UNIVERSITI PUTRA MALAYSIA

PROPERTIES AND ANTIMICROBIAL ACTIVITY OF STARCH/CNF/THYMOL FILMS FOR FOOD PACKAGING APPLICATION

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ABSTRACT

The application of starch films in the food packaging industry has been restricted due to the poor mechanical and barrier properties. However, the addition of cellulose nanofibers (CNF) to the starch films as a reinforcing agent, may improve the properties of the films. Furthermore, food shelf life which is an important factor of food products, can be extended by incorporating antimicrobial agents such as thymol into the starch-based films.

Starch/CNF films incorporated with different concentrations of thymol (3, 5, 7, and 10 wt%) were produced using the solvent casting method. The physical, mechanical, barrier, and antimicrobial properties of the films were evaluated. For the physical properties, the color and opacity of the films were determined using a colorimeter whereas the thickness of the films was determined using a digital micrometer. The mechanical properties were analyzed in terms of tensile strength (TS), elongation at break (EAB), and Young's modulus (YM) using a texture analyzer. Meanwhile, the barrier properties particularly water vapor permeability (WVP) was investigated using a modified dry cup method. For the antimicrobial activity of the films, in vitro study was conducted by observing the clear inhibition zone of the films against *Escherichia Coli.* Besides, in vivo test was conducted whereby the films were put in direct contact with fresh meat inoculated with *Escherichia Coli* suspension, and the population was counted on designated reading days (days 3,5, and 7).

It was found that the TS and YM of the films decreased while the EAB increased with the increase of thymol concentrations added into the films. Furthermore, the addition of thymol at high concentrations (7 and 10 wt%) improved the barrier properties of the films by reducing the WVP. Starch/CNF films incorporated with thymol were also found to exhibited antimicrobial activity against *Escherichia Coli.* Finally, it was demonstrated that starch/CNF films incorporated with thymol were suitable to be used as flexible active food packaging films

due to their improved properties in terms of EAB and barriers properties and the ability to inhibit microbial growth.

ABSTRAK

Pengunaan plastik berasaskan kanji dalam industri pembungkusan makanan amat terhad berikutan sifat mekanikal dan ketahanannya yang lemah. Penambahan nanofiber selulosa (CNF) pada plastik berasaskan kanji sebagai agen penguat boleh meningkatkan ciri-ciri yang diperlukan oleh pembungkus makanan. Selain itu, jangka hayat makanan merupakan salah satu ciri penting dalam pembuatan produk makanan. Penambahan timol ke dalam plastik makanan sebagai agen antimikrobial dapat meningkatan jangka hayat makanan. Oleh itu, kajian ini bertujuan untuk menghasilkan filem kanji/CNF yang mempunyai ciri-ciri yang lebih baik dan bersifat antimikrobial.

Dalam kajian ini, filem dihasilkan melalui kaedah *solvent casting*. Timol ditambahkan ke dalam filem kanji/CNF dengan kepekatan berbeza iaitu 3, 5, 7, dan 10%. Ciri-ciri fizikal, mekanikal, halangan air dan gas, serta antimikrobial filem dinilai. Untuk sifat fizikal, warna dan kelegapan filem ditentukan menggunakan colorimeter, manakala ketebalan filem diukur menggunakan mikrometer digital. Sifat mekanikal dianalisis dari segi kekuatan tegangan (TS), pemanjangan (EAB), dan Modulus Young (YM) menggunakan alat penganalisis tekstur. Sementara itu, sifat penghalang terutamanya kebolehtelapan wap air (WVP) disiasat menggunakan kaedah *dry*. Untuk ujian antimikcrobial filem, kajian *in-vitro* dilakukan untuk dengan memerhatikan zon perencatan bakteria. Selain itu, ujian *in vivo* terhadap sampel makanan sebenar dijalankan dengan kaedah sentuhan langsung antara filem dengan daging segar yang diinokulasi dengan larutan bakteri *E. coli*. Pertumbuhan bakteria dihitung pada hari 3, 5, dan 7.

Dapatan kajian menunjukkan TS dan YM filem menurun dan EAB meningkat berkadaran terus dengan peningkatan kepekatan timol yang ditambahkan ke dalam filem. Penambahan timol pada kepekatan tinggi (7 dan 10% berat) meningkatkan kebolehtelapan

wap air. Tambahan pula, filem kanji/CNF yang mengandungi timol juga didapati menunjukkan aktiviti antimikrobial terhadap *Escherichia coli.* Kesimpulannya, filem kanji/CNF yang digabungkan dengan timol sesuai digunakan sebagai filem pembungkusan makanan aktif yang fleksibel kerana sifatnya yang lebih baik dari segi EAB dan sifat kebolehtelapan air di samping kemampuan untuk menghalang pertumbuhan mikrob.

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CHAPTER 1

INTRODUCTION

1.1 Background

Plastic is often used in food packaging because it is inexpensive and durable. Plastic pollution is a very serious issue caused by huge plastic waste improperly disposed of in the environment. The chemical structure of most plastic makes them resistant to many processes of degradation. In the food packaging industry, petroleum-based plastics such as polyethylene (PE), polypropylene (PP), and polyamide (PA), are often used due to their good physicochemical and processing properties (Rhim et al., 2013). Unfortunately, these constitute a large percentage of global plastic pollution since they are inconvenient to be recycled because they are usually contaminated with food. The environmental impact of this issue has raised a huge concern and led to a growing interest in developing bio-based polymers as biodegradable films for food packaging applications. According to Rhim et al. (2013), biopolymers are polymeric materials whereby the degradation process is through naturally occurring mechanisms with the action of organisms such as bacteria, fungi, and yeast.

The most studied biodegradable polymers can be categorized into 3 main groups: natural biopolymers, synthetic biodegradable polymers, and biopolymers produced by microbial fermentation. Packaging materials from natural polymers such as proteins, lipids, and carbohydrates from plants and animals, have relatively good mechanical and barrier properties and are renewable and biodegradable at the end of their life (Rhim et al., 2013).

Among the natural polymers, starch is a saccharide that is highly promising in the formulation of biodegradable films (Santacruz, 2015). Starch is the main source of glucose in many types of plant organs such as seeds, tubers, roots, and fruits (Davoodi, 2017). Starch is not only widely available in a few natural sources such as corn, wheat, and potatoes, but starch can also be obtained from leftovers of harvesting and industrial processes (Campos-Requena, 2017). Starch is abundant, biodegradable, low cost, and exhibits a good film-forming property. (Zhang et al., 2015)

However, for packaging applications, starch-based films are limited by their poor mechanical and barrier properties (Shapi'i et al., 2020). To improve the granular structure and the functional properties of starch films, plasticizers, cross-linking agents, nanosized fillers or nanofillers, and natural antioxidants and/or antimicrobial agents can be incorporated into the starch films. (Davoodi et al., 2017). For instance, incorporating glycerol which is a plasticizer into the biopolymer may increase the elasticity of the starch films (Viera et al., 2011). Furthermore, the formulation of starch films mixed with nano-sized fillers to form bionanocomposites such as nanosized cellulose will increase the rigidity and flexibility of the films and improve the thermal, barrier, and mechanical properties (Rhim et al., 2013). In addition, there is good adhesion between cellulose and starch matrix in starch-based films that can be attributed to the similarity of the chemical structure of the two components, which facilitates the bonding (Shankar et al., 2015).

Moreover, the addition of the antimicrobial agent into food packaging films can inhibit and kill the microorganisms, thus extending the shelf life of the food product. Among the antimicrobial agents, thymol ($C_{10}H_{14}O$) has been a very good option in antimicrobial packaging for researchers due to the board spectrum of antimicrobial activity against foodborne pathogens such as *Salmonella Typhimurium, Listeria monocytogenes*, *Escherichia coli* (*E. Coli*), *Staphylococcus aureus*, and *Bacillus subtilis* (Issa et al., 2017). Thymol is a phenolic compound extracted from the aromatic plant thyme (*Thymus vulgaris*) and has been known as an excellent antimicrobial agent. In this work, the antimicrobial activity of starch/CNF films incorporated with thymol will be investigated against *E. Coli* on fresh meat samples.

1.2 Problem Statement

Starch is a widely used biopolymer in the food packaging industry as a base film but its application as food packaging material has been restricted due to its poor mechanical, thermal, and barrier properties (Shapi'i et al., 2020). The incorporation of CNF, a reinforcing agent to produce bionanocomposite, and glycerol as plasticizer can improve these limited properties.

Meanwhile, food products are very sensitive to handle since they can be easily spoiled by microorganisms. Consumers nowadays tend to demand food without preservatives but with long shelf life. Thymol, which is a component of plant essential oil is an efficient antimicrobial agent that can be incorporated in the starch films to extend the food shelf-life and prevent foodborne diseases. However, the addition of different concentrations of thymol may affect the physical, mechanical, barrier, and antimicrobial properties of the resulting films. Therefore, this research intends to investigate the physical, mechanical, barrier, and antimicrobial properties of the starch/CNF films incorporated with different concentrations of thymol (starch/CNF/thymol films).

1.3 Objectives

The objectives of this study are:

1. To produce and characterize physical, mechanical, and barrier properties of starch/CNF films incorporated with different thymol concentrations (3, 5, 7, and 10 wt%).

2. To investigate the antimicrobial activity of the produced films via in-vitro and in-vivo analysis.

1.4 Scope of the Work

For objective number one, the scopes of work are as the following:

i. Produce starch/CNF films incorporated with various thymol concentrations (3, 5, 7, and 10 wt%) via the solvent casting method.

ii. Characterize the physical properties of the films particularly thickness, color, and transparency.

iii. Characterize the mechanical properties of the films including tensile strength, elongation at break, and Young's modulus.

iv. Characterize the barrier properties of the films particularly water vapor permeability.

For objective number two, the scopes of work are as the following:

i. Produce starch/CNF films incorporated with various thymol concentrations (3, 5, 7, and 10 wt%) via the solvent casting method.

ii. Investigate the antimicrobial activity of the films via in vitro analysis by observing the inhibition zone of the films against *E. Coli*.

iii. Investigate the antimicrobial activity of the films via in vivo analysis particularly on fresh meat that is in direct contact with the produced films.

CHAPTER 2

LITERATURE REVIEW

2.1 Starch

2.1.1 Characteristics and properties of corn starch

Starch is the most abundant polysaccharide in plants and exists in the form of spherical granules within the plant cells (Shing, 2003). It exists in many food products either naturally (wheat flour, rice, potato, corn) or as an additive (Eliasson, 2010). According to Eliasson (2010), the shape, size, and size distribution of starch granules vary depending on the source of the starch. There are some granules classified as small with a diameter of less than 5 μm and others are very large with diameters up to 110 μm. The starch granule surface is either smooth like potato starch or shows pores as in the case of corn starch (Eliasson, 2010). Corn starch constitutes 80% of the world's starch production (Ke et al., 2019).

Naturally, starch is a semicrystalline and the levels of crystallinity vary following the source of starch (Shinget al., 2003). Starch consists of two main components which are the glucose polymers, namely amylose and amylopectin. Amylose is a linear polymer with glucopyranose compounds linked by the α-D-(1-4) glycosidic linkages whereas amylopectin is one of the longest branched polymers with the highest molecular weight (Shing et al., 2003). The crystallinity is mainly associated with the amylopectin component while the amylose is what exclusively makes the amorphous regions (Shing et al., 2003). According to Shing et al. (2003), corn starch has the highest amylose content which can vary from 22.4 to 67.8% depending on whether it is a normal corn or a high amylose content corn. Given that the film-forming properties of the starch are dependant on the amylose content in the starch, films produced with corn starch are more rigid and stronger (Nordin et al., 2020). Moreover, because of its hydrophilic properties and its degradation capability, corn starch enables the incorporation of various polymers into, its resulting different types of thermoplastics (Fonseca-García et al., 2021).

2.1.2 Starch as food packaging material

Starch is one of the most interesting biodegradable polymers that has received growing attention in the formulation of food packaging films due to its properties such as total compostability without the formation of toxic residues (Abreu et al., 2015). The starch gelatinization process occurs when excess water is available in the presence of starch granules. An irreversible order-to-disorder transition of starch leads to the disruption of hydrogen bonds in the amorphous and crystalline regions caused by water ingress (Guo et al., 2020). By the end of the gelatinization process, granule swelling, amylose leaching, uncoiling of double-helix, and loss of birefringence take place. Solvent casting, which is one of the most studied film preparation methods for biopolymer-based films, consists of dissolving the polymer into an adequate solvent, casting it onto a suitable mold, and leaving it to dry until complete evaporation of the solvent (Takkaltar et al., 2019). Then, a thin film is obtained. According to Ratnayake and Jackson (2007), the corn starch gelatinization process peak is reached when the temperature is approximately equal to 85°C.

However, the resulting films display some limitations due to the structure of starch molecules. The high intermolecular forces cause film brittleness and weaken the mechanical and barrier properties of the film (Mali et al., 2005). According to Wu et al. (2017), good food packaging material should necessarily provide sufficient mechanical strength, barrier properties, thermal stability, biodegradability, and antimicrobial and antioxidant properties.

Therefore, to overcome the limitations of the starch-based films, plasticizers are often added to improve the flexibility and uphold the barrier properties. Glycerol is the polyol that has been the most used and studied as a plasticizer for starch film production (Mekonnen et al., 2013). It is low cost, non-toxic for food packaging usage, and has a high boiling point. Adding glycerol reduces the polymer-polymer interactions thereby reducing the rigidity of the three-dimensional structure, thus allowing deformation without rupture (Mekonnen et al., 2013). The logical consequence is that the film becomes more processible and flexible. In a study conducted by Santacruz et al. (2015), it was stated that films incorporated with glycerol exhibited a high capacity to absorb water which can be attributed to the hydrophilic property of the glycerol. Therefore, the resulting films are usually thicker due to the hydrophilic nature that helps retaining water in the film matrix. However, this property tends to alter the mechanical properties of the starch-based films for instance by lowering the tensile stress of the films (Mali et al., 2005).

2.2 Bionanocomposites

2.2.1 Inclusion of nanosized fillers into starch

The shortcomings of biopolymers can be overcome by nanotechnology particularly by applying the nanocomposite concept. The inclusion of nanomaterials or nanosized fillers into starch films to form bionanocomposites is a promising way to improve the films in terms of mechanical, thermal, and barrier properties. Examples of such nanosized fillers include cellulose (Vigneshwaran, et al., 2011), clay (Avella et al., 2005; Shefera et al., 2014), chitin (Salaberria, et al., 2015), montmorillonite (Kumar, et al., 2010; Zhang et al., 2017), graphene oxide (Zhang et al., 2017), zinc oxide (Marvizadeh, et al., 2017) and chitosan (Hosseini, et al., 2016; Li et al., 2017). The nanosized fillers can fill in the space between the polymer matrix, thus improving the flexibility and rigidity of the bionanocomposite (Rhim et al., 2013). Table 1 shows the results of the study by Rhim et al. (2013), precisely on the tensile strength, elongation at break, and tensile modulus of poly(butylene succinate) (PBS)/Cloisite 30B nanocomposites. These properties were measured for different concentrations of Cloisite 30B, which is an organically modified clay, added into PBS. Tensile strength and Young's modulus increased gradually with the increase of filler content while tensile strain decreased with the increase of filler content.

Table 1: Mechanical properties of PBS/Cloisite 30B nanocomposites.

(Source: Rhim et al., 2013)

Content of Cloisite 30B(Wt%)	Tensile strength (Kgf/cm^2)	Elongation at break (%)	Tensile modulus (Kgf/cm^2)
0	131.7	12.45	106.7
1	139	12.25	112.3
3	144.1	11.95	114.4
5	149.8	11.40	118.2
10	157.7	10.90	129.4
20	190.8	11.30	144.4
30	213.5	12.25	173.8

2.2.2 Cellulose nanofiber (CNF) as a reinforcing agent

Many different types of nanosized fillers have been used to reinforce starch films. Among them, cellulose is favorable due to its natural abundance on earth. Fruit wastes such as the skins, husks, seeds, and other fruit residues are rich in cellulosic materials which can be exploited to be used as reinforcing materials and composites. In food packaging, cellulose nanocomposites could withstand stress and mechanical properties (Sinha, 2003). It has attractive properties such as renewable and non-toxic to the environment (Harini & Sukumar, 2019). It posses a large number of hydroxyl groups that provide active sites thus, allowing the control of the size, shape, and dispersion of the nanofibers (Abreu et al., 2015). Studies on reinforcement with CNF have been conducted on several polymer matrices such as natural rubber, poly(styrene-co-butyl acrylate), polylactic acid (PLA), polyvinyl alcohol (PVA), and polycaprolactone (PCL) (Lin et.al., 2009). In most cases, the mechanical properties of bionanocomposites produced have been substantially improved, depending on the amount and homogeneity of CNF in the matrix. This was due to the intermolecular interactions between CNF and biopolymers. However, a too high concentration of CNF might end up giving the opposite effect which is poor dispersion and non-uniform distribution of stress in the film matrix.

CNF, when added to polymer-based packaging films, tends to impart high rigidity. In a work conducted by Carvalho et al. (2018), there was a significant increase in the TS and a decrease in the EAB especially with the incorporation of 2% w/w of CNF in the whey protein-based films. However, when incorporated with 4% w/w of CNF, the TS and EAB were quite the same as the control film. This was because the addition of a low concentration of CNF resulted in the optimum mechanical properties of the films. When added with CNF with concentrations beyond the optimum, CNF tends to agglomerate, thus forming hydrogen bonds and reducing the desired effect (Carvalho et al., 2018).

Figure 1 shows the mechanism of barrier properties improvement of the films incorporated with CNF. Nanosized fillers or nanofillers with a uniform dispersion will enhance the barrier properties of the resulting films (Carvalho et al., 2018). Moreover, Balakrishna et al. (2017) found that when 1 to 4% w/w of CNF were incorporated into starch nanocomposites, the WVP reduced due to the formation of hydrogen bonds between the CNF starch that obstruct the water molecules from passing through the films. However, higher concentrations resulted in an increase of WVP due to the agglomeration of the fibers in the films (Balakrishna et al., 2017).It can be observed that the tortuosity path for the diffusion of molecules such as water vapor and oxygen increases when it is filled with nanofillers. This is due to the crystalline nature of CNF and its ability to form a good network with the polymer matrix (Sorrentino et al., 2007).

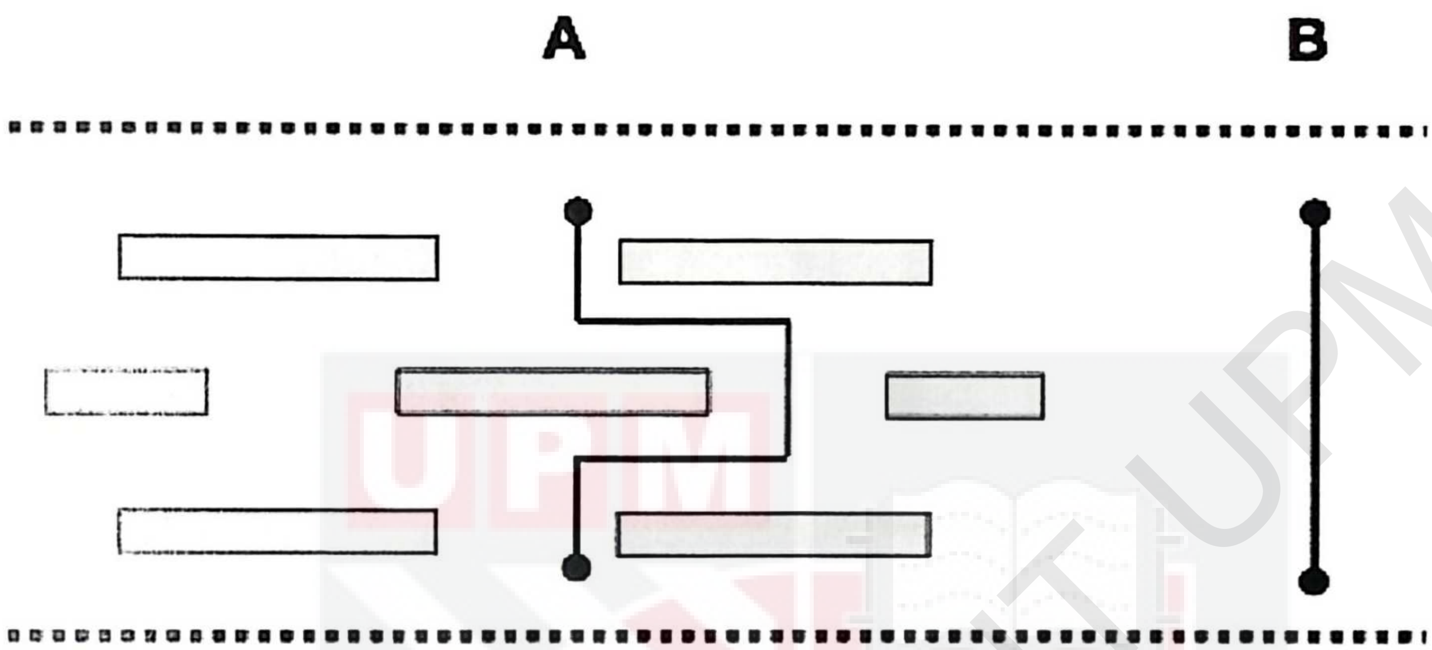


Figure 1. Schematic illustration of the tortuosity of a polymer matrix (A) Filled with nanofiller, (B) Unfilled polymer.

(Source: Sorrentino et al., 2007)

To sum up, CNF is a good reinforcing agent and that optimum concentration of CNF resulted in a film with better mechanical, and barrier properties.

2.3 Antimicrobial packaging

There is a growing interest in recent times to develop food packaging materials that exhibit antimicrobial properties to improve the safety and shelf-life of food products (Tripathi, Mehrotra, & Dutta, 2009). Antimicrobial packaging is one of the best active packaging systems that are meant to effectively protect food products from microbial contamination (Zhang et al., 2015).

Various antimicrobial agents have been incorporated into the packaging system, which include chemical antimicrobials, antioxidants with antimicrobial function, antimicrobial polymers, or natural antimicrobials (Han, 2003). For starch-based films, the most favorable antimicrobials used are chitosan, natural extracts, and essential oils due to their non-toxic properties ?. Due to the different antimicrobial mechanisms and different physiologies of microorganisms, the effectiveness of the antimicrobial agents might vary. The use of some of the antimicrobial agents can in fact alter the color, odor, stability, mechanical, and barrier properties of the food packaging materials (Zhang et al., 2015).

Table 4 shows the antimicrobial activity of starch-based films incorporated with different concentrations of oregano essential oil (EO), on different types of bacteria (Šuput et al., 2016). It can be deduced that different concentrations of EO added into the films have different affect on the antimicrobial activity of the films and that the antimicrobial activity increased with the increase in concentrations of EO added into the films.

Table 2: Antimicrobial activity of starch-based films with essential oils.

(Source: Šuput et al., 2016)

Sample	Inhibition zone(mm)		
	Salmonella Typhimurium	Escherichia Coli	Listeria monocytogenes
Essential Oil 0.5%	-	-	-
Essential Oil 1%	12	22	37
Essential Oil 2%	33	37	39

Among the many antimicrobial agents, thymol is a promising agent because as a natural extract, it is easily accessible, safe, and non-toxic (Lin et al., 2018). Thymol also has satisfying antimicrobial effects on a large range of bacteria (Cui et al., 2016).

2.3.1 Thymol

Thymol is a very popular antimicrobial agent that can be extracted from the aromatic plant thyme (*Thymus vulgaris).* It is the main component of the essential oils of thyme and oregano (*Oreganum sp.*). Thymol is an isomer 2 isopropyl-5-methylphenol and is a white crystalline substance. Figure 2 shows the chemical structure of thymol.

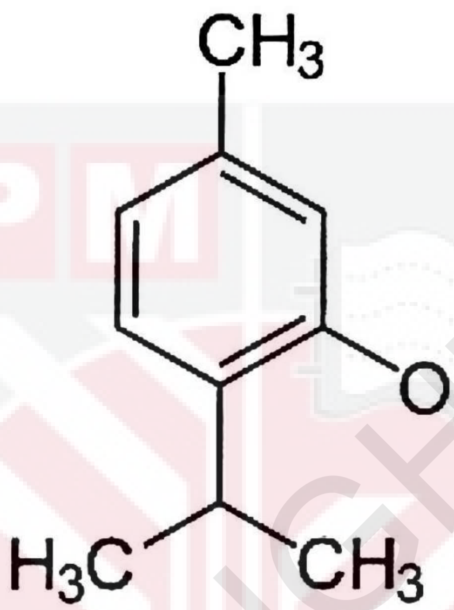


Figure 2. Chemical structure of thymol.

Thymol has been a very good option in antimicrobial packaging for researchers due to the board spectrum of antimicrobial activity against foodborne pathogens such as *Salmonella Typhimurium, Listeria monocytogenes*, *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus subtilis* (Issa et al.,2017). Thymol can be used in contact with food products and like other volatile antimicrobials (AM) agents, thymol can provide the food in the packaging with continuous antimicrobial activity (Tawakkal et al., 2014). Besides, this compound has been categorized under Generally Recognized as Safe (GRAS) by the Food and Drugs Administration (FDA), thus it is very suitable to be used in the food industry (Castillo et al., 2014).

The mechanism of action of thymol is the disruption of the cytoplasmic membrane of the bacteria. Thymol has strong hydrophilic capacities which help it dissolves the microbial membrane and impair it. Thymol disturbs the membrane integrity, increases membrane permeability, and causes the leakage of protons and potassium, finally leading to the loss of membrane potential (Xu et al., 2008). Figure 3 shows the mechanism of action and target sites of thymol on microbial cells at the level of the fatty acids profile, the proteins, the adenosine triphosphate (ATP), and the cell morphology.

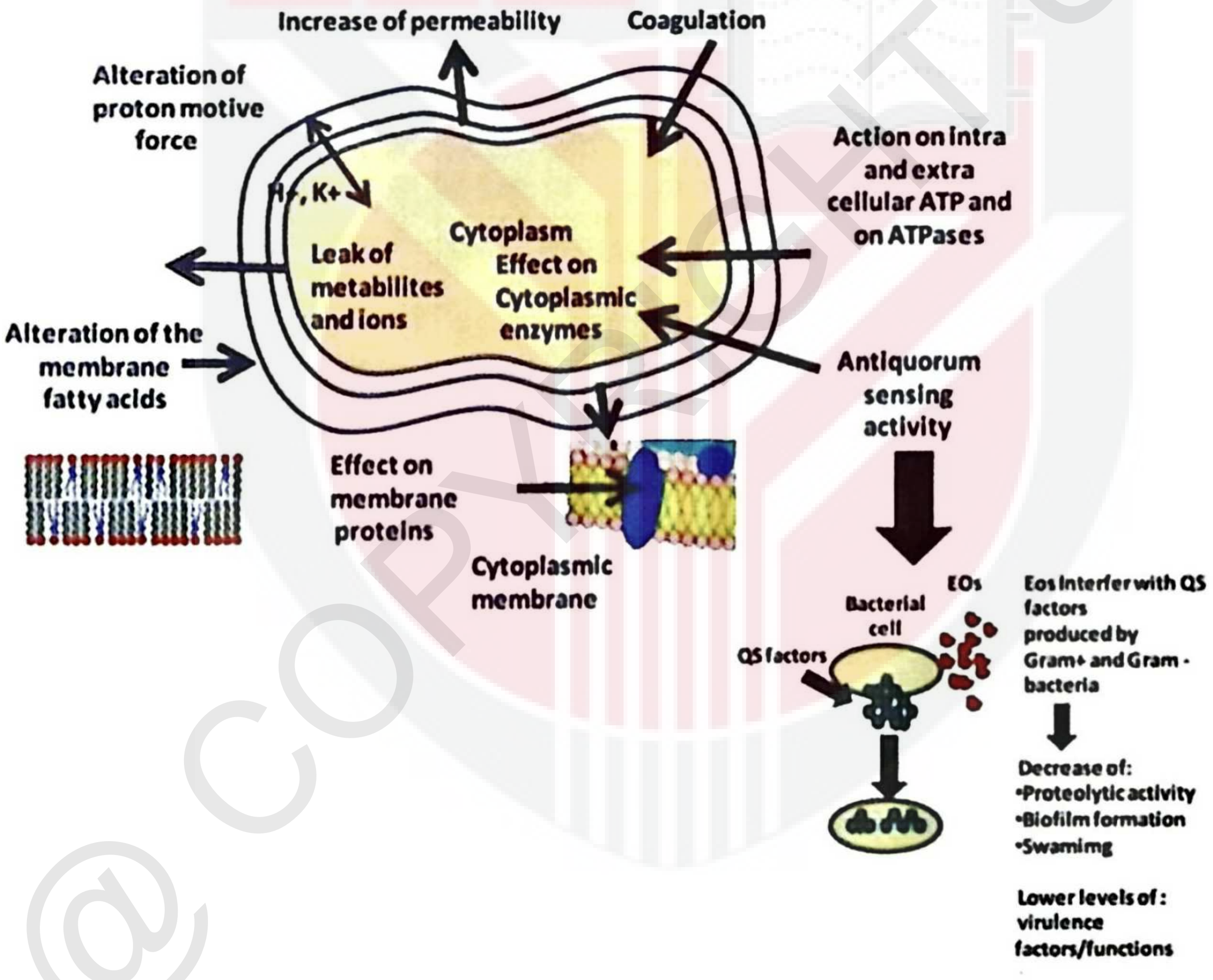


Figure 3. Mechanism of action and target sites of thymol on microbial cells.

(Source: Nazzaro et al., 2013)

In a study done by Medina et al. (2019), whereby thymol with a concentration ranging from 0.1 to 0.3 mg.mL^{-1} was added into chitosan-quinoa protein films, they found that the films with thymol had a greater inhibitory effect on microorganisms such as *Staphylococcus aureus* and *Salmonella Typhimurium*. They also found that films with the addition of thymol exhibited improved water barrier properties without affecting the elongation at break. On the other hand, Tawakkal et al. (2017) stated that when the concentration of thymol was increased from 10 to 30% w/w in PLA/kenaf films, the population of *Escherichia. Coli* was significantly reduced.

2.3.1.1 Physical properties of films incorporated with thymol

The incorporation of thymol may affect the physical appearance of the polymer-based films in terms of thickness, color properties, and opacity. According to Li et al. (2020), the thickness seems to increase with the addition of thymol into gelatin films due to the resulting higher dry concentration. Also, the higher the concentration of thymol in the gelatin films, the higher the thickness. The plasticizing effect of thymol can also induce to thicker films (Reddy & Rhim, 2014). However, the work by Almasi, Azizi, and Amjadi (2020) found that incorporating essential oil emulsions into pectin films did not significantly affect the thickness of the films owing to oil losses while preparing the films.

The color of a wrapping film can affect food's appearance through the consumer's eyes. Acevedo-Fani et al. (2015) reported that alginate films comprising thymol emulsions showed a greenish-yellowish tone due to the presence of phenolic compounds. They also found that the alginate-based films containing thyme essential oil presented a homogeneous surface due to the good entrapment of oil inside the matrix but their opacity increased slightly. According to Li et al. (2020), the effect of essential oil on the color of the films depends on the essential oil concentrations. Generally, a high concentration of essential oil will affect the color and opacity of the films.

2.3.1.2 Mechanical properties of films incorporated with thymol

Table 3 shows how the TS, which is the maximum resistance of a material can give when subjected to tension; the YM, which is the stiffness of the material; and the EAB, which indicates the elongation of the material before rupture, were influenced by the addition of different amounts of thymol into PBS films. Petchwattana and Naknaen (2015) found that adding thymol to PBS reduced both the TS and YM but increased the EAB. Various works of literature such as Kavoosi et al. (2013) and Ramos et al. (2014) have found the plasticizing effect of thymol s on food packaging material. Thymol alters the intermolecular bondings in the film's structure and makes the films less stiff and easily deformed (Li et al., 2020). However, the mechanical properties of the films are very much dependent on the amounts of thymol added into the films

Table 3: Effect of thymol on the mechanical properties of PBS films.

(Source: Petchwattana and Naknaen, 2015)

Thymol content(wt%)	Young's modulus (GPa)	Tensile strength (MPa)	Elongation at break (%)
Neat PBS	0.72 ± 0.07	39.98 ± 1.25	17.46 ± 1.46
2	0.73 ± 0.11	35.64 ± 0.90	17.10 ± 1.56
4	0.64 ± 0.78	31.05 ± 0.87	19.30 ± 1.35
6	0.58 ± 0.50	26.95 ± 0.95	21.85 ± 1.60
8	0.53 ± 0.41	24.77 ± 0.67	21.05 ± 1.67
10	0.48 ± 0.60	23.03 ± 0.49	22.58 ± 2.90

Table 4 shows the range of values of the tensile strength, percent elongation, and the Modulus of elasticity of a few common plastics used in food packaging. The values can be taken as a reference in determining whether or not, the developed film material is suitable for food packaging applications.

Table 4: Mechanical properties of common food plastics

(Source: Chin, 2010)

Material characteristic	#1 PET	#2 HDPE	3# PVC	4# LDPE	5# PP	6# PS
Tensile strength (MPa)	48.3-72.4	21.1-31.0	40.7-50.7	8.3-31.4	31.0-41.4	35.9-51.7
Percent elongation (%)	30-300	10-1200	40-80	100-650	100-600	1.2-2.5
Modulus of elasticity (GPa)	2.67-4.14	1.08	2.41-4.14	.172-.282	1.14-1.55	2.28-3.28

2.3.1.3 Barrier properties of films incorporated with thymol

The presence of thymol in biopolymer-based films has an impact on the barrier properties of the films. Table 4 shows the WVP of peanut protein isolate (PPI) based films incorporated with different concentrations of thymol (Zhong et al., 2017). It was found that WVP decreased from 2.1 $g.mm.kPa^{-1}.h^{-1}.m^{-2}$ for the control film to 1.15 $g.mm.kPa^{-1}.h^{-1}.m^{-2}$ for films containing 2% of thymol. Zhang et al. (2020) also found similar findings when incorporating thyme essential oil into curdlan films and pointed out that the decrement in the WVP was due to the hydrophobicity of essential oils. The repulsion forces between the water molecules and thymol reduce the barrier performance. Thymol improves the tortuosity factor of the vapor diffusion path through the films (Kazemi-Pasarvi et al., 2020). Nonetheless, the barrier properties of the films are also very much dependent on the amounts of thymol added into the films

Table 5: WVP of PPI modified films incorporated with different amounts of thymol.

Thymol (% w/v)	WVP (g.mm. $kPa^{-1}.h^{-1}.m^{-2}$)
0 (control)	2.10 ± 0.10^a
0.5	1.61 ± 0.08^b
1	1.48 ± 0.06^b
1.5	1.17 ± 0.08^c
2	1.15 ± 0.08^c

Different letters in the same graph indicate a statistically significant difference (P<0.05).

2.3.2 Antimicrobial packaging applied to fresh meat

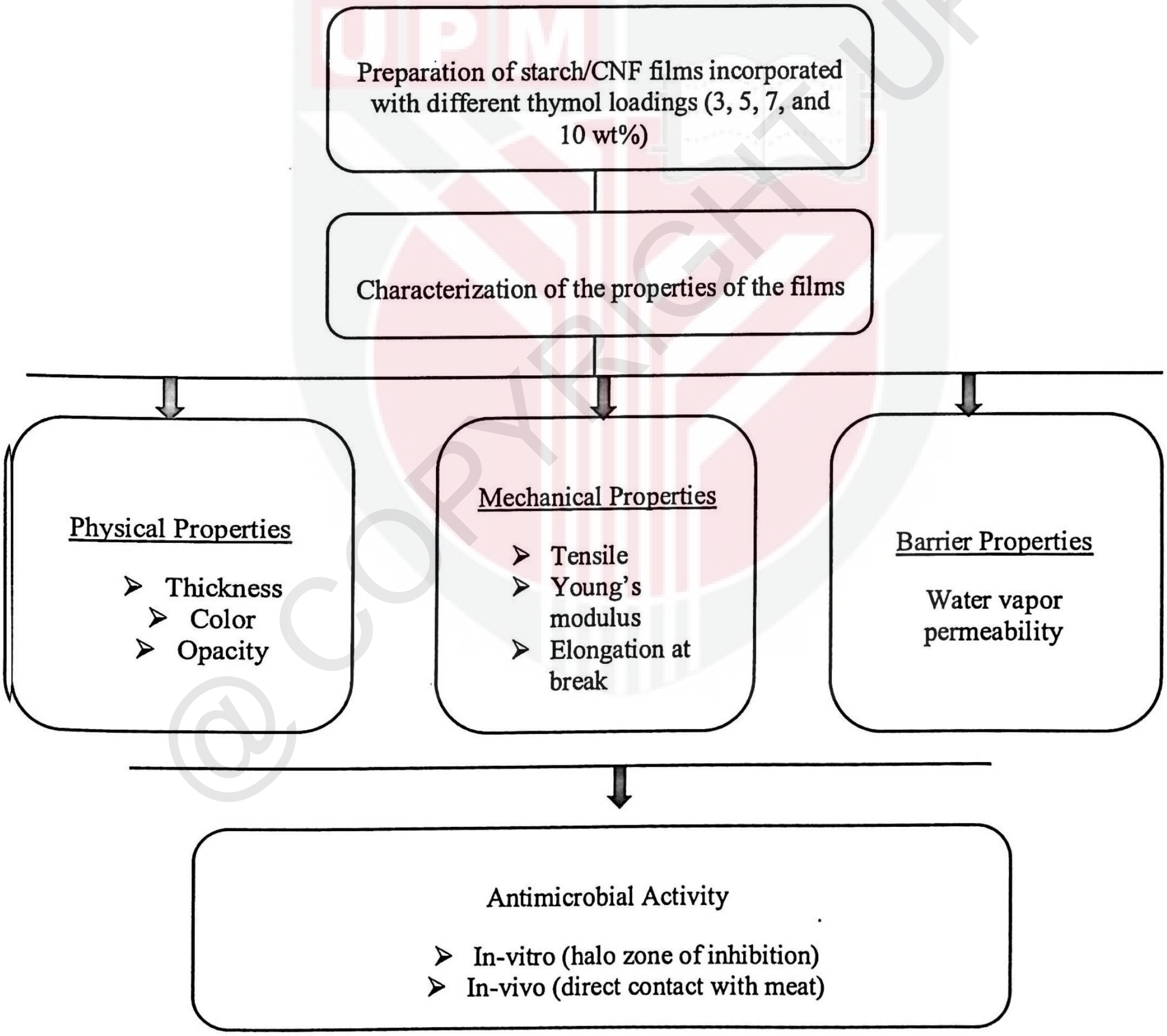
Meat is sensitive to handle and deteriorates easily during storage. Therefore, its packaging is an important matter to take into account. A packaging is classified as antimicrobial when its system can prevent and limit the microbial growth and inhibit contamination and spoilage of the product. Pathogenic microorganisms can easily grow on fresh meat because fresh meat is rich in nutrients and water, which is in favor of their growth.

Total bacterial counts analysis is a method to quantify the colonies of microorganisms on an agar plate 24 hours after inoculation with dilutions of a solution containing the product packaged with the antimicrobial packaging. Wang et al. (2020), have developed chitosan/potato starch/linseed oil/zinc oxide nanoparticles (ZnO NPs) films and analyzed the antimicrobial activity of the films on fresh raw meat. They found that the total bacterial count of the control group was higher than the maximum acceptability limit for raw meat at day 5 of storage at 4°C, and below the maximum acceptability limit for the samples packed in the developed films even after 7 days of storage at 4°C. In addition, the microbial growth of *Escherichia Coli* on fresh meat was significantly inhibited when packed with curdlan/polyvinyl alcohol films incorporated with thyme essential oil (Zhang et al., 2020). Zhang et al. (2020) found that the meat packed with control film without the incorporation of thyme essential oil could be stored at 4°C for only up to 6 days while the shelf life of meat packed with curdlan/polyvinyl alcohol films incorporated with 1.5 and 2% thyme essential oil was extended up to 14 days.

CHAPTER 3

METHODOLOGY

This chapter discusses the materials and the methods used in detail. First, the materials and methods used to produce and characterize (physical, mechanical, and barrier properties) starch/CNF films incorporated with different thymol concentrations are explained. Then, the materials and methods used to investigate the antimicrobial activity of the films are covered. Below is a summary of the experimental work.



3.1 Materials

Materials used in this study are grade corn starch (33% amylose, 67% amylopectin) from R&M Materials (Malaysia), CNF purchased from *Institut Perhutanan Tropika Dan Produk Hutan* (INTROP), Universiti Putra Malaysia, thymol crystal purchased from R&M Marketing (Essex, UK), Tween 80 (polyoxyethylene-20-sorbitan monooleate) purchased from Sigma Aldrich (St Louis, MO, USA), and analytical standard glycerol purchased from R&M Marketing (Essex, UK).

3.2 Preparation of starch/CNF/thymol films

Starch/CNF bionanocomposite films were prepared using the solvent casting method. For the preparation of 200 mL film-forming solution, an amount of 8 g corn starch was dissolved in 160 mL of distilled water, containing glycerol (fixed at 25 wt.% of starch, thus 2 g) and the solution was heated for 30 minutes and stirred constantly using a hotplate magnetic stirrer (DAIHAN, Indonesia) at 700 rpm until the mixture gelatinized at 90°C. Then, the film-forming solution was left to cool down to 50°C at ambient temperature. In the meantime, CNF/thymol emulsion was prepared by adding various amounts of thymol (3, 5, 7, and 10 wt.% of starch) into 40 mL of distilled water containing 1.5 wt.% of CNF (6 mL) at a temperature of 50°C. Thymol is known to be volatile, therefore a temperature of 50°C is fixed in order to minimize the loss of thymol during the preparation. Subsequently, a fixed amount of Tween 20 (20wt.% of thymol) was added as a surfactant and the emulsion was kept stirred for 15 minutes using a digital hotplate stirrer. The 1.5wt.% concentration of CNF was chosen based on the previous studies whereby 1.5 wt.% concentration resulted in the optimum properties improvement of the starch films. The CNF/thymol emulsion was added to the gelatinized starch film, in droplets using pipettes, after the film-forming solution was cooled down. The solution then underwent ultrasonication (QSonica, 500W, 20 kHz) for 10 minutes at 50% amplitude to produce a homogenous solution. The solution was then cast in a 140 mm petri dish (35 mL each) and dried in an air-conditioned room (25°C) for 48 hours. All the films were then placed in a ventilated oven (Memmert universal oven UN110, Germany) at 45°C for 15 minutes to dry. The dried films were peeled off from the casting plate and conditioned in a dry cabinet (WEIFO, Malaysia) set at 25°C and 55% relative humidity (RH) for 48 hours before further characterization.

3.3 Characterization of starch/CNF/thymol films

The characterization of the films was done by conducting analyses on the physical, mechanical, and barrier properties. Next, their application as the antimicrobial film was investigated by conducting in-vitro analysis on solid media and in-vivo analysis on fresh meat.

3.3.1 Physical properties

3.3.1.1 Thickness

The thickness of the films was measured using a digital micrometer (Mitutoyo, Japan). The thickness was read at 6 random points on the film and the average was calculated.

3.3.1.2 Color properties

The color the films were determined using a color spectrophotometer (Hunterlab, Ultrascan Pro, USA) by measuring the CIELAB coordinates (L*, a*, and b*). The equipment was calibrated following the scale L*=0 (black) to L*=100 (white), -a* (greenness) to +a* (redness) and -b* (blueness) to +b* (yellowness). The total color difference (ΔE) was calculated using the following equation:

$$\Delta E = [(L^* - L)^2 + (a^* - a)^2 + (b^* - b)^2]^{1/2} \quad \text{(Equation 1)}$$

The color values were averaged based on five different readings.

3.3.1.3 Opacity

The light transmittance of the films was recorded using the color spectrophotometer (Hunterlab, Ultrascan Pro, USA) from a wavelength of 200 nm to a wavelength of 700 nm. The transmittance value measured at 600 nm was used to calculate the opacity using the following equation (Sarojini et al., 2018):

$$\text{Opacity} = A_{600} / L \qquad \text{(Equation 2)}$$

where A_{600}: the value of absorbance at wavelength 600 nm

L: film thickness (mm)

3.3.1.4 Physical appearance

Images of the different films were taken on a black background to observe the appearance of the films.

3.3.2 Mechanical properties

The films were cut into rectangular strips (100 mm x 15 mm) after conditioning for 48 hours. Texture analyzer (TA.XT2 Stable Micro Systems, UK) was used to determine the maximum tensile strength, maximum elongation at break, and Young's Modulus according to ASTM standard method D882 (ASTM,2012). The films were stretched at a speed of 20 mm/min. A microcomputer was used to record the stress-strain curve with a minimum of three replicates of each film tested. TS, EAB, and YM were calculated according to the following equations:

$$TS = F/A \qquad \text{(Equation 3)}$$

where F: Force at specimen break (N)

A: Cross-sectional area of the film (mm^2)

$$EAB = [(l_f - l_o)/ l_o] \times 100 \quad \text{(Equation 4)}$$

where l_f: final length at specimen break

l_o: initial length at specimen break

$$YM = \sigma/\varepsilon \quad \text{(Equation 5)}$$

where σ: tensile stress (MPa)

ε: tensile strain

3.3.3 Barrier properties

The barrier properties of the films were determined in terms of WVP. WVP was determined using the dry cup method according to ASTM E96 (Risyon et al., 2020) but with slight modification. First, the film samples were cut into circular shapes using a cutter with a diameter of 7 cm and a transmission area of 28 cm^2. The test dish inside the permeability cup was filled with 10 g of calcium chloride to ensure a 0% RH below the film. The film was placed on top of the permeability cup and closed with a ring cover. The cup and the ring were sealed with a mixture of paraffin wax and bee wax (8:2) that was melted by heating using the magnetic stirring hotplate beforehand. The cup was then placed in a desiccator containing a sodium bromide saturated solution to provide RH of 54% at 25°C. Inside the desiccator, the temperature and relative humidity were monitored using a digital temperature humidity meter (Proskit NT-312, Techno Tools & Equipment Sdn Bhd, Malaysia). The weight of the permeability cup was measured every 24 hours for 10 days and the weight versus time graph was plotted. The water vapor permeability rate (WVTR) and the WVP of the starch/CNF/thymol films were calculated using Equations 6 and 7 respectively (Risyon et al, 2020):

WVTR = (g/t)/A (Equation 6)

WVP = WVTR x L/ΔP (Equation 7)

Where

(g/t): the slope of the straight line from the graph of weight changes versus time (g/day)

A: the transmission area of the film (m^2)

L: the average thickness of the film (m)

ΔP: vapor pressure difference between the inside and outside of the cup (RH = 54% - RH = 0%), ΔP = 2.29 x 10^6 Pa.

3.4 Antimicrobial activity of starch/CNF/thymol films

3.4.1 In-vitro

The effect of incorporating the films with different thymol concentrations on the antimicrobial activity of the films against the microorganism *E. Coli* (Gram-negative) was investigated by a disc diffusion assay on Mueller-Hilton Agar (MHA). *E. Coli* is a common microorganism that exists when it comes to perishable food products like fresh meat. *E. Coli* is an indicator of nonhygienic conditions and fecal contamination. *E. Coli* was cultivated on nutrient agar and incubated for 24 hours at 37°C in the incubator (Thermo Scientific Heraeus Function Line incubator, China) . Then, a suspension of the *E. Coli* bacteria was prepared in peptone water such that the concentration was approximately $10^7 – 10^8$ CFU/mL. An amount of 0.1 mL of the bacteria suspension was spread over the prepared MHA surface in a petri dish. Films samples were cut into circles (6 mm diameter) using a cutter and sterilized for 30 minutes under UV light of the laminar flow (Esco Global, Singapore). Under the laminar flow, all the discs were carefully placed into the Petri dishes that were previously inoculated. The test was performed in triplicate for each sample. The plates were incubated for 24 hours at 37°C in the incubator. The presence of a clear halo inhibition zone around each of the film disc indicates the antimicrobial activity in the films. After the incubation time, the diameter of the halo inhibition zone was measured.

3.4.2 In-vivo

To assess the applicability of the active starch/CNF/thymol bionanocomposites as potential antimicrobial packaging material, the films were analyzed on a real food product, specifically fresh meat whereby each film was placed in direct contact with the meat. Fresh meat was chosen because *E. Coli* commonly lives in the intestines of cattle and can contaminate beef products during the slaughtering or subsequent handling. The method for in-vivo analysis following the previous studies by Guo et al. (2015). The meat and the films were cut aseptically into 3 cm x 3 cm and 5 cm x 5 cm, respectively. First, the meat portions were sprayed with 75% ethanol to kill any presence of bacteria. The meat samples were then inoculated with 0.1 mL of *E. Coli* suspension and the inoculum was spread evenly on the meat upper surface using a sterile spreader. All the steps were conducted under a laminar flow to prevent external contamination and to facilitate bacteria attachment. The portion of meat was placed perfectly at the center of the film. As a control, the inoculated meat samples in the absence of films were prepared. The samples were each put in a petri dish and kept in a chiller (13°C) to stimulate mild temperature abuse (Tawakkal et al., 2017). Then, on different reading days (1, 3, 7), the meat that was previously in direct contact with the film was placed in a stomacher bag and macerated with 400 mL of 0.1% peptone water for 2 minutes. The meat samples were filtered and serially diluted in peptone water (10^1 to 10^7). Then, 1 mL of the final dilutions were plated onto MHA medium and incubated at 37°C for 24 hours. Finally, the number of colonies formed was counted, taking into account the dilution factor.

CHAPTER 4

RESULTS AND DISCUSSION

This chapter presents the results and the discussion of the findings. Section 4.1 correlates with the first objective and consists of the results and discussion on the characterization of starch/CNF films incorporated with different concentrations of thymol, such as physical, mechanical, and barrier properties. Section 4.2 presents the results and discussion on the antimicrobial properties of the starch/CNF/thymol films.

4.1 Characterization of starch/CNF/thymol films

4.1.1 Physical properties

4.1.1.1 Thickness of the films

Figure 4 shows the thickness of starch/CNF films containing varying concentrations of thymol. When preparing the films, a volume of 35 mL of the film-forming solution was set to cast for all the films in the Petri dish. However, it can be seen from Figure 4 that the thickness increases with the increase in thymol concentration. It was found that the thickness increased slightly from 0.0859 mm for the films with 0 wt% thymol to 0.0945mm for the films incorporated with 10 wt% thymol.

The increase in the thickness which is proportionate to the concentration of thymol can be related to the addition of both glycerol and thymol. Due to the plasticizing effect of both glycerol and thymol, the intermolecular polymer chains are restructured and this resulted in voids that lead to thicker films (Nordin et al., 2020). This finding correlates with the work of Li et al. (2020) who found that the thickness of gelatin films increased with the addition of thymol nanoemulsions. The films also show similar behavior to that described by Lozano-

Navarro et al., (2017), whereby adding the natural extract from oregano to chitosan-starch films resulted in an increasing trend of the thickness of the films. This was due to the presence of compounds such as polysaccharides, carboxylic acids, and antioxidants in the extract, which resulted in a more complex matrix. Hence, in this study, the presence of thymol which is a natural extract increased the dry content, thus increasing the thickness of the films especially at higher concentrations.

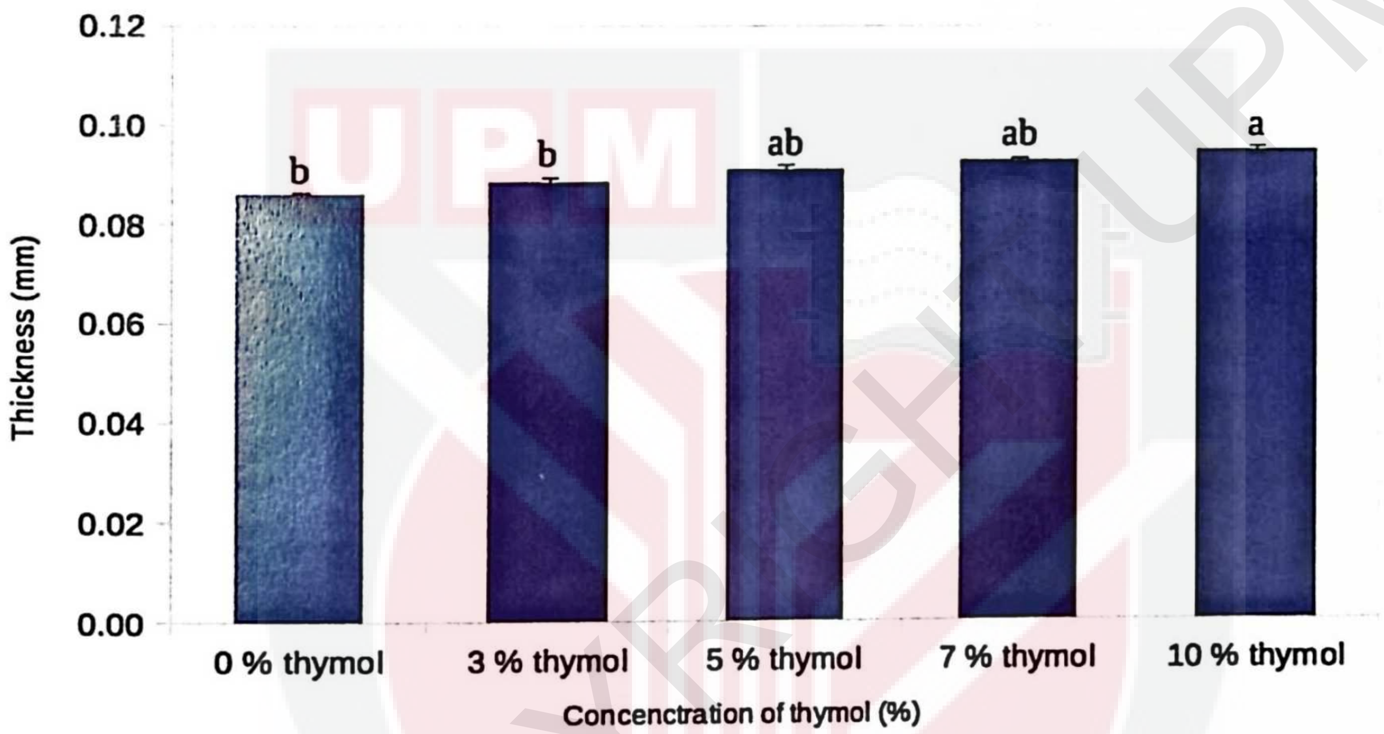


Figure 4. Thickness of starch/CNF films incorporated with different concentrations of thymol. Different letters in the same graph indicate a statistically significant difference ($P<0.05$).

4.1.1.2 Color properties

The color properties of the films were investigated in terms of L*, a*, b*, and ΔE values. As shown in Table 6, there were only slight differences in L* and a* values but no significant differences in the b* values for the starch/CNF films incorporated with various thymol concentrations indicating that all the films were practically almost similar in color, thus suitable for food packaging applications. It was found that the color difference, ΔE increased slightly with the addition of thymol and that increase in thymol concentration did not significantly affect the ΔE. The slight increment in ΔE with the addition of thymol was due to the white crystalline color of thymol. These findings seem to be consistent with the previous study of Hosseini et al. (2009) where they found that ΔE values of chitosan-based films incorporated with thyme essential oil increased with the incorporation of essential oils.

Table 6: Colour properties of starch/CNF films incorporated with different concentrations of thymol. Different letters in the same graph indicate a statistically significant difference ($P<0.05$).

Concentration of thymol (%)	L*	a*	b*	ΔE
0%	92.11 ± 0.26^{b}	-0.93 ± 0.01^{b}	5.46 ± 0.19^{a}	-
3%	93.02 ± 0.34^{ab}	-0.89 ± 0.01^{a}	4.25 ± 0.64^{a}	1.514 ± 0.06^{b}
5%	93.53 ± 0.27^{a}	-0.88 ± 0.01^{a}	4.15 ± 0.75^{a}	1.933 ± 0.10^{a}
7%	93.61 ± 0.51^{a}	-0.95 ± 0.02^{b}	4.07 ± 0.87^{a}	2.045 ± 0.11^{a}
10%	93.68 ± 0.76^{a}	-0.95 ± 0.01^{b}	4.12 ± 0.25^{a}	2.064 ± 0.04^{a}

4.1.1.3 Opacity

The opacity of starch/CNF films incorporated with different thymol concentrations (3, 5, 7, and 10 wt%) was determined and tabulated in Table 7. A high opacity value indicates low transparency of the films and vice versa. Transparency of food packaging material is a crucial factor because it can help suppliers to keep track of the product quality through distribution and storage as well as help to appeal consumers (Wang et al., 2020). According to Garavand et al.

(2017), for food packaging, film transparency varies depending on the formulation of the films and is preferably high.

It was found that the addition of thymol into the films increased the opacity of the films and that the increment becomes more pronounced at higher thymol concentrations. Increasing thymol loadings in the starch/CNF films resulted in an increasing trend of the opacity from 8.824 $A.mm^{-1}$ for the films without thymol to 9.678 $A.mm^{-1}$ for the films containing 10 wt% of thymol. These results indicate that the transparency of the films reduced with the increase in thymol concentrations. The films incorporated with the highest concentration of thymol (10 wt%) exhibited the highest opacity, therefore, the lowest transparency. These findings are consistent with the work of Zhong et al. (2017) and attributed to the presence of phenolic compounds in the thymol that form an emulsion. Nordin et al. (2020) stated that the degree of homogeneity of the film components can directly influence the opacity. It is worth mentioning that although essential oils resulted in less transparent films, they might prevent light-sensitive food products from oxidizing too early (Klangmuang & Sothornvit, 2016).

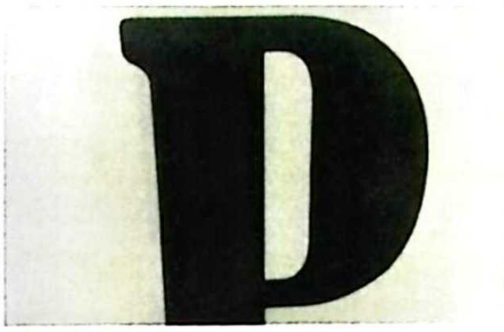
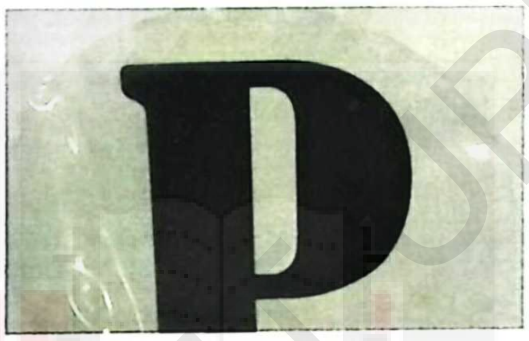
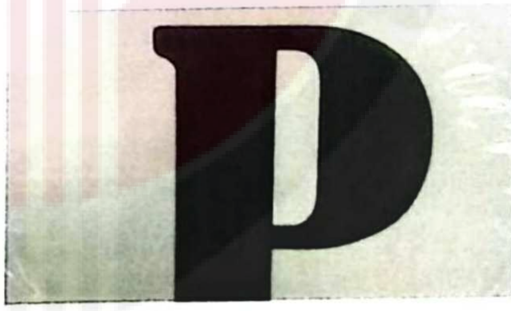
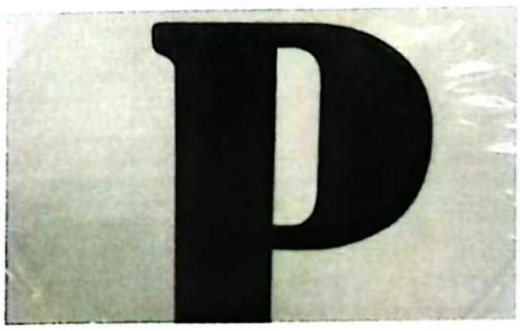
Table 7. Opacity of starch/CNF films incorporated with different concentrations of thymol. Different letters in the same graph indicate a statistically significant difference ($P<0.05$).

Concentration of thymol (wt%)	Opacity ($A.mm^{-1}$)
0%	8.824 ± 0.63^{e}
3%	8.891 ± 0.64^{d}
5%	9.209 ± 0.40^{c}
7%	9.410 ± 0.39^{b}
10%	9.678 ± 0.65^{a}

4.1.1.4 Physical appearance

Table 8 shows the physical appearance of the films. No major changes can be observed in terms of the physical appearance of the films. As can be seen, the opacity of the films increased slightly or the transparency of the films reduced slightly with the increase in thymol concentrations whereby the P letter when observed through the films becomes slightly whitish at high concentration of thymol (10 wt%) thus proving the previous findings. However, the reduction in the transparency was only slightly noticeable indicating that the films still preserve high transparency and that they are suitable to be used for food packaging applications. Thymol which is white in color nature slightly affected the appearance of the films.

Table 8: Physical appearance of starch/CNF films incorporated with different concentrations of thymol.

Starch/CNF film + 0% thymol	
Starch/CNF film + 3% thymol	
Starch/CNF film + 5% thymol	
Starch/CNF film + 7% thymol	
Starch/CNF film + 10% thymol	

4.1.2 Mechanical Properties

4.1.2.1 Tensile strength (TS)

Figure 5 shows the variation of the TS of the starch/CNF films incorporated with different concentrations of thymol (3, 5, 7, and 10 wt%). The TS of the films decreased with the addition of thymol and the decrement becomes more pronounced at higher concentrations of thymol. The TS values decreased from 10.63 to 6.30 MPa for films without the addition of thymol and films containing 10 wt% of thymol, respectively. The percentage of decrement was 40.73%. Referring to Table 4 in Chapter 2, the resulting films containing 3 to 10 wt% thymol exhibit TS in the range of that of low-density polyethylene (LDPE), a common food plastic material which ranges from 8.3 to 31.4 MPa, thus proving that the developed films have the potential to be used as food packaging material. Thymol that is a hydrophobic agent, may penetrate between the polymer chains and reduce the intermolecular forces (Petchwattana & Naknaen, 2015). Hence, the presence of thymol made the matrix to become heterogeneous. The consequence of this is the decrease in TS due to the increase in flexibility and a decrease in the rigidity of the films. Other works including Kavoosi et al. (2013) have found the same trend of results when they added thymol into gelatin films and explained that the trend of findings was due to the plasticization effect of thymol that makes the film softer. Furthermore, it was found that when thymol was incorporated into polybutylene succinate (PBS) based films, the tensile strength was reduced by 10 to 40% depending on the thymol content (Petchwattana & Naknaen, 2015).

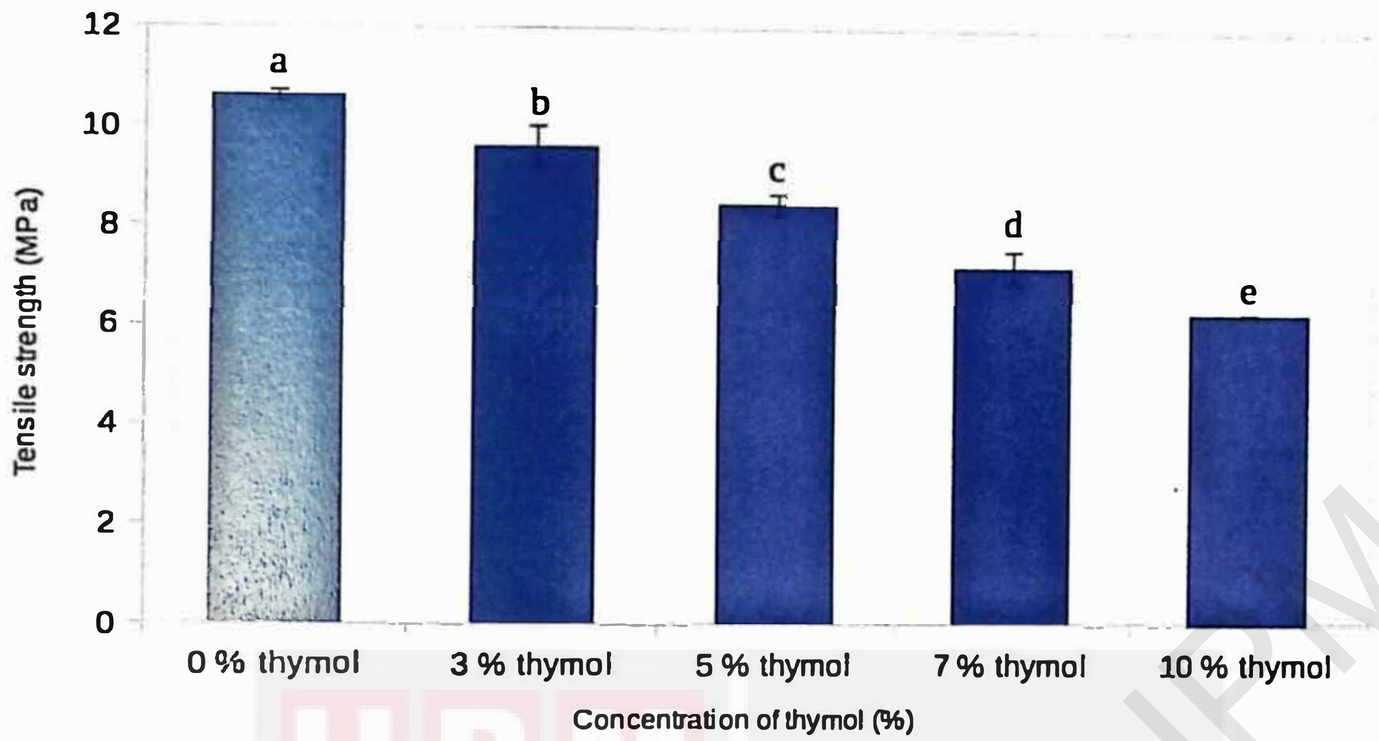


Figure 5. Tensile strength of starch/CNF films incorporated with different concentrations of thymol. Different letters in the same graph indicate a statistically significant difference (P<0.05).

4.1.2.2 Elongation at break (EAB)

EAB of the films provides information on the ability of the films to stretch, particularly indicates its flexibility. Figure 6 shows the effects of adding different concentrations of thymol into starch/CNF films on the EAB values. It can be seen that EAB value increased with the addition of thymol and there is a trend of an increase in EAB with the increase in thymol concentrations. The trend of the EAB values is reciprocal to the trend of the TS values, as expected. The starch/CNF films without thymol exhibited the minimum EAB value (110.587%) whereas the films incorporated with 10% of thymol exhibited the maximum EAB value (123.537%) which was 11.7% higher than the control films. All the resulting films exhibit EAB values in the range of that of polyethylene terephthalate (PET), high-density polyethylene (HDPE), LDPE, and polypropylene (PP), common food plastic materials which ranges from 30 to 300, 10 to 1200, 100 to 650, and 100 to 600%, respectively (Refer Table 4), thus proving that the developed films have the potential to be used as food packaging material.

A similar trend of results was also reported by Arfat et al. (2014) who produced fish skin gelatin films incorporated with basil leaf essential oil, and Ansorena et al. (2016), who incorporated thyme oil into Polyethylene wheat gluten films whereby the EAB increased with the increase in essential oil concentrations from 3.5 wt% of thymol. According to Wu et al. (2014), these results may be explained by the changes in the crystallinity and the increase in ductile properties of the polymer caused by the incorporation of thymol. Thymol, which acted as a plasticizer hinders chain-to-chain interactions in the films' matrix and provides flexible domains in the film (Wu et al., 2021).

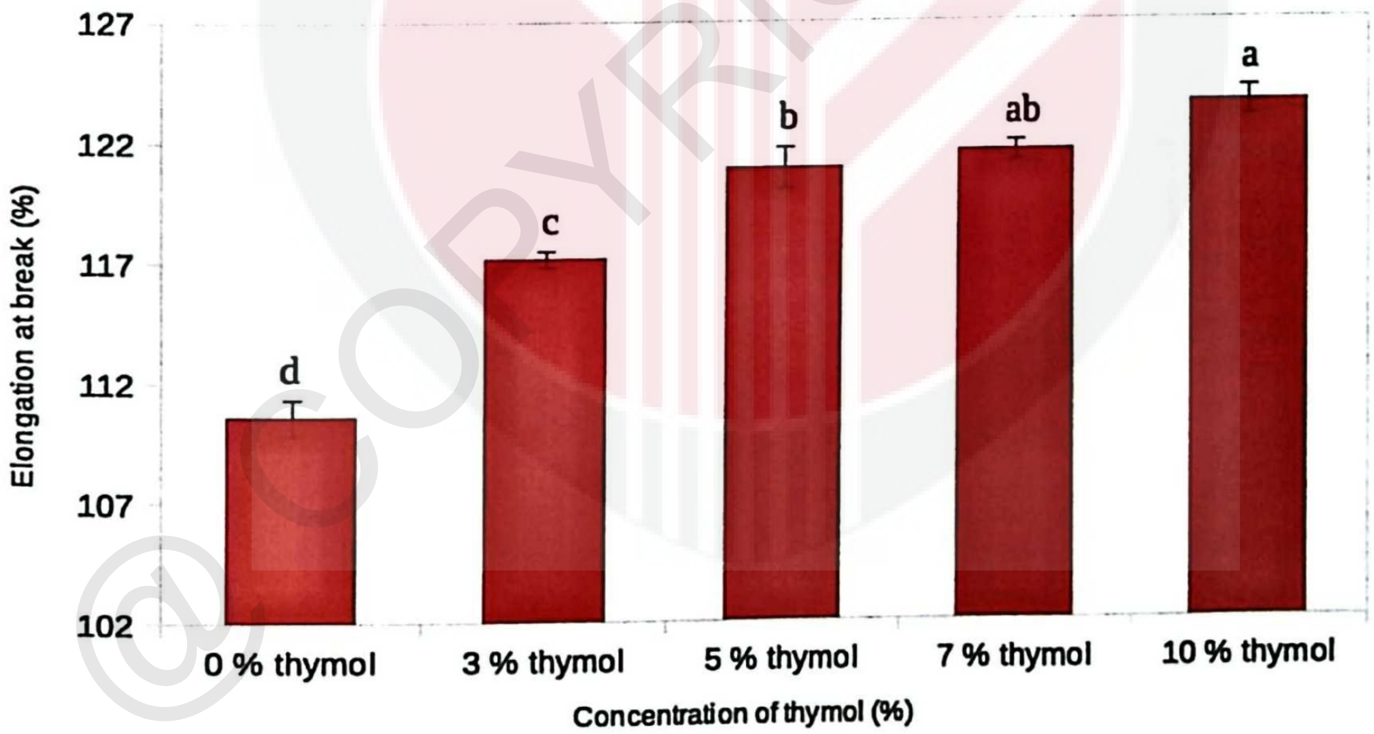


Figure 6. EAB values of starch/CNF films incorporated with different concentrations of thymol. Different letters in the same graph indicate a statistically significant difference ($P<0.05$).

4.1.2.3 Young's modulus (YM)

Figure 7 shows the YM of the starch/CNF films incorporated with different concentrations of thymol. It can be observed that the incorporation of thymol caused a decreasing trend in YM values of the films whereby the YM decreased from 436.85 to 209.77 MPa for the films without the addition of thymol and for the films with the addition of 10 wt% thymol, respectively indicating the reduction in stiffness of the films. This result is consistent with the findings reported by Ramos et al. (2012) whereby the addition of 8 wt% thymol into PP films decreased significantly the YM from 851 to 677 MPa compared to the control film. The YM values of the starch/CNF/thymol films incorporated with 7 and 10 wt% thymol in this study is consistent with the range of YM values of LDPE material whereby the range is 172 to 282 MPa (Refer Table 4).

The reduction in YM values with the addition of thymol can be explained by the plasticizing effect of thymol that led to a less stiff and more easily deformed film. The addition of thymol to the polymer matrix caused a modification of the crystallinity and decreased the ductile properties, thus improving the flexibility of the films (Khairuddin et al., 2020), hence the decrease in YM.

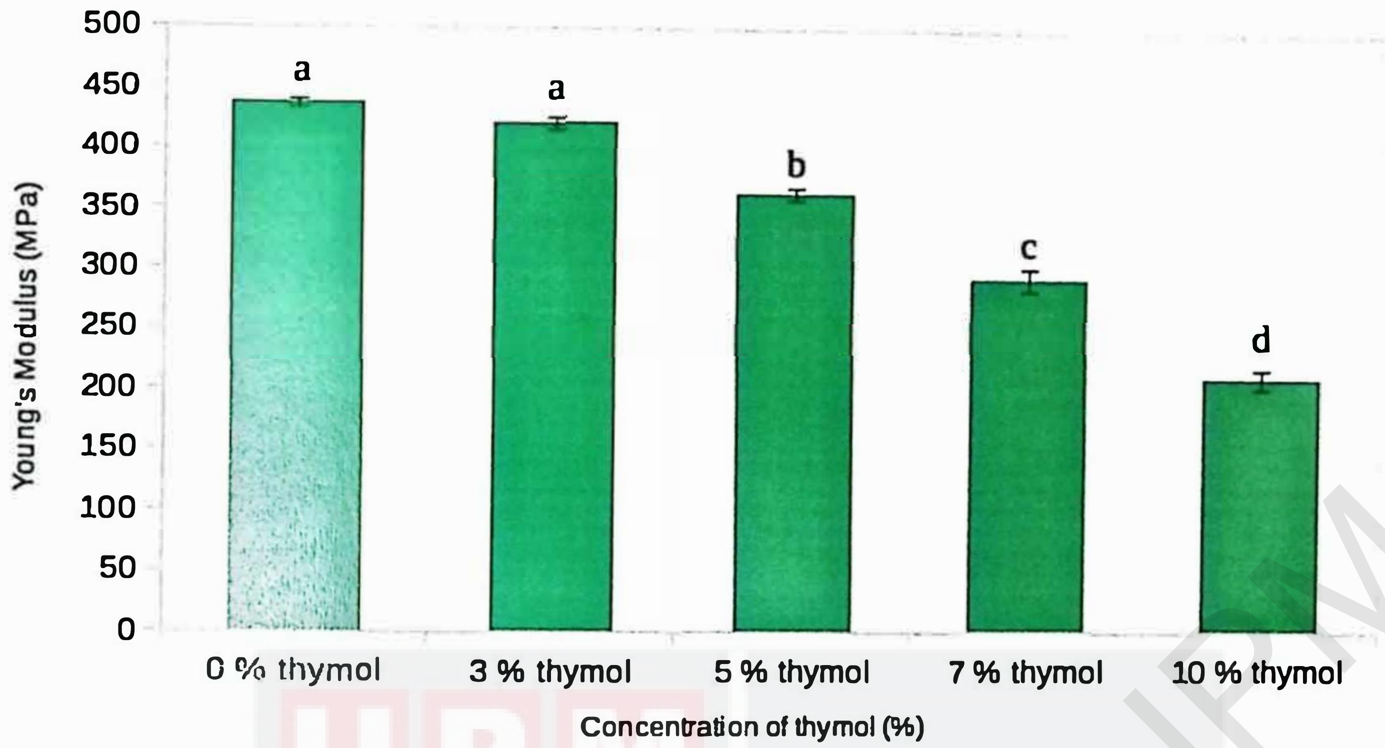


Figure 7. Young's modulus values of starch/CNF films incorporated with different concentrations of thymol. Different letters in the same graph indicate a statistically significant difference (P<0.05).

4.1.3 Barrier properties

Water vapor permeability (WVP) indicates the water barrier efficiency of the films and is a crucial factor to monitor since it can affect the quality of the food packaged with the films especially during distribution and storage (Vahedika et al., 2019). Figure 8 shows the WVP of the starch/CNF films incorporated with different concentrations of thymol. It was found that at lower concentrations (<7%), thymol content is not high enough to cause significant changes in the WVP. However, at higher concentrations (≥7%), thymol presence caused a decrease in the WVP of the films. The WVP of the films is dependent on the hydrophobic-hydrophilic balance of the components that form the film and the degree of cross-linking. Many works explained that the incorporation of oils led to an increase in hydrophobicity of the polymer-based films and thus, to a decrease in the WVP (Almasi et al., 2020).

In this work, it was found that WVP decreased significantly when 7 and 10 wt% thymol was added into the starch films. This result seems to be consistent with the work of López-Mata et al. (2013), who incorporated carvacrol into chitosan films, whereby the WVP of the films decreased with the increment of carvacrol content. This can be explained by the hydrophobic nature of the phenolic compounds that become dominant. For certain types of food such as dry food (cereal, bread, etc.), it is favorable to reduce the WVP of food packaging materials since the food packaging materials should not facilitate water transfer between the food product and the surroundings to enhance the shelf life of food products. However, from the findings in this work, it seems like there is a certain range of concentration where that resulted in the reduction of WVP.

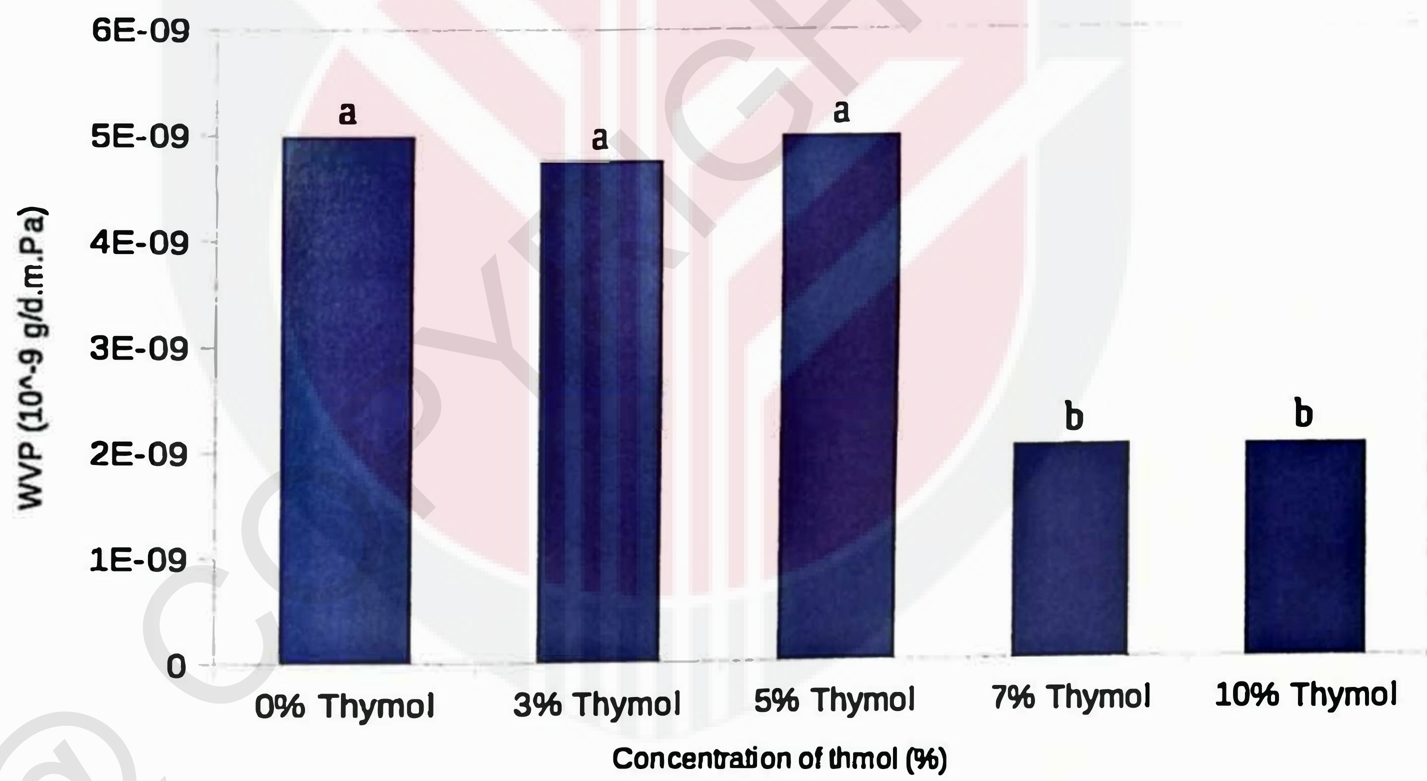


Figure 8. WVP of starch/CNF films incorporated with different concentrations of thymol. Different letters in the same graph indicate a statistically significant difference (P<0.05).

4.2 Antimicrobial properties

4.2.1 In-vitro

The antimicrobial properties of the starch/CNF films incorporated with different concentrations of thymol (3, 5, 7, and 10 wt%) were investigated through the disc diffusion method against *E. Coli* (gram-negative bacteria). The inhibition zones that correspond to a halo around the films are shown in Figure 9.

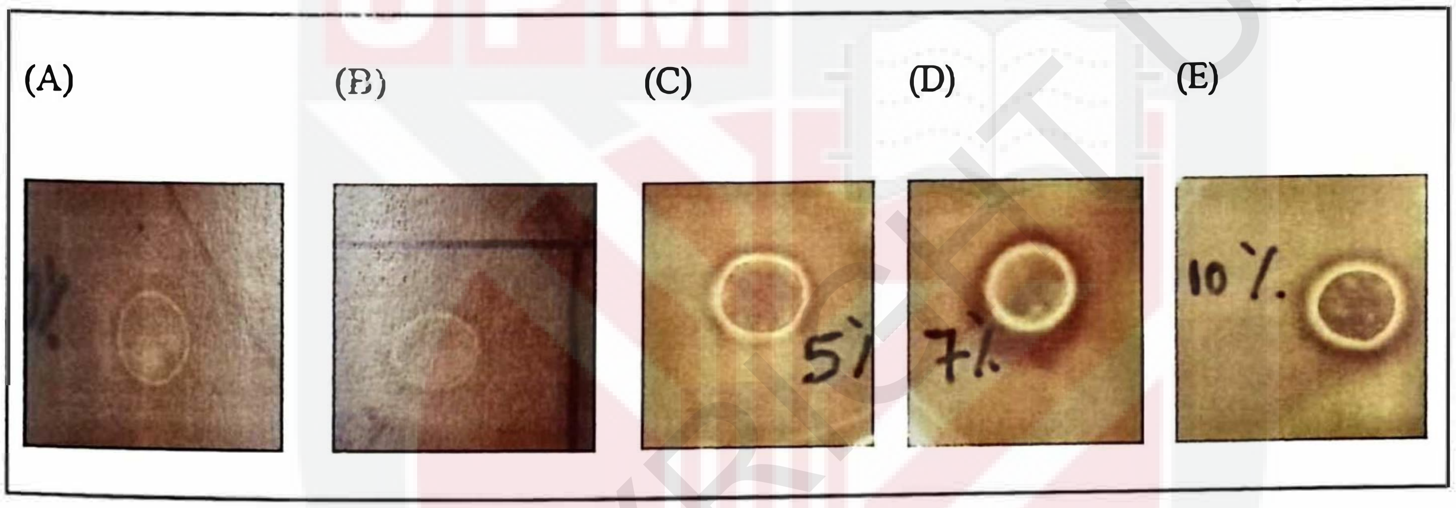


Figure 9. Antimicrobial activity of starch/nanocellulose films after 24 h of incubation at 37°C: (A) 0 wt% thymol, (B) 3 wt% thymol, (C) 5 wt% thymol, (D) 7 wt% thymol, and (E) 10 wt% thymol.

To further investigate the effect of concentrations of thymol on the antimicrobial activity of the films, the diameter of the clear zone of inhibition including the film was measured and tabulated in Table 9.

Table 9: Inhibition zone diameter of the starch/CNF films incorporated with different concentrations of thymol.

Concentration of thymol (%)	Inhibition zone diameter (mm)
0%	ND
3%	ND
5%	9.5 ± 0.5
7%	11.5 ± 0.5
10%	12.5 ± 0.5
ND: No inhibition zone was detected.	

The results in Figure 9 and Table 9 demonstrated that the area in direct contact with starch/CNF films without thymol, and with the addition of 3 wt% thymol exhibit no inhibition zone of *E. Coli*. Nonetheless, the addition of 5 wt% thymol into the films resulting in clear inhibition around the films, and the diameter of the inhibition zone against *E. Coli* becomes more pronounced with the increase in thymol concentrations. The starch/CNF film incorporated with 10 wt% thymol exhibited the strongest antimicrobial effect demonstrated by the largest clear zone of inhibition around the film. The starch/CNF films incorporated with 5 wt% of thymol exhibited a diameter of 9.5 mm whereas the films incorporated with 10 wt% of thymol had a diameter of 12.5 mm zone of inhibition. These results are consistent with the findings of Tawakkal et al. (2017) whereby when the concentration of thymol was increased from 10 to 30% in the PLA/kenaf films, the diameter of the inhibition zone increased from 7.5 mm to 20.6

mm at 20% kenaf content. This was attributed to the volatile nature of thymol (Zhong et al., 2017). The release of the thymol, which is an antimicrobial agent from the films to the agar medium inhibits the growth of *E. Coli*. Meanwhile, the films incorporated with 3 wt% thymol might not exhibit the inhibition zone due to the low concentration of thymol that was not enough to inhibit the growth of *E. Coli*.

These findings were consistent with the work of Petchwattana and Naknaen (2015) where they found that the minimum concentration of thymol in the gelatin films that resulted in the inhibition zone of *E. Coli* was 10 wt%. However, when tested against *Staphylococcus Aureus* (gram-positive bacteria), the antimicrobial activity of the gelatin films was evident at 6 wt% of thymol concentration. According to Zhong et al. (2017), gram-negative bacteria are less sensitive to essential oils and their components compared to gram-positive bacteria. Indeed, the cell membrane of the gram-negative bacteria is stronger than that of gram-positive bacteria (Kavoosi et al., 2013). Therefore, higher amounts of the antimicrobial agent are required to tackle this limitation.

4.2.2 In-vivo

To evaluate the effectiveness and applicability of the starch/CNF films incorporated with thymol as food packaging material, the application of starch/CNF films with 0 wt% and 10 wt% thymol was demonstrated on fresh meat samples. The starch/CNF films with 10 wt% thymol were investigated as starch/CNF films with 10 wt% thymol exhibited the optimum results in the in-vitro analysis. Table 10 shows the population of *E. Coli* in terms of log CFU/m^2 on the surface of the inoculated meat samples stored at 13°C on three different reading days (days 3, 5, and 7).

The results in Table 10 confirmed that the antimicrobial activity of the films was the highest for the films with the addition of thymol whereby the *E. Coli* population was the lowest for that films compared to control and the films without the addition of thymol throughout the 7 days of storage. Indeed, from day 3, the population of *E. Coli* was higher for the control (fresh meat sample without films) and for the meat sample that was in direct contact with the starch/CNF films without the addition of thymol compared to that of the starch/CNF films with the addition of 10 wt% thymol.

Table 10: Antimicrobial activity of starch/CNF films incorporated with thymol against *E. Coli* on fresh meat slices.

	E. Coli counts (log CFU/cm^2)		
Films	Day 3	Day 5	Day 7
Control (no films)	9.52	9.51	8.34
Starch/CNF/0% thymol	9.31	9.29	8.32
Starch/CNF/10% thymol	8.13	6.12	2.58

The population of *E. Coli* for the meat sample without film decreased slightly from day 3 to day 7. This was most probably due to the low-temperature environment (13°C) that was not optimum for the growth of *E. Coli*. Mild temperatures like 37°C are more favorable for the metabolism of *E. Coli*. However, *E. Coli* has the ability to adapt and survive at low temperature (Lee et al., 2019), hence the slight decrement in the *E. Coli* population. Meanwhile, the population of *E. Coli* in the meat sample that was in direct contact with starch/CNF films without the addition of thymol was slightly lower than the control films due to the existence of the films that act as a barrier against the meat samples and the surrounding. On the other hand, the population of *E. Coli* decreased significantly for the meat samples in direct contact with the starch/CNF films incorporated with 10 wt% thymol whereby on day 7, the population was 2.58 log CFU/cm^2 of *E. Coli*, due to the antimicrobial properties of thymol in the films.

These results were consistent with the findings of Zhang et al. (2020) whereby thyme essential oil was blended in curdlan/polyvinyl alcohol composites and the resulting films were tested on chilled meat. They observed that the microbial growth was inhibited and the storage period was extended with the incorporation of thyme essential oil into the films. The bacterial count of the control group, on the other hand, increased with the storage time, and the meat was deteriorated by the 6th day. Furthermore, in a study conducted by Tawakkal et al. (2017), they found that the population of *E. Coli* decreased significantly upon increasing the thymol concentration from 10 to 30% in PLA/kenaf films. For instance, by day 19 of reading, there were no colonies detected for the samples wrapped in the films containing 20% kenaf and 30% w/w thymol. This was due to the death of the bacteria caused by the destruction of their phospholipid membrane resulting in an imbalance of water (Gómez-Esteca et al., 2010).

As shown in Figure 10, the physical appearance of the meat changed over time depending on whether or not the meat was in direct contact with the films, as well as the addition of thymol into the film. It is clear that on day 1, all of the meat samples were reddish, attesting to their freshness. However, on day 3, the control meat sample started to become a bit darker in color, indicating a slight deterioration in the freshness. On day 7, the color of the meat sample became even darker compared to day 3 demonstrating a high reduction in the freshness. The meat sample that was in direct contact with the starch/CNF film without thymol also started to present changes in freshness as can be observed from the change in color on day 3 and the changes become more pronounced on day 7. On the other hand, the meat sample that was in direct contact with the starch/CNF film incorporated with 10 wt% thymol looked quite fresh and reddish on day 0 and day 3. However, the color started to change to darker color on day 7 indicating the reduction in the freshness of the meat sample.

The shelf life of the meat sample that was in direct contact with starch/CNF incorporated with 10% thymol was extended due to the presence of thymol as an antimicrobial agent that inhibits the growth of bacteria that can spoil the meat. These findings seem to be consistent with the findings obtained from the in-vitro and in-vivo (*E. Coli* counts) analyses which proved that the starch/CNF films incorporated with thymol have the potential to inhibit microbial growth and thus, extend the shelf life of food products.

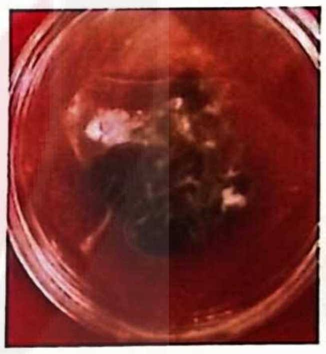
Films	Day 1	Day3	Day 7
Control			
Starch/nanocellulose/ 0 wt% thymol			
Starch/nanocellulose/ 10 wt% thymol			

Figure 10. Physical appearance of the meat in direct contact with the films, stored in a chiller, on days 1, 3, and 7.

CHAPTER 5

CONCLUSIONS AND RECOMMENDATION

In this work, the physical, mechanical, and barrier properties of starch/CNF films incorporated with different concentrations of thymol (3, 5, 7, and 10 wt%) were investigated, as well as their antimicrobial activity. The conclusions and recommendations for future studies are presented in this chapter.

5.1 Characterization of starch/CNF/thymol films

It was found that the thickness of the films increased with the addition and with the increase in the concentration of thymol (P<0.05). The thickness increased significantly from 0.0859 mm for the films with 0 wt% thymol to 0.0945 mm for the films incorporated with 10 wt% thymol. This can be explained by the increase of dry content matter in the biopolymer matrix which comes from thymol and the plasticizing effect of thymol. In terms of color, the L*, a*, and b* values were quite similar for all the films. However, there was a slight increase in the ΔE value as thymol was incorporated into the films, which was due to the white color nature of thymol. The opacity of the films increased slightly with the increase in thymol concentrations in the starch/CNF films indicating a slight reduction in transparency of the films.

The addition of thymol into the starch/CNF films altered the mechanical properties of the films in terms of TS, EAB, and YM whereby TS and YM decreased proportionally with the increase of thymol concentration in the starch/CNF films while EAB increased with the increase in thymol concentration in the starch/CNF films. The lowest TS value was recorded for the films containing 10 wt% of thymol (6.30 MPa) in opposition to the value of 10.63 MPa for the films without the addition of thymol. The starch/CNF films incorporated with 10 wt% of thymol exhibited the lowest YM value (209.77 MPa) in comparison to that without thymol (436.85 MPa). Meanwhile, the highest value of EAB was obtained when 10 wt% of thymol was incorporated in the films (123.54%). The starch/CNF films without the addition of thymol exhibited the lowest EAB value of 110.59%. These findings were related to the improvement of the flexibility and the decrease in the rigidity of the films as a consequence of the presence of thymol which exhibits a plasticizing effect. It was found that the mechanical properties of the starch/CNF/thymol films produced in this study were in accordance with that of some common food plastic materials, thus proving that they are suitable, promising, and have a high potential for food packaging applications.

In terms of barrier properties, the incorporation of thymol at high concentrations (≥7%) into the starch/CNF films led to a significant decrease in the WVP. The films without thymol exhibited a WVP value of 4.98 E-09 g/Pa.d.m. The value decreased to 2.01E-09 g/Pa.d.m with the addition of 10 wt% thymol into the films due to the hydrophobic nature of the phenolic compounds in thymol.

5.2 Antimicrobial activity of the starch/CNF/thymol films

Antimicrobial properties of the prepared films were investigated through in vitro (disc diffusion method) and in vivo (direct contact of the films with fresh meat) analyses. The inhibition zone against *E. Coli* was observed when the thymol concentration was at 5 wt% and higher. The diameter of the inhibition zone increased with an increase in thymol concentration. These findings prove the antimicrobial properties of the films incorporated with thymol. Also, the in-vivo analysis demonstrates that the starch/CNF films incorporated with 10 wt% of thymol inhibited the microbial growth and reduced the bacterial count on fresh meat samples. The designated films exhibit the potential to be used as active packaging because of the effectiveness of the thymol as an antimicrobial agent.

5.3 Recommendations

The recommendations for the future study are as the following:

1. Investigate and compare the different methods of starch-based films production, and use the most adequate and promising for industrial application.
2. The overall properties of the films could be improved by adding other agents that have specific functions such as antioxidant agents, vitamins, or enzymes.
3. Investigate the thermal properties of the films in terms of thermal stability and degradation temperature of the films. This is particularly important for applications.
4. Study the chemical bonding and the nanosized filler distribution in the designated films by conducting Fourier Transform InfraRed spectroscopy (FTIR) and transmission electron microscope (TEM) analysis.
5. Demonstrate the application of the starch/CNF films incorporated with thymol as antimicrobial packaging to a larger scale and investigate the antimicrobial activity of the films against different types of microorganisms that are relevant to food.
6. Investigate the migration and release of thymol over time and under different temperatures, as thymol is known to be volatile.

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APPENDIX

APPENDICES A: EQUIPMENTS & MATERIALS

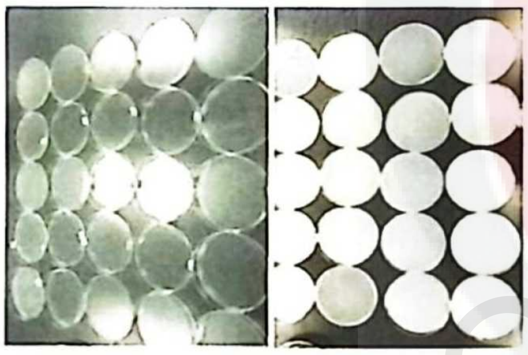
Preparation of 200 mL film-forming solution: 8g corn starch dissolved in 160 mL of distilled water, containing 2g glycerol (fixed at 25 wt.% of starch)

The solution was heated for 30 minutes and stirred constantly using a hotplate magnetic stirrer until the mixture gelatinized at 90°C. Then cool down at ambient temperature.

Add various amounts of thymol (3, 5, 7, and 10 wt.% of starch) into 40 mL of distilled water containing 1.5 wt.% of CNF (6 mL). A fixed amount of Tween 20 (20wt.% of thymol) was added as surfactant

CNF/thymol emulsion was added to the gelatinized starch film, in droplets using pipettes

The solution underwent ultrasonification for 10 minutes at 50% amplitude

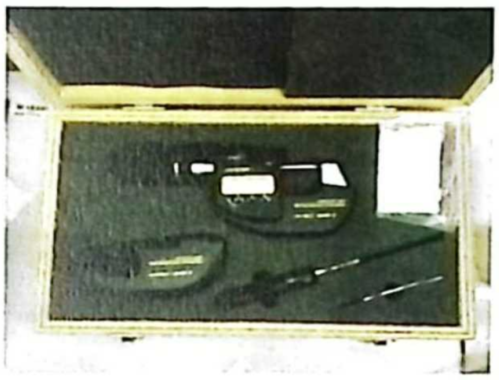


Cast in a 140 mm petri dish and dried in an air-conditioned room (25°C) for 48 hours.

Dried films were peeled off from the casting plate and conditioned in a dry cabinet set at 25°C and 55% RH.

Characterization of starch/CNF/thymol films

a) Physical properties
   - Thickness : A digital micrometer **(Mitutoyo, Japan)**

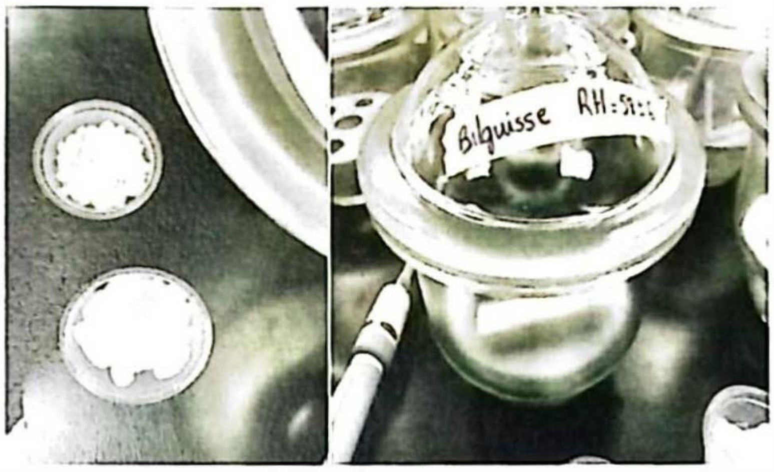


   - Color properties & transmittance : A color spectrophotometer **(Hunterlab, Ultrascan Pro, USA)**

b) Mechanical properties

TS, EAB and YM were measured using **Texture analyzer (TA.XT2 Stable Micro Systems, UK) in** accordance with ASTM standard method D882 (ASTM,2012)

c) Barrier properties



WVP was determined using the modified dry cup method according to ASTM E96 (Risyon et al., 2020)

Antimicrobial activity of starch/CNF/thymol films

❑ IN-VITRO

The antimicrobial activity was investigated by a disc diffusion assay on Mueller-Hilton Agar (MHA)

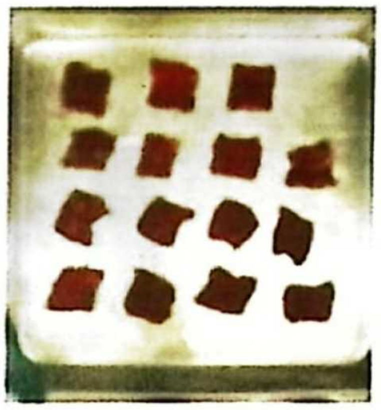
A suspension of a cultivated *E. Coli* bacteria was prepared in peptone water such that the concentration was approximately $10^7 - 10^5$ CFU/mL.

0.1 mL of the bacteria suspension was spread over the prepared MHA surface in a petri dish.

Films samples were cut into circles (6 mm diameter) and sterilized for 30 minutes.



❑ IN-VIVO



Incoculated meat samples were wrapped with films and stored in a chiller (13°C) up to 7 days

→

On reading day: Meat was placed in stomacher bag and macerated with 400mL of 0.1% peptone water for 2minutes

→

Samples was filtered and serially diluted in peptone water(10^1 to 10^7)

→

Samples was plated onto MHA medium then incubated at 37°C for 24 hours

→

The number of colonies formed was counted (considering the dilution)

APPENDICES B: DATA ANALYSIS

In-vivo test: Results on petri dish on day 7 of reading