

## Antimicrobial Effectiveness of Biobased Film Against *Escherichia coli* O157:H7, *Listeria monocytogenes* and *Salmonella typhimurium*

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**Abstract:** Antimicrobial packaging, an active packaging concept, can be considered challenging technology that could have a significant impact on food safety of meat and meat products. The feasibility of polylactic acid (PLA)-based film was evaluated for its application as a material for antimicrobial film. A bio-based commercial polylactic acid (PLA) product, Ecovio<sup>®</sup>, was used as an environmentally friendly polymer matrix. The PLA based film was incorporated with lactic acid or sodium lactate by extrusion film-blowing process. The antimicrobial activity of films against *Escherichia coli* O157:H7, *Listeria monocytogenes* and *Salmonella enterica* Serovar Typhimurium (*S. Typhimurium*) were evaluated. Antimicrobial film incorporated with lactic acid packaging film was found to be highly effective in inhibiting *L. monocytogenes*. In contrast, no inhibitory activity was observed against *E. coli* O157:H7 and *S. Typhimurium*. This is consistent with Minimum Inhibitory Concentration (MIC) studies which indicated that undissociated lactic acid was more efficient in inhibiting *L. monocytogenes* than enterobacteria. This preliminary study shows the potential use of bio-based film as one hurdle technology in combination with good manufacturing practices and adequate storage temperatures. The use of antimicrobial packaging may contribute to improve the safety in minimally processed foods. Further work is required to improve the mechanical properties of the material in order to meet industry requirements.

**Key words:** Bio-based film, food safety, innovative antimicrobial film, polylactic acid

### INTRODUCTION

Today's world is almost unimaginable without plastics, but most of these materials are derived from fossil fuels and face potential problems with rising costs, potential scarcity and customer demands for non-fossil alternatives. These concerns have triggered research and development into alternative raw materials for use in bioplastics. Food packaging offers both convenience and increased safety assurance from contamination by microorganisms, biological as well as chemical changes, and prolonging shelf life for packaged foods. Packaging has thus become a critical component in food manufacturing, and the industry has undergone remarkable growth and development over the past 20 years (Brody *et al.*, 2008).

In response to current consumer demands and market trends, active packaging is becoming increasingly important (Jamshidian *et al.*, 2010). Consumers are also

demanding consumer-friendly packaging (Cutter, 2002; Lopez-Rubio *et al.*, 2004). One of the most promising renewable plastic is polylactic acid (PLA). The use of PLA as bio-based material in food packaging has already received wide attention (Conn *et al.*, 1995; Haugaard *et al.*, 2002; Frederiksen *et al.*, 2003; Theinsathid *et al.*, 2011). What has become even more important today is that PLA is made out of plant based raw materials. This is important because CO<sub>2</sub> reduction has become a very important topic for the whole world.

The transition from hydrocarbon-based to plant-based plastics can play an important role in greenhouse gas mitigation strategies. Increasing demand for safe, minimally processed, fresh food products presents major challenges to the food packaging industry to develop packaging concepts for maintaining the safety and quality of packaged foods. Recent outbreaks of foodborne pathogens such as *Escherichia coli* O157:H7, *Salmonella* spp., and *L. monocytogenes* continue to drive a search for

innovative ways to inhibit microbial growth in foods while maintaining quality, freshness and safety (Rangel *et al.*, 2005; Teratanavat and Hooker, 2004). For example, *L. monocytogenes* is a public health concern in many countries including the United States and European countries (Scharff, 2010).

Post processing contamination of ready-to-eat (RTE) processed meat products by *L. monocytogenes* represents a serious health risk and a major concern for the RTE meat processing industry (Ericsson *et al.*, 1997; Barmpalia *et al.*, 2005; Norton and Braden, 2007). As an additional hurdle to non-thermal processes, there has been growing recent interest in developing packaging materials and having antimicrobial properties which help to improve food safety and shelf life. Antimicrobial packaging can play an important role in modifying retail and distribution practices driven by globalization, new consumer product logistics, new distribution trends and stricter requirements regarding consumer health and safety (Vermeiren *et al.*, 1999; Sonneveld, 2000; Cooksey, 2005; Quintavalla and Vicini, 2002; Appendini and Hotchkiss, 2002; Cutter, 2002; Coma, 2008).

First developments and commercial packages intended to present an actively role in the protection of the product were based on independent devices which were incorporated with the product in a conventional package. This is the case of sachets and other devices. The potential misuse by the consumer of that device, the need to include an extra processing operation in the packaging lines and the evidence of the presence with the product of a foreign object, are driving to the development of packaging structures which include the active agents. In the field of food packaging, antimicrobial biodegradable films are generally obtained by using the solvent casting technique or modification of surface composition of the polymer. However, as an industrial and adoption technology point of view, active film obtains from tradition processes with existing equipment such as extrusion processing is preferred (Suyatma *et al.*, 2004; Sebastien *et al.*, 2006; Jin and Zhang, 2008; Hoang *et al.*, 2010). Despite practical point of view for extrusion processing, limited information is available for using bio-based polymer based on extrusion process. The main reason due to extrusion processing require high temperature, high shear rates and lead to deteriorate stability of antimicrobial compounds and the fact that the condition reduce their efficacy on its own antimicrobial properties. Lactic acid and sodium lactate are known to inhibit the growth of gram positive and gram negative bacteria and its antilisterial effect have been widely reported in various food products (Tompkin, 2002). Both of the materials have been used for several years in the meat industry because of its ability to extend shelf life and microbiological safety of these products (Koo, 1992; Houtsma *et al.*, 1993). The objective of this work is to develop innovative antimicrobial PLA based film for its

suitability as a bio-based material for antimicrobial film by extrusion film-blowing method. Herein, best of our knowledge we report for the first time that of antimicrobial PLA based films (Ecovio®) containing either lactic acid or sodium lactate. The method used in this study was developed with technology that is suitable for industrial application. The antimicrobial efficiency of the films were assayed through *in vitro* tests against *L. monocytogenes*, *Salmonella Typhimurium* and *E. coli* O157:H7.

## MATERIALS AND METHODS

**Materials:** Bio-based commercial polylactic acid (PLA) product, Ecovio® from BASF was used as an environmentally friendly polymer matrix and natural antimicrobial compounds were used to produce the tested active films. Lactic acid and sodium lactate were supplied by Purac Biochem B.V, The Netherland.

**Film preparation:** PLA-based films (Ecovio, BASF) of 50-60 µm in thickness, with and without lactic acid or sodium lactate, were prepared from Ecovio pellets. Five kilograms of pellets and three different concentrations of lactic acid (5, 10 and 15% (w/w)) and sodium lactate (10, 20 and 30% (w/w)) were accurately weighted and mixed the pellets. A pre-blended master batch of pellets containing lactic acid or sodium lactate was then subject to produce into films by using the same extruder. The extrusion film-blowing was conducted at Petroleum and Petrochemical College, Chulalongkorn University. Films without lactic acid or sodium lactate were used as controls and were prepared under similar conditions as the films containing the active agents.

**Tensile testing:** Tensile testing was carried out by using the Instron Universal Testing Machine at load cell 1N, crosshead speed 500 mm/min and initial grip distance 50 mm in accordance with ASTM D-882 (ASTM, 2002). The testing was performed at 21±1°C and 50% ±5 RH. Tensile Strength (TS), Young's modulus, and elongation at break (EB) were obtained. Standard method D882 (ASTM, 2002) was used to measure the tensile properties of films.

Films were cut into strips with a test dimension 165 mm x 19 mm with ASTM D638-02 (ASTM, 2002). Before testing, all of the films were conditioned for 48 h at 23±2°C and 50% ± 5 RH. Films were uniaxially stretched at a constant velocity of 7.5 mm/min. Sample width and thickness was measured before testing.

**FTIR spectra:** The FTIR spectra have been taken on a Perkin-Elmer 2000 spectrometer, with the samples in self-supporting pellets pressed at 4×10<sup>3</sup> kg/cm<sup>2</sup>.

**Culture preparation:** *L. monocytogenes* (ATCC 19115), *S. Typhimurium* (DMST 0562) and *Escherichia coli*

Table 1: Experimental conditions used for the agar disc diffusion test on packaging films

Test organism	Medium (h)	Time	Temperature (°C)
<i>Escherichia coli</i> O157:H7 (DMST12473)	Tryptic Soy Agar	24	37
<i>Listeria monocytogenes</i> (ATCC19115)	Tryptic Soy Agar	24	37
<i>Salmonella</i> Typhimurium (DMST0562)	Tryptic Soy Agar	24	37

O157:H7 (DMST12473) was obtained from the Department of Medical Science, Ministry of Public Health Thailand. All of the microbiological testing was conducted at National Center for Genetic Engineering and Biotechnology (BIOTEC). The organisms were stored at -80°C in tryptic soy broth (TSB; Merck) containing 10% (w/w) glycerol. Before use, the microorganisms were activated. A colony of each microorganism was picked and streaked on a Tryptic Soy Agar (TSA) plate and a colony was picked and suspended into a tube of TSB with 0.6% yeast extract (TSBYE) and incubated for 24 h. at 37°C on a shaker at 200 r.p.m. Both microorganisms then further suspended one more time in TSB and incubated under the same condition to reach a final concentration of approximately 10<sup>9</sup> CFU/mL. Before testing, the concentration of each organism was adjusted to 10<sup>7</sup> CFU/ml with sterile 0.1% (w/w) peptone water.

**Antimicrobial efficacy of the films against *L. monocytogenes*, *S. Typhimurium* and *Escherichia coli* O157:H7:** For qualitative evaluation of antimicrobial activity, the films were tested for their inhibition against the target microorganisms: *L. monocytogenes* (Gram-positive bacteria), *S. Typhimurium* (Gram-negative bacteria), and *Escherichia coli* O157:H7 (Gram-negative bacteria) by using an agar diffusion method. This is to simulate a test for solid food packaging (Siragusa *et al.*, 1999). Each film sample (2.0×2.0 cm<sup>2</sup>) was placed on Tryptic Soy agar (Merck, Germany) medium overlaid with a semi-soft agar (1% (w/v) agar). The density of overlay was approximately 10<sup>6</sup> CFU/mL of *E. coli* O157:H7, *L. monocytogenes*, *S. Typhimurium* separately on each plate (Table 1). The agar plates were incubated at 37°C for 24 h. A clear zone underneath the film indicates antimicrobial diffusion from the film and subsequent growth inhibition. The diameters of the growth inhibition zones were used to determine antimicrobial activity of each film sample.

For quantitative evaluation, antimicrobial activity of films was determined under dynamic contact condition according to the ASTM E-2149-10 standard method

(ASTM, 2004). Each film sample (4.0×4.0 cm<sup>2</sup>) was placed in a screw-cap bottle containing a 50±0.1 ml of buffer solution and test organisms, *Listeria monocytogenes*, *Salmonella* Typhimurium and *E. coli* O157:H7. Lactic acid at 1% put into the buffer solution was used as positive control for the experiment. The concentration of each tested organism was 1.5-3.0×10<sup>5</sup> CFU/mL at the start. The flasks were incubated at appropriate temperature for each bacterium under dynamic conditions up to 24 h. The number of viable cells was detected by standard plate count techniques after 37°C at 24 h. The results are presented as log reduction. The antimicrobial effects of the treated films were tested against three different test organisms. In instant effect study, the number of viable cells was detected at 0, 6 and 24 h at 37°C. The antimicrobial activity of each film can be presented in percent reduction according to the following formulation:

$$\text{Reduction (\%)} = \frac{(B-A)}{B} \times 100$$

where;

**B:** Number of bacteria before the addition of films sample (at time 0 h) **A:** Number of bacteria recovered after dynamic contacted to the films for 24 h. Minimum Inhibitory Concentrations (MICs) assay of antimicrobial film: The MIC assay was determined according to the method described by Castellano *et al.* (2001).

The tested microorganisms were *Listeria monocytogene*, *Salmonella* Typhimurium and *E. coli* O157:H7, respectively. The tested microorganisms were prepared by diluting overnight cultures to an optical density [OD] of 0.8 at 600 nm. Briefly, 96-well plates containing two-fold serial dilutions of a sample with a known amount of the indicator strains were prepared, and incubated until the control culture (with no added sample) had reached the stationary phase (about 24 h). The changes in cells proliferation of each indicator tested were detected using a microplate reader at a wavelength of 600 nm.

The MIC<sub>50</sub> was defined as the lactic acid concentration (mmol/L) causing a 50% growth inhibition. Culture plates were covered with lids to avoid evaporation of water during incubation at 37°C and to prevent contamination of the plates (Castellano *et al.*, 2001). Lactic acid released from films:

A film (4.0×4.0 cm<sup>2</sup>) was placed into flask contains 50 mL of buffer at pH 6.8 and was incubated with shaking. At each sampling time (0.5, 1, 3, 6, 24 h), 1 mL of buffer solution was collected in order to determine the amount of lactate released by using the YSI -7100 MBS Biochemical Analyzer. The control was a flask containing film without lactic acid.

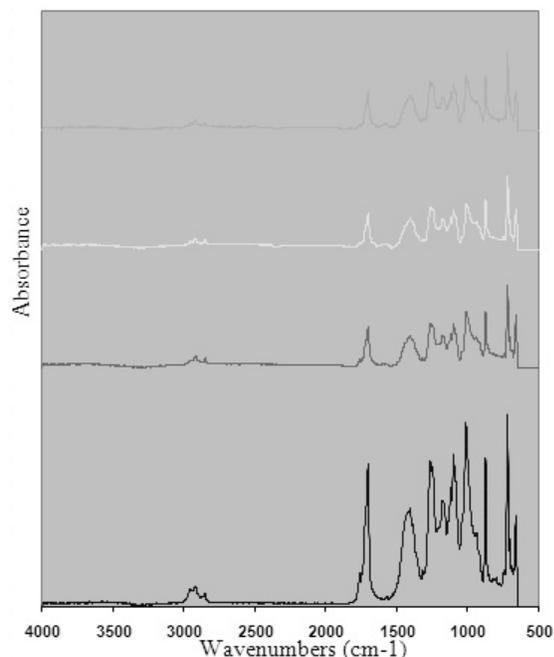


Fig. 1: The spectra of the control film incorporated sodium lactate at various levels used in the study

**Statistical analysis:** Data were subjected to analysis of variance (ANOVA) and mean comparisons were carried out using Duncan's Multiple Range Test. SPSS statistic program (Version 11.5, 2002) was used for data analysis.

## RESULTS AND DISCUSSION

**Fabrication of the films:** A pre-blended master batch of lactic acid and sodium lactate were separated mixing by dry blending with Ecovio® at different concentrations before transferred to blow film extrusion. In the production of antimicrobial films by blow-film extrusion, the temperature profile was used at 160°C. The efficiency of two antimicrobials, lactic acid and sodium lactate are not loss at this used temperature (Tam *et al.*, 1997; Bowmer *et al.*, 1998). However, unexpectedly, The addition of the lactic acid at 10 % (w/w) and 15% (w/w) film dry basis into Ecovio® film dramatically dropped mechanical properties as a result of unable to go, extrusion process.

**Mechanical properties:** The mechanical resistance of films was studies according to three parameters: Tensile Strength (TS), Young's modulus (Y) and Ultimate Elongation at break (UE). With the 5% (w/w) film dry basis lactic acid in the film, the mechanical properties were not determined due to the results were obviously shown that the film incorporated with lactic acid increased the rigidity and the brittleness of the film and

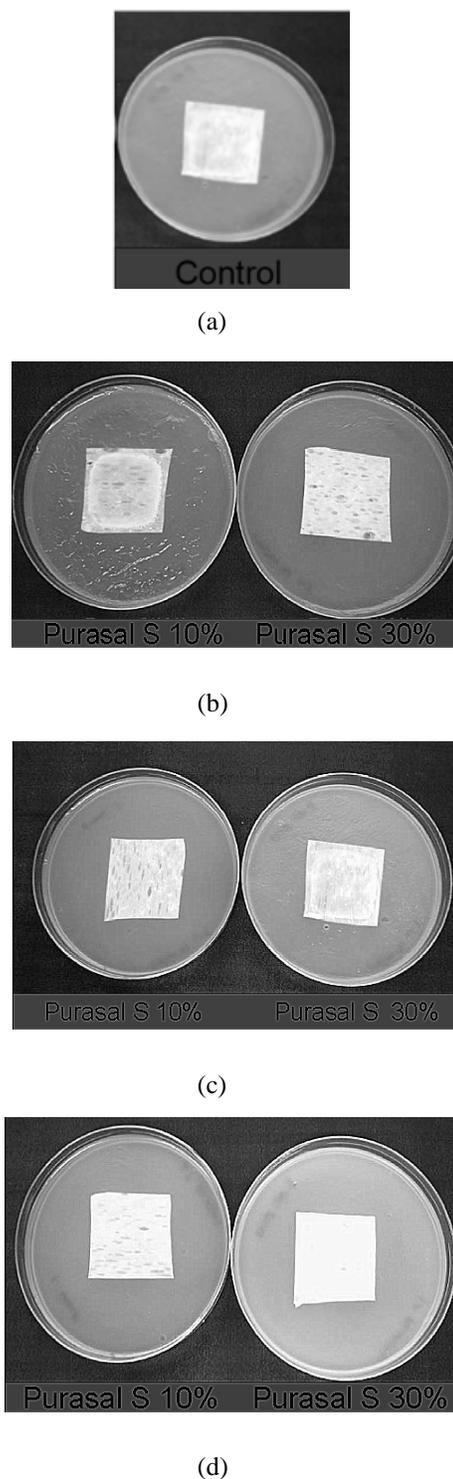


Fig. 2: Qualitative antibacterial activity evaluation of film containing; (a) untreated film and sodium lactate at 10% and 30% against; (b) *Listeria monocytogenes* (ATCC19115); (c) *E. coli* O157:H7 (DMST12473) and (d) *Salmonella* Typhimurium (DMST0562)

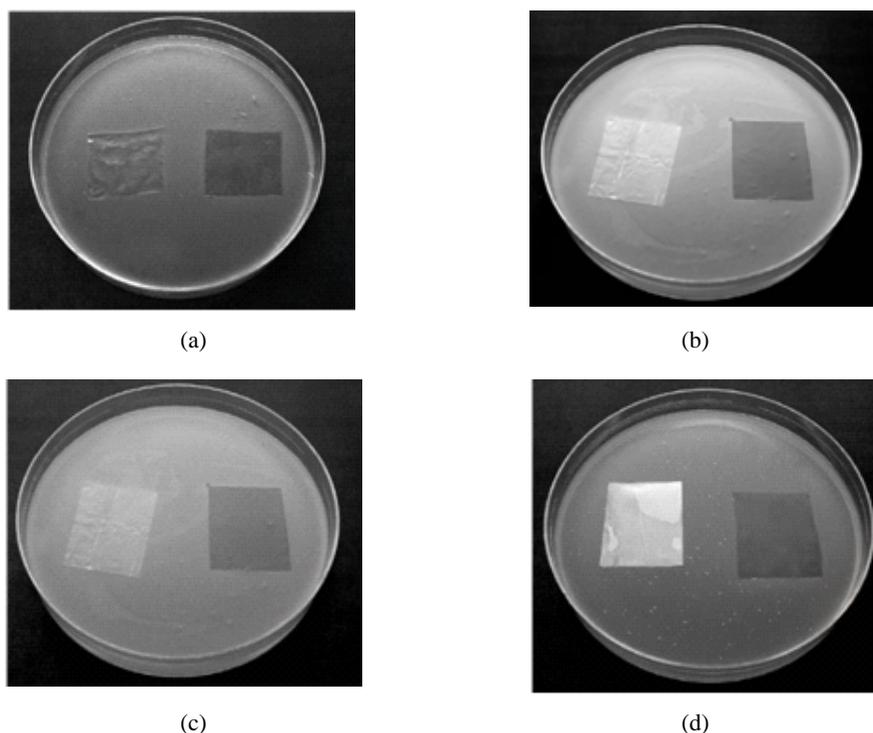


Fig. 3: Qualitative antibacterial activity evaluation of film containing lactic acid against (a) untreated film (b) antimicrobial film against *E. coli* O157:H7 (DMST12473) (c) antimicrobial film against *Listeria monocytogenes* (ATCC19115) and (d) *Salmonella Typhimurium* (DMST0562)

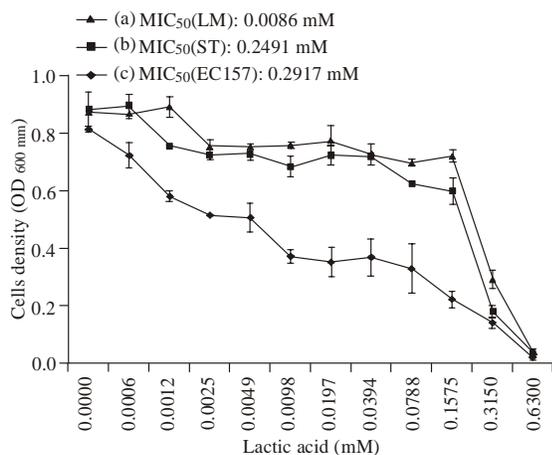


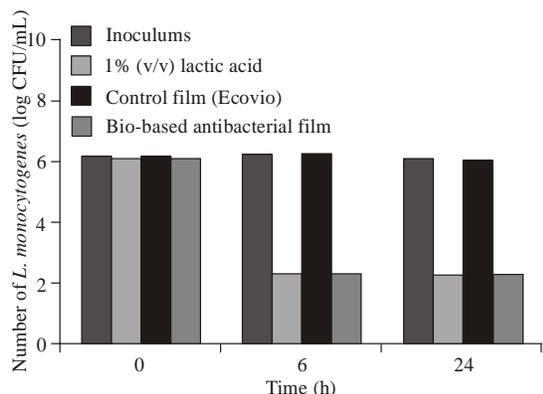
Fig. 4: Inhibition effect of lactic acid on growth of bacterial indicator. (a): *Listeria monocytogenes* ATCC19115; (b): *Salmonella Typhimurium* (DMST 0562) and (c): *E. coli* O157:H7 (DMST 12473), respectively

dramatically decreasing the film's mechanical properties. The transparency of the film contains lactic acid in this study decrease significant compared to the control film. On the other hand, the mechanical properties of the

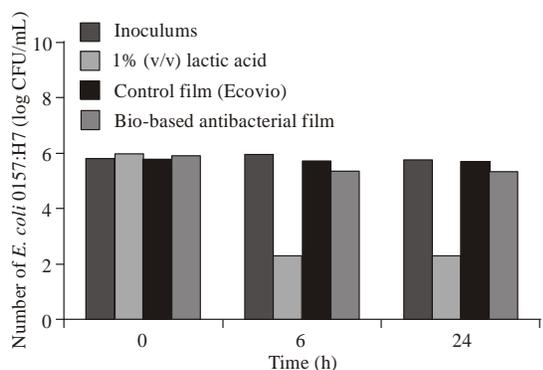
antibacterial film incorporated with sodium lactate were comparable to those of control film (data not shown), when the content of sodium lactate was between 10.0% (w/w) and 30.0% (w/w) film dry basis.

**FTIR analysis:** The FTIR was used to study the interaction between film and antimicrobial agents incorporated. Figure 1 depicts the spectra of the control film incorporated sodium lactate at varying levels used in the study. The spectra of control film and antimicrobial films incorporated with different level of sodium lactate showed the same pattern on their informative peaks as the control film. As expected, this behaviour could be considered to be because of no specific interaction between active groups of sodium lactate with functional group of control film.

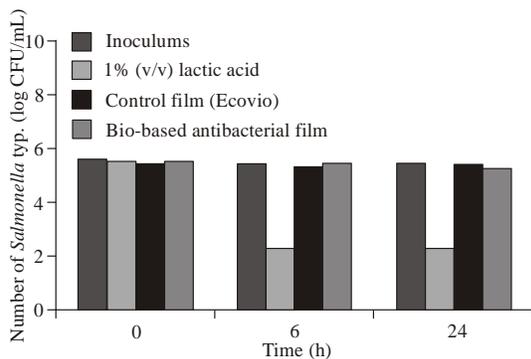
**Antimicrobial activity evaluation of the coated LAE on the PLA films:** For qualitative evaluation, the two types of films containing different antibacterial agents were tested with agar diffusion testing method (Siragusa *et al.*, 1999). Figure 2 shows the results of qualitative antibacterial activity evaluation of film containing sodium lactate at 10% (w/w) and 30% (w/w) against *E. coli* O157:H7, *L. monocytogenes* and *S. Typhimurium*.



(a)



(b)



(c)

Fig. 5: The inhibition of *L. monocytogenes*, (a) *E. coli* O157:H7; (b) and *S. Typhimurium*; (c) by the bio-based film containing antimicrobial compound in a liquid culture medium at 37°C by ASTM E2149-10 Method

Colonies of tested microorganisms could be viewed underneath film samples meaning no activity against the tested microorganisms was observed for the films with and without sodium lactate. The result is consistent with observations made by Kristo *et al.* (2008), which found Means  $\pm$  standard deviation (n = 3) are significantly different

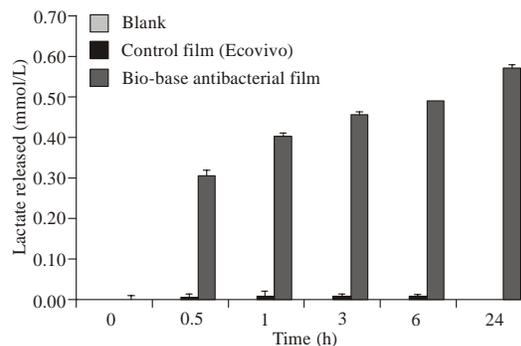


Fig. 6: Migration of lactic acid from antimicrobial film

$p \leq 0.05$  that the incorporation of 10% sodium lactate into antimicrobial films does not enhance the effectiveness of the films against *L. monocytogenes* when compared to the efficacy of sodium lactate directly added into the TSA medium. The films containing sodium lactate were slightly effective in this respect and found that only at the higher concentration (40% w/w film dry basis) (Silva *et al.*, 2009).

Although the activity of film containing 20% (w/w) sodium lactate was not determined, the film incorporated with sodium lactate at 30% (w/w) was investigated, and no antimicrobial activity was observed. It should be logical to assume that 20% (w/w) would also have no activity comparable to the testing at 30% (w/w) where no activity was observed. Consequently, it appeared that sodium lactate shows no efficacy on surface application. This result is consistent with other researchers reported that sodium lactate shows very high efficacy when direct addition into the product formulation rather than surface application (Mehyar *et al.*, 2005; Kalchayanand *et al.*, 2008).

The prepared films containing 5% (w/w) lactic acid exhibited a significantly positive antimicrobial activity against all test microorganisms in the agar disc diffusion test (Fig. 3). Colonies of *E. coli* O157:H7, *L. monocytogenes* and *S. Typhimurium* could not be viewed underneath film samples whereas such colonies were formed all over the control plates. Briefly, antimicrobial activities of films incorporate with lactic acid and sodium lactate are shown in Table 2. The microbial inhibition indicates that a portion of lactic acid was released from the extruded film sample and diffused into the agar layer, the development of microbial cells in the agar. Lactic acid and lactate have a wide action spectrum against bacteria, like food borne bacteria for example *E. coli* O157:H7, *L. monocytogenes* and *S. Typhimurium*. This is confirmed by the previous study that lactic acid shows high efficiency of surface decontamination rather than sodium lactate (Netten *et al.*, 1994). Siragusa and Dickson (1992) previously reported

Table 2: Antimicrobial activity of sodium lactate and lactic acid incorporate into Ecovio films against *E. coli* O157:H7, *Listeria monocytogenes*, *S. Typhimurium*

Antimicrobial agents	Conc. (% w/w)	<i>E. coli</i> O157:H7 inhibitory effect <sup>a</sup>	<i>L. monocytogenes</i> inhibitory effect <sup>a</sup>	<i>S. Typhi</i> inhibitory inhibitory effect <sup>a</sup>
Control	0	-	-	-
Sodium lactate	10	-	-	-
	30	-	-	-
Lactic acid	5	+	+	0

a: (+) inhibitory effect and (-) no inhibitory effect

lactic acid incorporated into calcium alginate gel showed inhibitory effect on beef tissue against *L. monocytogenes*. Hence the film incorporated with 5% (w/w) lactic acid was selected for further studies in quantitative evaluation.

For quantitative evaluation, survival of the pathogens was studied by means of the bacterial counts after contact time of 0, 6, and 24 h at 37°C. Antimicrobial film incorporate with 5% (w/w) lactic acid was highly effective in reduction of 4 log units for *L. monocytogenes* within 6 h with respect to the control (Fig 5a). The similar result was also found with positive control at 1% (w/w) lactic acid. These data indicated that the lactic acid incorporated into the Ecovivo polymer was responsible for the antibacterial activity against *L. monocytogenes*. However, there were less than 0.5 log differences between the Ecovio/lactic acid sample and the control sample at 6 and 12 h against *L. monocytogenes*. For films incorporated with lactic acid, the growth of *E. coli* O157:H7 and *S. Typhimurium* were reduced by 0.4 and 0.2 log unit respectively within the same time period at 6 and 24 h (Fig. 5b and c). While positive control, 1% (w/w) lactic acid showed 4 log reduction against both tested microorganisms. Although the different was still statistically significant, however, the difference was not sufficient to real-world situations. From this quantitative evaluation, inhibition of *L. monocytogenes* by the AM film was clearly observed. This is in consistent with the Minimum Inhibitory Concentration (MIC) studies indicated that undissociated lactic acid was more efficient in inhibiting *L. monocytogenes* than enterobacteria (Charlotta and Lindgren, 1993).

**Minimum Inhibitory Concentration (MIC) determination:** In order to confirm the above quantitative result, MICs testing were conducted for investigation of lactic acid activity against tested microorganisms. Figure 4 showed the lactic acid activity of *Escherichia coli* O157:H7, *L. monocytogenes* and *S. Typhimurium* represented by MIC values. MIC values of *L. monocytogenes*, *S. Typhimurium* and *E. coli* O157:H7 are 0.0086 mM, 0.2491 mM and 0.2917 mM respectively. This is clearly showed that undissociated lactic acid was more efficient in inhibiting *L. monocytogenes* than enterobacteria. This data can support the finding that the

film is effective against *L. monocytogenes* rather than *S. Typhimurium* and *E. coli* O157:H7. The MIC data for the lactic acid in our study were in agreement with the findings reported by other researchers (Charlotta and Lindgren, 1993).

**Lactate released from films:** Quantitation of the L-lactic acid by a YSI biochemical analyser indicated the release of lactic acid from the film which corresponded to approximately 0.6 mM under the tested condition. Lactate migration was detected from the antimicrobial film and the level increase with the testing period (Fig. 6). The concentration of lactate was released at 0.31, 0.40, 0.46, 0.49 and 0.57 mmol/L at 0.5, 1, 3, 6, and 24 h, respectively.

## CONCLUSION

The finding of the present study demonstrate lactic acid can be incorporated into biobased polymer and retain their inhibitory effect against microbial growth in model study. The bio-based film incorporated with lactic acid offers a clear advantage in preventing the growth of *L. monocytogenes* rather than films containing sodium lactate on surface application. Difficulties are however encountered in producing bio-based antimicrobial film containing lactic acid because of high brittleness of the film. With this mechanical property, therefore, the use of the AM film may be useful in application such as antimicrobial pad by putting at the bottom of tray. This antimicrobial pad may provide an alternative intervention for decontamination of food product surface (such as shrimp). This preliminary study offers a starting point to determine the potential and limitations of biobased/lactic acid films for antimicrobial packaging applications. However, additional research and development work is required to improve the mechanical properties of the material in order to meet industry requirements.

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