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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 package matrices and applying a bio switch concept. A starch-based film was prepared and incorporated with an antimicrobial agent, *i.e.* lysozyme with EDTA as a 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 as a clear zone formation in the culture agar test. The film appearance showed that lysozymes could give a better inhibition to the growth of *E. coli* and to *B. subtilis,* at a satisfying inhibition rate. From the broth test, the decreased in the optical densities were found to be 65.83% and 91.30%, suggesting an effective growth inhibition of *E. coli* and *B. subtilis,* respectively. Physically, the film which was incorporated with lysozymes was found to be slightly different from the control film. The moisture content of the film, with lysozymes, was found to be below 10.5%, as compared to the control, after 24 hours of formation in the storage at ambient temperature.

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

INTRODUCTION

Starches are polymers which naturally occur in a variety of botanical sources such as wheat, corn, potatoes and tapioca (Avella *et al.*, 2005; Fama *et al.*, 2005). It is a naturally abundant nutrient carbohydrate, $(C_6H_{10}O_5)_n$, which is found mainly in seeds, fruits, tubers, roots, and stems pith of plants, particularly in corn, potatoes, wheat, and rice, and this varies widely in appearance, depending on its sources; however, it is commonly prepared as a white amorphous tasteless powder. Starch is composed of repeating 1,4-α-D glucopyranosyl units: amylose and amylopectin (Avella *et al.*, 2005; Fringant, Desbrieres and Rinaudo, 1996; Mali *et al.*, 2006), where relative amounts of amylose and amylopectin, depending on the plant source (Avella *et al.*, 2005). In industry, it is used in the manufacture of adhesives, paper and textiles (Kim, Na and Park, 2003). The chemical structure of simple starch is presented in *Fig. 1.*

As a packaging material, starch alone does not form films with adequate mechanical properties (high percentage elongation, tensile, and flexural strength), unless it is first treated by either plasticization, blending with other materials, genetic or chemical modification or combinations of the above approaches. Plasticizing agents such as glycerol, sorbitol or polyethylene glycol,

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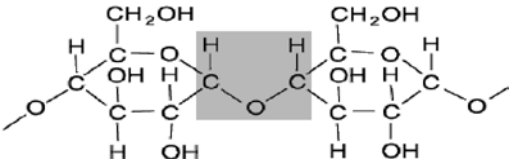

Fig. 1: Chemical structure of starch

mono-, di- or oligosaccharides, fatty acids, lipids and derivatives, are usually used to overcome film brittleness and improve its flexibility and extensibility (Flores, in press).

Antimicrobial packaging (AM) is a form of active packaging which also acts to reduce, inhibit or retard the growth of microorganisms which may be present in the packed food or packaging material itself (Appendini and Hotchkiss, 2002). Among common antimicrobial substances for food products are preservative organic acids, antimycotics (fungicide), enzymes, oxygen absorber, alcohol, etc. (Han, 2000). Many AMs are incorporated, at 0.1%-5% w/w of the packaging material, particularly films (Appendini and Hotchkiss, 2002).

Lysozyme (*Fig. 2*) is 129 amino acid residue enzyme (EC 3.2.1.17) which catalyse the 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 secretions. The enzyme is antibacterial because it degrades the polysaccharide 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 arrow (a glycosidic bond), as indicated in *Fig. 3*. This hydrolysis breaks the chain at that point. Some investigations reveal that compounds like 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 which must be penetrated before the antimicrobial compound can reach the effective membrane suite. Penetration through the outer membrane can be accomplished by the use of a chelating

Fig. 2: The primary structure of egg white, lysozymes

agent (*i.e.* EDTA) or by osmotic shock (Padgett *et al.*, 1998). A previous study showed that the outer membrane of Gram-negative bacteria possesses divalent cations which stabilize the lipopolysaccharide association within the membrane, which is believed to hinder the ability of nisin and other molecules to reach the cytoplasmic membranes (Padgett *et al.*, 1998; Hancock, 1984). However, an addition of EDTA to edible film, containing nisin or lysozymes, had little inhibition effect on *E. coli* (Padgett, 2000) and *S. typhimurium* (Natrajan and Sheldon, 2000).

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

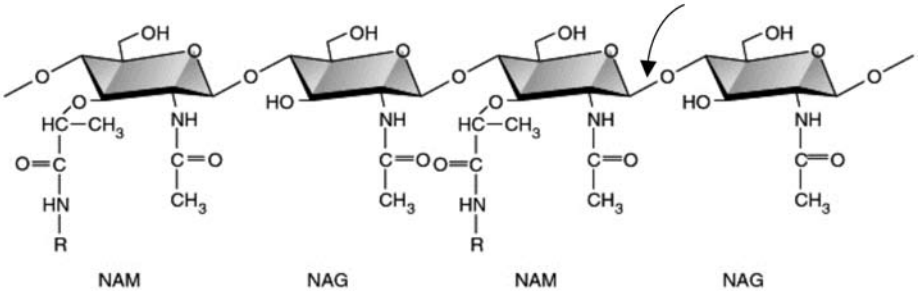

Fig. 3: Bacterial polysaccharides consists of long chains of alternating amino sugars; N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM)

MATERIALS AND METHODS

The Preparation of Antimicrobial Starch-Based Film

Starch-based films were prepared by dissolving and stirring 8.35 g starch in 80 mL of 20% ethanol. After the solution was completely dissolved, 3.8 mL glycerin (HmbG Chemicals) was added as plasticizer and the mixture was slowly heated to a mild boiling. For antimicrobial incorporated films, antimicrobial agents, lysozymes (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.

The Inhibition of Escherichia coli *and* Bacillus subtilis *on Agar Plate Test*

The strain selection represented the typical spoilage organism groups which commonly occur 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* such as *Salmonella*; and (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 particularly in cereals. As 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 which was spread with bacteria (0.1 mL per plate). The same tests were performed using a control film. Duplicate agar plates were also prepared for each type of films and control films. The agar plates were incubated for 48 hours at 37°C in

the appropriate incubator. The plates were visually examined for the "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 sample squares were immersed in 20 mL nutrient broth (Merck, Germany) in a 25 mL universal bottle. The medium was inoculated with 200µL of *E. coli / B. subtilis* in its late exponential phase, and then transferred to an orbital shaker and rotated at 30°C at 200 rpm. The culture was periodically sampled (0, 2, 4, 8, 12, 24 hours) during the incubation to obtain the profiles of the microbial growth. 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 was according to the method proposed by Finkenstadt and Willet (2004). A Moisture Determination Balance FD-620 was used to determine the moisture content (MC) of the starch products by gravimetric methods using the following equation:

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

For powder and thin sheet samples, the sample was heated for 25 min at 110°C. The determination of moisture content was performed in 3 replicates and the average was then reported.

RESULTS AND DISCUSSION

Antimicrobial Starch-based Film Formation

In general, a translucent starch-based film, incorporated with lysozymes which presented a good flexibility than the purely starch-based film, was formulated and formed *(Fig. 4)*. The average thickness of different films, obtained by this procedure, changed between film thicknesses which ranged from 0.16 and 0.18 mm (almost 10 measurements were conducted at different points of each kind of films with a micrometer).

The Inhibition of Escherichia coli *and* Bacillus subtilis *on Agar Plate Test*

All the samples were examined, for possible inhibition zones, after incubation at 37°C for 48 hours. *Fig. 5* shows the agar plate which contained AM incorporated film in comparison to the control film which did not consist any AM compound at all. From the observations, the AM-incorporated films revealed a clear zone which was formed on the agar plate after being in contact with the microbe colonies. For this test, the measurement of inhibition zones on/around the film squares on the inoculated bacteria was determined.

Table 1 lists the calculated inhibition area for each plate test. The control films showed that no inhibition area and colonies were formed all over the plate. The AM film showed the inhibitory growth of both *E. coli* and *B. subtilis*. Obviously, the inhibition area of *E. coli* was 27.29% larger than *B. subtilis*. The incorporation of EDTA into the film was found

Fig. 4: A translucent starch-based film incorporated with lysozymes

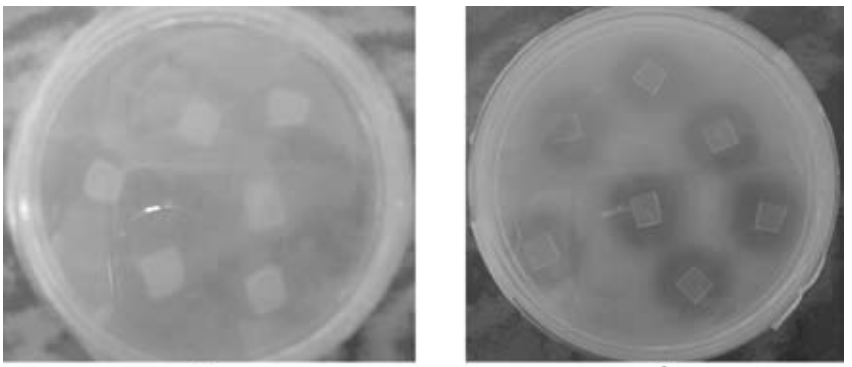

Fig. 5: A comparison of the inhibition area of (a) control film, and (b) AM incorporated film

to enhance the effectiveness against *E. coli*. The EDTA alters the outer membrane of the bacteria cell by disrupting the magnesium ions which make it stable (Dawson *et al.*, 1996).

Liquid Culture Test

In this particular test, the decrease in the optical turbidity showed that the AM had inhibited the bacteria growth. *Fig. 6a* shows the inhibition of *E. coli,* by the AM films, in a 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 lysozymes incorporated film (OD_{600nm} = 0.463). *Fig. 6b* depicts the inhibition of *B. subtilis,* by the AM starch-based film, in a liquid culture broth at 37°C. Similarly, the decrease in turbidity showed that the starch-based film, containing lysozymes, had inhibited 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 as compared to the cell concentration in the medium containing AM film (OD_{600nm} = 0.098). Clearly, the inhibition of *B. subtilis* was found to be higher than *E. coli* because lysozymes is known as active against

TABLE 1
The inhibition of *E. coli* and *B. subtilis* on the agar plates, expressed as an area (cm^2) of inhibition zone

Film	*B. subtilis* (48 hours @ 37°C)	*E. coli* (48 hours @ 37°C)
Control	NI	NI
AM Film	15.00	20.63

NI = No inhibitory effect

Gram-positive bacteria and could target this particular bacteria type when combined with chelating agents (*i.e.* EDTA). The EDTA altered the outer membrane of the bacterial cell by disrupting the magnesium ions which stabilized the membrane (as previously reported) to increase permeability (Padgett, 1998).

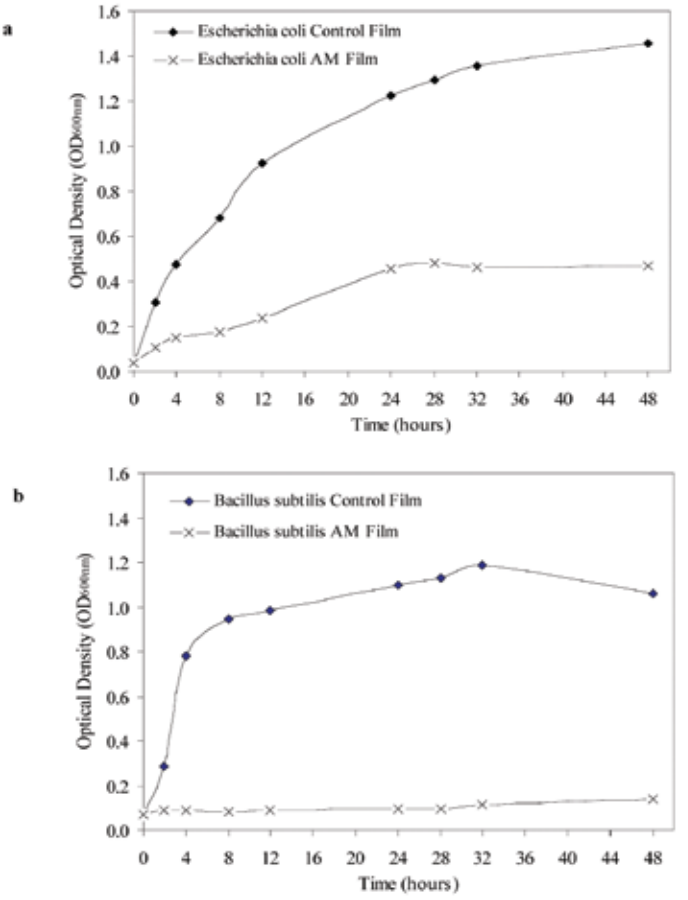

Fig. 6: The inhibition of the microbial growth by the starch-based film containing AM agents: (a) in a liquid culture medium containing E. coli *at 37°C, (b) in a liquid culture medium containing* B. subtilis *at 37°C*

Moisture Content Determination

The results showed a decrease of 10.5% in the moisture content 24 hours after the samples were incorporated with lysozyme, as compared to the control film which contained no AM agent *(Fig. 7)*. A previous study suggested that the increase in the crystalline phase of a semi-crystalline material was highly related to or associated with the decrease in its moisture content (Chang *et al.*, 2000). Consequently, the increase in the crystalline fraction with the addition of antimicrobial, and perhaps moisture or water molecules are used as the carrier to diffuse out the AM substances from the film matrices to obtain the inhibition action, which was significantly observed for the moisture content in this study. Therefore, the percentage of the moisture content was shown to decrease for the film with antimicrobial agent (Famal *et al.*, 2006).

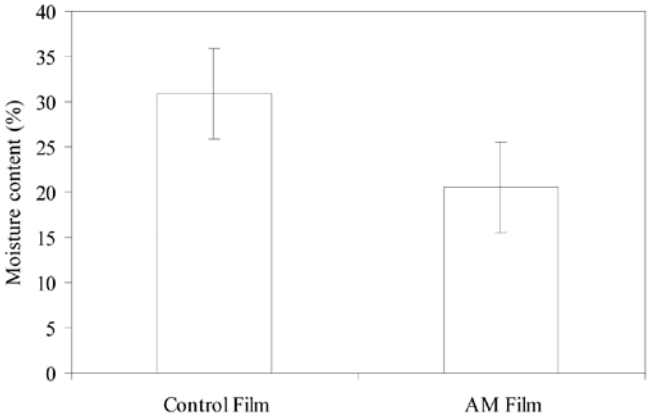

Fig. 7: A comparison of the moisture content (%) between control starch-based film and AM starch-based film

CONCLUSIONS

From the above discussion, it can be concluded that lysozymes, when combined with EDTA, enable the inhibition of both bacteria growth, but this was found to be more effectively in the inhibition of *E. coli*. As a chelating agent, EDTA plays an important role for the antimicrobial to function in the film matrix. The reduction in the moisture content of the AM film indicated the relationship between lysozymes and water molecules in the diffusion mechanism throughout the film matrices.

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