Inhibition of *Bacillus subtilis* and *Escherichia coli* by Antimicrobial Starch-Based Film incorporated with Lauric Acid and Chitosan

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Abstract. *Food safety is one of key issues of public health. One option is to use packaging to provide an increased margin of safety and quality. Active packaging technologies are being developed as a result of these driving forces. Active packaging is an innovative concept that can be defined as a mode of packaging in which the package, the product and the environment interact to prolong shelf life or enhance safety or sensory properties, while maintaining the quality of the product (Suppakul, Miltz, Sonneveld & Bigger, 2003). Antimicrobial (AM) packaging is one of the most promising active packaging systems. Starch-based film is considered an economical material for antimicrobial packaging.*

This study aimed at the development of food packaging based on wheat starch incorporated with lauric acid and chitosan as antimicrobial agents. The purpose is to restrain or inhibit the growth of spoilage and/or pathogenic microorganisms that are contaminating foods. The antimicrobial effect was tested on B. substilis and E. coli. Inhibition of bacterial growth was examined using two methods, i.e. zone of inhibition test on solid media and liquid culture test (optical density measurements). The control and AM films (incorporated with chitosan and lauric acid) were produced by casting method. From the observations, only AM films exhibited inhibitory zones. Interestingly, a wide clear zone on solid media was observed for B. substilis growth inhibition whereas inhibition for E. coli was only revealed underneath the film discs. From the liquid culture test, the AM films clearly demonstrated a more effective inhibition against B. substilis than E. coli.

Keywords. Antimicrobial packaging, Lauric acid, Chitosan, *B. subtilis*, *E. coli*.

Introduction

Antimicrobial packaging (AM) is gaining interest from researchers and industry due to its potential to provide quality and safety benefits. Interest in antimicrobial packaging films has increased in recent years due to a concern over the risk of foodborne illness, desire for extended food shelf life, and advances in the technology of film production. Slowing the growth of spoilage bacteria will reduce the losses of product to spoilage and extend shelf life. Reduction of pathogen growth will reduce the risk of foodborne illness caused by those products (Dawson, Carl, Acton & Han, 2002). It can effectively control the microbial contamination of various solid and semisolid foodstuffs by inhibiting the growth of microorganisms on the surface of the food, which normally comes into direct contact with the packaging material. Antimicrobial function of the packaging system can be achieved by incorporating active substances into the packaging system by various ways (Han, 2003). The antimicrobial packaging is conducted by (1) the addition of antimicrobial containing sachets or pads into food packages; (2) the coating, immobilization or direct incorporation of antimicrobials into food packaging materials or (3) the use of packaging materials that are inherently antimicrobial (Appendini & Hotchkiss, 2002).

Nowadays, about 150 million tons of plastic are produced annually all over the world, and the production and consumption continue to increase (Parra, Tadini, Ponce & Lugao, 2004). Most of these plastics are crude oil based. In addition, handling of plastic waste associated with serious environmental pollution problem due to waste disposal and undegraded polymers. Therefore, the use of agricultural biopolymers that are easily biodegradable not only would solve these problems, but would also provide a potential new use for surplus farm production (Okada, 2002; Pavlath & Robertson, 1999; Scott, 2000). Because of the environmental concerns and technological problems such as denaturing effects of thermal polymer processing methods, extrusion and injection molding, the incorporation of biopreservatives into biodegradable films is more suitable than incorporation into plastic films (Appendini and Hotchkiss, 2002; Han, 2000; Suppakul et al., 2003).

Most of biodegradable films are edible and their film formation occurs under mild conditions. Different edible films incorporated with biopreservatives includes cellulose derivatives, carrageenan, alginate, protein films include casein, collagen, corn zein, gelatin, soy protein, whey proteins and wheat gluten (Padgett, Han & Dawson, 1998; Cha, Choi, Chinnan & Park 2002; Han, 2000; Quintavalla & Vicini, 2002; Suppakul et al., 2003).

In the food packaging sector, biodegradable polymers based on natural polysacaccharides, particularly starch has gain more attention owing to its availability as agricultural surplus raw material, abundant, can be produced at low cost and at large scale, nonallergic and thermoprocessable. Several studies are concentrated on the development of starch-based materials for the above-mentioned reasons.

Starch based materials reduce nonrenewable resources use and environmental impact associated with increasing emissions as CO_2 and other products. Starches are polymer that naturally occurs in a variety of botanical sources such as wheat, sago, corn, yam, potatoes and tapioca. Starches can interact with many additives or components of the food (Famá, Rojas, Goyanes & Gerschenson, 2004). However starch presents some major drawbacks such as the strong hydrophilic behaviour (poor moisture barrier) and poorer mechanical properties than the conventional non-biodegradable plastic films used in the food packaging industries.

In the last years much research has been done concerning the use of biodegradable films as a way of supporting antimicrobials in food products. Several researchers have previously reported on coating food contact surfaces with antimicrobial compounds. Nisin and lauric acid are two food-grade antimicrobials shown to be effective in food applications. Dawson et al. (2002), incorporated lauric acid and nisin singly and together into thermally compacted soy films. Nisin and lauric acid films were equally effective in reducing *L. monocytogenes* in 1% peptone water after 48h exposure. However, the combination of nisin and lauric acid in corn zein cast films was found to be more effective in reducing *L. monocytogenes* in peptone water than when each used singly (Hoffman, Han & Dawson, 2001). The advantage in having a film material carrying a biocide is that continued inhibition can occur during storage or distribution of the food product.

A widespread trend worldwide is the movement towards "natural" food products. In effort to meet this demand, there has been increased interest in the food industry in using antimicrobial preservatives that are perceived as more "natural". Future work will focus on the use of biologically active derived antimicrobial compounds bound to biopolymers. However many natural antimicrobials have a limited spectrum of activity and are effective only at very high concentrations. The need for new antimicrobials with wide spectrum activity and low toxicity will increase. A possible solution may be using combinations of antimicrobials (Sofos, Beuchat, Davidson & Johnson, 1998). Instead of concentrating on development of new antimicrobial, it could be more practical to combine the antimicrobial agents that already being researched.

Lauric acid, a medium length- long chain fatty acid is found in the form of glycerides in a number of natural fats, coconut oil and palm-kernel oil. It offers advantages in food processing as it acts as a kind of preservative, staving off oxidation and spoilage. Lauric acid has been shown to have an antimicrobial effect against gram positive bacteria and yeasts (Beuchat & Golden 1989; Kabara 1993). Beuchat & Golden (1989) suggested that fatty acids were bacteriostatic and may be potential microbial inhibitors in foods using a systematic approach with other antimicrobials. Based on Padgett, Han & Dawson (2000), nisin instantaneously kills *L. plantarum* cells whereas lauric inhibits more slowly but steady inhibitory effect. The incorporation of lipid compounds such as fatty acid to a starch film decreases the moisture transfer due to their hydrophobic properties (Coma, Sebti, Pardon, Deschamps & Pichavant, 2001). Fatty acids, such as lauric acid were found to be effective in limiting water vapor transfer through edible film (Gennadios, Weller & Testin, 1993; Greener & Fennema 1989 a, b; Kamper & Fennema 1984; Kester & Fennema 1989).

A packaging material with a wide antimicrobial spectrum would be necessary and desirable for universal use to improve the storage stability of variety of foods. For this purpose, the incorporation of another antimicrobial agent into the packaging materials would be useful. Besides, the choice of chitosan in preparing the antimicrobial packaging films was based on the fact that it has good film forming properties. Ban, Song, Argyropoulos & Lucia (2005), reported that chitosan can also play an important role in the enhancement of starch-based film strength.

Chitosan, a polysaccharide of β1, 4 linkages and a deacetylated form of chitin, appears as a natural antimicrobial candidate for the incorporation because it can inhibit the growth of a wide variety of fungi, yeasts and bacteria (Rhoades & Rastall , 2000; No, Park, Lee & Meyers, 2003; Tsai, Su, Chen & Pan, 2002; Sagoo,Board & Roller, 2002).

The objective of the research was to determine the effectiveness of lauric acid and chitosan as antimicrobial agent incorporated into starch-based film against test strain of Gram-positive (*B. substilis*) and Gram-negative bacteria (*E. coli*).

2. MATERIALS AND METHODS

2.1 Materials

Wheat starch and acetic acid (glacial 100%) that used to dissolve chitosan was purchased from Mersk (Malaysia). Medium molecular weight chitosan was from Sigma-Aldrich (Malaysia). Lauric acid was 99% pure purchased from Fluka Chemika (Malaysia) and glycerol as a plastisizer was bought from HmbG chemicals (Malaysia).

2.2 Film preparation

A starch based film was formed using casting process following previous work by Famá et al. (2004). A control film, without lauric acid or chitosan was formed using mixtures of starch (5.0g), glycerol (2.5g) and water (92.5g).

Chitosan was dispersed in 400ml of distilled water to which 20 ml of glacial acetic acid was added to dissolve the chitosan. The solution of starch and chitosan with different mixing ratios [9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9 starch/chitosan (w/w)] were prepared by adding glycerol (half amount of the starch) and 8% lauric acid (was added based on the percentage of starch (g fatty acid per g starch). The solution was mixed by gentle stirring with a magnetic stir bar until starch dissolved. The solution was then homogenized for about 15 min with addition of slow heating. Stirring and heating were ended when the solution reaches temperature of 80-86°C.

The 10 ml of the film forming solution was pipette and spread evenly into a petri dish bottom (100x 15 mm) and allowed to air-dry at room temperature overnight.

After casting, 5 measurements were made on each sample using an electronic micrometer (model Mitutoyo) and the mean thickness was calculated to the nearest 0.002 mm.

2.3 Testing Antimicrobial Effectiveness of AM Starch-Based Film

2.3.1 Agar Diffusion Method (Zone Inhibition Assay)

Antimicrobial activity test was carried out using agar diffusion method. Indicator cultures were *Bacillus subtilis* and *Eschericha coli*, representing Gram-positive and Gram-negative bacteria. One hundred microliters of the inoculum solution was added to 5 ml of the appropriate soft agar, which was overlaid onto hard agar plates.

Each film was cut into squares (1cm x 1cm) and was placed on the bacterial lawns. Duplicate agar plates were prepared for each type of film and control film.The plates were incubated for 48 h at 37 °C in the appropriate incubation chamber (aerobic chamber for *E.coli*). The plates were visually examined for zones of inhibition around the film disc, and the size of the zone diameter was measured at two cross sectional points and the average was taken as the inhibition zone. This method was slightly modified from Padgett et al. (1998).

2.3.2 Liquid Culture Test (Optical Density Measurements)

For the liquid culture test (Chung, Papadakis & Yam, 2002), each film was cut into squares (1cm x 1cm). 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 *Eschericha coli/ B. subtilis* in its late exponential phase, and then transferred to an orbital shaker and rotated at 37°C at 200 r.p.m. 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 λ = 600nm using a spectrophotometer (Model UV-160, Shimadzu, Japan).

3. RESULTS AND DISCUSSION

3.1. Antimicrobial Starch-Based Film Formation

In general a translucent starch-based film incorporated with lauric acid and chitosan presented good flexibility than purely starch-based film was formulated and formed as can be seen in fig. 1. Film thickness ranged from 0.03 to 0.04 mm, with an average 0.0346 ± 0.002 mm.

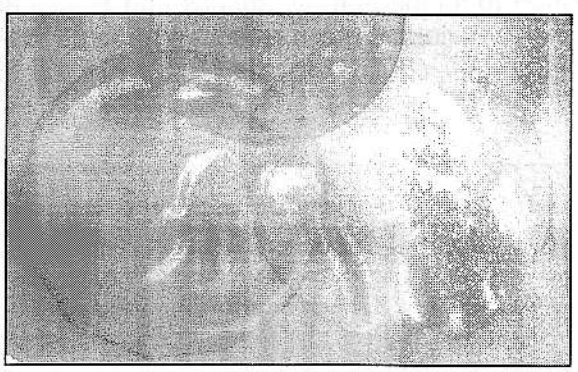


Figure 1: A translucent starch-based film incorporated with lauric acid and chitosan

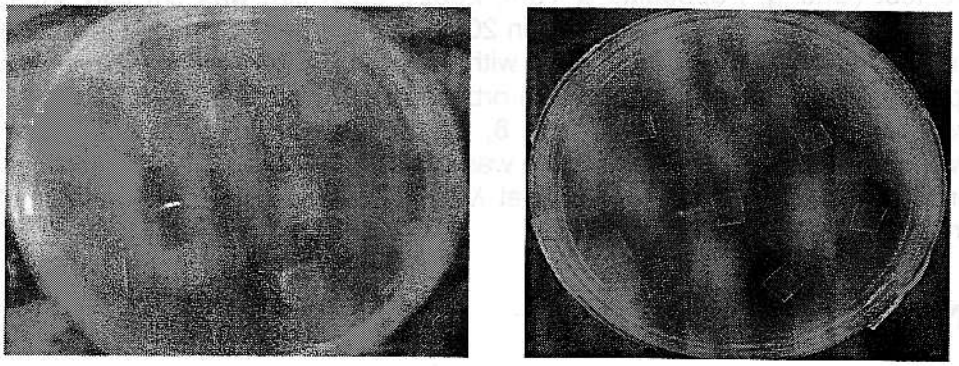
3.2. Antimicrobial Effectiveness of AM Starch-Based Film

3.2.1. Inhibition of E. coli and B. subtilis on Agar Plate Test

The details of antimicrobial effectiveness of starch-based film incorporated with chitosan and lauric acid are shown in fig. 2- 5 and table 1. The inhibitory activity was measured based on the average diameter of the clear inhibition zone. If there was no clear zone surrounding as revealed in fig. 2, it was assumed that there was no inhibitory effect, and was assigned as NI with a value of 0.00.

After 24 hours incubation at 37°C for starch film only (S), there was no inhibition occurred for both *B. subtilis* and *E. coli*. Bacteria colonies also occurred at the top of film sample. On the

contrary, chitosan film only (C), showed a better inhibition than starch film only for both *B. subtilis* than *E. coli* inhibition. However the effect was not as good as combination of chitosan and lauric acid as shown in fig. 2.



a b

Figure 2: Comparison of inhibition area of (a) control film and (b) AM incorporated film

Starch and chitosan different mixing ratio (S:C) 8:2, revealed the best inhibition on *B. subtilis* which is Gram- positive bacteria compared to other S:C ratios. In contrast, S:C ratio 9:1 showed a very good inhibition on *E. coli* (Gram-negative bacteria). From fig. 3, the results indicated that S:C ratio 8:2 is the best formulation to inhibit both *B. subtilis* and *E. coli* effectively followed by S:C ratio 9:1. S:C ratio from 1:9-3:7 obviously more effective towards inhibition of *E. coli* than S:C 4:6-7:3.

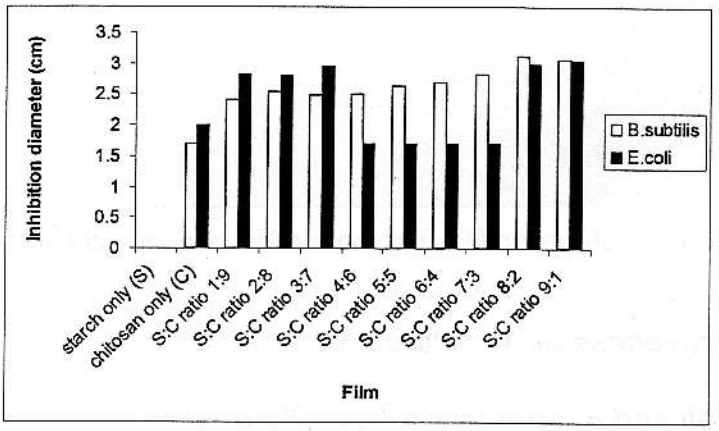


Fig. 3: Inhibition of *B. subtilis* and *E. coli* On Agar Plates Figure Based On Average Zone Diameter Expressed As An Area (cm) of Inhibition Zone

Table 1: Inhibition of *B. subtilis* and *E. coli* On Agar Plates Based On Average Zone Diameter Expressed As An Area (cm) of Inhibition Zone

Film	*B. subtilis*	*E. coli*	Remarks
Starch only (S)	NI	NI	NI = No inhibitory effect (all area on plates and film covered by bacteria)
Chitosan only (C)	1.7	2.0	
S:C ratio 1:9	2.423	2.825	
S:C ratio 2:8	2.55	2.8	
S:C ratio 3:7	2.488	2.95	
S:C ratio 4:6	2.5	1.7	
S:C ratio 5:5	2.631	1.7	
S:C ratio 6:4	2.7	1.7	
S:C ratio 7:3	2.825	1.7	
S:C ratio 8:2	3.125	3.00	
S:C ratio 9:1	3.075	3.05	

3.2.2 Liquid Culture Test ($O.D_{600\ nm}$ Measurement)

S:C ratio 8:2 is the most effective formulation to inhibit *B. subtilis* as can be seen in fig. 4. Meanwhile, S:C ratio 9:1 is the best formulation to inhibit *E.coli* (fig. 5). Although there were inhibition for both *B. subtilis* and *E.coli*, the antimicrobial starch-based film incorporated with lauric acid and chitosan were more effective against Gram-positive bacteria than the Gram-negative bacteria studied.

Lauric acid alone only has antimicrobial effect against Gram-positive bacteria and yeasts (Beuchat & Golden 1989; Kabara 1993). The incorporation of lipid compounds such as fatty acid to a starch film decreases the moisture transfer due to their hydrophobic properties (Coma et al., 2001). This will be observed for the future work on physical and mechanical properties of the antimicrobial starch-based film. As well as incorporation of chitosan, besides inhibit *E. coli* and increase the film effect on *B.subtilis* inhibition, it helps to enhance the antimicrobial starch-based film strength (Ban et. al., 2005).

In fact, one of the reasons for the antimicrobial character of chitosan it's positively charged amino group which interacts with negatively charged microbial cell membranes, leading to the leakage of proteinaceous and other intracellular constituents of the microorganisms (Shahidi,

Arachchi & Jeon, 1999). In the Gram-positive bacteria, the major constituent of its cell wall is peptidoglycan and there is very little protein. The cell wall of Gram-negative bacteria also has an outer membrane, which constitutes the outer surface of the wall (Zheng & Zhu, 2003). Study from (Jiang, Bi, Wang, Xu & Jiang, 1997), observed that from electron micrographs for Gram-positive and Gram-negative bacteria in the presence of chitosan show the cell membrane of Gram-positive bacteria was weakened or even broken, while the cytoplasma of Gram-negative bacteria was concentrated and the interstice of the cell were clearly enlarged. This study indicated that the mechanisms of the antimicrobial activity of chitosan were different between Gram-positive and Gram-negative bacteria. Additionally, the antimicrobial mechanism of chitosan might differ from that of other polysaccharides because there are positives charges on the surface of chitosan (Jiang et al., 1997).

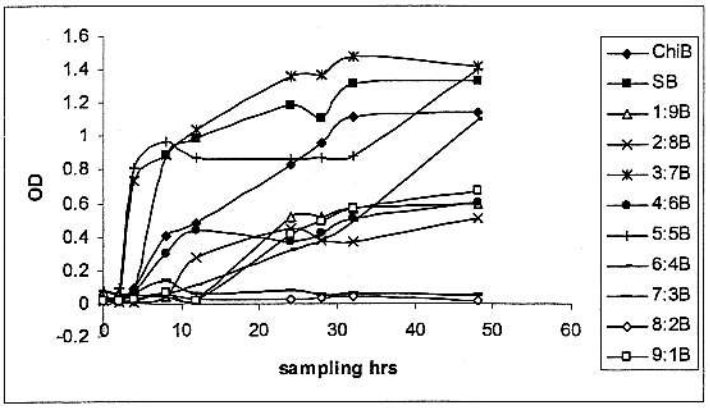


Fig. 4: Inhibition of Controls (starch only and chitosan only) and Starch (S): Chitosan (C) on *B. subtilis* in Liquid Culture Test

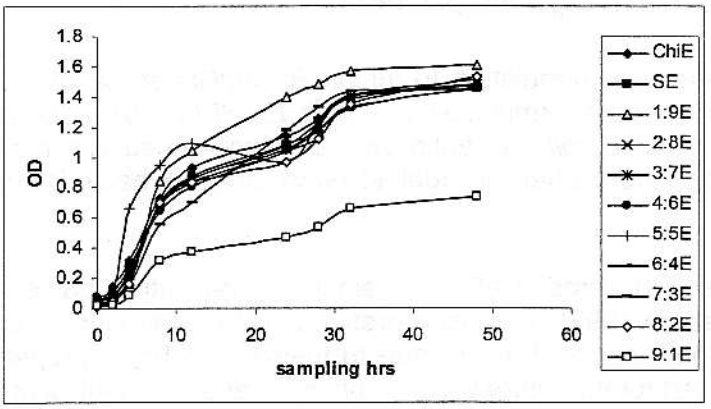


Fig. 5: Inhibition of Controls (starch only and chitosan only) and Starch (S): Chitosan (C) on *E. coli* in Liquid Culture Test

Conclusions

Incorporating chitosan and lauric acid into starch based film showed obvious effects towards inhibition of *B. subtilis* and *E. coli* indicated that the film had synergistic antimicrobial effect when chitosan and lauric acid were combined. The antimicrobial starch-based film demonstrates more effective antimicrobial ability against *B. subtilis* than *E. coli*. The solution of starch and chitosan with different mixing ratio (w/w) 8:2 and 9:1 were the most effective mixing ratio which had greater inhibition on both *B. subtilis* and *E. coli* than others solution as revealed in agar plate test and liquid culture test. Thus, the antimicrobial starch-based film may have potential applications for both fluid and semisolid foods by inhibiting bacterial growth and extended the shelf-life and improve the food safety.

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