ผลการต้านจุลินทรีย์และลักษณะของฟิล์มเอ็กโซพอลิแซ็กคาไรด์จาก *Aureobasidium pullulans* YTP6-14 ร่วมกับซินนามอลดีไฮด์

Antimicrobial Effect and Characterization of Exopolysaccharide Film from *Aureobasidium pullulans* YTP6-14 with Cinnamaldehyde

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บทคัดย่อ

งานวิจัยนี้ศึกษาสมบัติการต้านจุลินทรีย์ของฟิล์มดัดแปลงซึ่งเตรียมโดยการผสมสารซินนามอลดีไฮด์ (Cinnamaldehyde, CH) ลงในสารละลายเอ็กโซพอลีแซคคาไรด์ (Exopolysaccharide, EPS) โดยสาร EPS ผลิตจาก *Aureobasidium pullulans* YTP6-14 สารซินนามอลดีไฮด์ที่ความเข้มข้นต่ำสุดในการยับยั้ง *Staphylococcus aureus* เท่ากับ 1.484 mg/mL และยับยั้ง *Bacillus subtilis* เท่ากับ 0.093 mg/mL เนื่องจากซินนามอลดีไฮด์เป็นสารระเหยง่าย ความเข้มข้นของสารจะมีผลต่อคุณลักษณะของแผ่นฟิลม์ ในการศึกษาจึงเลือกสารละลายฟิล์ม EPS ที่มี CH ผสมอยู่ 24 and 30 mg/mL มาใช้ในการเตรียมฟิล์ม พบว่าฟิล์มที่ไม่ได้ผสม CH มีลักษณะบาง เรียบ สม่ำเสมอ ไม่มีสี และไม่มีกลิ่น ในขณะที่ฟิล์มที่ผสม CH มีความหนาไม่สม่ำเสมอ มีสีเหลืองอ่อน ขุ่นกว่าฟิล์มที่ไม่ผสม CH และได้กลิ่นซินนามอน ฟิล์มดัดแปลงจากพอลีแซคคาไรด์ที่ผสม CH อยู่ 30 mg/mL มีสมบัติการให้การซึมผ่านของไอน้ำต่ำ ระหว่างการเก็บรักษาพบว่าปริมาณสาร CH ลดลงไปประมาณ ร้อยละ 70 และลดลงทุกสัปดาห์แต่ยังมีความเข้มข้นมากกว่า ความเข้มข้นต่ำสุดที่ใช้ในการยับยั้งจุลินทรีย์ จนถึง 5 สัปดาห์ ฤทธิ์การต้านจุลินทรีย์ของฟิล์มพบทั้งในการทดสอบในส่วนที่เป็นอาหารเลี้ยงเชื้อแบบเหลวและแข็ง และฟิล์มที่มีการผสม CH ในปริมาณ 30 mg/mL แสดงผลการต้านจุลินทรีย์สูงสุดต่อทุกเชื้อจุลินทรีย์

คำสำคัญ: ฟิล์มพอลีแซคคาไรด์ ซินนามอลดีไฮด์ บรรจุภัณฑ์ชนิดแอคทีฟ *Aureobasidium pullulans*

ABSTRACT

This study describes antimicrobial properties of modified films prepared by incorporation of cinnamaldehyde (CH) into the solution of exopolysaccharide (EPS). EPS was produced by *Aureobasidium pullulans* YTP6-14 and partial physical properties were investigated. CH exhibited high antimicrobial activity against bacterial and fungal at low concentration. The highest and the lowest minimum inhibitory concentration (MIC) was observed against *Staphylococcus aureus* and *Bacillus subtilis* at 1.484 and 0.093 mg/mL, respectively. Film forming solutions containing CH 24 and 30 mg/mL were chosen to form the films. The film without CH was thin, smooth, homogeneous, colorless and odorless while the incorporated film was slightly thicker and inhomogeneous in the matrix, exhibited more yellow tinge, and less transparent with cinnamon odor. Moreover, the modified polysaccharide film with 30 mg/mL CH exhibited lower water vapor permeability. Amount of CH (%CH) was lost during film preparation (approximately 70%) and gradually decreased every week but remained higher than MIC value for at least 5 weeks. The antimicrobial activity of the films was observed in both culture broth and agar plate. The polysaccharide film with CH 30 mg/mL showed the highest antimicrobial activity against all strains.

Keywords: polysaccharide based film, cinnamaldehyde, active packaging, *Aureobasidium pullulans*

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INTRODUCTION

Food spoilage is one of the main reasons for the economic loss in the food industry. Polysaccharide based film coating has been reported as one of the ways to preserve and maintain the quality and safety of the foods [1]. Nowadays, customers demand for natural products and environmental concerns are increasing. This leads to possibility of using biopolymers to replace synthetic polymers. Exopolysaccharide or extracellular substances (EPS) are biopolymers synthesized by microorganisms, algae, plant microorganisms (MO) and animals [2].

There is growing interest for MO polysaccharides to be produced at industrial scale. This technology offers many advantages such as short production time, reduce production cost (able to produce by agricultural waste) [3-4], independence of climate and require only small production area [5]. There are many polysaccharides that have the capability of forming a biodegradable film including starch, chitosan, kefiran, alginate, carrageenan, pullulan and pectin [6-9]. Moreover, many studies indicate that the polysaccharide film can prevent the moisture loss, aroma loss, gas diffusion or water absorption in the product matrix [10-11].

Aureobasidium pullulans is a black yeast- like fungus widely found in many environments such as soil, sea shore, plant, etc. with different morphologies such as yeast cell, septate mycelium and chlamydospore. This fungus exhibit different biochemical characteristics bring about to an accessible source for biotechnological applications. They can secrete EPS, well known as pullulan, consists of maltotriose repeating unit connected with **α**-(1, 6) linkage, the internal glucose units link by **α**-(1, 4)-glycosidic bond [12]. Pullulan has many promising properties such as edibility, water solubility, tasteless and odorless. It also has ability of film forming which flexible, heat and pH stability, and exhibit excellent adhesiveness to the surface when dry [13].

However, pullulan does not exhibit antimicrobial properties [14-15]. Hence, combination with antimicrobial agent is necessary to inhibit the microbial growth. However, the use of chemicals is hardly acceptance by consumer. Essential oils are complex mixtures of natural substances from vegetable matter extraction. Cinnamaldehyde (CH), a natural and edible product was selected to serve as antimicrobial agent for this purpose. CH is one of the main active compounds of the cinnamon oil in the group of essential oils, widely accepted with 'generally recognized as safe' (GRAS) status [16]. It is classified as a natural preservative and currently popular in research [17-19]. It has pale yellow color, cinnamon odor and is generally used as a flavoring agent in many kinds of food. CH has a broad spectrum of inhibition of many food spoilage microorganisms including bacteria and fungi [20].

This study was focusing on the effects of the addition of plant CH into the polysaccharide based film. There is no study on the addition of CH to polysaccharide film or pullulan from *Aureobasidium pullulans* YTP6-14 so far. The aims of the study were 1) Characterization of exopolysaccharide film appearance after blending with CH, 2) Quantitation of the remaining CH in the EPS based film and control-

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release profile, and 3) Influence of modified film on tested MOs growth or antimicrobial activity.

MATERIALS AND METHODS

1. Reagents and tested microorganism

Cinnamaldehyde (purity >95%, Food Grade), Pullulan standard from *Aureobasidium pullulans* (HPLC Grade), Pullulanase microbial, maltotriose (purity >95%, HPLC Grade) and glucose (purity ≥ 99.5%, HPLC Grade) were purchased from Sigma-Aldrich (USA). Yeast extract was obtained from Biospringer (France). Malt extract, Bacto™ Peptone, Potato Dextrose broth/agar (PDB/PDA), Mueller Hinton broth/agar (MHB/MHA) were procured from Difco Laboratories (USA). Hydrochloric acid (HCL), acetic acid, sulfuric acid, sodium hydroxide (NaOH), water (HPLC Grade), acetonitrile (HPLC Grade) and sodium acetate trihydrate were purchased from Merck (Germany). Magnesium nitrate hexahydrate (AR Grade) was supplied by QREC (New Zealand). *Aureobasidium pullulans* YTP6-14 which can produce biosurfactant was isolated from Koh Si Chang (Chonburi, Thailand) [21]. *Staphylococcus aureus* ATCC 6538P, *Escherichia coli* ATCC 8739, *Salmonella* Typhimurium MSCU 0492, *Bacillus subtilis* ATCC 16643, *Aspergillus niger* MSCU 0361 and *Aspergillus flavus* MSCU 0580 were obtained from culture collection of the Department of Microbiology, Faculty of Science, Chulalongkorn University (Bangkok, Thailand).

2. Polysaccharide production

A. pullulans YTP6-14 was pre-cultured on yeast extract malt extract (YM) agar plate (yeast extract 3 g/L, malt extract 3 g/L, peptone 5 g/L, glucose 10 g/Land agar 20g/L), 30 °C for 3 days and then cultivated in YM broth with shaking at 30 °C, 200 rpm until optical density (600 nm) in range 0.8-1.0 was obtained [22]. Then 10% (v/v) inoculum was transferred into production medium (yeast extract 3 g/L, malt extract 3 g/L, peptone 5 g/L and sucrose 50 g/L) and the cell culture was incubated under the same conditions for 3 days. Culture broth was centrifuged at 8000 *g* for 40 min at 4 °C to remove the cells. Supernatant was collected and 2 volumes of 95% cold ethanol were added. The solution was kept at 4 °C overnight and subsequently centrifuged to precipitate EPS and removed the ethanol waste. The EPS was kept at -20 °C and dry weight was measured after freeze drying with lyophilizer (model N-100; Eyela, Japan). The dried EPS was in kept in a desiccator for further experiments. EPS is characterized and confirmed by use enzymatic method by employing pullunase and confirm the present of monomer by HPLC as previously described [23].

3. Determination of MIC of CH

The MIC of the tested strains was measured by broth microdilution in a 96 well plate method [24]. CH was diluted in range of 95-0.0232 mg/mL with MHB (serial 2 fold dilution, 12 concentrations) for bacteria and PDB for fungi. The bacterial inoculum was prepared with MHB overnight cell-cultured and diluted to $5x10^5$ CFU/mL. For the fungal spore suspension, the quantity of spore was counted under hemocytometer corresponding to $5x10^4$ CFU/mL in physio water (NaCl 8.5 g/L, Tween 80 0.1 g/L). Resazurin was used as a redox indicator to detect the viable cells [25]. The 96 well plates were incubated at

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37 ºC, 16-24 h for bacteria and 25ºC, 48-72 h for fungi. The lowest concentration of CH was recorded as MIC value that completely inhibited growth of microbial strains. This experiment was performed with six replicates.

4. EPS film forming solutions incorporated with CH

Dried EPS 7 g was dissolved in sterile water (100 mL) and glycerol (1.5 g) was added as a plasticizer and mixed to homogeneity with magnetic stirrer at room temperature. Since CH is a volatile substance. The concentration of the substance will have an effect on the characteristics of the film. Then the concentration of CH at 1.5 (MIC of *S. aureus*), and 3, 6, 12, 24, 30, 36, 42 and 48 mg/ mL were selected for incorporated and blended into the film solutions for 2 h or until the solution is clear. The control EPS film solution was prepared in the same way except for adding CH.

Antimicrobial activity of film solutions was determined using agar well diffusion method [26]. Bacterial strains were cultured overnight in MHB at 37 ºC. The Mueller-Hinton Agar plates (20 mL) were smeared with overnight cultured of bacterial suspension at 10^8 CFU/mL or 0.5 McFarland standard by using sterile cotton swap. Meanwhile, spore suspension of mold strains was prepared by grown cells on potato dextrose agar (PDA) at 25 ºC for 3 days suspended in physio water (0.85% NaCl plus Tween80) and then spores were count by hemocytometer. The 10^5 CFU/ mL of spore suspension was spread evenly on the PDA plates (20 mL). After the surfaces were dried, the agar plate was punched with cork borer No. 4 (diameter 8 mm) at the center. EPS film forming solutions with CH were loaded into the wells and incubation at 37 ºC, 16-24 h and 25 ºC, 48-72 h for bacteria and fungi, respectively. The inhibition zone was measured and the control test was EPS solution without CH. The test of activity of film solutions was carried out in triplicate.

5. Characterization of EPS film with CH

CH Film solutions which contained 24 and 30 mg/mL of CH were mixed and 30 mL of film solutions were transferred to the acrylic trays (10 × 15 cm). The EPS films were dried in hot air oven (45-50 ºC) for 3-4 h then peeled off and stored at room temperature in a desiccator with magnesium hexahydrate for RH 53% condition for 48 h before next experiments [27].

For film structure determination, the structure of modified and control EPS films was observed under the field emission scanning electron microscope (FE-SEM, model JSM-7610F and X-MaxN 20; JEOL, Japan). Samples were coated with gold for improving the image of the film surface. The films were broken in liquid nitrogen before the gold coating for cross section observation.

For the thickness of the films, both, control and modified film, was measured by using digimatic micrometer (Model MDC-25SX; Mitutoyo Corporation, Japan). Various points (≥ 25) of the film were measured to determine the mean and standard deviation of the thickness.

The color of the EPS films obtained was described in three dimensions of the Hunter color space, which are L* (+ brightness, - darkness), a* (+ red, - green), and b* (+ yellow,

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- blue) or CIELAB color (International Commission on Illumination). The EPS films were measured with a Minolta Chromameter (Model CR-300; Minolta Camera Corporation, Japan) by detecting color at various points of the film. Mean and standard deviations were calculated. The total color difference value (ΔE) was calculated using equation (1) [28].

$$\Delta E = [(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2]^{0.5} \quad \text{(Equation 1)}$$

Water vapor permeability (WVP) of the EPS films was determined following McHugh et al. (1993) [29]. The films were cut in the circular shape with 6 cm diameter and coated over the glass cups containing silica gel. The samples were pre-weighed and stored in an incubator at 25°C with 75% RH. The film coated cups were weighed at 1, 2, 4, 6 and 8 h. The water vapor transmission rate (WVTR) was calculated using equation (2) [2] from the slope of cups' weight gain (Δw) during the time (Δt) and with film area (A). WVP was calculated by using equation (3) [4], WVTR value was used to calculate WVP with film thickness (x) and the partial water vapor between inner and outer (Δp) film surface.

$$WVTR = (\Delta w)(A\Delta t)^{-1} \quad \text{(Equation 2)}$$

$$WVP = (WVTR)(x)(\Delta p)^{-1} \quad \text{(Equation 3)}$$

where the unit for water vapor permeability is g mm h^{-1} m^{-2} kPa^{-1}

6. Quantitation and antimicrobial activity of CH in the EPS films

Quantitation of CH in the EPS films was carried out by gas chromatography [30]. Dried EPS with incorporated 24 and 30 mg/mL CH were cut into 4×4 cm squares. The films were transferred into the screw cap tube with 10 mL of dimethyl sulfoxide (DMSO). The tubes were shaken at 200 rpm for 4 h in order to dissolve the film. Cleaning of the film debris and dirt from the solutions was performed with 0.2 µm pore filter. The samples were measured by GC (model GC-2010, Shimadzu, Japan) with STX-5MS column (serial number 97137, length 30 m, thickness 0.25 µm), flow rate of 1.34 mL/min with the injection volume 1.0 µl. Helium was the carrier gas and the injector temperature was set at 260 °C. The CH content of the films was measured at 0, 1, 2, 3, 4 and 5 weeks. The amount of CH was calculated compared to the graph of the CH standard ($R^2 \geq 0.99$) and the peak area converted into net CH quantity.

The preliminary test of antimicrobial activity of EPS films with incorporated CH was modified by using disc diffusion method. The films were cut into small discs with 1.2 cm diameter (with an area of approximately 1 cm^2). Bacterial inoculums were culture in MHB overnight; the cultures broth were adjusted to 0.5 McFarland standard. For the fungal spore suspension, the quantity of spore was counted corresponding to 10^5 CFU/mL in physio water. The bacterial and spore suspensions were placed by cotton smear or spread evenly on MHB and PDA plates, respectively. The EPS film disc was placed at the center of the plate after the surface had been dried. The EPS film without CH was used as a control. The MHAs were incubated at 37 °C for 16-24 h whereas the PDAs were stored in an incubator at 25 °C for 48-72 h. The clear zones were measured and recorded for the inhibitory activity of the modified films.

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In order to evaluate the bactericidal activity, the film with incorporated CH was cut into 1×1 cm squares and kept overnight in 5 mL of broth cultures of bacterial strains (5×10^5-10^6 CFU/mL). The tubes were incubated at room temperature (25-30°C) and total viable cells were counted after 0, 2, 4, 8, 24 and 48 h. The samples were diluted 10-fold in 0.85% normal saline and transferred onto MHA and incubated at 37 °C for 24 h and reported as $\log_{10}$CFU. The film without CH and the tube without any film were considered as the control sets. All experiments were carried out in triplicate.

RESULTS AND DISCUSSION

1. Antimicrobial properties of CH

As shown in Table 1, the most susceptible strain was *B. subtilis* ATCC 16633 with the lowest MIC of 0.0928 mg/mL while *S. aureus* ATCC 6538P exhibited the strongest resistance to the CH at 1.4840 mg/mL. *E. coli* ATCC 8739 and *S.* Typhimurium MSCU 0492 were completely inhibited at 0.3711 mg/mL of CH. The results are consistent with previously research [16] which reported that *B. cereus* was the most susceptible strain to CH inhibition while *S. aureus*, *E. coli* and *K. pneumonia* were more tolerant. CH at 0.1855 mg/mL was providing effective inhibition of *A. flavus* MSCU 0580 and *A. niger* MSCU 0361. The MIC of fungi was lower than bacteria as reported by Ooi et al. (2006) [31] who observed that the concentration of both cinnamon oil and CH for filamentous fungi ranged from 0.075-0.150 mg/mL while higher for bacteria that between 0.075-0.6 mg/mL. CH is commonly known as a strong antifungal agent which can decrease the spore germination and significantly change in morphology of hypha [32]. This because it acts as an ATPase and cytokine-involved enzyme inhibitor leading to cell wall synthesis disruption, membrane perturbation and specific enzyme activity blockage [20].

Table 1 The minimum inhibitory concentration (MIC) of cinnamaldehyde (CH) against tested bacteria and fungi

Strains	MIC (mg/mL)
Staphylococcus aureus ATCC 6538P	1.4840
Bacillus subtilis ATCC 16633	0.0928
Escherichia coli ATCC 8739	0.3711
Salmonella Typhimurium MSCU 0492	0.3711
Aspergillus flavus MSCU 0580	0.1855
Aspergillus niger MSCU 0361	0.1855

The 7% EPS with glycerol film solution appeared as a viscous, homogenous, odorless and colorless liquid when dissolved in water. The film solution with incorporated CH was observed an initial separation as a droplet during initial blending. According to previous report [32], CH is slightly soluble in water therefore it can be separated when mixing in

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aqueous solution. This is because pullulan consists of maltotriose repeating unit connected with **α**-(1, 6) linkage and the internal glucose units link by **α**-(1,4)-glycosidic bond. However, after blending the solution for 2 h the shear force broke droplet of the CH into tiny droplet and disperse uniformly in the film solution.

The antimicrobial activity of film solutions was performed to determine suitable CH concentration for microbial inhibition by agar well diffusion method. Low CH concentration in the film solution (at 1.50 mg/mL) was found to cover MIC of every tested strains and the highest concentration used in this experiment was observed at 48 mg/ mL CH in EPS film solution as shown in table 2.

Table 2 Effects of CH incorporated in EPS film forming solutions on tested strains

Strain	Concentration of CH in EPS film solution (mg/mL)									
	control (0)	1.5	3	6	12	24	30	36	42	48
	Diameter of inhibition zone (cm ± SD)									
S. aureus	ND	ND	2.43±0.10^{f}	3.05±0.10^{e}	3.50±0.00^{d}	3.93±0.10^{c}	4.13±0.19^{bc}	4.23±0.10^{b}	4.55±0.06^{a}	4.35±0.13^{ab}
B. subtilis	ND	ND	1.64±0.05^{d}	2.35±0.17^{c}	2.86±0.05^{b}	3.00±0.00^{b}	3.70±0.08^{a}	3.63±0.05^{a}	3.65±0.06^{a}	3.60±0.00^{a}
E.coli	ND	ND	1.00±0.00^{e}	1.38±0.10^{d}	1.78±0.03^{c}	2.29±0.02^{b}	2.65±0.06^{a}	2.65±0.06^{a}	2.75±0.06^{a}	2.70±0.00^{a}
S. Typhimurium	ND	ND	1.35±0.06^{e}	1.70±0.00^{d}	2.19±0.10^{c}	2.78±0.05^{b}	3.15±0.06^{a}	3.08±0.05^{a}	3.08±0.10^{a}	3.10±0.08^{a}
A. flavus	ND	ND	3.50±0.10^{e}	5.08±0.31^{d}	6.47±0.32^{c}	7.88±0.48^{b}	8.50±0.01^{a}	8.32±0.39^{ab}	8.50±0.00^{a}	8.50±0.00^{a}
A. niger	ND	ND	1.70±0.00^{c}	2.90±0.11^{b}	3.22±0.15^{b}	3.15±0.29^{b}	4.15±0.27^{a}	3.97±0.16^{a}	4.00±0.23^{a}	3.90±0.32^{a}

ND – antimicrobial activity was not detected, diameter of agar well was 8 mm.

The values are mean ± standard deviation (SD) of three separate experiments. The different superscript letters in the same row show a significant ($P < 0.05$) difference between the means.

The mean values were compared by one-way ANOVA, Tukey test.

The inhibition zone can measure when film solution containing 3 mg/mL CH was used. EPS may have diluted and detained CH in its matrix resulting in the retardation of CH antimicrobial activity [33]. The control without CH has not shown any antimicrobial property as also found in a previous report [15]. The highest level of growth inhibition for bacteria were found for *S. aureus* and *B. subtilis.* The lowest inhibition effect of CH film solution was observed with *E. coli* which indicated that it is more resistance to CH than the other bacteria. Gram-negative bacteria are resistance to CH due to its cell wall is complex. Although, its peptidoglycan layer is thinner than cell wall of Gram- positive bacteria. It has an outer membrane (OM) lies outside of the thin peptidoglycan layer. OM composed of a phospholipids bilayer that is linked to inner membrane by lipopolysaccharides (LPS). LPS consists of lipid A, the core polysaccharide, and the O- side chain, which provides the obstruction that allows Gram-negative bacteria to be more resistant to essential oils and other

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natural extracts with antimicrobial active. For this reason, Gram- negative bacteria are relatively resistant to hydrophobic antibiotics and toxic drugs [34-35]. The structure of the Gram- positive bacteria cell wall allows hydrophobic molecules to easily penetrate the cells and act on both the cell wall and the cytoplasm. Phenolic compounds, which are also present in the essential oil, generally show antimicrobial activity against Gram- positive bacteria. *A. flavus* was the most sensitive organism to the modified film solutions since no growth was observed on PDA plate with 30 to 48 mg/mL of CH in the film solutions. CH at 24 and 30 mg/mL were thus selected to prepare the EPS films since there was no significant inhibition zone at higher concentrations.

2. Characterization of EPS film with incorporated CH

Control sample, 7% w/v EPS film was transparent, colorless, odorless and smooth. The films with incorporated CH were less transparent, soft, exhibit yellow color and cinnamon odor. The films were stored in a desiccator RH 53%. The EPS film structure was observed under FE-SEM as shown in Figure 1. The control film without CH appeared smooth and homogenous at the surface and in cross section. In contrast, the modified films contained dispersed oil droplets at the surface and in cross section. The increasing amount of oil droplet are related to the increasing amount of CH.

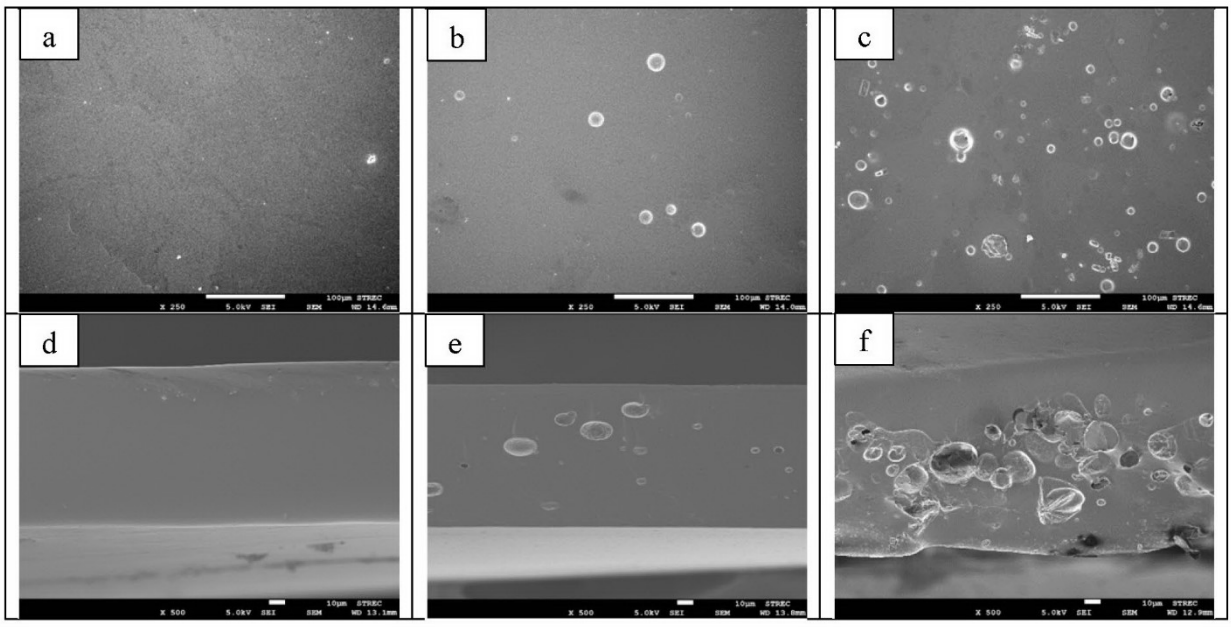

Figure 1 FE-SEM image of the surface and the cross section of EPS film with incorporated CH: a) surface of a control film, b) surface of a film with 24 mg/mL CH, c) surface of a film with 30 mg/mL CH, d) cross section of the control film, e) cross section of the film with 24 mg/mL CH and f) cross section of the film with 30 mg/mL CH.

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Thickness of the film exhibited no significantly different as shown in table 3. However, the thickness of the film with 30 mg/mL CH (0.12 mm) was slightly higher from the control film and that the film contained 24 mg/mL CH. Adding the plant extract or essential oil makes the film thicker [15,36]. This might be due to the encapsulation of the insoluble extract in the EPS matrix. Moreover, the addition of CH into the film caused color change. The control film showed brightness of 87.985±1.305, redness of -1.300±0.026 and yellowness of 3.330±0.187 while the modified film with 30 mg/mL CH exhibited the highest yellowness (b*) at 19.368±3.350 and the least redness (a*) at -3.964±0.272 or more green appearance. The yellowness and redness of film incorporated with 24 mg/mL CH was 10.585±2.304 -3.063±0.254. The brightness (L*) of all the EPS films was not significantly change comparing to the control. The color difference value (ΔE) of the films was calculated. It can be concluded that adding CH related to the color difference. The most significant color change was found in the EPS film with 30 mg/mL with a ΔE value of 19.080±3.638 followed by 24 mg/mL with a ΔE value of 9.668±2.338 and the control film with a ΔE value of 3.1623±0.920. The increasing amount of CH which contribute to yellowness and opaque liquid make the ΔE value significantly increased in the EPS film with 30 mg/mL compared to ΔE value of 24 mg/mL.

Table 3 Effect of CH on thickness and color of EPS film

CH in EPS film	control	24 mg/mL	30 mg/mL
Thickness^{ns}	0.10±0.014	0.11±0.013	0.12±0.017
*L**	87.985±1.305^{a}	88.148±0.405^{a}	86.788±0.484^{a}
*a**	-1.300±0.026^{a}	-3.063±0.254^{b}	-3.964±0.272^{c}
*b**	3.330±0.187^{c}	10.585±2.304^{b}	19.368±3.350^{a}
ΔE	3.1623±0.920^{c}	9.668±2.338^{b}	19.080±3.638^{a}

CH concentrations: the values are mean ± standard deviation (SD) of three separate experiments. ns : non-significant different

The different superscript letters in the same row show a significant ($P < 0.05$) difference between the means. The mean values were compared with one-way ANOVA, Tukey test.

The water vapor permeability (WVP) of the EPS film with incorporated CH at two concentrations was determined at 25 ºC (RH 75%). The results shown in Figure 2 indicate that addition of CH at 30 mg/mL had a significant effect on WVP. The 30 mg/mL CH film shows the lowest value of 1.295±0.010 g mm h^{-1} m^{-2} kPa^{-1}. The value increased to 1.431±0.082 g mm h^{-1} m^{-2} kPa^{-1} for EPS film with 24 mg/mL CH and the control film had the highest value of 1.499±0.046 g mm h^{-1} m^{-2} kPa^{-1}. The results might be explained by the hydrophobic CH being entrapped in the film matrix and thus preventing the water vapor from the EPS film.

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This agrees with the observation of previous research [36], which reported that addition of essential oil can improve WVP, tensile strength and elasticity of chitosan film.

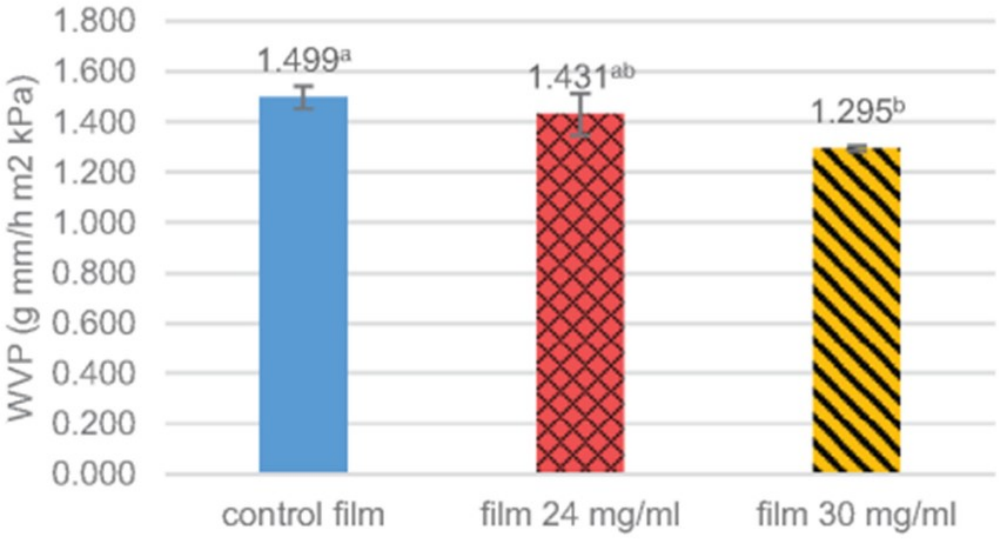

Figure 2 Water vapor permeability of the EPS film with incorporated CH. The charts represent the mean value ± standard deviation of three experiments. The different superscript letters on the bar show a significant difference ($P < 0.05$) between the means.

3. Content of CH in film

Table 4 shows that after drying the film contained CH both 24 and 30 mg/mL, the remaining amounts of CH were 28.53 and 32.87% of the initial amount, respectively. The CH reduction indicate that CH is a heat-labile substance. According to Wong (1989) [37], CH may be transformed into benzaldehyde and glyoxal in the presence of oxygen and heat. However, CH is more stable in cinnamon oil which contains eugenol comparing to pure CH. This might be the anti-oxidative property of eugenol that contributes to CH stability [30]. Therefore, drying temperature may need to be lowered or absent of oxygen during drying to improve remaining content of CH in the film. CH content has gradually decreased every week during storage at room temperature. However, the EPS film with incorporated 24 and 30 mg/mL still had a remaining CH content (3.60 and 5.38 mg/mL after at least 5 weeks) that was higher than the MIC value (1.5 mg/mL).

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Table 4 Percentage of CH remaining in the EPS film after drying and during storage for 5 weeks

Condition	EPS Film							
	CH 24 mg/mL				CH 30 mg/mL			
	CH (mg)	% remaining	CH (mg/mL)	CH/film area (mg/cm^2)	CH (mg)	% remaining	CH (mg/mL)	CH/film area (mg/cm^2)
Solution	720.00	100.00	24.00	4.80	900.00	100.00	30.00	6.00
Dried film	205.42	28.53	6.85	1.37	295.87	32.87	9.86	1.97
1st week	194.86	27.06	6.50	1.30	270.19	30.02	9.01	1.80
2nd week	175.48	24.37	5.85	1.17	246.81	27.42	8.23	1.65
3rd week	149.23	20.73	4.97	0.99	222.74	24.75	7.42	1.48
4th week	128.98	17.91	4.30	0.86	195.44	21.72	6.51	1.30
5th week	107.98	15.00	3.60	0.72	161.31	17.92	5.38	1.08

4. Measurement of the antimicrobial activity of EPS film

The film-disc diffusion method was used to confirm the antimicrobial activity of the film after the drying process. Figure 3 shows that films with CH exhibited the antimicrobial activity while the control film without CH did not show any inhibition. However, the results from the film-discs inhibition indicate that the activity of CH in the dried film was slightly lower than in the film solution (Table 2) at CH concentration of 24 mg/mL. This might be explained by the loss of CH during the film preparation process. However, the modified film with 30 mg/mL was the most effective among the tested concentration, especially on *S. aureus* and *A. flavus* with 5.62±0.25 and 8.50±0.00 cm, respectively. Although, there is a loss during film preparation but the evaporation of water during film formation may increase the concentration per area of CH and hence increase its activity.

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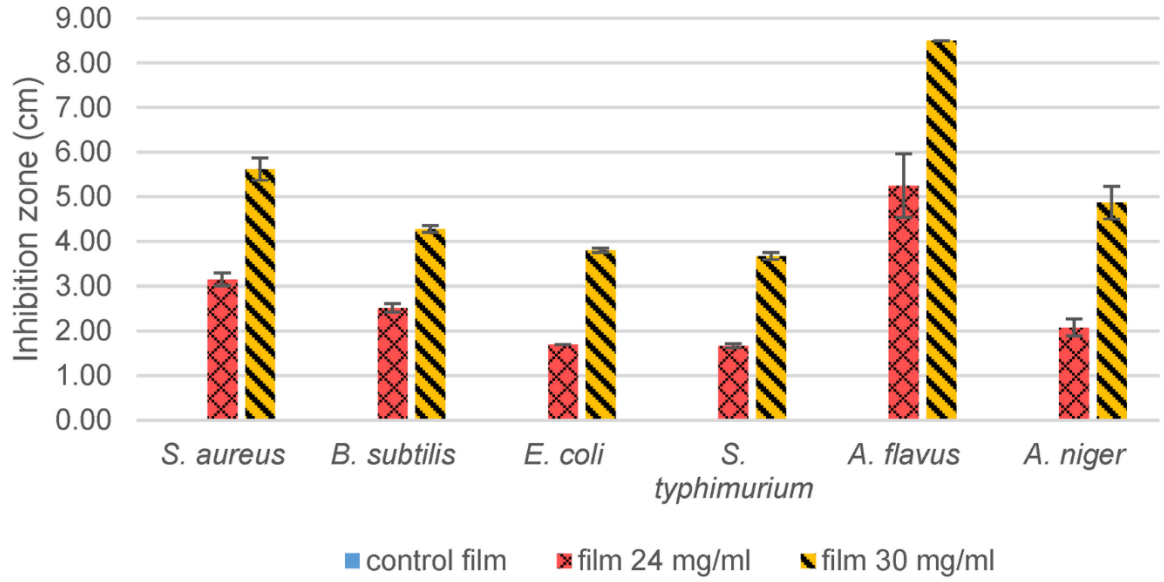

Figure 3 The inhibition zone of the tested strains with film-disc diffusion method with three different types of the film (control film, film with 24 mg/mL CH and film with 30 mg/mL CH). The values are mean ± standard deviation of three experiments.

The bactericidal activity of the modified EPS film was determined by observing the number of viable cell (Figure 4). The amount of bacteria remained unchanged during the initial 4 h of the experiment. This might due to the adaptation of bacterial cell when transferred to the new medium [38]. Moreover, it might due to the release rate of CH from the EPS film being low and thus unable to inhibit bacterial growth at this time. The film contained 30 mg/mL CH exhibited the largest bactericidal effect on every strain. This film reduced the number of the cell of *S. aureus* and *B. subtilis* by 3.3 log CFU/mL, of *E. coli* by 2.5 log CFU/mL and of *S.* Typhimurium by 4.7 log CFU/mL in comparison with the control. The film contained 30 mg/mL CH showed an obvious bacteriostatic effect on *B. subtilis* and control viable cell of all bacteria below the initial number at 48 h. The EPS control film revealed the highest number of bacteria among all tests, as the bacteria may use the EPS film as a carbon source having exhausted the substrate in the medium after 48 h. As for EPS film with incorporated 24 mg/mL CH, a low concentration of active compound or slow release rate result in lack of control over the proliferation of the bacteria [39].

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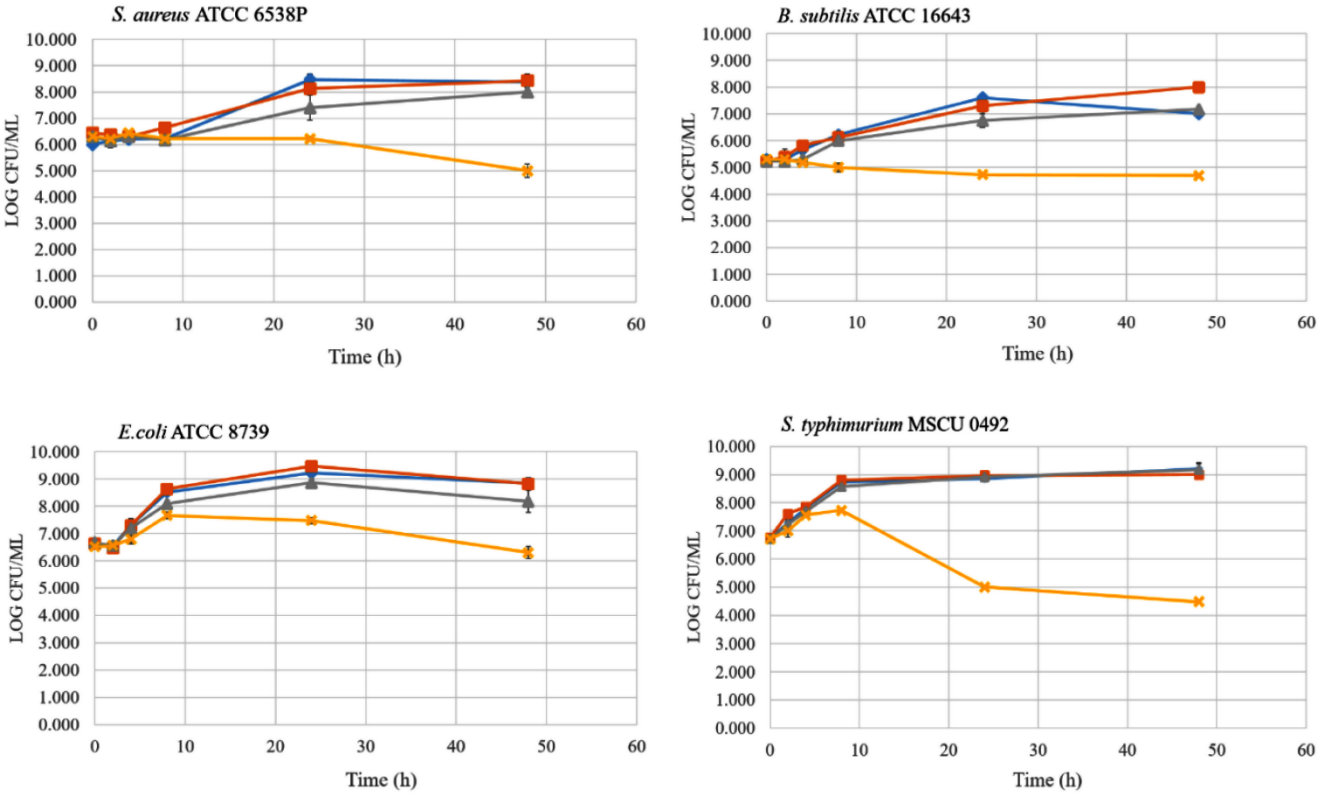

Figure 4 Effectiveness of CH released from EPS films. The values are mean ± standard deviation; *diamond* represents control without film, *square* indicates control EPS film, *triangle* represents EPS film with incorporated 24 mg/mL CH and *cross* indicates EPS film with incorporated 30 mg/mL CH.

CONCLUSIONS

CH exhibited high antimicrobial activity against both bacteria and fungi. The activity persisted after blending with the EPS solution although the release of CH was slow. Incorporation of CH into the EPS film did not affect the film brightness although a more green- yellow tinge could be detected. The modified EPS film with incorporated 24 mg/mL CH had the same thickness as the control film. The modified EPS film with incorporated 30 mg/mL CH had lower water vapor permeability. The film with incorporated CH maintained the antimicrobial activity after drying. The CH content was decreasing every week but remained higher than MIC value for least 5 weeks. The EPS film with incorporated 30 mg/mL CH can maintain the release ability of the active CH for a minimum of 48 h and hence this can prolong the shelf life of the product when the modified film is applied. Nevertheless, further studies including the gas permeability, mechanical strength and applications on the commercial products are required to devise the appropriate uses of the film.

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REFERENCES

[1] Diab, T., Biliaderis, C.G., Gerasopoulos, D. and Sfakiotakis, E. (2001). Physicochemical properties and application of pullulan edible films and coatings in fruit preservation. Journal of the Science of Food and Agriculture. 81(10): 988-1000.

[2] Shi, L. (2016). Bioactivities, isolation and purification methods of polysaccharides from natural products: A review. International Journal of Biological Macromolecules. 92: 37-48.

[3] López, C.G., Fernández, F.A., Sevilla, J.F., Fernández, J.S., García, M.C. and Grima, E. M. (2009). Utilization of the *cyanobacteria Anabaena* sp. ATCC 33047 in CO2 removal processes. Bioresource Technology. 100(23): 5904-5910.

[4] Thompson, J. C. and He, B. B. (2006). Characterization of crude glycerol from biodiesel production from multiple feedstocks. Applied Engineering in Agriculture. 22(2): 261-265.

[5] Donot, F., Fontana, A., Baccou, J. C. and Schorr-Galindo, S. (2012). Microbial exopolysaccharides: main examples of synthesis, excretion, genetics and extraction. Carbohydrate Polymers. 87(2): 951-962.

[6] Espitia, P. J. P., Du, W. X., de Jesús Avena-Bustillos, R., Soares, N. D. F. F. and McHugh, T. H. (2014). Edible films from pectin: Physical- mechanical and antimicrobial properties-A review. Food Hydrocolloids. 35: 287-296.

[7] Galus, S. and Kadzinska, J. (2015). Food applications of emulsion-based edible films and coatings. Trends in Food Science & Technology. 45(2): 273-283.

[8] Xu, Q., Chen, C., Rosswurm, K., Yao, T. and Janaswamy, S. (2016). A facile route to prepare cellulose- based films. Carbohydrate Polymers. 149: 274-281.

[9] Zolfi, M., Khodaiyan, F., Mousavi, M. and Hashemi, M. (2014). The improvement of characteristics of biodegradable films made from kefiran– whey protein by nanoparticle incorporation. Carbohydrate Polymers. 109: 118-125.

[10] Aider, M. (2010). Chitosan application for active bio-based films production and potential in the food industry. LWT-Food Science and Technology. 43(6): 837-842.

[11] Dutta, P.K., Tripathi, S., Mehrotra, G.K. and Dutta, J. (2009). Perspectives for chitosan based antimicrobial films in food applications. Food Chemistry. 114(4): 1173-1182.

[12] Leathers, T. D. (2003). Biotechnological production and applications of pullulan. Applied Microbiology and Biotechnology. 62(5-6): 468-473.

[13] Singh, R.S., Saini, G.K. and Kennedy, J.F. (2008). Pullulan: microbial sources, production and applications. Carbohydrate Polymers. 73(4): 515-531.

[14] Ravella, S.R., Quiñones, T.S., Retter, A., Heiermann, M., Amon, T. and Hobbs, P.J. (2010). Extracellular polysaccharide (EPS) production by a novel strain of yeast- like fungus *Aureobasidium*

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pullulans. Carbohydrate Polymers. 82(3): 728-732.

[15] Gniewosz, M., Synowiec, A., Krasniewska, K., Przybył, J. L., Baczek, K. and Weglarz, Z. (2014). The antimicrobial activity of pullulan film incorporated with meadowsweet flower extracts (Filipendulae ulmariae flos) on postharvest quality of apples. Food Control. 37: 351-361.

[16] Al-Bayati, F.A. and Mohammed, M.J. (2009). Isolation, identification, and purification of cinnamaldehyde from *Cinnamomum zeylanicum* bark oil. An antibacterial study. Pharmaceutical Biology. 47(1): 61-66.

[17] Gallucci, M. N., Oliva, M., Casero, C., Dambolena, J., Luna, A., Zygadlo, J. and Demo, M. (2009). Antimicrobial combined action of terpenes against the food borne microorganisms *Escherichia coli, Staphylococcus aureus and Bacillus cereus*. Flavour and Fragrance Journal. 24(6): 348-354.

[18] Inouye, S., Takizawa, T. and Yamaguchi, H. (2001). Antibacterial activity of essential oils and their major constituents against respiratory tract pathogens by gaseous contact. Journal of Antimicrobial Chemotherapy. 47(5): 565-573.

[19] Ye, H., Shen, S., Xu, J., Lin, S., Yuan, Y. and Jones, G. S. (2013). Synergistic interactions of cinnamaldehyde in combination with carvacrol against food-borne bacteria. Food Control. 34(2): 619-623.

[20] Shreaz, S., Wani, W. A., Behbehani, J. M., Raja, V., Irshad, M., Karched, M., et al. (2016). Cinnamaldehyde and its derivatives, a novel class of antifungal agents. Fitoterapia. 112: 116-131.

[21] Thaniyavarn, J., Jindamarakot, S., Am-in, S., Luepongpattana, S., Yoochang, T., Poomtien, J., et al. (2013). Yeast biodiversity in the coastal area of Koh Si Chang and their potential as biosurfactant producers. Proceedings of the 25th Annual Meeting of the Thai Society for Biotechnology and International Conference. Vol.25, pp. 265-274. October 16-19, 2013. Bangkok, Thailand.

[22] Duan, X., Chi, Z., Wang, L. and Wang, X. (2008). Influence of different sugars on pullulan production and activities of α-phosphoglucose mutase, UDPG-pyrophosphorylase and glucosyl transferase involved in pullulan synthesis in *Aureobasidium pullulans* Y68. Carbohydrate Polymers. 73(4): 587-593.

[23] Akepaopun, S. (2015). Production, characterization and film forming from exopolysaccharides from *Aureobasidium pullulans* YTP6- 14. Master Thesis, Department of Microbiology, Faculty of Science, Chulalongkorn University.

[24] Wiegand, I., Hilpert, K. and Hancock, R. E. (2008). Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nature Protocols. 3(2): 163.

[25] Ramaprasad, A.T., Latha, D. and Rao, V. (2017). Synthesis and characterization of polypyrrole grafted chitin. Journal of

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Physics and Chemistry of Solids. 104: 169-174.

[26] Valgas, C., Souza, S.M.D., Smânia, E.F. and Smânia Jr, A. (2007). Screening methods to determine antibacterial activity of natural products. Brazilian Journal of Microbiology. 38(2): 369-380.

[27] Xu, S., Chen, X. and Sun, D.W. (2001). Preservation of kiwifruit coated with an edible film at ambient temperature. Journal of Food Engineering. 50(4): 211-216.

[28] Pathare, P.B., Opara, U.L. and Al-Said, F.A.J. (2013). Colour measurement and analysis in fresh and processed foods: a review. Food and Bioprocess Technology. 6(1): 36-60.

[29] McHugh, T.H., Avena Bustillos, R. and Krochta, J.M. (1993). Hydrophilic edible films: modified procedure for water vapor permeability and explanation of thickness effects. Journal of Food Science. 58(4): 899-903.

[30] Friedman, M., Kozukue, N. and Harden, L.A. (2000). Cinnamaldehyde content in foods determined by gas chromatography– mass spectrometry. Journal of Agricultural and Food Chemistry. 48(11): 5702-5709.

[31] Ooi, L.S., Li, Y., Kam, S.L., Wang, H., Wong, E.Y. and Ooi, V.E. (2006). Antimicrobial activities of cinnamon oil and cinnamaldehyde from the Chinese medicinal herb Cinnamomum cassia Blume. The American Journal of Chinese Medicine. 34(3): 511-522.

[32] Xie, X.M., Fang, J.R. and Xu, Y. (2004). Study of antifungal effect of cinnamaldehyde and citral on Aspergillus flavus. Food Science. 25(9): 32-34.

[33] Balaguer, M.P., Lopez-Carballo, G., Catala, R., Gavara, R. and Hernandez-Munoz, P. (2013). Antifungal properties of gliadin films incorporating cinnamaldehyde and application in active food packaging of bread and cheese spread foodstuffs. International Journal of Food Microbiology. 166(3): 369-377.

[34] Vaara M. (1992) Agents that increase the permeability of the outer membrane. Microbiology Reviews. 56: 395–411

[35] Nikaido H. (1994) Prevention of drug access to bacterial targets: Permeability barriers and active efflux. Science. 264: 382–388.

[36] Zivanovic, S., Chi, S. and Draughon, A.F. (2005). Antimicrobial activity of chitosan films enriched with essential oils. Journal of Food science. 70(1): 45-51.

[37] Wong, D.W.S. and Falatko. (1989). Mechanism and theory in food chemistry (Vol. 115). Van Nostrand Reinhold, New York.

[38] Buchanan, R.E. (1918). Life phases in a bacterial culture. The Journal of Infectious Diseases. 23(2): 109-125.

[39] Han, J.H., Aristippos, G. (2005). Edible films and coatings- 15: A review. Elsevier Academic Press, Amsterdam, Netherlands.

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