formation of tortuous pathway in biopolymer matrix. According to the study reported by Azeredo et al. (2010), addition of at least 0.1% weight of cellulose nanofiber into mango puree film improved the water vapor barrier of the mango puree film. The nanofiller delays the permeation of volatile materials through the film and improved the barrier properties of the film (Sorrentino et al., 2007; Siracusa, 2012).

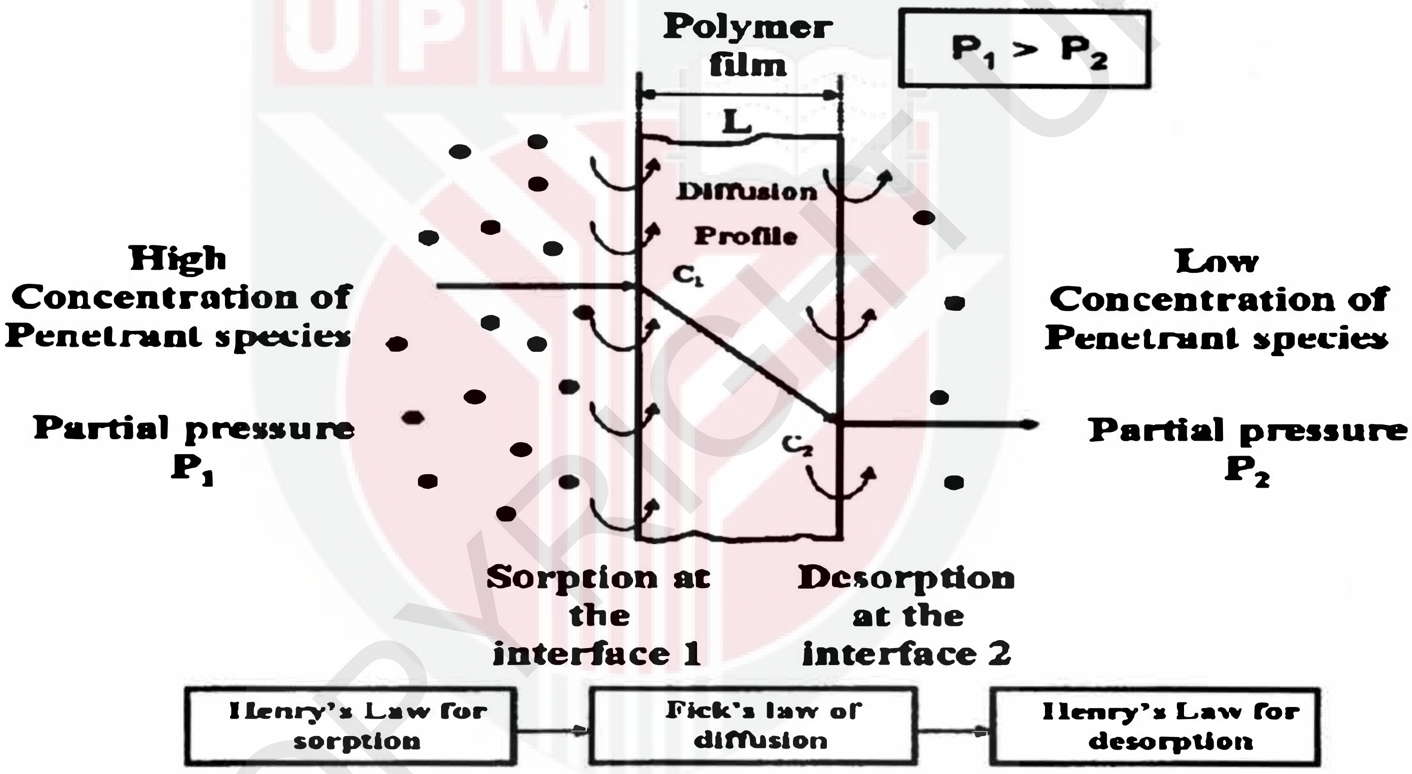


Figure 9. Permeation of penetrate species through polymer film thickness.

Source: Siracusa (2012).

2.6.5 Antimicrobial properties

Antimicrobial packaging is the packaging system that is capable of killing or inhibiting food contaminating spoilage and pathogenic microorganisms. When the packaging system acquires antimicrobial activity, the packaging system (or material) limits or prevents microbial growth by extending the lag period and decreasing the rate of growth or decreasing the number of live microorganisms (Han, 2000). Thymol, and its main natural source, thyme (Thymus vulgaris L.), are usually employed for their positive antioxidant, anti-inflammatory, local anaesthetic, antinociceptive, cicatrizing, antiseptic, antibacterial, and antifungal properties as well as for their beneficial effects on the cardiovascular system (Marchese et al., 2016)

One way to quantify the ability of antimicrobial agent to inhibit bacterial growth is by zone of inhibition analysis. It involves antimicrobial sample to be placed on the surface of agar plates seeded with the test bacterial strain. Diffusion of antimicrobial agent from these sources to the agar medium results in inhibition of bacterial growth near the source and the formation of distinct "zones" without bacterial lawn. The diameter of this zone increases with the increase in antimicrobial agent concentration (Bonev and Hooper, 2008). According to the study by (Petchwattana & Naknaen, 2015), poly(butylene succinate) (PBS)/thymol film effectively inhibit *Staphylococcus aureus* at 6 wt% while *Escherichia coli* did at 10 wt% thymol. Thymol was also found to inhibit the E. coli, S. aureus, Listeria, B. subtilis, and Salmonella.

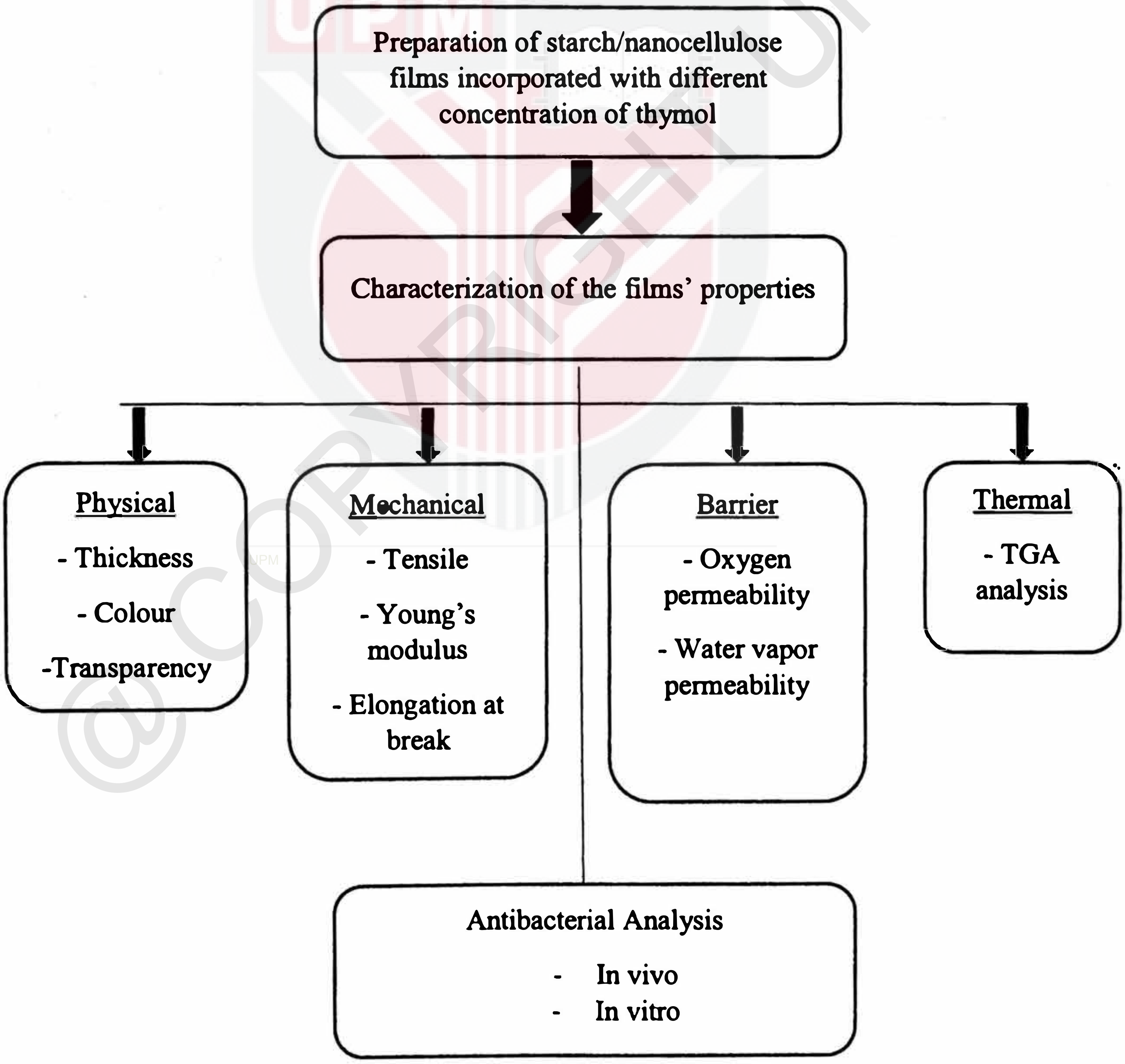
Besides that, appearance, colour, and texture of food products are crucial factors that consumers will consider prior to making a decision to buy fresh products and meat products. Most foods are highly perishable due to their biological and chemical compositions. This polymer-based film system can be applied to various types of food products such as meat, seafood, fruits, and vegetables, in order to maintain quality, enhance food safety, and prolong the shelf life of foods. In recent years, the applications of antimicrobial films on the real food system have been investigated.

For instance, Putsakum et al. (2018) developed 0.3% (w/v) of neem extract (*Azadirachta indica*)-contained gelatin films, applied on minced beef, and determined the quality of minced beef during storage 4 ± 1°C for 7 days. They found that minced beef wrapped with gelatine films containing Neem extract had lower in 2-ThioBarbituric Acid Reactive Substances (TBARS) value than the sample wrapped with polyvinyl chloride (PVC). According to study by Ramos et al. (2013), thymol has demonstrated potential to be used as active additive in PP films for food packaging applications by their controlled antimicrobial release to foodstuff and also by the possibility to protect food from degradation processes. They optimized thymol to increase the post-harvest shelf life of fresh strawberries and bread during storage.

CHAPTER 3

METHODOLOGY

This chapter discusses about the detail description of material used and the methods covered for the preparation to produce starch/nanocellulose films incorporated with thymol and characterization of the films. Apart from that, this chapter also covers the materials and methods used to investigate the antibacterial properties of the films. The overall approach for the methodology adopted in the present work is summarized below.



3.1 Materials

Materials used in this research were corn starch purchased from Thye Huat Chan Sdn Bhd (Brand Kapal ABC, Malaysia), nanocellulose purchased from Institut Perhutanan Tropika Dan Produk Hutan (INTROP) UPM, thymol crystal purchased from R&M Marketing (Essex, U.K), Tween 80 (polyoxyethylene-20-sorbitan monooleate) purchased from Sigma Aldrich,(St. Louis, MO, USA) and analytical standard glycerol purchased from R&M Marketing (Essex, U.K).

3.2 Preparation of neat starch films and starch/nanocellulose films

Neat starch films were prepared by dissolving 8g corn starch and 2g glycerol as plasticizer (25wt% of starch) in 180mL of distilled water. The suspension was heated to 85˚C and stirred constantly for 30 minutes using magnetic stirrer (FAVORIT, Indonesia) at 700 rpm stirring to obtain solubilization and gelatinization of the starch. The solution then underwent ultrasonication (QSonica, 500W, 20kHz) for 10 minutes at 50% amplitude to produce a homogenous solution. The 35ml solution was then casted in 140mm petri dish and dried in air-conditioned room at 21˚C for 48 hours.

Starch/nanocellulose films were prepared by dissolving 8g corn starch and 2g glycerol as plasticizer (25wt% of starch) in 180mL of distilled water. The suspension was heated to 85˚C and stirred constantly for 30 minutes using magnetic stirrer (FAVORIT, Indonesia) at 700 rpm stirring to obtain solubilization and gelatinization of the starch. Then, the film-forming solution was cooled down to 40°C. Meanwhile, thymol at different concentrations (0.1%, 0.3%, 0.5% of total volume) were prepared by dissolving 0.2g, 0.6g, 1g of thymol in 20ml distilled water with Tween 80 as surfactant (2.5wt% of thymol) and 6ml of nanocellulose (1.5wt% of starch). This concentration of nanocellulose was chosen because previous studies revealed that this concentration resulted to optimum properties of starch films. These solutions were stirred magnetic stirrer (FAVORIT, Indonesia) until soluble. Afterward, the suspension was cooled to 40°C and then mixed together with film forming solution by stirring for 5 minutes. The solution then underwent ultrasonication (QSonica, 500W, 20kHz) for 10 minutes at 50% amplitude to produce a homogenous solution. The 35ml solution was then casted in 140mm petri dish and dried in air-conditioned room at 21°C for 48 hours. The dried films were peeled off from the casting plate and conditioned in desiccator set at 25°C and 55% relative humidity for 48 hours before further characterization.

After drying, the film was peeled off from the petri dish and conditioned in the humidity cabinet (RH: 51%, Temp: 25°C) in sealed plastic bag (Xu et al., 2005). Figure 10 shows the transparent and smooth film produced after peeled off from the petri dish. The thickness of the film was measured using a digital micrometre (Mitutoyo, Japan) at five random positions around the film. The average values of the thickness were used to calculate TS, EAB, YM, WVP, and OP.

Figure 10. Starch/nanocellulose film incorporated with thymol produced by solvent casting method.

3.3 Characterization of Starch/Nanocellulose films

3.3.1 Physical properties

Physical properties of the films were determined in term of colour using Hunterlab Colorimeter (Hunterlab, Ultrascan Pro, USA). Colour properties of the films were analysed in term of L*, a*, b* values. A white tile was placed on the surface of white standard plate in order to calibrate the equipment. The equipment was calibrated using CIELAB colour scale for L*= 0 (black) to L* = 100 (white), -a* (greenness) to +a* (redness), and -b* (blueness) to +b* (yellowness). The colour measurement was performed by placing the film specimens over colorimeter and the colour parameters were read at least five randomly selected positions on each of the film. The total colour difference (ΔE*) of each film was determine using the following equation:

$$\Delta E = \sqrt{(L^{*} - L)^2 + (a^{*} - a)^2 + (b^{*} - b)^2} \qquad \text{(Equation 1)}$$

3.3.2 Mechanical properties

The film specimens were cut into rectangular strips (100mm × 15mm) after conditioning in 51% relative humidity and 25°Cfor 48 hours. Maximum tensile strength, maximum elongation at break and Young's Modulus were determined using Texture analyser (TA.XT2 Stable Micro Systems, UK) according to ASTM standard method D882 (ASTM, 2012). The initial grip separation and cross head speed were set to 65 mm and 8.30 mm/sec, respectively. A microcomputer was used to record the stress-strain curve with a minimum of three replicates of each film tested. Tensile strength (TS), Elongation at break (EAB), and Young's Modulus (YM) were calculated according to the following equations (Faridmehr et al., 2014):

$$TS = F/A \qquad \text{(Equation 2)}$$

where

F: force at specimen break (N)

A: cross-sectional area of the film (mm2)

$$EAB = [(l_f - l_o)/l_o] \times 100 \qquad \text{(Equation 3)}$$

where

l_f: final length at specimen break

l_o: initial length of the film

$$YM = \sigma/\varepsilon \qquad \text{(Equation 4)}$$

where

σ: tensile stress (MPa)

ε: tensile strain

3.3.3 Thermal properties

TGA test analysis was performed using a Mettler Toledo TGA Thermogravimetric Analyzer (Greifensee, Switzerland). Approximately 5 mg sample film was weighted in alumina pan and was heated from 20˚C to 500˚C at heating rate of 10˚C/min under inert nitrogen atmosphere (flow rate 60 mL/min). Data analysis was performed with STARe Thermal Analysis Software Version 12.10.

3.3.4 Barrier properties

3.3.4.1 Oxygen permeability

Oxygen transmission rate (OTR) was measured using an oxygen permeation system equipped with a calorimetric sensor (MOCON, USA), in accordance with ASTM F1927-07 (2007). The films (diameter: 50 cm^2) were pre-conditioned at 25˚C and 51% RH for three days. Pre-conditioned films were sealed tightly between two chambers of the oxygen permeation system. The oxygen in the chamber was flushed out using nitrogen gas until the percentage of oxygen in the chamber drop to 0%. Then, oxygen gas (12.5 kPa) was allowed to enter the chamber and transmitted through the film. The OTR value was recorded every 5 seconds until the line of OTR versus time curve became plateau. All the films were measured at least duplicate. The oxygen permeability coefficient (OP) were calculated using the following equation (Souza et al., 2012):

OP : OTR/L (Equation 7)

where

OTR : average value of OTR at plateau in OTR versus time curve (cm3/m2.d)

L : average of film thickness (mm)

3.3.4.2 Water vapor permeability

Determination of water vapor permeability (WVP) was carried out using modified dry cup method according to ASTM E96 (ASTM, 2005). The test cup was filled with 10 g of Calcium Chloride (desiccant) to produce a 0% relative humidity (RH) below the film. The mixture of paraffin wax and bee wax (8:2) was heated using magnetic stirring hotplate until the wax melted. The film was cut into circular shapes using the cutter (diameter: 7 cm) and the film was placed in between the cup and the ring cover of each cup and sealed using a bee wax solution. In order to maintain the surrounding humidity of the WVP cup, each cup was stored in a desiccator containing saturated $MgNO_3$ (magnesium nitrate) to provide a constant RH of 50% at 25°C. Digital temperature humidity meter (Proskit NT-312, Techno Tools & Equipment Sdn Bhd, Malaysia) was used to monitor the relative humidity and temperature inside the desiccator. Changes in the weight of the cup were recorded every 24 hours for 10 days and plotted to obtain the weight loss versus time graph. The water vapor transmission rate (WVTR) were calculated using the following equation (Abdollahi et al., 2012):

$$WVTR = (W/t)/A \qquad \text{(Equation 8)}$$

where

W/t: slope of weight changes versus time graph (g/h)

A: transmission area of the film (28 cm2).

The water vapor permeability (WVP) were calculated using the following equation (Abdollahi et al., 2012)

$$WVP = (WVTR \times L) / (P1-P2) \qquad \text{(Equation 9)}$$

where

L: average of film thickness (m)

P1: water vapor partial pressure in desiccator at RH=51%, 21.64 x 105 Pa

P2: water vapor partial pressure in the cup at RH=0%, 0

3.3.5 Application of starch/nanocellulose films incorporated with thymol as antimicrobial film

3.3.5.1 In-vitro

Disc diffusion assay (Pelissari et al., 2009) was done to investigate the effect of varying thymol concentrations in the films to inhibit the gram-positive bacteria *(S.Aureus)* and gram-negative bacteria. *(E-Coli).* The bacteria were cultivated in the nutrient agar and incubated in the incubator (Stuart, Cole Parmer, UK) at 37°C. After 24 hours, the cultured bacteria were diluted into peptone water until the concentration of bacteria were 10^8 CFU/mL. Then, 0.1 mL of bacteria in the peptone water was swabbed onto the petri dish containing Mueller Hinton agar.

The films were cut aseptically into 1.5 cm discs and sterilized under UV light before conducting the antibacterial analysis. All discs were placed carefully into the petri dish that contained bacteria and incubated at 37°C for 24 hours in the incubator. After 24 hours, the inhibition area of the film discs was examined to determine the ability of the film to inhibit bacterial growth. The analysis was carried out in triplicate for each sample.

3.3.5.2 In-vivo

To assess the applicability of the active starch/nanocellulose composites film on a real food sample, analysis were performed using fresh chicken meat where the composite film was placed in direct contact with the food sample. In addition, since thymol is a volatile substance, its release into the sample test was expected to provide antimicrobial activity. The coverage of bacteria on the surface of chicken samples was determined via visual observation.

Method used for this analysis was taken from previous studies by Putsakum et al. (2018) about application of gelatine film-neem extract on minced beef but with modification. In this work, the chicken meat was use instead of beef meat because chicken meat is easier to buy and cheaper. The chicken meat was first sprayed with 75% ethanol to kill any bacteria. Chicken meat and film were cut aseptically into 3cm×3cm and 5cm×5 cm respectively. Each portion of the sample was placed in the centre of the film sheet in direct contact with the film surface. The film was then folded and wrapped around the meat tightly without any sealing substances. The wrapped samples were then kept in a petri dish and sealed with parafilm. The airtight containers were used to prevent the loss of moisture and any uptake of oxygen, which could affect the weight and colour of the meat sample during storage. The petri dish was placed at room temperature for 5 days. The chicken meat was observed in terms of colour, edibility, and microbial growth.

CHAPTER 4

RESULTS AND DISCUSSION

This chapter presents the results and discussion on the findings of the present work, including the analysis and interpretation of data. Section 4.1 presents the results and discussion on the characterization of starch/nanocellulose films incorporated with different concentrations of thymol, including physical, mechanical, thermal, and barrier properties. Meanwhile, Section 4.2 presents the results and discussion on the antibacterial properties of the starch/nanocellulose films with and without addition of thymol.

4.1 Characterization of starch/nanocellulose films

4.1.1 Physical properties

4.1.1.1 Thickness and physical appearance of the films

Figure 11 shows the thickness values of neat starch and starch/nanocellulose films incorporated with different concentrations of thymol. During experiment, the volume for all the film-forming solutions were standardized to 35ml before the films underwent drying. It was found that thickness increased from 0.083mm to 0.096mm with the increase in concentration of thymol. This finding was consistent to the work of Benavides et al. (2012) whom found that the addition of oregano essential oil in alginate films increased the thickness of the alginate films (0.031–0.038 mm). According to Lozano-Navarro et al., (2017) addition of natural extract into chitosan films increased the thickness of the chitosan films due to the presence of compounds in the extract such as polysaccharides, carboxylic acids, antioxidants, etc., which created a more complex matrix. Thus, in this work the presence of compounds in thymol may be the reason for the increment in thickness of the starch films.

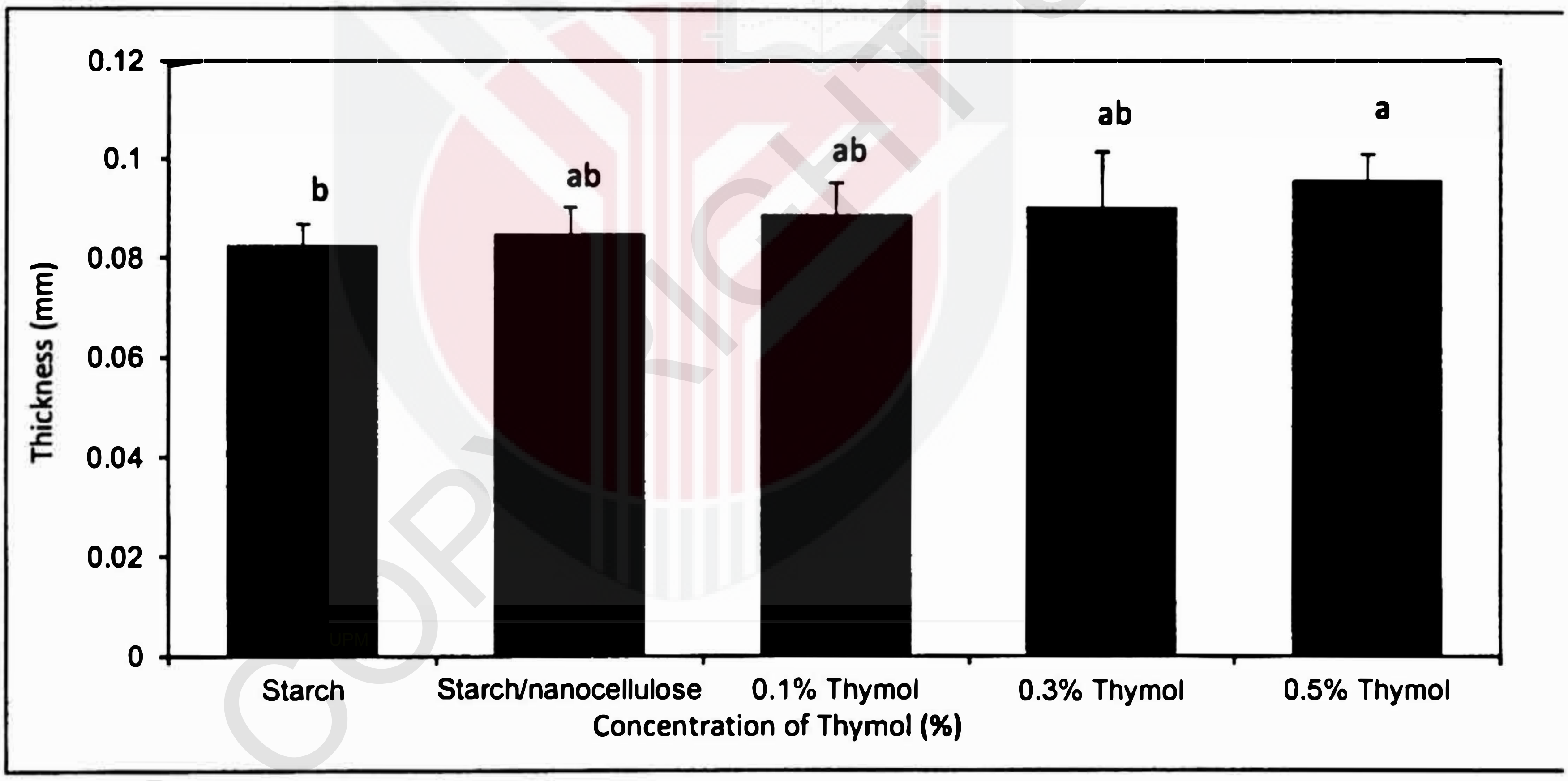
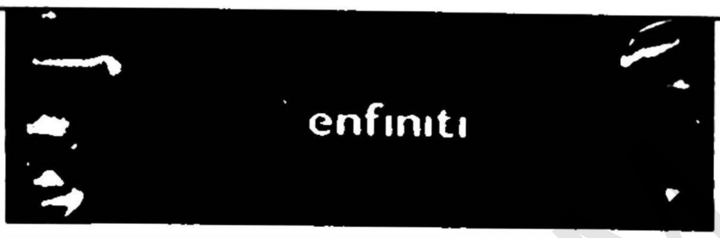


Figure 11: Thickness of starch and starch/nanocellulose films incorporated with different concentrations of thymol. Different Letters in the Same Graph Indicate a Statistically Significant Difference (P<0.05).

Table 3 shows the physical appearance of the films. All the were easily peeled off from the plate. It can be seen that the films had a homogeneous appearance except for starch/nanocellulose film incorporated with 0.5% w/v concentration of thymol. High concentration of thymol affected the physical appearance of the films due to the white colour nature of the thymol.

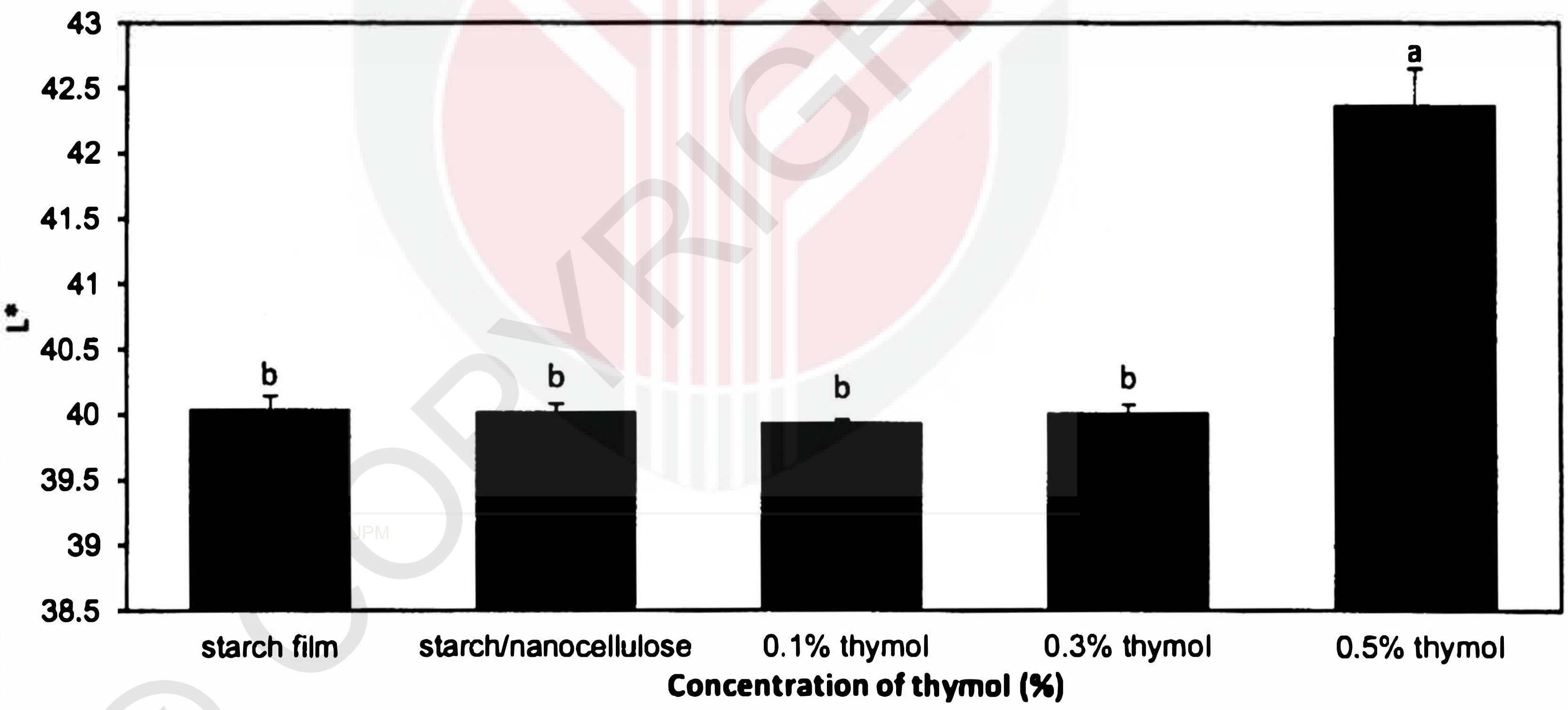
Table 3: Physical appearance of the films incorporated with different concentration of thymol.

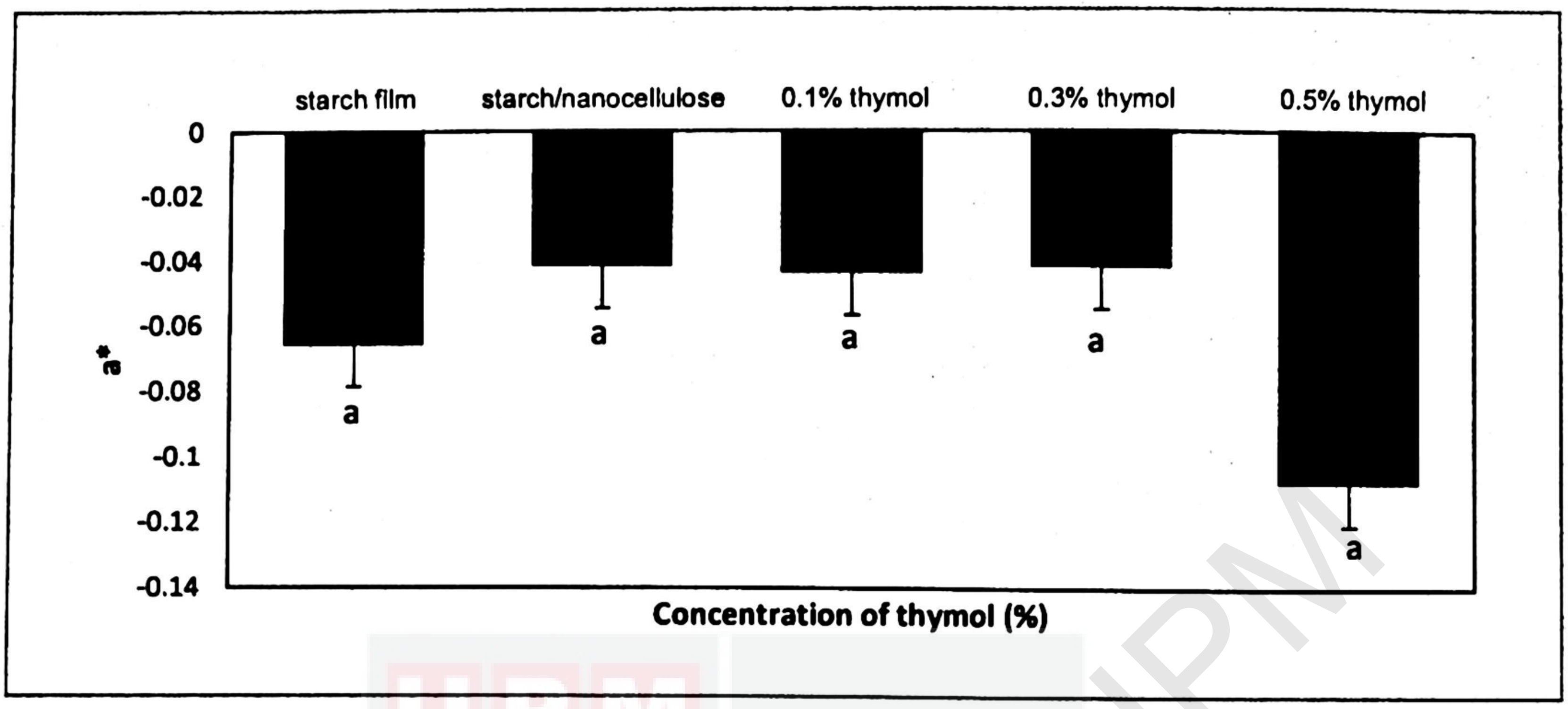
Starch/nanocellulose film	
Starch/nanocellulose with 0.1% thymol.	
Starch/nanocellulose with 0.3% thymol	
Starch/Nanocellulose with 0.5% thymol	

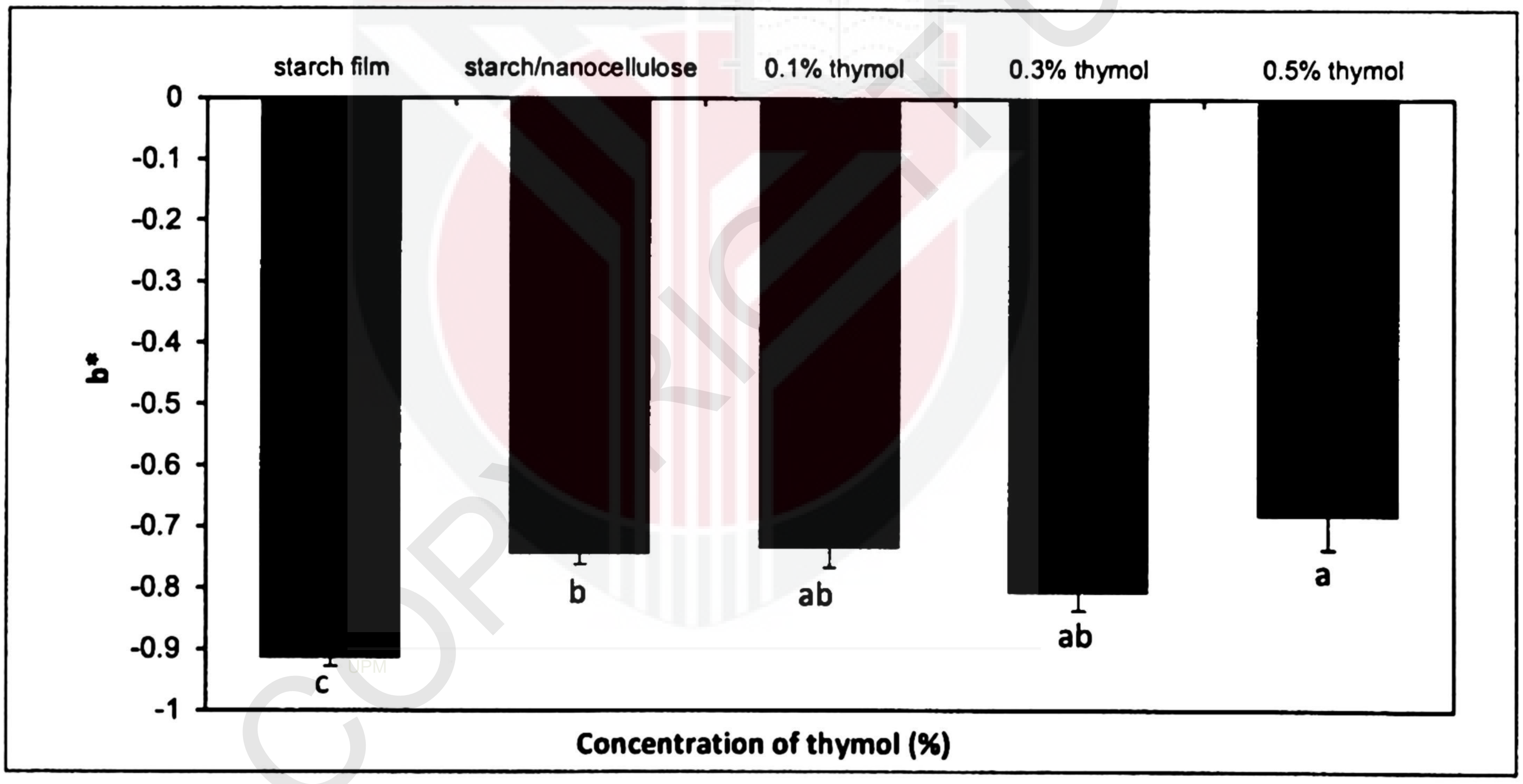
4.1.1.2 Colour properties

Food product appearance through packaging is an important factor that can attract customer acceptance towards buying the food product (Hosseini et al., 2013). Therefore, the physical property of food packaging material is one of the important aesthetical factors that needs to be determined. Colour parameters that include L*, a*, b* values and ΔE of starch/nanocellulose films incorporated with different concentrations of thymol (0.1%, 0.3%, and 0.5%) were determined and tabulated in Figure 12. It was found that starch/nanocellulose films incorporated with 0.5% thymol exhibited significant increase

($p<0.05$) of L*, b* and ΔE values but not significant ($p>0.05$) of a* values than other films. Incorporation of thymol especially at higher concentrations increased the whiteness of the films hence the increase in L* and ΔE value. However, there was no significant different among the L* and a* values of neat starch and starch/nanocellulose films incorporated with 0%, 0.1%, and 0.3% of thymol probably due to the no addition of thymol and very low concentration of thymol. These findings seem to be consistent to the previous study which demonstrated that the effects of essential oil on the colour of gelatine films were dependent on the type and concentrations of essential oil incorporated into the films (Ahmad et al, 2012; Tongnu et al, 2012).




starch film
starch/nanocellulose
0.1% thymol
0.3% thymol
0.5% thymol
0
-0.02
-0.04
-0.06
-0.08
-0.1
-0.12
-0.14
a*
a
a
a
a
a
Concentration of thymol (%)


starch film
starch/nanocellulose
0.1% thymol
0.3% thymol
0.5% thymol
0
-0.1
-0.2
-0.3
-0.4
-0.5
-0.6
-0.7
-0.8
-0.9
-1
b*
c
b
ab
ab
a
Concentration of thymol (%)

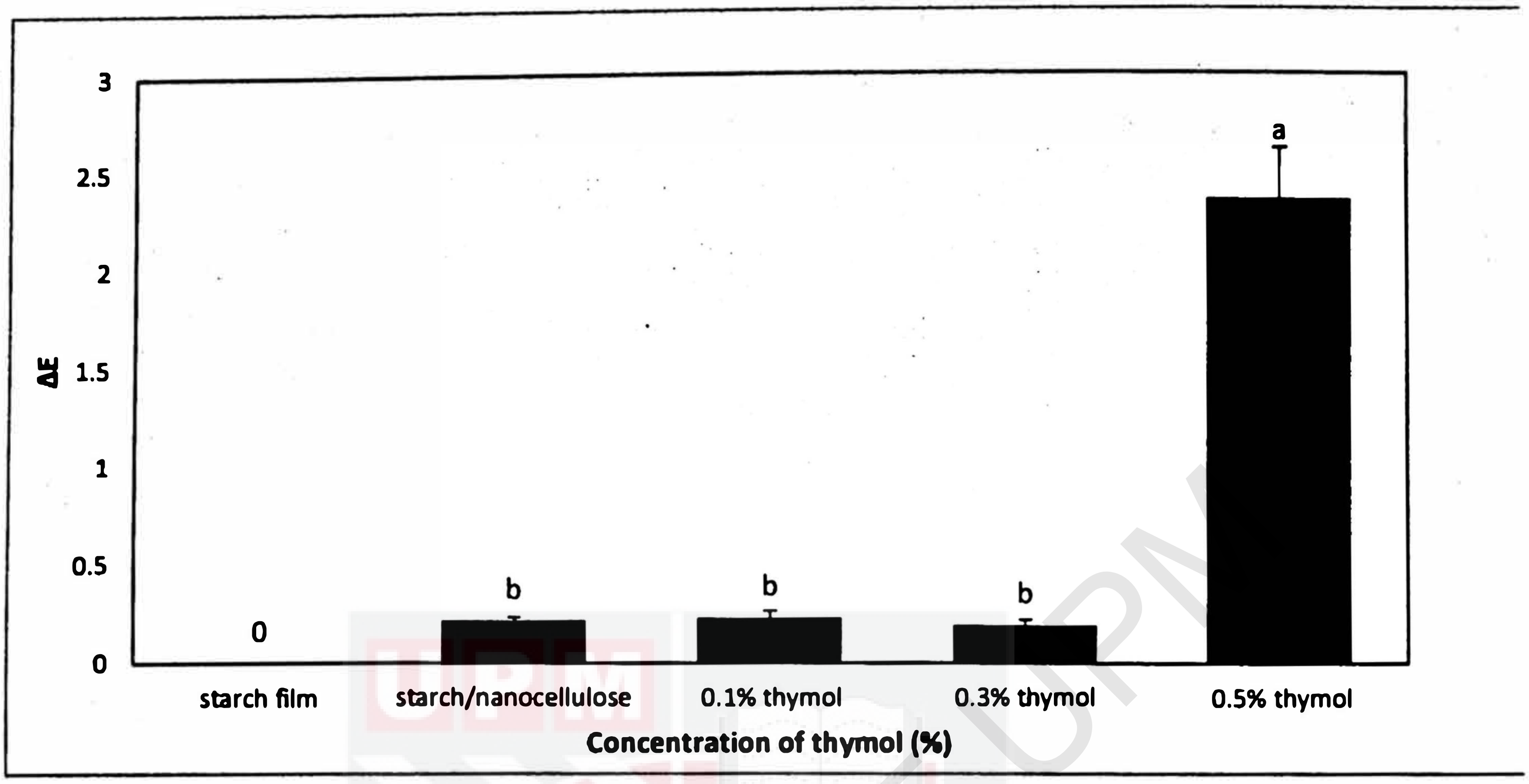


Figure 12. Colour parameters of L* a* b* and ΔE values of starch and starch/nanocellulose films incorporated with different concentrations of thymol. Different Letters Indicate a Significant Difference at ($P<0.05$).

4.1.2 Mechanical properties

The effects of thymol addition on mechanical properties of the films were determined by the evaluation of different parameters such as tensile strength, elongation at break, and elastic modulus. Mechanical properties indicate the strength, flexibility, and the ability of the films in order to ensure food integrity (Nouraddini et al., 2018).

4.1.2.1 Tensile strength (TS)

Figure 13 shows the effects of adding different concentrations of thymol on the TS of the starch/nanocellulose films. Addition of nanocellulose to neat starch films was found to increase the TS of the starch film due to the nanosized of cellulose that allowed effective contact area with the starch matrix and hence promoted strong intermolecular forces. The forces resulted to the rigidity of the film and therefore high TS. The results show significant decrease ($p<0.05$) of TS with the increase in thymol concentration. Starch/nanocellulose film incorporated with 0.5% of thymol (3.68 MPa) exhibited the lowest TS than 0.3% of thymol (4.71 MPa) and 0.1% of thymol (5.93 MPa). This can be attributed to the complex interaction form between the lipids from thymol and starch polymers which reduced the cohesion of the starch network forces and subsequently lowering the film strength. Consistent result was reported by Mastromatteo et al. (2009) whereby they found that addition of thymol in gelatine films increased the average pore size of the films, resulting to a decrease in TS. The increase in the flexibility and decrease in the rigidity of the films can be also accounted by the replacement of the original bonds among polymer chains with the new bonds among starch chains and thymol micelles. Through this, greater segmental mobility and sliding effects of starch chains increases the flexibility of the films (Davoodi, Kavoosi, & Shakeri, 2017).

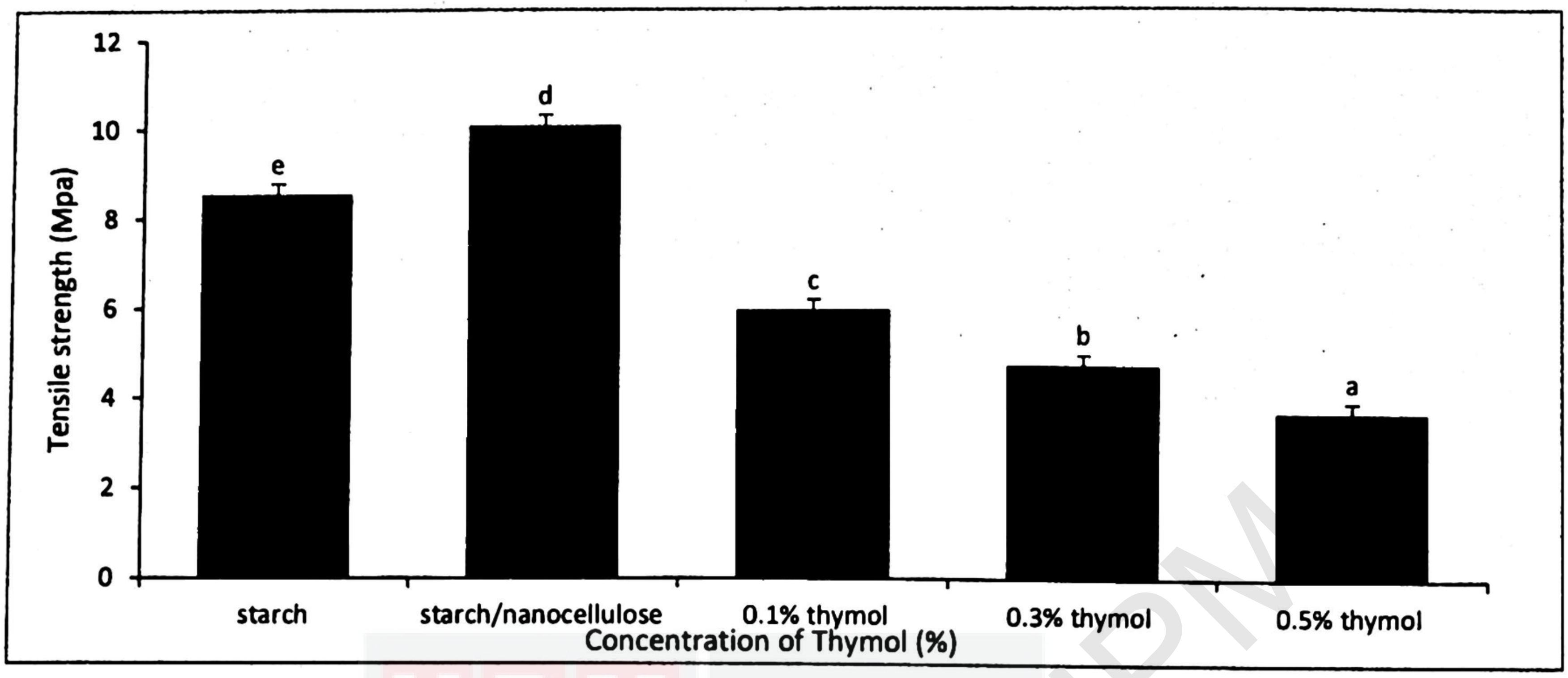


Figure 13. Tensile strength of starch and starch/nanocellulose films incorporated with different concentrations of thymol. Different Letters Indicate a Significant Difference at ($P<0.05$).

4.1.2.2 Elongation at break (EAB)

Figure 14 shows that incorporation of thymol in starch/nanocellulose films can also affected the EAB values of the films. It can be seen from the figure that the trend of the EAB values are reciprocal to the TS values, as expected. These results can be explained by the chemical and structural compatibility between the starch and cellulose chains (Averous and Boquillon, 2004). The glycerol partition between the starch matrix and fibers is also reported as a phenomenon that influences the elasticity modulus of the films due to the unplasticized starch matrix (Kunanopparat et al. 2008). Film with addition of 0.5% of thymol exhibited the maximum EAB value (113.53%) while film with addition of nanocellulose alone exhibited the minimum EAB value (95.22%). Elongation at break (EAB) of the film expresses the film's ability to stretch. The EAB increased with the increase in thymol concentration due to the plasticizing effect of the thymol resulting to the increase in ductile properties of the films. This also resulted to the changes in the crystallinity of the starch polymer. According to Kowalczyk et al., (2012), appropriate amount of thymol in the composites led to obvious improvements in the mechanical properties of the composites, but additional increase in the amount of thymol may not improve the mechanical properties any more. Limpisophon et al., (2010) had investigated properties of the gelatin films incorporated with citrus oil that mainly contains limonene and they found that films incorporated with essential oil exhibited lower tensile strength but higher elongation at break than the control films without incorporation of essential oil. Tongnuanchan et al., (2012) also reported that gelatine films have good mechanical properties but the properties changed slightly with the presence of thymol. Maizura et al. (2018) reported a decrease in tensile strength and an increase in the flexibility of sago starch-alginate composite film with the presence of lemongrass oil and glycerol. Pelissari et al. (2014) also found a decrease in tensile strength and an increase in the percent elongation of cassava starch-chitosan composite file enriched with oregano oil. The effects of essential oil on the mechanical behaviour of the films are dependent on the type and amount of essential oil. (Ortega-Toro et al., 2014).

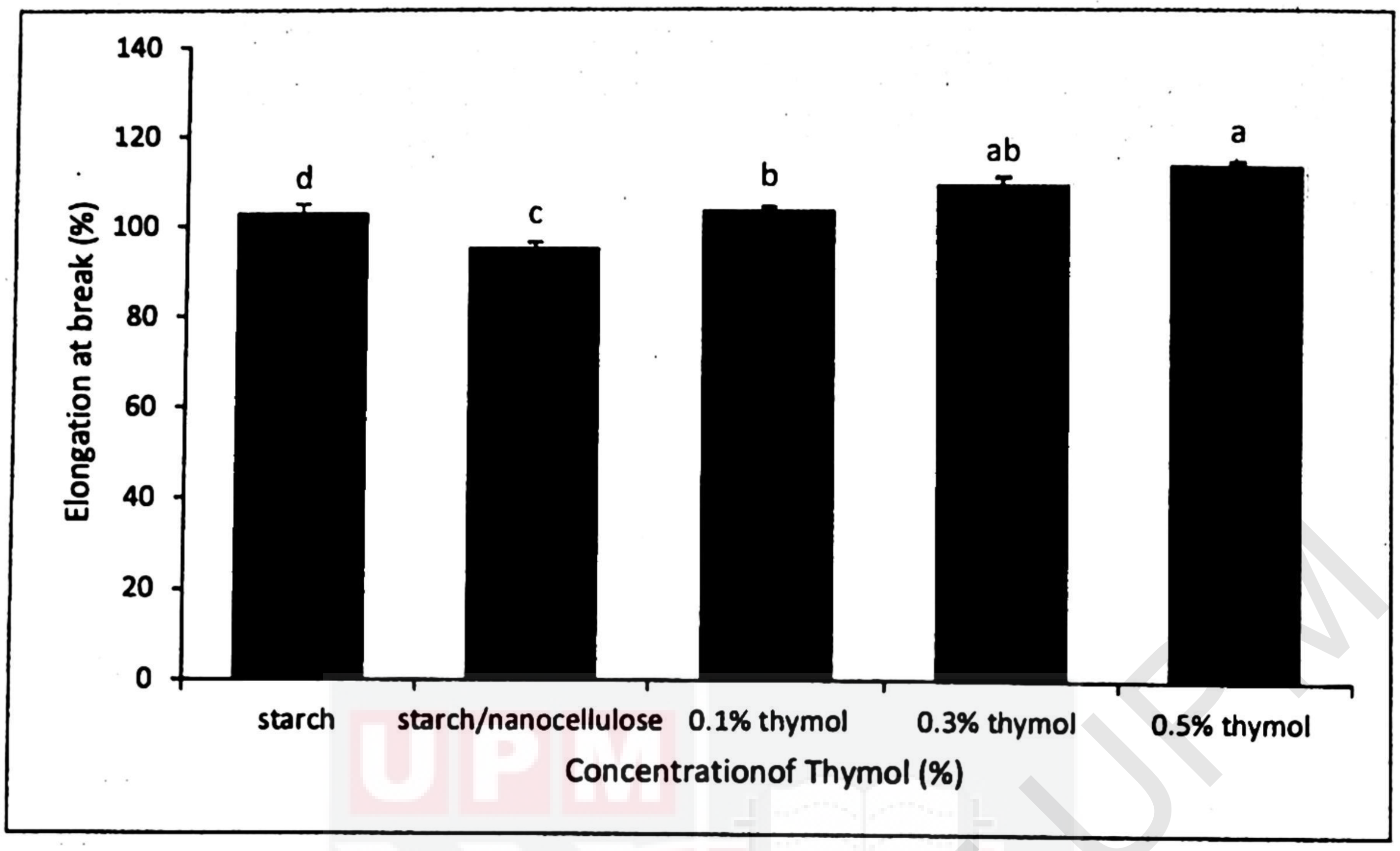


Figure 14: EAB value of starch and starch/nanocellulose films incorporated with different concentrations of thymol. Different Letters Indicate a Significant Difference at (P<0.05).

4.1.2.3 Young's modulus (YM)

Figure 15 shows the Young's modulus for neat starch and starch/nanocellulose films incorporated with different concentrations of thymol. It seems that addition nanocellulose and thymol decreased the Young's modulus of the films. The decrement became prominent with the increase in the thymol concentration. The film incorporated with 0.5% thymol exhibited the lowest YM value (26.86MPa) compared to 0.3% (53.33MPa) and 0.1% (69.93MPa) of thymol. The decreasing trend of YM result was consistent to the findings reported by Petchwattana and Naknaen (2015) whereby the addition of thymol to PBS reduced both the tensile modulus and tensile strength of the films by around 10% to 40% depending on thymol content. Addition of thymol made the films to become less stiff due to the plasticization effect derived from thymol. This allowed the films to be stretched or deformed easier than the unmodified film. As reported by Wu et al. (2014), thymol acted as plasticizer, reducing the intermolecular forces of polymer chains, thus improving the flexibility and extensibility of the films.

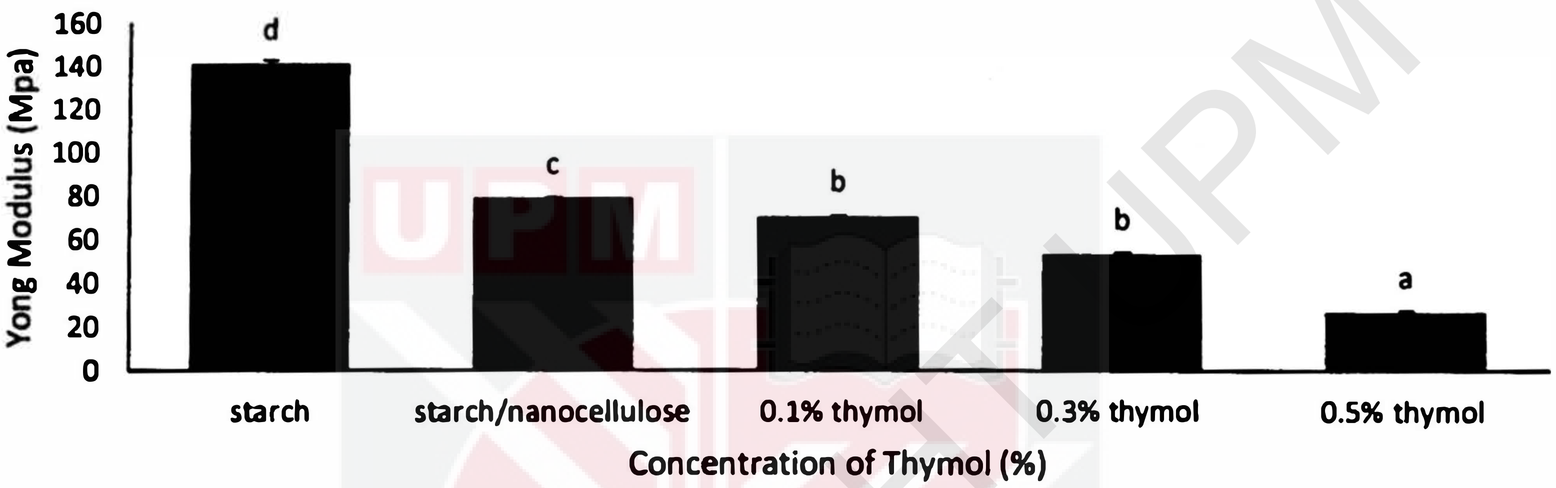


Figure 15. YM values of starch film and starch/nanocellulose films incorporated with different concentrations of thymol. Different Letters Indicate a Significant Difference at ($P<0.05$).

4.1.3 Thermal properties

Thermogravimetric analysis (TGA) was performed on the films incorporated without and with different concentrations of thymol in order to investigate the thermal stability and degradation temperature of the films. The thermogravimetric (TG) curves of the films are shown in Figure 16. TGA records the weight loss of all the samples over a range of temperature from 20-500°C.

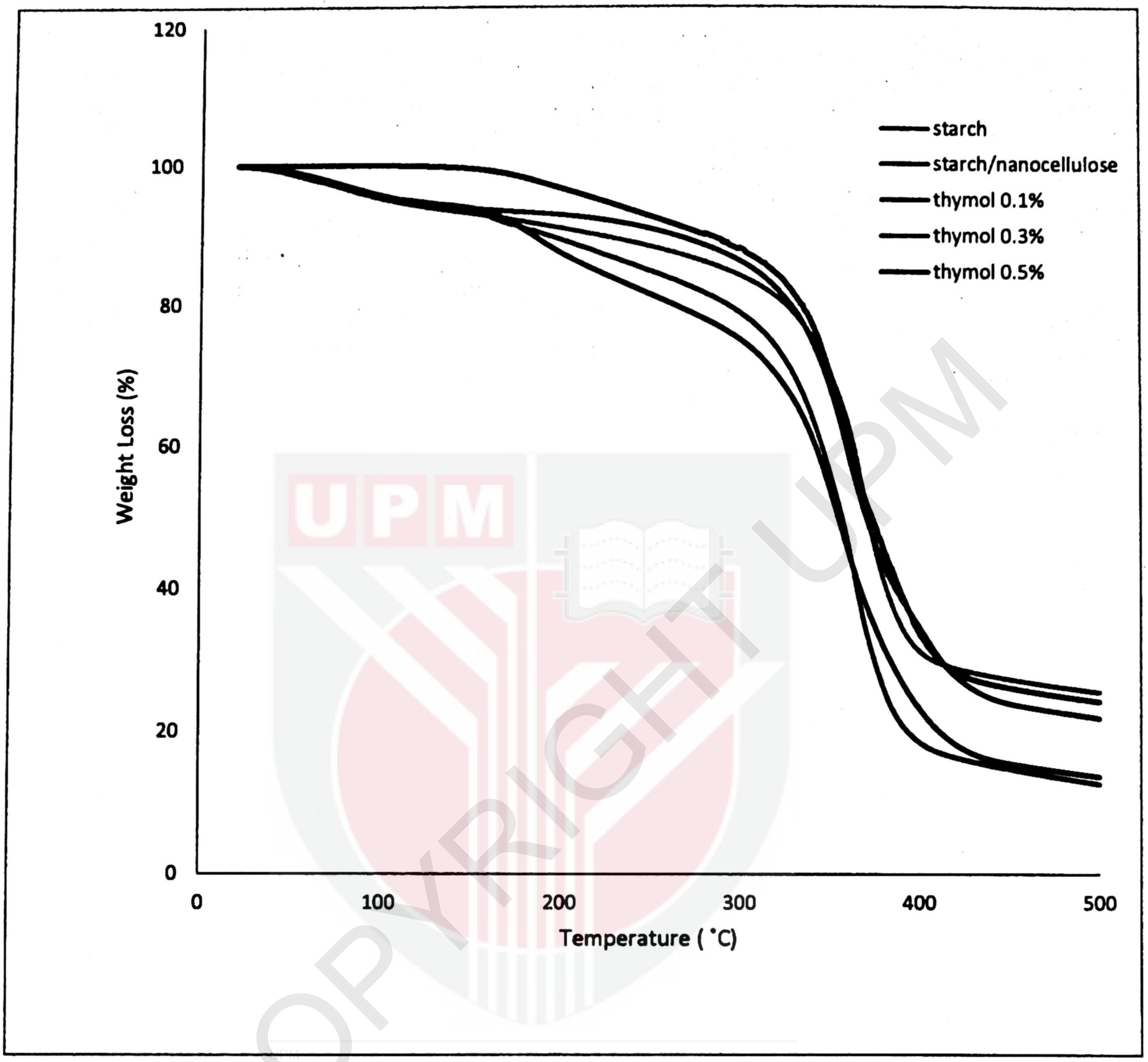


Figure 16. TGA curves of starch films and starch/nanocellulose incorporated with different concentrations of thymol.

Figure 16 shows the two stages of weight loss of all the films. The initial degradation temperature, T_{onset}(˚C) for first weight loss of all the films occurred around 143°C-174°C. This was due to the loss of moisture in the film which was easily evaporated at boiling point of water. The maximum rate temperature T_{max}(˚C) for second stage of weight loss was in the range of 320 to 325 °C which indicated degradation of starch due to the depolymerization of film compositions particularly starch, nanocellulose, and glycerol. Liu et al. (2016) also found that the dominant degradation temperature of neat corn starch and corn starch films incorporated with starch nanoparticles was in the range of 250 to 350 °C which seems to be consistent to the findings in this work. It was found that T_{max} increased from 318°C ,327°C, 337°C with the increase in the concentration of thymol content of 0.1%, 0.3% and 0.5% respectively attributed to a rearrangement in polymer structure after addition of higher concentration of thymol. The structure form more homogenous structure which increases the thermal stability. This result is consistent with study by Nooshin et al. (2017). They found that addition of GEO (ginger essential oils) to chitosan-carboxymethyl cellulose films resulted to the following: the lowest amount of GEO led to a lower thermal stability, but the addition of highest amounts of GEO (GEO 2% and GEO 3%) led to a higher thermal stability. According to Ramos et al. (2012), addition of carvacrol and thymol into starch polymer matrix did not significantly affected thermal degradation profile of the polymer in inert nitrogen atmosphere. It is expected that a certain amount of carvacrol and thymol would be lost during processing, that was when the materials were submitted to temperatures above the volatilization point of these additives. However, addition of more thymol resulted to the structure to become more homogeneous structure, which increased the thermal stability. This is in accordance with the increasing of crystallinity indexes when the concentration of essential oil increases.

4.1.4 Barrier properties

4.1.4.1 Oxygen permeability

Figure 17 shows the oxygen permeability (OP) of the films incorporated with different concentrations of thymol. Oxygen barrier properties of the films was investigated by determination of oxygen transmission rate per film thickness. The OP value of the pure starch film was found to be 29.31 × 10 /.m.day.Pa decreased to 21.03×$10^3 cm^3$/.m.day.Pa with the addition of nanocellulose indicating that addition of nanocellulose producing film with a relatively good oxygen barrier. The oxygen molecules penetrated much more slowly within film containing nanocellulose due to the higher fibril entanglements within the film which increased the tortuosity in the film (Belbekhouche et al., 2014). The improvement of oxygen barrier properties by addition of nanocellulose was attributed to the dense network formed by nanofibrils with small dimensions. This is because inorganic nanoparticles are impermeable to gases, at least compared to polymers and organic macromolecules, and their presence constitutes a solid barrier in the path of the gas molecules throughout the polymer bulk phase. Thus, gases are forced to follow a more tortuous path as they diffuse through the material. The more tortuous the path, the longer it takes for the gas to pass through the material resulting macroscopically in a reduced permeability. (Bikiaris & Triantafyllidis, 2013).

However addition of 0.1% and 0.3% thymol resulted to an increase in OP values to 90.92×$10^3 cm^3$/.m.day.Pa and 136.11×$10^3 cm^3$/.m.day.Pa. The increase in oxygen transmission for the films could be due to the modification of the polymer matrix structure in the presence of the additives, consequently reducing the resistance of films to oxygen diffusion through them (Sothornvit and Krochta, 2000). This may be due to swelling of the starch polymer matrix in the presence of volatile oils at these higher concentrations and increased pore to the films (Ahvenainen, 2003). This result was similar to (Cran et al., 2013). They found that the starch films containing essential oil, Zataria multiflora Boiss (ZEO) or Mentha pulegium (MEO) showed no significant differences ($P > 0.05$) in OP value at concentrations of 1% and 2% (v/v).These lower levels of essential oil concentration might not have been sufficient concentration to induce structural changes in the starch structure that would otherwise increase the molecular mobility of oxygen within the film structure. However, the addition of 3% (v/v) of either essential oil to the films significantly increased the OP values ($P<0.05$). This may be due to swelling of the starch polymer matrix in the presence of volatile oils at these higher concentrations (Ahvenainen, 2003).

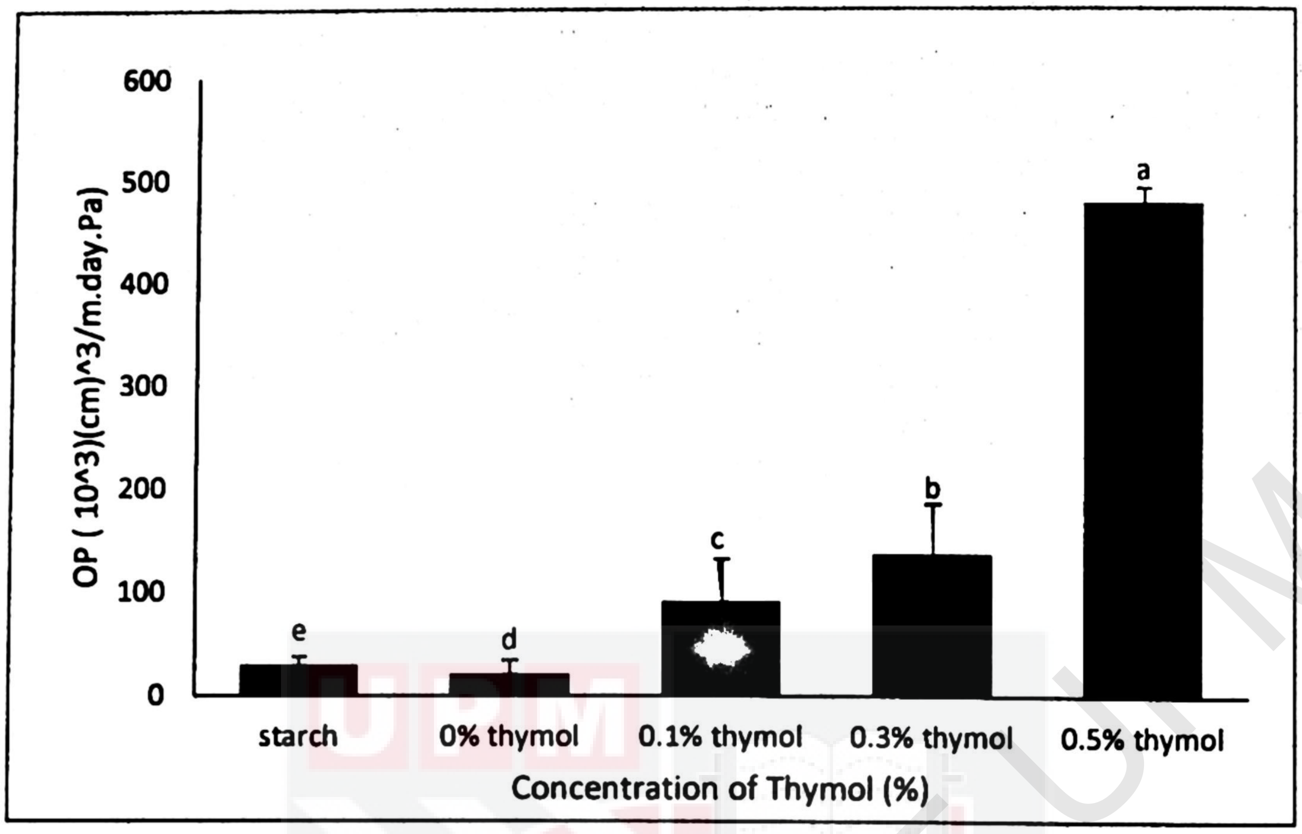


Figure 17. Oxygen permeability of starch film and starch/nanocellulose films incorporated with different concentrations of thymol. Different Letters Indicate a Significant Difference at ($P<0.05$).

4.1.4.2 Water vapour permeability (WVP)

Figure 18 shows the WVP for the films incorporated with different concentrations of thymol. Incorporation of thymol especially at higher concentration caused a significant increase in WVP of the film. Starch/nanocellulose film without thymol exhibited WVP value of 4.78E-08 g/Pa.h.m. The value decreased to 3.37E-08 g/Pa.m.h with the addition of 0.1% thymol. The presence of numerous -OH group make nanocellulose suitable nanomaterial to be hydrogen-bonded to the polymer producing high performance nanocomposite. However, addition of higher concentration of thymol (0.3% and 0.5%) increased the WVP to 5.00E-8 g/Pa.h.m and 1.01E-7 g/Pa.h.m respectively.

The water vapor transfer process in the films are dependent on the hydrophilic–hydrophobic ratio of the film constituents and the degree of cross-linking. Thymol at higher concentration may hinder polymer chain-to-chain interactions and reduced cross-linking. Consequently, WVP increased. According the work of Zivanovic et al. (2005), WVP of chitosan film enriched with oregano oil (mainly contains carvacrol and thymol) decreased due to hydrophobicity nature of the oil. The WVP of PLA films incorporated with 9 wt.% essential oil was significantly ($p<0.05$) higher than that of pure PLA film. Apart from that, the addition of Zataria multiflora essential oil to gelatin films also increased water vapor permeability of the films (Atarés,2015). Pranoto et al. (2005), also reported that the addition of garlic essential oil to alginate films increased the WVP value. The differences in WVP values are dependent on the transfer process that is relevant to the hydrophilicity/hydrophobicity index of compounds in the films.

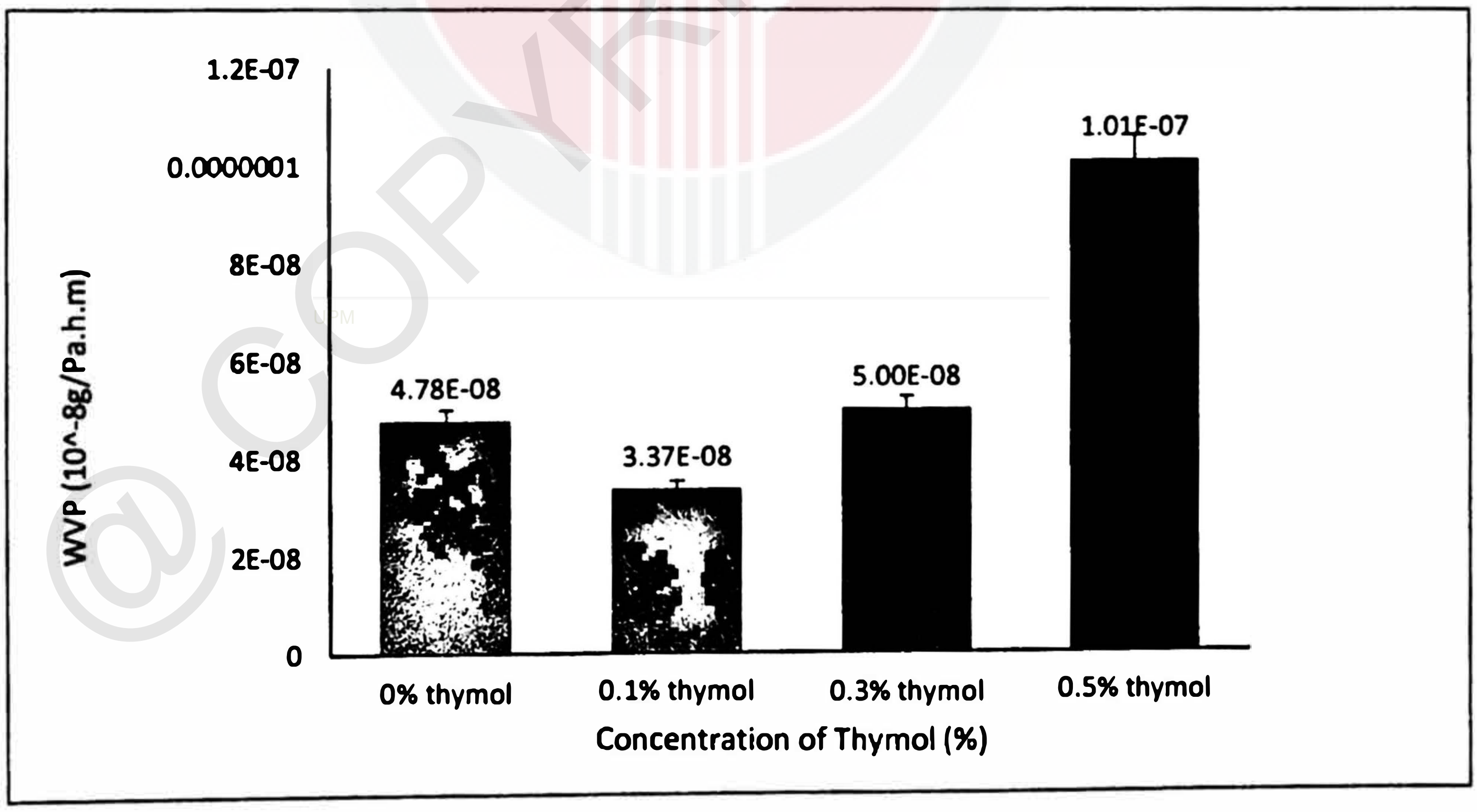


Figure 18. WVP values of starch/nanocellulose films incorporated with different concentrations of thymol.

4.2 Antibacterial properties

4.2.1 In-vitro

The antibacterial properties of the films incorporated with various concentrations of thymol (0.1, 0.3, 0.5% w/v) against 2 different types of bacteria *S. aureus* (gram-positive bacteria) and *E. coli* (gram-negative) was investigated using disc diffusion method. Growth inhibition zones around the films (inhibition halos) were visually examined as shown in Figure 19.

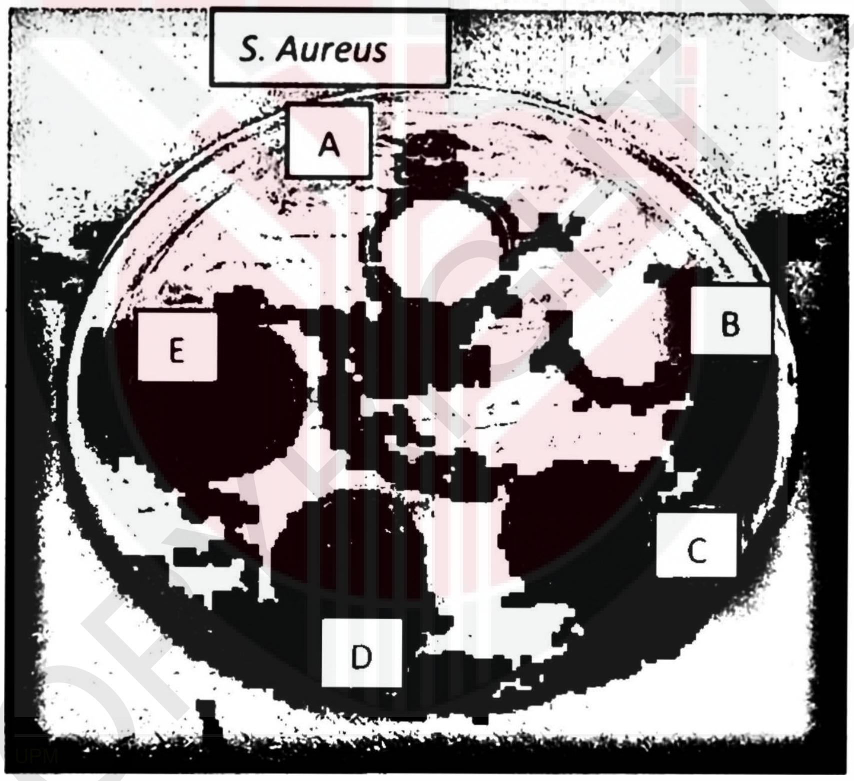


Figure 19. Inhibition zone of (A) starch (B) starch/nanocellulose (C) starch/nanocellulose incorporated with 0.1% thymol, (D) starch/nanocellulose incorporated with 0.3% thymol, and (E) starch/nanocellulose incorporated with 0.5% thymol films against gram-positive bacteria *S. Aureus*.

Figure 19 shows that the neat starch (A) and starch/nanocellulose films (B) without thymol show no inhibition zone for *S. Aureus* (gram-positive bacteria), which indicated that the bacteria colonies were able to grow under the films. This was due to the absence of thymol as antibacterial agent, thus no antibacterial activity against bacteria was observed. However, as expected, addition of thymol which released from starch/nanocellulose films into the agar resulted to the inhibition zone around the films. This can be seen for all sample films added with different concentrations of thymol (0.1%,0.3%, and 0.5%). The inhibition zone became significant with the increase in thymol concentration (0.3% and 0.5%). This demonstrated that increase in thymol concentration contributed to the improvement in efficiency of the antibacterial activity.The inhibition of gram-positive bacteria was attributed to the formation of an impermeable layer on the surface of the bacteria cell by thymol, thus prevent the nutrients to diffuse through the membrane of the bacteria cell (Kong et al., 2010). Bacteria cell inhibited the bacterial growth when the bacteria received lack of nutrient.

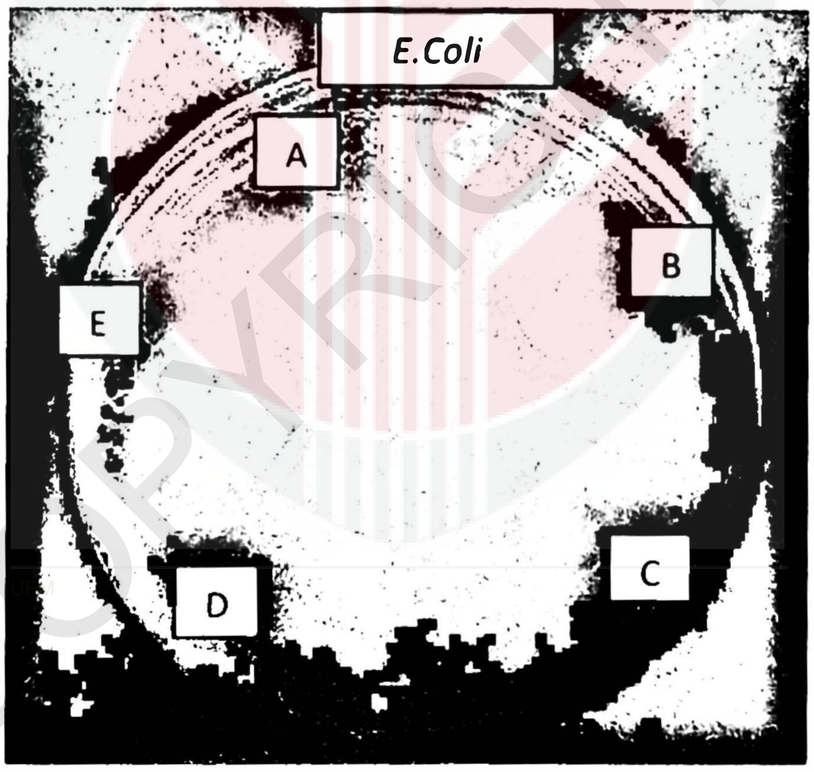


Figure 20. Inhibition zone (A) starch (B) starch/nanocellulose (C) starch/nanocellulose incorporated with 0.1% thymol, (D) starch/nanocellulose incorporated with 0.3% thymol, and (E) starch/nanocellulose incorporated with 0.5% thymol films against Gram-negative bacteria *E. Coli*.

It can be observed from Figure 20 that there were no inhibition zone of E. coli (gram-negative bacteria) for neat starch film and for starch/nanocellulose films incorporated with different concentrations of thymol. This result indicates that thymol was not efficient to inhibit gram-negative bacteria compared to gram-positive bacteria. This was expected because of the outer membrane of the gram-negative bacteria which are stronger than gram-positive bacteris. To overcome this feature, larger amounts of antimicrobial agent is required to inhibit the gram-negative bacteria such as E. coli.. This result was consistent to the finding reported by Petchwattana and Naknaen (2015). The antimicrobial activity of the gelatin film against Staphylococcus aureus was evidence at 6 wt% thymol concentration while E. coli at 10 wt% thymol concentration. According to Kavoosi et al. (2013), the antibacterial activity of the gelatin films containing thymol was the greatest against Staphylococcus aureus followed by Bacillus subtilis followed by Escherichia coli and then by Pseudomonas aeruginosa. Most of the antimicrobial films are more effective against Gram-positive bacteria than Gram-negative bacteria. This is due to the cell wall lipopolysaccharides or the protection of outer membranes of Gram-negative bacteria which could inhibit the diffusion of antimicrobial agent into the cell, thus decreasing the microbial growth inhibition (Han,2017)

4.2.2 In-vivo

Figure 21 shows the images of chicken meat that was in direct contact to the films on day 1 and day 5. To investigate the function of thymol as antimicrobial agent in extending the shelf life of food, the chicken meat was stored at room temperature of 25°C and 55% relative humidity for 48 hours to accelerate the microbial growth. It can be seen that on day 1, all chicken meat was pinkish in colour and looked fresh. On day 5, it can be seen that many maggots came out from chicken meat which was in direct contact with starch and starch/nanocellulose films indicating bacteria growth.. However, there were no maggot coming out from chicken meat that was in direct contact with starch/nanocellulose films incorporated with thymol. This finding proved the antibacterial properties of the films and that starch/nanocellulose films incorporated with thymol has the potential to extend shelf life of food product.

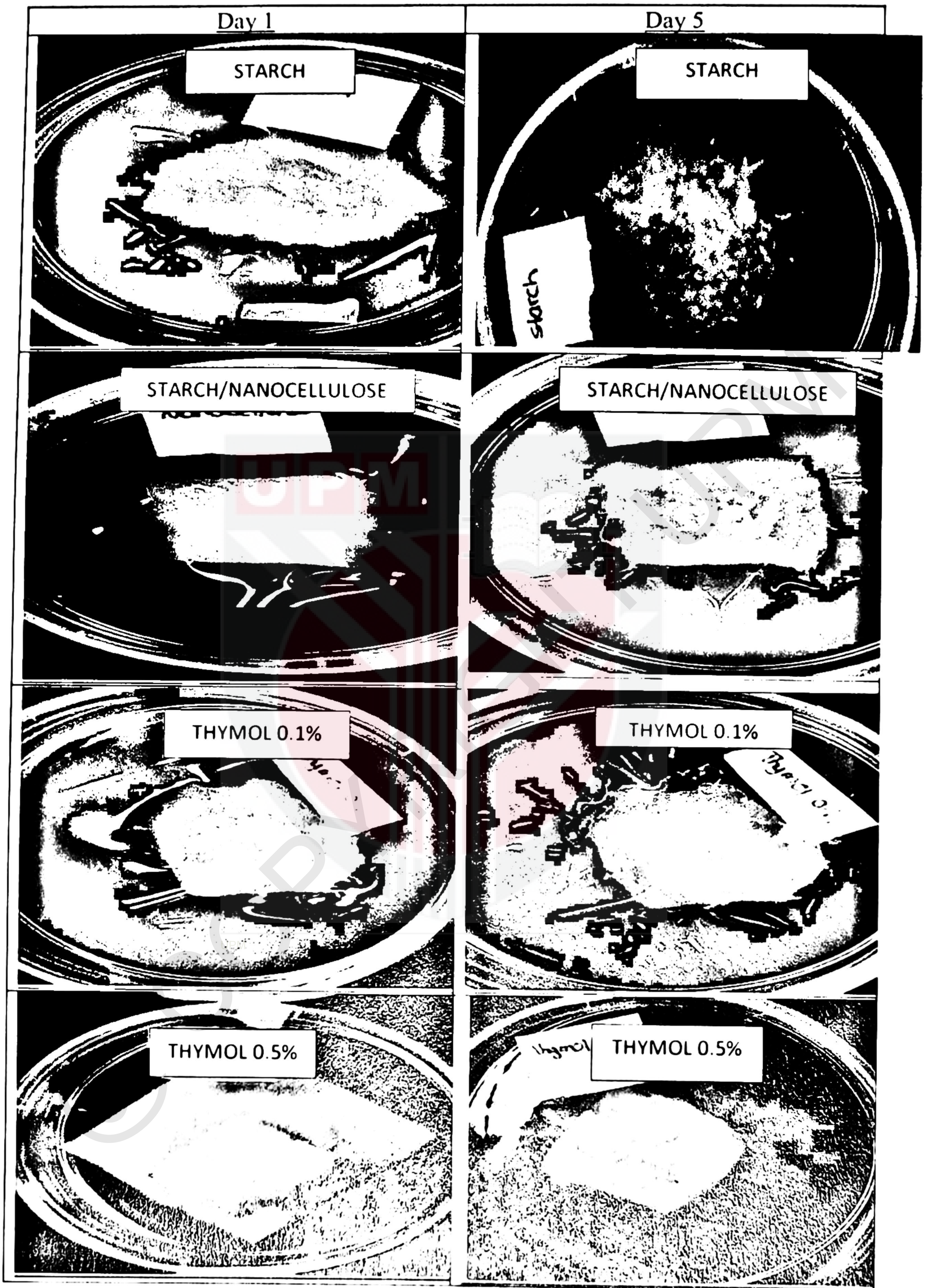


Figure 21. Physical appearances of chicken meat in direct contact with the films on day 1 and day 5.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

In this study, the physical, mechanical, thermal, barrier, and antibacterial properties of starch and starch/nanocellulose films containing different concentrations of thymol were investigated.

5.1.1 Characterization of starch/nanocellulose films

It was found that thickness increased from 0.083mm to 0.096mm with the increase in concentration of thymol. The increment in thickness became significant ($p<0.05$) with the increase in concentration of thymol. Besides that, starch/nanocellulose film incorporated with 0.5% w/v concentration of thymol was observed to exhibit changes in the physical appearance. High concentration of thymol affected the physical appearance of the films due to the white colour nature of the thymol. In term of colours, starch/nanocellulose films incorporated with 0.5% thymol exhibit significant increase ($p<0.05$) of L^*, b^*. and ΔE values but not significant ($p>0.05$) of a^* values compared to other films. Incorporation of thymol especially at higher concentrations increased the whiteness of the films hence the increase in L^* and ΔE value.

TS and YM decreased but EAB increased with the addition of thymol in starch/nanocellulose films. TS value for film incorporated with 0.5% of thymol (3.68 MPa) exhibited the lowest TS than 0.3% of thymol (4.71 MPa) and 0.1% of thymol (5.93 MPa). Starch/nanocellulose film incorporated with 0.5% of thymol has maximum EAB value (213.53%) and starch/nanocellulose without addition of thymol exhibited the minimum EAB value (195.22%). The starch/nanocellulose films incorporated with 0.5% concentration of thymol exhibited the lowest YM value (26.86MPa) compared to 0.3% of thymol (53.33MPa) and 0.1% (69.93MPa) thymol. These findings suggested that the films were suitable to be used as plastic wrapping for food packaging application due to high EAB value.

The initial degradation temperature, T_{onset} (°C) for first weight loss of all the films occurred around 143°C- 174°C. This was due to the loss of moisture in the film which was easily evaporated at boiling point of water. The maximum rate temperature T_{max} (°C) for second stage of weight loss was in the range of 320 to 325°C which indicated degradation of starch due to the depolymerization of film compositions. It was found that T_{max} increased from 318°C ,327°C, 337°C with the increase in the concentration of thymol content of 0.1%, 0.3% and 0.5% respectively the concentration of thymol content attributed to the rearrangement in polymer structure after addition of higher concentration of thymol. The structure form more homogenous structure with the addition of higher concentration of thymol which increases the thermal stability.

The OP value of the pure starch film was found to be $29.31\times10^3 cm^3$/.m.day.Pa decreased to $21.03\times10^3 cm^3$/.m.day.Pa with the addition of nanocellulose, indicating that this film exhibited a relatively good oxygen barrier. However, addition of 0.1% and 0.3%

thymol resulted to the increase in OP values to 90.92×10^3 $(cm)^3$/.m.day.Pa and $136.11\times10^3(cm)^3$/.m.day.Pa. The increase in oxygen transmission for the films could be due to the modification of the polymer matrix structure in the presence of the additives, consequently reducing the resistance of films to oxygen diffusion through them.

Incorporation of thymol especially at higher concentration caused a significant increase in WVP of the film. Starch/nanocellulose film without thymol exhibited WVP value of 4.78E-08 g/Pa.h.m. The value decreased to 3.37E-08 g/Pa.m.h with the addition of 0.1% thymol. However, addition of higher concentration of thymol (0.3% and 0.5%) increase the WVP to 5.00E-8 g/Pa.h.m and 1.01E-7 g/Pa.h.m respectively. Thymol at higher concentration may hinder polymer chain-to-chain interactions and reduced cross-linking.

5.1.2 Antibacterial properties of the starch/nanocellulose films containing thymol

Antibacterial properties of starch /nanocellulose films containing thymol was analyzed using disc diffusion method and inhibition zone was observed against gram-positive (S. aureus) but not on gram-negative bacteria (E. coli). Besides that, in-vivo test show that starch /nanocellulose films containing thymol was able to prolong the shelf life of chicken meat. Starch/nanocellulose films incorporated with thymol produced in this work exhibit high potential to be used as antimicrobial food packaging application since exhibited positive inhibition towards bacterial growth.

5.2 Recommendations

The following recommendations are made for future study:

1. Explore and compare the different methods in the production of starch films, particularly extrusion and hot press, which is more promising in industrial application compared to solvent casting method.
2. Add other additive to improve films' properties and functions such as cross-linked agent and antioxidant agent.
3. Study the biodegradability and shelf-life of starch/nanocellulose films incorporated with thymol at various storage conditions such as different relative humidity, temperature, and light.
4. Moisture sorption analysis is also important to determine the stability of starch/nanocellulose containing thymol films towards moisture as the films exhibit hydrophilic properties.
5. In order to ensure the application of starch/nanocellulose films incorporated with thymol is safe for end-user, it is also suggested to investigate the genotoxicity of starch/nanocellulose films containing thymol film.

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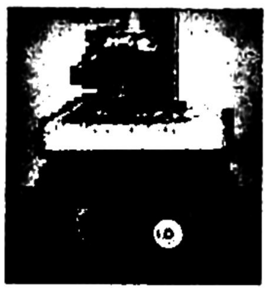
APPENDIX

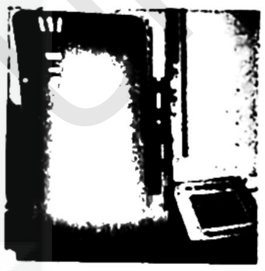
APPENDICES A: EQUIPMENT

Dissolved 8g corn starch + 2g glycerol in 180mL distilled water



Gelatinization (30 min, 85°C)

Dissolved thymol (0.1%, 0.3%, 0.5% w/v) in 20ml distilled water with 2.5% tween 80 and 1.5% NCF



Ultrasonication in ice bath (50% amp, 10mins)

Films casting (21°C for 48 hours)

Pre-conditioning: 25°C,RH 55%,48hrs

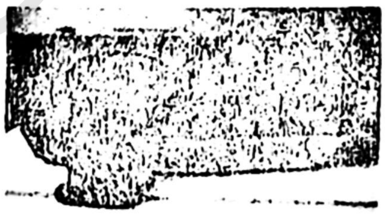
Characterization of physical, mechanical, thermal and barrier properties of film

a) Physical properties

- Thickness - Digital micrometer (Mitutoyo, Japan)

Thickness was measured at five random positions around the films.

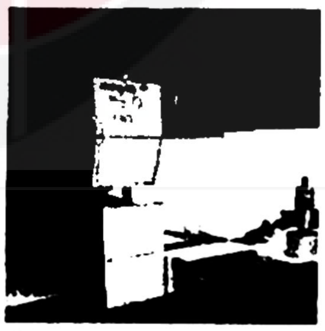
- Optical properties – Hunterlab Colorimeter (Hunterlab, Ultrascan Pro, USA).



L*, a* and b* values were determined.

b) Mechanical Properties

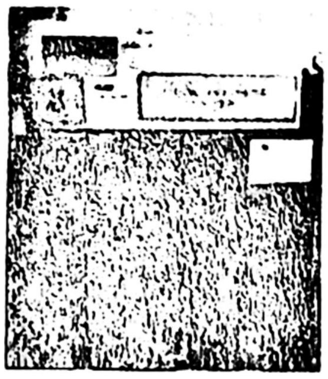
TA.XT2 Stable Micro Systems, UK



Head grip:65mm
Speed:8.30 mm/sec

Maximum TS, EAB and YM value was determined used D882 (ASTM,2012).

c) Thermal Properties



TGA-Greifensee, Switzerland
5mg sample was weighted in alumina pans (70ml) heated from 20°c to 500°C at heating rate of 10 °C/min under inert nitrogen atmosphere (flow rate 60 mL/min)

d)Barrier Properties

Water Vapor Permeability (WVP)

Sample was placed in between the cup and the ring cover

Sealed using a bee wax solution

Stored in a desiccator containing saturated MgNO3, RH of 51% at 30 ºC

Oxygen Permeability-(MOCON OpTech Oxygen Analyzer , USA)

Calibrated using Platinum CalCard

Placed the film sample for test.

OP value was calculated using formula: OTR/average thickness

In vitro

Bacteria was cultivated in the nutrient agar and incubate at 37 °C

Cultured bacteria was inoculated in 0.1% peptone water

Bacteria in the peptone water was spread onto the petri dish containing nutrient agar

All films were placed carefully into the petri dish that contained bacteria, incubated at 37 °C for 24 hours

In vivo

Chicken meat and film were cut aseptically into 3cm×3cm and 5cm×5 cm respectively

Sprayed with 75% ethanol and then dried it.

All film were placed on the chicken meat in petri dish, and placed at room temperature for 5 days .

APPENDICES B: DATA ANALYSIS

Table 4. Thickness reading of films

THICKNES READING	STARCH	0% Thymol	0.1% Thymol	0.3% Thymol	0.5% Thymol
1	0.084	0.086	0.088	0.076	0.098
2	0.084	0.083	0.095	0.11	0.095
3	0.083	0.088	0.087	0.084	0.098
4	0.081	0.085	0.087	0.08	0.101
5	0.08	0.087	0.088	0.114	0.09
6	0.083	0.088	0.092	0.09	0.098
7	0.086	0.083	0.093	0.08	0.094
8	0.083	0.08	0.093	0.1	0.095
9	0.08	0.088	0.084	0.083	0.096
10	0.082	0.082	0.082	0.088	0.096
AVERAGE	0.0826	0.085	0.0889	0.0905	0.0961
SD	0.00189737	0.002867442	0.004228212	0.013159281	0.002960856
SE	0.00435588	0.00535485	0.00650247	0.011471391	0.005441375

Table 5. Tabulated result of colour analysis

TYPE FILM	TS	EAB	YM	seTS	seEAB	seYM
starch	8.531159	102.855	140.67	0.259613	1.962462	1.728446
starch/nanocellulose	10.076	95.215	78.53	0.244037	1.046908	0.306784
0.1% thymol	5.929667	103.4313	69.93367	0.243275	0.818854	0.535718
0.3% thymol	4.709333	109.0653	53.33067	0.22617	1.83781	0.766052
0.5% thymol	3.683667	113.526	26.85967	0.237595	0.762295	1.087303

Table 6. Tabulated result of mechanical analysis.

TYPE FILM	TS	EAB	YM	seTS	seEAB	seYM
starch	8.531159	102.855	140.67	0.259613	1.962462	1.728446
starch/nanocellulose	10.076	95.215	78.53	0.244037	1.046908	0.306784

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0.5% thymol	3.683667	113.526	26.85967	0.237595	0.762295	1.087303