Physicochemical and Thermal Characterization of Hydroxyethyl Cellulose - Wheat Starch Based Films Incorporated Thymol Intended for Active Packaging

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ABSTRACT

Biodegradable packing materials with antimicrobial properties have been a concern for years because of its positive environmental implications. The present work aimed to develop the formulation of hydroxyethyl cellulose (HEC)/wheat starch based film in which the active compound, thymol (0.5, 1, 1.5, 2, and 2.5% w/w) were incorporated into the polymeric material. Solution casting method was used for the film preparation while thymol was incorporated prior to casting. The physical and chemical properties of the developed film were determined. SEM was found to have a smooth and homogeneous with a small amount of thymol which grows coarser with 1.5% or higher thymol content. FTIR was used to find the chemical property of the film and suggested that the carbonyl functional group was unchanged in the film, however; -OH groups increased substantially with increased amount of thymol. Thermal properties were profiled through thermogravimetric analysis and differential scanning calorimeter where the AM film containing 1.5% (w/v) of thymol shows the highest thermal stability and decomposes less in comparison to other samples. The inhibitory capability of the film was tested against a list of microbial contamination and was found to successfully inhibit the growth of selected gram positive and gram negative bacteria in a wide range of studied concentration. The mechanical properties of the films were improved by 60.3% with an optimum tensile strength at thymol concentration of 1.5% w/w. It can be concluded that the film properties are retained chemically whereas mechanical properties, strength, flexibility and function of the film are being enhanced remarkably by the incorporation of thymol.

Keywords: Active packaging; hydroxyethyl cellulose; thymol; wheat based film

INTRODUCTION

In recent years, the industry of food packaging has gained numerous improvements mainly due to the increased demands on the safety of packaged food, shelf-life extension, economical benefit, environmental concerns and consumer convenience. Surveys cond HEC uted by Brody et al. (2001) have shown that active packaging technologies involve interactions between the food, the packaging material, and the internal gaseous atmosphere. Food packaging materials used to provide only barrier and protective functions. However, various kinds of active substances can now be incorporated into the packaging material to improve its functionality and give it new or extra functions. Such active packaging technologies are
designed to extend the shelf life of foods while maintaining their nutritional quality and safety (Han 2003). The most promising active packaging systems are oxygen scavenging systems (Gibis & Rieblinger 2011) and antimicrobial systems (Malhotra et al. 2015). The effectiveness of antimicrobial (AM) film to inhibit the microbial growth has been reported by many researchers (Abreu et al. 2015; Moreno et al. 2015; Salleh et al. 2014; Zhang et al. 2015).

The rising cost of petroleum, and the eventual depletion of the oil reserves, has cause many research efforts to focus on developing renewable and biodegradable films. Thus, for years, there has been a renewed interest in films made from renewable and natural polymers such as starch (Mali et al. 2006). Starches are polymers that occur naturally in a variety of botanical sources such as wheat, corn, potatoes, and tapioca (Avella et al. 2005; Famá et al. 2006). Its composed of repeating 1,4-α-D glucopyranosyl units: amylose and amylopectin where the relative amounts of amylose and amylopectin depend upon the plant source (Avella et al. 2005; Rodríguez et al. 2006). Meanwhile, cellulose is the most abundantly occurring natural polymer on earth and is an almost linear polymer of anhydroglucose. Because of its regular structure and array of hydroxyl groups, it tends to form strongly hydrogen bonded crystalline microfibrils and fibres and is most familiar in the form of paper or cardboard in the packaging context. Besides, for the regular -OH groups in cellulose are replaced by ethyl groups to produce more viscous substance that can be used for thickening agent. This product which is known as hydroxyethyl cellulose (HEC) is commonly used commercially for thickening purpose.

The natural product of the essential oil of the *Thymus vulgaris* L., thymol, is a phenolic monoterpene that has received great interest as a possible AM agent (Del Nobile et al. 2008; Mistry 2006; Tippayatum & Chonhenchob 2007). Similar to carvacrol, thymol antimicrobial activity results in structural and functional alterations in the cytoplasmic membrane that can damage the outer and inner membranes; it can also interact with membrane proteins and intracellular targets (Sikkema et al. 1995).

The objectives of this research was to evaluate the physical, chemical and thermal effect of thymol as AM agent in HEC-wheat based film.

**MATERIAL AND METHODS**

The main base of the films which were wheat starch (C\(_6\)H\(_{10}\)O\(_5\)) and hydroxyethyl cellulose were supplied by Merck. Thymol (C\(_7\)H\(_8\)O) was purchased from Sigma-Aldrich (Malaysia); methyl red, bromothymol blue were purchased from Fluka; Glyoxal was purchased from Merck. Thymol (C\(_7\)H\(_8\)O) was purchased from Sigma-Aldrich (Malaysia); methyl red, bromothymol blue were purchased from Fluka; Glyoxal was purchased from Merck. Thymol (C\(_7\)H\(_8\)O) was purchased from Sigma-Aldrich (Malaysia); methyl red, bromothymol blue were purchased from Fluka; Glyoxal was purchased from Merck. Thymol (C\(_7\)H\(_8\)O) was purchased from Sigma-Aldrich (Malaysia); methyl red, bromothymol blue were purchased from Fluka; Glyoxal was purchased from Merck. Thymol (C\(_7\)H\(_8\)O) was purchased from Sigma-Aldrich (Malaysia); methyl red, bromothymol blue were purchased from Fluka; Glyoxal was purchased from Merck.

**PREPARATION OF HEC/STARCH/THYMOL (AM) FILM**

The films were prepared by slightly modifying the method described by Gennadios et al. (1993). In this research, 0.5 g thymol was dissolved in 20 mL of absolute ethanol. The solution was then added into 80 mL of distilled water containing 4 g of HEC and 5 g of wheat starch. After the solution was completely dissolved, 5 mL of glycerol (HmbG Chemicals) and 5 mL of glyoxal was added and the mixture was heated slowly to a mild boiling. Glycerol act as plasticizer whilst, glyoxal acted as cross-linking agent. Films were casted into square plate (20 × 20 cm). The casting plate was placed for 24 h in an oven (Memmert) set at 60°C. The same step was repeated for the preparation of 1, 1.5, 2, and 2.5% (w/v) with additional of 1, 1.5, 2 2.5 g of thymol, respectively. The control film was prepared with no thymol being added to the film solution.

**PHYSICAL CHARACTERIZATION**

**Surface Study**

Scanning electron microscopy analysis was done using the method suggested by Cao et al. (2008) with minor modification. It has resolution in VP mode of up to 2 nm at 30kV with high probe current (up to 20 nA) and high stability better than 0.2%h for analytical applications. Scanning electron microscope (SEM, Zeiss Supra 35 VP, Germany) was used to evaluate the surface characteristic and the cross section of films under magnification 50×, 500×, and 1000×. The in-lense detector option was used for a clear topographic imaging in high vacuum mode which were later analyzed to study surface morphology of the produced films.

**Mechanical Strength of Film**

The mechanical properties of films were determined using Lloyd LRX materials testing (Lloyd Instruments Ltd, Fareham, UK). This test method conforms to the standard of ASTM D-638-03. The measurements were performed using a 2000 N load cell and a cross-head speed of 5 mm/min. The films were cut into 9.53 mm × 3.18 mm. Thickness of films was measured using a Digimetric Micrometer (Mitutoyo, Japan) to the nearest 0.001 mm at 10 random positions around the strip film, and the average values were used in calculations. Five parallel determinations were made for each sample. Average values for Young’s modulus (YM) and tensile strength (TS) were determined.

**CHEMICAL CHARACTERIZATION USING FOURIER TRANSFORM INFRA-RED (FTIR) ANALYSIS**

Perkin-Elmer Spectrum One FT-IR Spectrometer was used to study the chemical composition and chemical bonding presence in the prepared films. Before the FTIR analysis, the prepared films were dried overnight in an oven at 40°C. Then, each of the dried film was grounded into fine powder. 4 mg of grounded films were mixed with 10 times as much KBr powder. Hydraulic press was used to form the sample pellets under a pressure of 500 kg/cm². Preared samples were analyzed using Perkin-Elmer Spectrum One FT-IR Spectrometer with a resolution of 4 cm\(^{-1}\) in the range of 4000 – 400 cm\(^{-1}\) and was averaged over 16 scans.
Thermal Characteristic of AM/AMI Films by Thermo Gravimetric Analysis (TGA)

TGA analysis was done using the method suggested by Ramos et al. (2012) with minor modification. The analysis was performed with a Mettler Toledo thermal analyser, model TGA/SDTA 851, USA. Approximately 5 mg samples were heated at 10°C min⁻¹ from 0°C to 600°C under nitrogen atmosphere (flow rate 20 mL min⁻¹). Initial degradation temperature (T₅) was determined as the temperature at which 5% mass loss was observed.

Thermal Properties Study using Differential Scanning Calorimetry (DSC)
The thermal properties of the starch-based films were investigated using a Perkin-Elmer DSC-7 differential scanning calorimeter (DSC) in accordance with ASTM Method D3417-83. Film samples approximately 5 mg were weighed into an aluminium pan (40 μL). Nitrogen was used as the purging gas for all the DSC measurements. An empty aluminium pan was used as a reference. The crystalline melting temperature (Tₘ) was determined by heating the samples from 25°C to 300°C at a rate of 10°C min⁻¹ and with a flow rate of nitrogen of 50 mL min⁻¹. The Tₘ of each sample was determined from the temperature axis on its thermogram and Tₘ was taken at the maximum of the endothermic peak.

Microbiological Study of AM/AMI Starch Based Film Using Agar Diffusion Method (Zone Inhibition Assay)

The present sub-topic discussed the method to determine the efficacy of thymol incorporated into starch-based film to inhibit the growth of microorganism. The antimicrobial activity testing was based on the agar diffusion method.

The agar diffusion test was carried out using the method described by Dawson et al. (1996). The strain selection represented typical spoilage organism groups commonly occurring in various kinds of food products. The strains were as follows: *Escherichia coli*, a conventional hygiene indicator organism, a Gram-negative rod belonging to the same family of *Enterobacteriaceae* as for example *Salmonella*, *Bacillus subtilis*, a Gram-positive rod capable of forming heat-resistant spores. Spores and vegetative cells of *Bacillus* species are widely distributed in nature and are common for example in cereals (Nozieana et al. 2019). For the agar plate test, the starch-based films containing AM agent were cut into six squares (0.5 cm × 0.5 cm). Six sample squares were then placed onto the plate which was spreaded with bacteria (0.1 mL per plate). The same tests were performed using other film containing, stated: thymol in various concentrations. Duplicate agar plates were prepared for each type of film and control film. The agar plates were incubated at 37°C for 48 h.

Statistical Analysis

Data obtained from experiments were analyzed by using SPSS statistical analysis software version 17.0. Statistical analysis was performed using ANOVA as it could determine whether the differences among two or more means greater than that would be expected from sampling. The statistical analysis was focussed on one factor effect and the analysis was obtained via one-way ANOVA. The data were presented as mean values ± standard deviation (SD). The statistical significance of the results was evaluated using Duncan’s multiple range test (DMRT) at a significance level of R = 0.05. Significance between means represents the validity of different value level in a set of data.

Results and Discussion

Physical Appearance of AM Films

Understanding the film-forming characteristic is of practical importance in producing starch films or coating with desirable microstructures that determine their performance (Liu & Han 2005). Knowledge on physicochemical properties of a film would allow a more efficient selection of films for their specific and suitable applications. Characterization of film in this study has been divided into two categories: the physical- and chemical characterizations.

Figure 1(a)-1(f) show the appearance of all formulations of AM films incorporated with 0 (control), 0.5, 1, 1.5, 2, and 2.5% w/v of thymol, respectively. The control film produced without thymol being added was completely transparent, smooth, and glossy (Figure 1(a)). The presence of thymol (Figure 1(b), 1(c), & 1(d)) in all concentrations (0.5, 1 and 1.5 % w/v) did not greatly affect the films’ appearance. No changes were observed in the films containing low concentrations of thymol compared to the control one; they were still found to be translucent and clear. Higher concentrations of thymol (2 and 2.5% w/v) in the films contributed to a change in the colour of the film to light creamy, and to its dull surface (Figure 1(e) and 1(f)). These results, in fact, were found to be similar to the reported findings by other researchers where they also reported the changes in colour of the film with the variation of thymol concentration (Gniewosz & Synowiec 2011).

Physical Characterization

Generally, physical characterization study of the prepared film will give information on the physical properties, shape, look, esthetical, and strength of the film. In this study, the opacity and morphological structure of film were determined by the SEM analysis. Information on the mechanical properties of the film was obtained from the tensile strength analysis whereas the water uptake analysis and moisture content determination will present valuable information on the film’s behaviour towards water.

Microstructure Analysis for Surface Study

Figure 2(a) - 2(f) display the results of surface micrograph under magnification of 500×. A better surface study of control and thymol-incorporated films are shown in the
figures. Although more compact surfaces could be perceived as the amount of thymol increased, the micrograph of film surfaces still showed a relatively smooth and continuous surface with no differences due to the inclusion of thymol. This is in agreement with previous study by Salarbashi et al. (2013) who concluded that based on their SEM analysis, the micrograph for all films containing essential oils were homogenous, without signs of phase separation between components. Likewise, Ramos et al. (2014) as well observed homogenous surface morphologies for all materials with no apparent effect over the addition of thymol and AG-Nps to their PLA matrix. Besides, Salleh et al. (2014) also suggested that strong interactions were present between the hydroxyl groups of starch and the amino groups of natural AM (chitosan) resulting in a good miscibility between starch and chitosan in the blended films. A small white spot that were observed on the films could be attributed to small particles produced during the film fracture before observation that was deposited on the film’s surface (Salarbashi et al. 2013).

The control film with no thymol being added as in Figure 2(a) shows a relatively smooth and homogenous surface; verified a good film forming properties. It proves that the control film solution without the addition of AM agent used for the formation of the film examined was homogenous and constituted a homogenous grid of the film after drying. This is due to a good homogeneity with a continuous matrix without any pores or cracks with a good structural integrity (Chen et al. 2008).

Although a smooth and continuous microstructure was observed for the pure starch based film, AM-combined films showed discontinuities, even to a lesser extent due to the presence of the lipid dispersed phase in the polymer matrix. Generally, in the films containing a lower concentration of thymol (Figure 2(b) & 2(c)), oil droplets were intimately integrated within the film matrix and were less evident on the film surface.

However, coarser microstructures were observed in the films that incorporated 1.5, 2.0, and 2.5% w/v of thymol (Figure 2(d) – 2(f)) are due to the fact that higher lipid content favours the flocculation rate. These results are in accordance with recent study by Sanchez-Gonzalez et al. (2009) who reported that a slight enlargement in lipid droplets may be caused by the deformation forces that act during the polymer chain aggregation during the solvent evaporation.

**Mechanical Strength of Film**

Tensile properties such as tensile strength (TS) and Young’s modulus (YM) have been evaluated from the experimental stress-strain curves obtained for all prepared films.
Figure 3(a) presents the result obtained from the tensile analysis data. The addition of 0.5 to 1% w/v of thymol had no significant effect on the tensile strength. This is in agreement with previous study by Ramos et al. (2014) who found that the addition of thymol and carvacrol in polypropylene film caused a slight modification on tensile properties. However, in contrast to the results obtained for 1.5% to 2.5% w/v of thymol, a slight increase was perceived in tensile properties of the films. This is in contrast with previous study that detected decreasing in tensile strength properties (Li et al. 2012; Shojae-Aliabadi et al. 2014; Wu et al. 2014). In fact, decreasing of tensile strength may be linked to the carrageenan ability to interact with other components such as essential oils. However, the strong electrolyte property of carrageenan may cause a decrease in intermolecular polymer interactions due to the polar-non polar barrier of the different components (Karbowiak et al. 2006). Considering the fact that the interactions between polymer and phenolic compounds depends on the characteristics of the phenolic compounds and the individual phenolic constituents, the effectiveness of thymol in increasing tensile strength is probably linked

FIGURE 3. (a) Tensile strength and (b)Young’s modulus of AM film with different concentration of thymol
to its capacity for interaction with the starch based matrix (Kroll & Rawel 2001).

Figure 3(b) depicted the Young’s Modulus for control film (no thymol being added) and AM films, containing thymol in the range of 0.5 to 2.5% w/v. These results are in accord with recent studies indicating that the Young’s Modulus of biobased materials ranges from 2500 - 4000 MPa. It can be seen that there is no significant difference of the Young’s Modulus for control film compared with AM film containing 0.5, 1 and 2% w/v of thymol. However, AM film containing 1.5% w/v of thymol shows a slight increase of Young’s Modulus by 16.52%. Whilst, a significant decrease of Young’s Modulus by 20.91% was perceived from the AM film containing 2.5% w/v of thymol. Previous study by Ramos et al. (2012) also suggested that the addition of the highest concentration of carvacrol and thymol to polypropylene film resulted in a significant decrease of the elastic modulus of the film in compared to the control (PP0) film by 20.45%. This behaviour could be explained by some plasticizing effect caused by the addition of both additives to the polymer matrix resulting in the increase in ductile properties, which would also result in changes in the materials crystallinity.

CHEMICAL CHARACTERIZATION

FTIR was used to determine the effect of incorporation of substance on the structural changes of film (Kroll & Rawel 2001). The infra-red spectra of control film and AM films are shown in Figure 4. Interestingly, the result implies the consistency of the chemical composition and structure of the AM film compared to the control film. This clearly indicates that the addition of thymol into the HEC/starch/thymol based film did not affect or alter the carbonyl functional group of the starch-based film. This indicates that there is no chemical interaction occurred between thymol and starch matrix.

For the spectrum of control film, the strong and broad absorption peak at 3381.60 cm\(^{-1}\) was assigned to the characteristic absorption peak of the stretching vibration of \(-\text{OH}\). The bands at 1155.68 cm\(^{-1}\) and 1112.68 cm\(^{-1}\) were attributed to the stretching vibration of C-O in C-O-H groups and the band at 1042.32 cm\(^{-1}\) was attributed to the stretching vibration of C-O in C-O-C groups. The bands detected on the starch-based film were in agreement with the previous study of starch-based film (Soares et al. 2005; Xiong et al. 2008).

Figure 4 shows overlapping spectra for control film and AM films, indicating no alteration of the absorbance spectra of the HEC-wheat starch based films due to the inclusion of thymol. This is in agreement with previous study by Marcos et al. (2010) who found that the addition of AM into zein films did not alter carbonyl groups. It can be seen that no significant changes were monitored for film containing 0.5% w/v of thymol compared to control film. However, it can also be seen from Figure 4 that the addition of thymol resulted in a considerable increase in intensity of the O-H peaks from the films containing 1 - 2.5% w/v of thymol. Interestingly, the increase of the intensity was related to the presence of phenolic group band from 3200 – 3650 cm\(^{-1}\) corresponding to C-O bending from thymol (Torres et al. 2014). Besides, it is possible to observe the characteristic bands for thymol on the bending vibration of =C-H in the range of 900 to 690 cm\(^{-1}\).
and a pair band of aromatic C=C stretches at 1600 – 1585 and 1500 – 1400 cm\(^{-1}\) (Hasnah et al. 2000).

**THERMAL CHARACTERISTIC OF AM FILMS**

*Thermal Characteristic of AM Films by Thermo Gravimetric Analysis (TGA)*

Thermal analyses of the films were carried out to study the influence of the addition of thymol on the thermal stability and morphology of the polymer under nitrogen atmosphere. A better understanding on the thermal degradation of control (without the additional of thymol) and AM films was summarized in Figure 5. As shown in Figure 5, the control AM gives the lowest thermal stability whilst, the addition of thymol shifted the thermal stability of the films to a higher temperature. The AM film containing 1.5% (w/v) of thymol shows the highest thermal stability and decomposes less in comparison to other samples.

The thermogram of control film shows three consecutive weight loss steps. The first weight loss step was about 19.22 wt. % at 50°C to 70°C which was responsible for the loss of moisture content indicating its hygroscopic nature. The second degradation step was about 37.82 wt. % in the range of 142°C to 215°C is attributed to chemisorbed water through hydrogen bonds due to the presence of glycerol (Cerqueira et al. 2014; Quijada-Garrido et al. 2007). The third degradations step (maximum peak from 268 - 315°C) is related to decomposition of the samples including depolymerisation, and pyrolytic decomposition of the polysaccharide backbone as was confirmed previously by Cerqueira et al. (2014) and Zohuriaan and Shokrolahi (2004).

As can be seen in Figure 5, the thermograms curves suggested that the addition of thymol slightly improved the thermal stability of the polymer due to the antioxidant character of thymol. As indicated by Torres et al. (2014), polymer thermal degradation was protected by the presence of alkyl radicals, thymol which play a role as the radical scavenger in the matrix. TGA thermogram of the film containing 0.5% w/v of thymol shows similar behaviours as the control film, with the presence of three thermal events. The first minor one was due to the loss of water from 50°C to 80°C with a weight loss of 8.990%. The second and third decomposition happened from 139°C to 211°C and from 268°C to 311°C with weight loss of 30.06% and 41.58%, respectively. The second step was due to chemisorbed water through hydrogen bonds due to the presence of glycerol (Quijada-Garrido et al. 2007). Surprisingly, the second degradation step was believed to be associated to the degradation of thymol as suggested in previous research by Ramos et al. (2014, 2012) where in their study, losses associated with thymol degradation was observed at 120°C and continued up to 280°C. The third degradation step (maximum peak from 268 - 311°C) is related to decomposition of the samples as being discussed in previous paragraph.

Similar behaviours of TGA patterns were monitored for all the studied films. No significant differences were observed for T\(_{\text{max}}\) values in all samples. These results show consistency along with the findings reported by other researchers which found that the addition of thymol to the polymer matrix did not significantly affect its thermal degradation profile in inert nitrogen atmosphere (Ramos et al. 2014, 2012; Torres et al. 2014; Wu et al. 2014).
**Thermal Properties Study using Differential Scanning Calorimetry (DSC)**

DSC analyses were carried out with the aim of investigating the effect of thymol addition on thermal properties of HEC-wheat-starch based films. DSC thermogram depicted in Figure 6 shows thermal property of control film (no thymol being added) and AM films.

As can be seen from Figure 6, for higher temperatures, the broad endotherm is accompanied by a peak above 270°C as due to a degradation of the samples. These results are consistent with data obtained from TGA analyses as reported in previous sub section. Bershtein and Egorov (1994) described a possible explanation for this might be related to the evaporation of water present in the samples that occur over a large temperature interval (about 150°C for starch).

The results of this study indicate that 1.5% w/v of thymol shows the highest thermal stability and decomposes less in comparison to other samples. This is in agreement with previous result in thermal characteristic of AM films by thermo gravimetric analysis (TGA) section and seems to be consistent with other research which found that the addition of 3.8% (w/w) thymol in the formulation caused a decrease in the $T_m$ compared to the control TPS film (Kuorwel 2011). The same researcher also suggested that the addition of essential oils like thymol into the TPS film decreased the intermolecular interaction within the film that in turn, caused the decrease in the degree of crystallinity. According to Garcia et al. (2009), the addition of additives into a polymeric material may interfere with chain association and cause a possible decrease in the film crystallinity.

**ANTIMICROBIAL ACTIVITY OF DEVELOP FILM HEC/STARCH/THYMOL (AM FILM) USING THE AGAR DIFFUSION METHOD**

Antibacterial activity of AM film against two pathogenic bacteria was expressed in terms of zone inhibition. The agar diffusion test simulates the wrapping of foods and therefore can be used to estimate how much the antimicrobial agent migrates from the film to the food when the film contacts the contaminated surfaces (Appendini & Hotchkiss 2001; Han 2006). This simple technique was based on the measurement of the clear zone caused by growth inhibition produced by a film disk containing antimicrobial agent when placing in direct contact with a bacterial culture (Ramos et al. 2012; Weerakkody et al. 2010).

All samples were examined for possible inhibition zones after incubation at 37°C for 48 h. Table 1 lists the calculated inhibition area for each plate test after 48 h of incubation. Surprisingly, control film with no thymol being added shows inhibition zone after 24 h for all types of bacteria. However, after 48 h of incubation, colonies of bacteria were spotted on the agar. A possible explanation for this might be that the addition of glyoxal to the film solution acts as a crosslinker. Kittinavarat and Kantuptim (2005) combined glyoxal with chitosan to acquire a better antibacterial activity. Glyoxal has been proven to have a fair antibacterial property. However, glyoxal alone in the film failed to inhibit the growth of gram-positive and gram-negative bacteria after 48 h of incubation.

Clear zones on the petri dish indicated that the inhibitory action of thymol against the growth of bacteria tested was observed around the films incorporated with thymol as AM agents. With an increase in the thymol content in the film, a successive increase was perceived in diameters of the inhibition zones. The addition of 1 to 2.5% w/v of thymol to the HEC-wheat starch based film solution significantly affected the size of the $E. coli$ inhibition zones formed by those films. This finding is in line with the previous study by Gniewosz and Synowiec (2011) who reported that the size of the formed zones was significantly affected by the addition of 1.2%, 1.5%, and 3% of thymol to the pullulan film solution films. In fact, there are quite many of researches that confirmed the antibacterial activity...
of thymol against gram negative bacteria, e.g. E. coli (Helander et al. 1998; Matias & Beveridge 2005; Trombetta et al. 2005). E. coli, a gram negative bacteria, consist of thin peptidoglycan layer about 5 - 10 nm thickness that surrounds the cytoplasmic membrane and is bounded by an outer membrane composing of phospholipids on the inner leaflet and lipopolysaccharide on the outer leaflet. Helander et al. (1998) have described in their previous research that thymol disintegrated the outer membrane and increased the permeability of the cytoplasmic membrane to ATP of Escherichia coli and Salmonella typhimurium cells.

| Concentration of thymol in each film (w/v) | Diameters of growth inhibition zone (mm ± SD) |
|-------------------------------------------|---------------------------------------------|
|                                           | E. coli                                     |
|                                           | B. subtilis                                 |
|                                           | A. niger                                    |
| 0.0%                                      | nd                                         |
| 0.5%                                      | 38.7 ± 0.5a                                 |
|                                           | 41.3 ± 0.59b                               |
|                                           | 7.0 ± 0.06a                                |
| 1.0%                                      | 42.3 ± 0.21a                               |
|                                           | 41.3 ± 0.15b                               |
|                                           | 5.0 ± 0.006a                               |
| 1.5%                                      | 41.0 ± 0.106a                              |
|                                           | 40.0 ± 0.26a                               |
|                                           | 10.5 ± 0.07a                               |
| 2.0%                                      | 41.0 ± 0.066a                              |
|                                           | 40.3 ± 0.066a                              |
|                                           | 8.5 ± 0.07a                                |
| 2.5%                                      | 43.7 ± 0.606a                              |
|                                           | 48.7 ± 0.316a                              |
|                                           | 5.5 ± 0.076a                               |

nd: no detection of inhibition zones; *means in the same column with the same letter are not significantly different (P>0.05)

B. subtilis, a gram positive bacterium is composed of a cytoplasmic membrane bound by a thick peptidoglycan layer, which has a thickness between 20 and 80 nm (Matias & Beveridge 2005). A similar effect of inhibition activity was monitored for all concentrations of thymol indicated the ability of thymol to inhibit the growth of B. subtilis in a wide range of concentration. This is not surprising as thymol has a strong antibacterial activity towards gram positive bacteria (Delgado et al. 2004; Ettayebi et al. 2000; Nazzaro et al. 2013; Trombetta et al. 2005). The cell wall structure of the Gram-positive bacteria allows hydrophobic molecules to easily penetrate the cells and act on the cell wall and within the cytoplasm. Phenolic compounds, which are also present in the EOs, generally show antimicrobial activity against Gram-positive bacteria (Nazzaro et al. 2013). Their effect depends on the amount of the compound present; at low concentrations, they can interfere with enzymes involved in the production of energy while at higher concentrations, they can denature proteins (Tiwari et al. 2009).

Aspergillus niger is a ubiquitous filamentous fungus found in grains, fruits, forage, mouldy vegetables, and dairy products. It is also a pathogenic mould that can cause ootomycosis and aspergillosis (Arras & Usai 2001; Bulpa et al. 2007). Table 1 lists the calculated inhibition area of plates containing A. niger in diameter. The control films showed no inhibition area and colonies were formed all over the plate. However, the films containing thymol shows an inhibition towards the growth of A. niger. This is in agreement with previous study that showed the ability of thymol to inhibit the growth of A. niger (Ayala-Zavala et al. 2009). Numpaque et al. (2011) suggested that the antifungal nature of thymol is caused by the ability of thymol to alter the hyphal morphology and cause hyphal aggregates, resulting in reduced hyphal diameters and lyses of hyphal wall. Additionally, thymol is lipophilic, enables it to interact with the cell membrane of fungus, altering the cell membrane permeability by permitting the loss of macromolecules (Šegvić Klarić et al. 2007).

The main purpose of this current section was to investigate the effect of antibacterial agents towards the inhibition of microbial activity. This study has found that generally, thymol has successfully inhibits the growth of selected gram positive and gram negative bacteria in a wide range of studied concentration. Thymol has also been proven to show the ability to inhibit the growth of A. niger.

### CONCLUSION

The present study discussed the antibacterial properties of the AM films incorporated with thymol in the range of 0.5% to 2.5% (w/v). As supported by previous study, essential oils have a synergistic effect towards inhibiting the microbial growth in a wide range of concentration even in a small amount. The findings indirectly provide a low cost manufacturing of films because a small amount of thymol could be selected for production of AM films. Physical, chemical and thermal characterization of the present research suggested that 0.5 to 1% of thymol did not give significant changes on its properties, whilst 1.6 to 2.5% gave a slight change of the physical, chemical, and thermal properties. The microbial inhibition analysis had shown that film containing 1.5% (w/v) of thymol gave the best inhibition activity towards all selected microorganisms; E. coli, B. subtilis and A. niger.

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