ANTIMICROBIAL EFFECTS ON STARCH-BASED FILMS INCORPORATED WITH LYSOZYMES

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ABSTRACT

An antimicrobial (AM) Active Packaging can be made by incorporating and immobilizing suitable AM agents into food packages and applying a bio switch concept. A starch-based film was prepared and incorporated with antimicrobial agents, i.e. lysozyme and EDTA as chelating agent. This film was then inoculated with the bacteria Escherichia coli and Bacillus subtilis to carry out the microbial contamination study. The inhibition of both E coli and B subtilis by the AM film was clearly observed in the broth and culture agar test. The decreased of optical density (O.D600nm) showed the inhibition of both E coli and B subtilis growth. While, the clear zones formed on the film appearance showed that AM agents give good inhibition to the growth of E coli and B subtilis with satisfying inhibition rate. The moisture content was determined to observe the differences between control film and antimicrobial film incorporated with lysozyme.

Keywords: *Antimicrobial agent, antimicrobial film, lysozyme, bio-switch concept, Escherichia coli, Bacillus subtilis*

INTRODUCTION

Starch (Figure 1) is a naturally abundant nutrient carbohydrate, $(C_6H_{10}O_5)_n$, found chiefly in the seeds, fruits, tubers, roots, and stems pith of plants, notably in corn, potatoes, wheat, and rice, and varying widely in appearance according to source but commonly prepared as a white amorphous tasteless powder. Wheat (Triticum spp.) is a grass that is cultivated worldwide. Globally, it is the most important human food grain and ranks second in total production as a cereal crop behind maize; the third being rice. Wheat is widely cultivated as a cash crop because it produces a good yield per unit area, grows well in a temperate climate even with a moderately short growing season, and yields a versatile, high-quality flour that is widely used in baking.

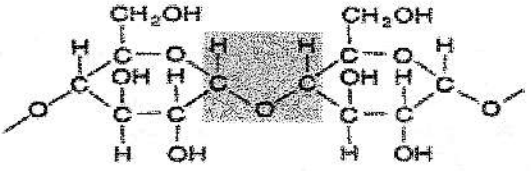

Figure 1: Chemical structure of starch

As a packaging material, starch alone does not form films with adequate mechanic properties (high percentage elongation, tensile, and flexural strength) unless it is first treated by either plastization, blending with other materials, genetic or chemical modification or combinations of the above approaches. Plasticizing agents such as glycerol, sorbitol or polyethylene glycol, mono-, di- or oligosaccharides, fatty acids, lipids and derivatives, are usually used to overcome film brittleness and improve its flexibility and extensibility [1].

Antimicrobial packaging is a form of active packaging which acts to reduce, inhibit or retard the growth of microorganisms that may be present in the packed food or packaging material itself [2]. Common antimicrobial substance for food products are such preservatives as organic acids, antimycotics (fungicide), enzymes, oxygen absorber, alcohol etc. [3]. Many AMs are incorporated at 0.1%-5% w/w of the packaging material, particularly films [2].

Lysozyme (Figure 2) is 129 amino acid residues enzyme (EC 3.2.1.17) which catalyze hydrolysis of 1,4-beta-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in a peptidoglycan and between N-acetyl-D-glucosamine residues in chitodextrins. Lysozyme is an enzyme found in egg white, tears

and other secreations. The enzyme is antibacterial because it degrades the polysaccharide that is found in the cell walls of many bacteria. It does this by catalyzing the insertion of a water molecule at the position indicated by the red arrow (a glycosidic bond) (Figure 3). This hydrolysis breaks the chain at that point. It has been investigated that compounds as lysozyme is active against Gram-positive bacteria and can target Gram-negative bacteria when combined with chelating agents (i.e. EDTA). Gram negative bacteria possess an outer cell membrane that must be penetrated before the antimicrobial compound can reach the effective membrane suite. Penetration through the outer membrane ncan be accomplished by the use of a chelating agent (i.e. EDTA) or by osmotic shock [4]. Previous study showed that the outer membrane of gram-negative bacteria possesses divalent cations that stabilize the lipopolysaccharide association within the membrane, which is believed to hinder the ability of nisinand other molecules to reach the cytoplasmic membranes [4 – 5]. Addition of EDTA to edible film containing nisin or lysozyme, however, had little inhibition effect on *E. coli* [6] and S. *typhimurium* [7]

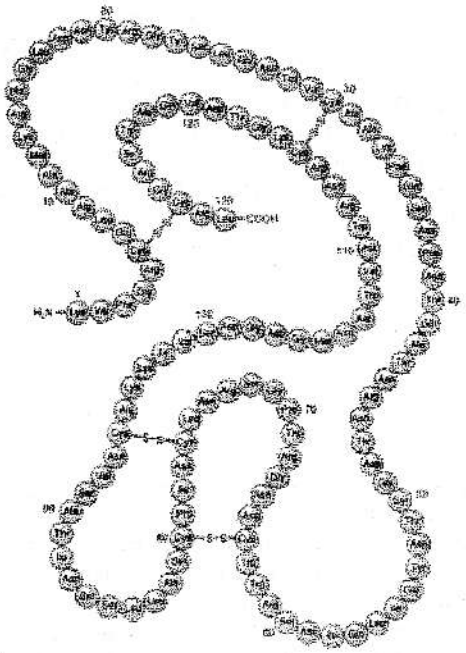

Figure 2: The primary structure of egg white lysozyme

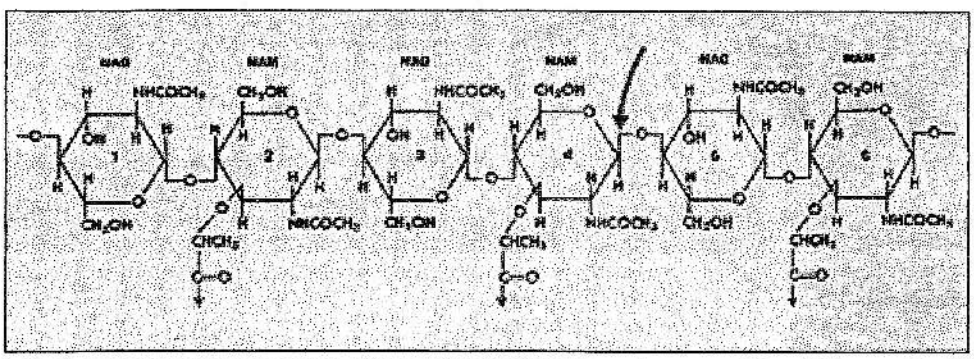

Figure 3: Bacterial polysaccharides consists of long chains of alternating amino sugars; N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM)

The present paper discussed the inhibitory effect of starch based film incorporated with lysozyme against test strains of gram-positive bacteria and gram-negative bacteria. The moisture content of the film was also determined to observe differences of control film and AM film.

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MATERIALS AND METHODS

Preparation of Antimicrobial Starch-Based Film

Starch-based films were prepared by dissolving 8.35 g starch in 80 mL of 20% ethanol with stirring. After the solution was completely dissolved, 3.8 mL glycerin (HmbG Chemicals) was added as plasticizer and the mixture was heated slowly to a mild boiling. For antimicrobial incorporated films, antimicrobial agents; lysozyme (Fluka) were mixed with 10 mL of the film solution in a separated beaker just before casting. Five milliliters of the film mixture was pipetted into petri dishes (100 mm diameter by 15 mm depth). The petri dishes were placed for 24 hour in an oven (Memmert) set at 70°C.

Inhibition of Escherichia Coli and Bacillus Subtilis on Agar Plate Test

The strain selection represented typical spoilage organism groups commonly occurring in various kinds of food products. The strains were as follows: (1) *Escherichia Coli*, a conventional hygiene indicator organism, a Gram-negative rod belonging to the same family of *Enterobacteriaceae* as for example *Salmonella*. (2) *Bacillus subtilis*, a Gram-positive rod capable of forming heat-resistant spores. Spores and vegetative cells of *Bacillus* species are widely distributed in nature and are common for example in cereals. For the agar plate test, the AM starch-based films were cut into six squares (0.5 cm x 0.5 cm). Six sample squares were then placed onto the plate spreaded with bacteria (0.1 mL per plate). The same tests were performed using a control film. Duplicate agar plates were prepared for each type of film and control film. The agar plates were incubated for 48 hours at 37°C in the appropriate incubator. The plates were visually examined for "zones of inhibition" around the film and the results were recorded.

Enumeration

For the liquid culture test, each film was cut into squares (1 cm x 1 cm). Three samples squares were immersed in 20 mL nutrient broth (Merck, Germany) in a 25 mL universal bottle. The medium was inoculated with 200 [*Escherichia Coli* in its late exponential phase, and then transferred to an orbital shaker and rotated at 30°C at 200 rpm. The culture was sampled periodically (0, 2, 4, 8, 12, 24 hours) during the incubation to obtain microbial growth profiles. The same procedure was repeated for the control starch-based film. The optical density ($o.d._{600}$) was measured at $\lambda = 600_{nm}$ using a spectrophotometer (Model UV-160, Shimadzu, Japan).

Moisture Content Determination

The determination of moisture content in this study followed method from Finkenstadt et al., (2004) [8]. A Moisture Determination Balance FD-620 was used to determined the moisture content (MC) of the starch products by gravimetric methods using

$$MC = \frac{M_f - M_i}{M_i} \times 100 \quad (1)$$

For powder and thin sheet samples, the sample was heated for 25 min at 110°C. Determination of moisture content was performed on 3 replicates and the average is reported.

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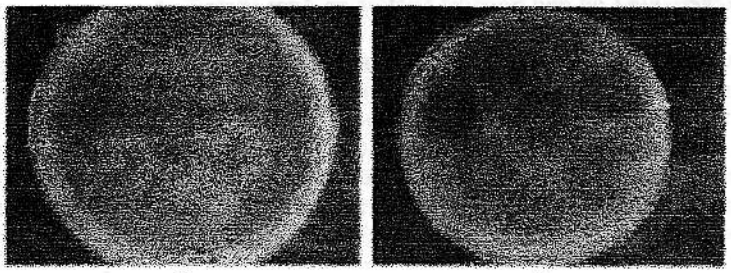
RESULTS AND DISCUSSIONS

Inhibition of Escherichia Coli and Bacillus Subtilis on Agar Plate Test

All samples were examined for possible inhibition zones after incubation at 37°C for 48 hours. Figure 4 shows the agar plate contained AM incorporated film in comparison to control film that contain no AM compound at all. From the observations, the AM-incorporated films showed clear zone formed on the agar plate after in contact with the microbe colonies. For this test a measurement of inhibition zones on/around film squares on inoculated bacteria was determined.



a b

Figure 4: Comparison of inhibition area of (a) control film and (b) AM incorporated film

Table 1 lists calculated inhibition area for each plate test. The control films showed no inhibition area and colonies were formed all over the plate. The AM film showed inhibitory growth of both *Escherichia Coli* and *Bacillus subtilis*. Incorporation of EDTA into the film enhanced the effectiveness against *E coli*. EDTA alters the outer membrane of the bacteria cell by disrupting the magnesium ions that make it stable [9].

Table 1: Inhibition of Escherichia coli and Bacillus subtilis on agar plates expressed as an area (cm^2) of inhibition zone

Film	*Bacillus subtilis* (48 hours @ 37°C)	*Escherichia Coli* (48 hours @ 37°C)
Control	NI	NI
AM Film	15.00	20.63

NI = No inhibitory effect

Liquid Culture Test

In this test the decrease in optical turbidity shows that the AM inhibits the bacteria growth. Figure 5a shows the inhibition of *E. coli* by the AM films in liquid culture broth at 37°C. At the stationary growth phase, the cell concentration in the control medium (OD_{600nm} = 1.355) was about 3 times higher than the cell concentration in the medium containing lysozyme incorporated film (OD_{600nm} = 0.463). Figure 5b shows the inhibition of *B. subtilis* by the AM starch-based film in a liquid culture broth at 37°C. Similarly, the decrease in turbidity shows that the starch-based film containing lysozyme inhibits the growth of *B. subtilis*. At the stationary growth phase, the cell concentration in the control medium (OD_{600nm} = 1.127) was about eleven times higher then the cell concentration in the medium containing AM film (OD_{600nm} = 0.098). Clearly, inhibition of *B. subtilis* was higher than *E. coli* because lysozyme was known as active against Gram-positive bacteria and can target Gram-negative bacteria when combined with chelating agents (i.e. EDTA). The EDTA alters the outer membrane of the bacterial cell by disrupting the magnesium ions that stabilize the membrane, previously reported, to increase permeability [4].

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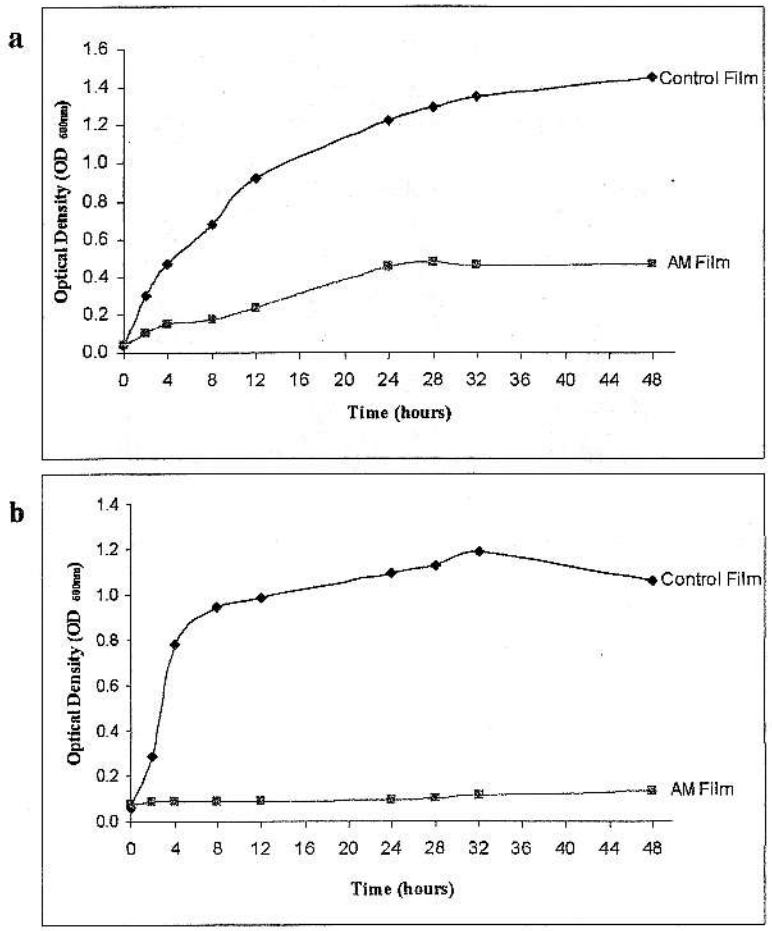

Figure 5: Inhibition of microbial growth by the starch-based film containing AM agents: (a) in a liquid culture medium containing E. coli at 37°C (b) in a liquid culture medium containing B. subtilis at 37°C

Moisture Content Determination

The results showed a decrease in moisture content of the samples incorporated with lysozyme compared to control film which contain no AM agent (Figure 6). Previous study suggested that the increase in the crystalline phase of a semi-crystalline material is highly linked with the decrease in its moisture content [10]. Consequently, the increase in crystalline fraction with addition of antimicrobial, maybe the caused of trend observed for moisture content in this study. Therefore, it is shown that the percentage of moisture content decrease for the film with antimicrobial agent [11]

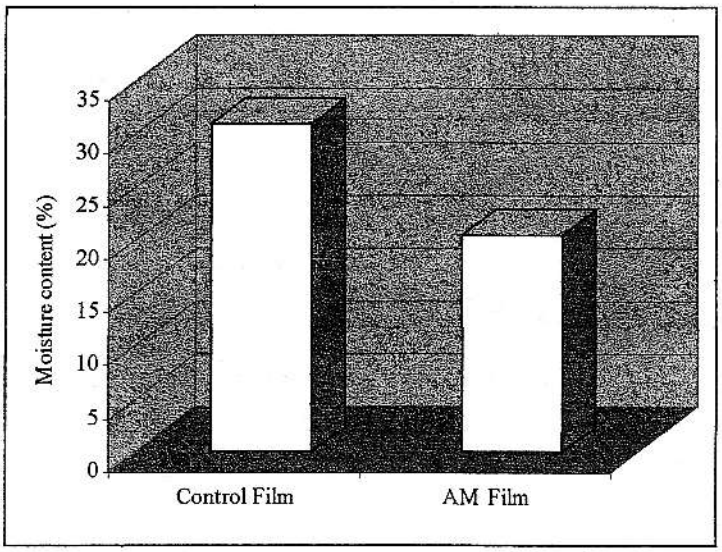

Figure 6: Comparison of moisture content (%) between control starch-based film and AM starch-based film

CONCLUSIONS

From the above discussion, it can be concluded that lysozyme combined with EDTA enable inhibition of both bacteria growth. As a chelating agent, EDTA plays an important role for the antimicrobial to function in the film matrix.

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NOMENCLATURE

M_i	initial weight of the sample	(g)
M_f	final weight after drying	(g)