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To cite this article: Nozieana Khairuddin *et al* 2019 *IOP Conf. Ser.: Mater. Sci. Eng.* **607** 012007

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Microbial study of pH sensitive starch based film using agar diffusion method (zone inhibition assay)

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Abstract. Active and smart packaging is a promising form of food packaging that offers a great economical potential due to consumer demand for a packaging that accommodate a hectic way of life. An antimicrobial film with pH colour indicator (pHF) can be made by incorporating suitable antimicrobial (AM) agent and colour indicator into food package matrices whilst applying a bio switch concept to inhibit the pathogenic microorganisms and respond automatically to changes (external stimuli) in the environment. The present work aimed to study the developed formulation of hydroxyethylcellulose (HEC)/wheat-starch based pHF film in which the active compound, thymol (0.5, 1, 1.5, 2, and 2.5% w/w) and 50:50% w/w bromothymol blue and methyl red (as the colour indicator) against microbial growth. A solution casting method was used in the film preparation while thymol and colourant were incorporated prior to casting. The effect of thymol showed a range of microbial inhibition zones of 16.3 - 26.4% and 22.1 - 39.9% towards *E. coli* and *B. subtilis*, respectively. Whilst, a lower inhibition zone of 0.4 - 5.1% was demonstrated for fungus *A. niger*.

Keywords: Active and smart packaging; antimicrobial; thymol; hydroxyethylcellulose; methyl red; bromothymol blue

1. Introduction

The food and beverage packaging has dramatically shifted from traditional to advanced packaging. Traditional packaging only addresses issues related to protection from external factors. However, advanced packaging interacts internally (active packaging) and externally (intelligent packaging) with the environment and enhances the visual appeal of the products. Therefore, manufacturers of food and beverages and packaging manufacturers are looking into the way the physical, chemical and microbial textures change inside the packaged food. The requirement over smart or intelligent packaging is changing rapidly due to the world awareness on environment. The concerns are not only on the materials and productions, but also on legal side related to the environmental concerns.



The general concept of bio-switch describes a system with capability to detect and respond automatically to changes (external stimuli) in the environment. For instance, the external stimulus may be a change in pH, or the presence of certain metabolites from biological activity. The bio-switch converts this stimulus into a particular functionality. Materials with ability to entrap compounds with a specific function which are released on an external stimulus from the environment are created. In order to be used in active packaging, biopolymer-based particles containing antimicrobial will only be released in the case of initial microbial contamination. The bio-switch particles monitor the releasing system by the stimulus of a microbial contamination that actively add or emit compounds i.e. antimicrobials, antioxidants, and preservatives to the packaged food or onto the surface of the package [1].

The current work is a novel combination of active packaging technology and smart packaging concept where the stimulus of a microbial contamination is further incorporated with an indicator to signal the conditions in the packaged food. The present work aimed to study the developed formulation of hydroxyethylcellulose (HEC)/wheat-starch based pHF film in which the active compound, thymol and the duo-colour indicator against microbial growth.

2. Materials and Method

2.1. Materials

The main based of the films which were wheat starch ($C_6H_{10}O_5$)_n and hydroxyethyl cellulose were supplied by Merck. Thymol ($C_{10}H_{14}O$) was purchased from Sigma-Aldrich (Malaysia), methyl red (MR), bromothymol blue (BB) were purchased from Fluka, Glyoxal was purchased from Merck and glycerol was purchased from HmbG chemicals.

2.2. Film Preparation

In this research, 0.5 g thymol was dissolved in 20 ml of absolute ethanol. Then 0.01 g of bromothymol blue and methyl red was added to the solution respectively and stirred well. The solution was then filtered using filter paper. The filtrate was added to the 80 ml 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. Films were casted into square plate (20 x 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) of thymol. The control film was prepared with no thymol being added to the film solution.

2.3. Microbiological Study of 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 strain selection represented typical spoilage organism groups commonly occurring in various kinds of food products. The strains were as follows: (1) *Escherichia coli*, a conventional hygiene indicator organism, a Gram-negative rod belonging to the same family of *Enterobacteriaceae* as for example *Salmonella*. (2) *Bacillus subtilis*, a Gram-positive rod capable of forming heat-resistant spores. Spores and vegetative cells of *Bacillus* species are widely distributed in nature and are common for example in cereals. For the agar plate test, the starch-based films containing AM agent were cut into six squares (0.5 cm x 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 hours.

3. Results and Discussion

3.1 Film Appearances

Figure 1 shows the visual aspect of all formulations of pH sensitive films incorporated with 0, 0.5, 1, 1.5, 2, and 2.5 % w/v of thymol for film pHF-0, pHF-0.5, pHF-1, pHF-1.5, pHF-2, and pHF-2.5 respectively with the addition of colour indicator. The control film produced without thymol being added was completely translucent, smooth, and glossy (pHF-0). No visual changes were observed in the films containing low concentrations of thymol, compared with the control film; they were still translucent and clear.

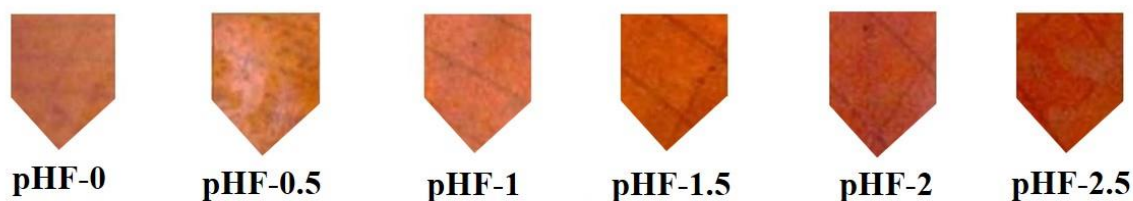


Figure 1. Visual appearance of the films with different concentration of thymol

Figure 2 shows the visual colour changes of the prepared indicator solutions. A reliable colour changes are required for production of indicator label as it will be a suitable communication-tool for the consumers [2]. The main purpose for applying colorimetric mixed-dye-based indicators to food packaging is to easily and reliably monitor the level of food spoilage of packaged food products in a non-destructive manner during distribution and retail sale [3]. It was found that indicator solutions showed a clear spectrum from bright light orange to dark blue when exposed to a pH level range of 1 - 14. However, similar colour spectrum could be monitored with naked eyes for indicator solutions in the range of pH 1 till 5. All the solutions changed into bright light orange, red-orange and orange.

An obvious transformation of the colour indicator was observed in the range of pH 5 till pH 8 (Figure 2). It was found that indicator solutions showed a clear spectrum from bright orange, red orange, yellow, green, and blue when exposed to pH 5, 6, 7, and 8 respectively. This is not surprising as the colour indicator of MR best works in the range of pH 4.4 to 6.2, while BB best works in the pH ranges from 6 to 7.6 [4]. The most obvious finding emerged from the analysis is that the most obvious colour change of the indicator solutions found in this study falls in the range of most pH for food deterioration. Moreover, it is also in the range of optimum pH for microbial growth which was the main factor of food spoilage [5].

Whereas, a similar blue spectrum could be observed with unaided eye for all indicator solutions in the pH ranges of 9 to 14. This is due to the effect of BB which changed to blue when reacted with pH more than 7.6. These findings have significant implications for the understanding of how the indicator solution will work in the HEC-wheat starch based film incorporated with thymol.

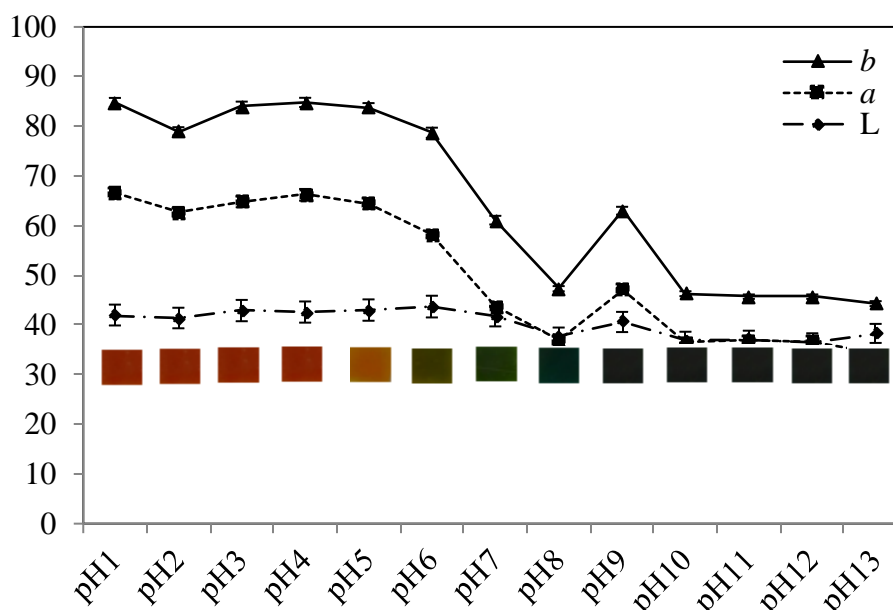


Figure 2. Change in colour of indicator solutions in response to pH buffer

3.2 Microbiological assessment of pHF film using agar diffusion method

The present test focus on the efficacy of the films to inhibit the growth of selected Gram positive food pathogenic bacteria i.e. *Bacillus subtilis* and Gram negative food pathogenic bacteria i.e. *Escherichia coli*. Agar plate test, also known as zone inhibition assay was performed as a preliminary step to screen the antibacterial activity of all films formulations, in an effort to select film formulations with high antibacterial activity against the test bacteria. The inhibitory zone in agar diffusion test can be affected by the solubility and diffusion rate of the test compounds in agar medium, thus agar diffusion test does not accurately reflect the antimicrobial effectiveness of the test compounds [6, 7].

All samples were examined for possible inhibition zones after incubation at 37°C for 24 hours. Table 2 lists the calculated inhibition area for each plate test. A similar finding to the previous research had been acquired. The control films showed no inhibition area and colonies were formed all over the plate for both types of microorganisms: the gram positive and gram negative bacteria. It can therefore be assumed that the colour indicator (MR and BB) did not have any antibacterial properties. Hence, the antibacterial activity provided by the films is primarily derived from thymol.

Table 1. Analysis of the zone of inhibition data in agar plate test for *E. coli*, *B. subtilis* and *A. niger* at 37°C in the presence of pH sensitive film incorporated with thymol

Films	Concentration of thymol in each film (w/v)	Diameters of growth inhibition zone (mm ± SD)		
		<i>E. coli</i>	<i>B. subtilis</i>	<i>A. niger</i>
		48 h	48 h	48 h
pHF-0	0.0%	nd	nd	nd
pHF-0.5	0.5%	41.0 ± 0.20 ^{a,b}	44.3 ± 0.31 ^b	22 ± 0.00 ^d
pHF-1	1.0%	39.0 ± 0.00 ^a	42.3 ± 0.21 ^{a,b}	19.5 ± 0.07 ^{d,e}
pHF-1.5	1.5%	39.3 ± 0.11 ^a	42.3 ± 0.06 ^{a,b}	19.5 ± 0.35 ^{d,e}
pHF-2	2.0%	38.0 ± 0.00 ^a	45.0 ± 0.85 ^c	17.5 ± 0.07 ^f

pHF-2.5	2.5%	36.3 ± 0.06^a	45.3 ± 0.32^b	6.5 ± 0.07^s
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nd: no detection of inhibition zones.

*means in the same column with the same letter are not significantly different ($P > 0.05$)

Clear zones on the agar indicating the inhibitory action of thymol against the growth of bacteria tested were observed around films incorporated with thymol as AM agents (Figure 3). pH sensitive films containing thymol effectively inhibit the growth of *E. coli* and *B. subtilis*. However, no significant effect on the concentration of thymol caused the inhibition effect. Yet again, as discussed in previous research by Tiwari and friends in 2009, thymol has the ability to kill the microorganism in wide range of concentration including a low concentration (0.5 % w/v) as chosen in this study [8].

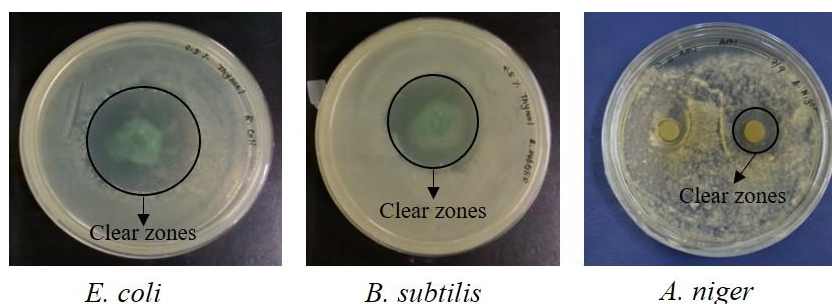


Figure 3. Inhibition of *Escherichia coli*, *Bacillus subtilis* and *Aspergillus niger* on solid media by pHF film incorporated with thymol after incubation for 24 hours at 37°C.

Conclusion

The present paper discussed the antibacterial properties of the pHF 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. In conclusion, pH colour indicators give no significant effect on the ability of thymol to inhibit the microbial growth compared to pHF-0 film. It is not surprising to perceive an effective inhibition of the growth of selected gram positive (*B. subtilis*) and gram negative (*E. coli*) bacteria in wide range of studied concentration of thymol. pHF films containing thymol has also proven to have the ability to inhibit the growth of *A. niger*.

Acknowledgement

The authors would like to thank the Ministry of Education for FRGS grant (FRGS/1/2018/TK05/UPM/02/8) and Research Management Centre UPM for their support of this study.

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