Development and Use of Antimicrobial Durian Starch-Carrageenan/Carvacrol Films

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Abstract

Durian starch (DS) was isolated from durian seeds by aqueous extraction with 0.5% $NaHSO_3$. The percent yield and purity of the starch were 11.42 and 42%, respectively. The DS-carrageenan (CG) films containing different concentrations (0, 4, 6, 8, and 10%) of carvacrol were prepared. The antimicrobial activity of the DS-CG/carvacrol films was evaluated against Staphylococcus aureus BIOTECH 1582 using disk diffusion assay. Results revealed that the zone of inhibition increased with carvacrol concentration and application of prediffusion treatment. Films containing 8% carvacrol had a zone of inhibition of 15.89 mm. The DS-CG/carvacrol films were found effective in controlling the growth of S. aureus in commercial durian candy. After 24 h of storage at 4 °C, the microbial count was reduced by 0.79 log cycle (83.6% reduction). The findings of this research suggest the potential of the DS-CG/carvacrol films as an active biodegradable packaging system to prevent the growth of S. aureus.

***Keywords**:* antimicrobial films, carrageenan, carvacrol, durian starch

1. Introduction

Food safety is a growing public health concern. Every year, there are cases of food poisoning which poses a threat to public safety. In the Philippines, 209 cases of foodborne outbreaks were reported from 2008 to 2015 (Azanza *et al*., 2019). There are several ways of mitigating this problem such as implementation of good manufacturing practices by food companies, proper hygiene, and appropriate food storage. Food packaging plays an important role in maintaining the quality of food during storage and distribution. Traditional food packaging acts as a physical barrier to protect the product from physical, chemical, and biological contamination (Youssef and El-Sayed, 2018). On the other hand, active packing is an innovative approach that is developed to address the needs of the consumers. This involves the incorporation of

substances into the polymer material or in the packaging system in order to maintain the quality or lengthen the shelf life of food.

Antimicrobial film is a type of active packaging that is designed to reduce or inhibit the growth of microorganisms (Rocha *et al*., 2013). It allows the slow release of antimicrobials on the food surface. This strategy is more effective than direct addition of antimicrobial since proliferation of microorganisms generally occurs on the surface (Guarda *et al*., 2011). Also, these antimicrobials may have an effect on the sensory attributes when directly added to food (Huang *et al*., 2019). Among the antimicrobials used in food packaging are enzymes (Bayarri *et al*., 2014; Ataide *et al*., 2017), organic acids (El-Fawal, 2014; Wu *et al*., 2017), polysaccharides (Ponce *et al*., 2016; Wang *et al*., 2018), bacteriocins (Abdollahzadeh *et al*., 2018; Eghbal *et al*., 2019), and essential oils (Bonilla *et al*., 2018; Chaiwarit *et al*., 2018; Xu *et al*., 2018).

Carvacrol (2-methyl-5-(1-methylethyl)phenol) is the major component present in the essential oils from Origanum and Thymus plants. It is generally recognized as safe (GRAS) by the United States Food and Drug Administration (Campana and Baffone, 2018). The antimicrobial activity of carvacrol can be explained by its ability to disrupt the cell membrane. This results in an increase in permeability leading to cellular leakage (Nazzaro *et al*., 2013). Furthermore, carvacrol acts as a proton exchanger thereby disrupting the proton motive force which is important for ATP synthesis (Ultee *et al*., 2002; Nostro and Papalia, 2012). This compound is proven to exert bacteriostatic and bactericidal activities against *Campylobacter jejuni*, *Escherichia coli*, *Salmonella* and *Staphylococcus aureus* (Suntres *et al*., 2015).

Durian (*Durio zibethinus*), also known as the "King of Fruits", grows abundantly in the Davao Region, Philippines. Durian seeds are by-products in the durian industry, which generates a large amount of waste that ends up in landfills. According to W. Pimpa and C. Pimpa (2015), durian seeds account for 20 to 25% of the fruit and contain 56% starch. This starch can be a suitable material for the preparation of starch-based films. However, such films are brittle and have poor mechanical properties. These properties can be improved by blending starch with synthetic or natural polymers. Carrageenan (CG) is a polysaccharide present in the cell wall and intercellular matrix of seaweed plants. Kappa and iota carrageenan are capable of forming thermo-reversible gels due to the buildup of a three-dimensional polymer network (Yuguchi *et*

al., 2002). Studies have shown that the mechanical properties of starch films can be enhanced when blended with carrageenan (Larotonda *et al*., 2005; Lafargue *et al*., 2007; Abdou and Sorour, 2014).

In this study, a durian starch (DS)-CG film containing carvacrol was prepared. The effectiveness of the film in controlling the growth of *S. aureus* in commercial durian candy was evaluated.

2. Methodology

Durian seeds were procured from Lola Abon's Durian Candy Factory, Davao City, Philippines. Carvacrol and k-carrageenan were purchased from Sigma Aldrich, USA. *S. aureus* BIOTECH 1582 was obtained from the Philippine National Collection of Microorganisms, National Institute of Molecular Biology and Biotechnology, University of the Philippines Los Baños. Bacterial culture media were procured from HiMedia Laboratories, USA. The Megazyme total starch assay kit was kindly donated by Dr. Juma Novie Alviola of the University of the Philippines Mindanao, Davao City, Philippines.

2.1 Extraction of DS

The extraction of starch from durian seeds was based on the method of Pimpa *et al*. (2012) with some modifications. The seeds were washed thoroughly with water, and the seed coating was removed and discarded. The endosperm was ground and mixed with an equal amount of 0.5% $NaHSO_3$ in a blender. Seed fibers were removed by pressing the slurry through a cheesecloth. The suspension was allowed to stand for two days at 4 °C and centrifuged for 15 min. The resulting starch was washed with distilled water and oven dried at 40 °C for 48 hours (h). The sample was ground and sifted through a 100 mesh sieve. The percentage yield was obtained and the purity of the starch was determined using the Megazyme Total Starch Assay kit.

2.2 Preparation of Carvacrol Emulsion

A volume (0, 40, 60, 80 and 100 µL) of carvacrol was mixed with 100 µL of Tween 80 using a vortex mixer. One milliliter of distilled water was added and mixed until an emulsion was formed. This emulsion was used in the preparation of films.

2.3 Preparation of DS-CG/Carvacrol Films

Films were prepared based on the method described by Abdou and Sorour (2014) with some modifications. One gram of DS and CG (70:30) was mixed in 30 mL distilled water until slightly boiling. Glycerol was slowly added to the mixture to obtain a final concentration of 30% v/w. The mixture was allowed to cool with gentle mixing followed by the addition of the carvacrol emulsion to achieve the desired concentration (0, 4, 6, 8, and 10%). The solutions were then poured in disposable petri plates and oven dried at 50 °C. The films were peeled off from the petri plates and stored in the desiccator until further use. DS-CG films with no carvcarol served as the control.

2.4 Antimicrobial Activity Test

2.4.1 Disk Diffusion Assay

Bacterial lawns of *S. aureus* (10^8 CFU/mL) were prepared on Mueller Hinton Agar plates by lawn streak method. Films were cut into 6 mm disks and surface-sterilized under UV light for 3 min on both sides. The films were placed on the bacterial lawns. The plates were then placed at refrigeration temperature (4 °C) for 0, 24, and 48 h to allow pre-diffusion of carvacrol followed by incubation at 37 °C for 24 h. Zones of inhibition were determined by measuring the average zone diameter at two cross-sectional points.

2.4.2 Antimicrobial Efficacy of DS-CG/Carvacrol films on Commercial Durian Candy

The antimicrobial activity of the films on commercial durian candy was tested based on the method described in another study (Barbosa *et al*., 2013) with some modifications. Commercial durian candy slices (1 cm x 1 cm) were sterilized under UV light for 3 min on each side. The slices were spike-inoculated with 20 µL of 0.1% peptone solution containing approximately 10^8 cells/mL of *S. aureus*. The inoculated samples were covered with DS-CG/carvacrol films and stored at 4 °C for 24 h. Film without carvacrol was used as a control. The microbial analyses were performed after 8, 16, and 24 h. The samples were manually homogenized in 0.1% peptone solution using a vortex mixer. The solutions were serially diluted and spread onto the Baird Parker agar. The plates were incubated for 36 h at 37 ºC and the colonies formed were expressed in terms of log CFU/mL. The initial count (inoculated sample without film) and microbial count of untreated (un-inoculated) samples were also determined.

2.5 Statistical Analysis

All experiments conducted were done in three trials with three replicates. The results were subjected to two-factor analysis of variance (ANOVA) at 0.05 level of significance. Tukey's HSD test was used to determine the significant difference between the treatments.

3. Results and Discussion

3.1 Extraction of DS

Crude starch was extracted from durian seeds by aqueous extraction using 0.5% $NaHSO_3$. This method yielded 11.43% starch, contrary to the study of Pimpa and Pimpa (2015), which obtained 56% starch. Factors that may have contributed to the low starch yield are variety and maturity of the durian fruit. The amount of starch is affected by the physiological maturity of seeds. Starch is degraded during seed development which results to lower amount of starch during maturity (Egli and Bruening, 2001). The starch obtained was a white and fine powder (Figure 1) and had a characteristic durian odor. Starch had a purity of 42%. Other components that may be present are protein, fiber, fat and ash (Ocloo *et al.*, 2010).

Figure 1. The extracted durian starch

3.2 Antimicrobial Susceptibility test of DS-CG/Carvacrol Films

The DS-CG films with different concentrations of carvacrol (0, 4, 6, 8, and 10%) were investigated for their antimicrobial activity against *S. aureus* using disk diffusion assay. Table 1 shows the effect of the different concentrations of carvacrol on the antimicrobial property of the DS-CG films. Higher concentration of carvacrol resulted in greater antimicrobial activity. It is noted that films subjected to 24 h prediffusion resulted in significantly larger zones as compared to those without pre-diffusion (0 h). In DS-CG films with 8% carvacrol, the zone of inhibition increased from 15.89 to 22.45 mm. Pre-diffusion treatment allows the antimicrobial to diffuse into the agar before the onset of bacterial growth resulting to a larger zone of inhibition (Frimodt-Moller *et al*., 1986; Lalpuria *et al*., 2013).

Table 1. Antimicrobial activity against *S. aureus* of DS-CG films with different concentrations of carvacrol

Concentration of carvacrol (% v/w)	Zone of inhibition[1] (mm) Pre-diffusion time[2]	
	0 h	24 h
Control	0^{cX}	0^{cX}
4	2.11 ± 3.66^{bcX}	3.22 ± 5.58^{bcX}
6	8.78 ± 5.048^{bX}	9.54 ± 2.80^{bX}
8	5.89 ± 3.36^{aX}	22.45 ± 4.14^{aY}
10	18.89 ± 5.74^{aX}	25.78 ± 2.12^{aY}

[1]Treatment means within a column followed by the same letter are not significantly different at α=0.05.
[2]X-Y: compares significant differences between columns.

The control films with and without prediffusion did not exhibit a zone of inhibition indicating that neither DS nor CG contributed to the antimicrobial activity towards *S aureus*. Hence, the antimicrobial activity can be attributed entirely to carvacrol. At the highest concentration of carvacrol (10%), a nearly total inhibition of microbial growth occurred after the 24 and 48 h prediffusion.

3.3 Antimicrobial Efficacy of DS-CG/Carvacrol Films on Commercial Durian Candy

The antimicrobial activity of DS-CG/carvacrol films on commercial durian candy was evaluated. The *S. aureus* count of the spike-inoculated food sample covered with films was monitored after 8, 16, and 24 h of storage at 4 °C. The test was performed on samples without film (initial), covered with control

film, and film with carvacrol. DS-CG films with 8% carvacrol were used since, in the previous experiment, these exhibited larger inhibition zones which were not significantly different from the films with 10% carvacrol.

Table 2 shows the effect of the DS-CG/carvacrol films on the *S. aureus* count of durian candy at different storage times. No growth was observed for the untreatedsample indicating the absence of *S. aureus* prior to inoculation (data not shown). The initial count of the spike-inoculated sample was 4.52 log CFU/mL. The observed decrease in the microbial count of the control compared to the initial count can be attributed to two factors: (1) adherence of the test microorganism to the film and (2) effect of the preservatives initially present in durian candy. The microbial counts of the control at different storage times were not significantly different from each other, except at 8 h.

Table 2. *S. aureus* count of durian candy covered with DS-CG films containing 8% carvacrol after different storage times at 4 °C

Treatment	Initial Log (CFU/mL)	*S. aureus* count[1] (log CFU/mL) Storage Time[2]		
		8 h	16 h	24 h
DS-CG (control)	4.52 ± 0.01^{W}	4.24 ± 0.03^{bX}	3.98 ± 0.12^{bY}	3.84 ± 0.05^{bY}
DS-CG/carvacrol		3.88 ± 0.14^{cX}	3.37 ± 0.26^{cY}	3.05 ± 0.39^{cZ}

[1]Treatment means within a column followed by the same letter are not significantly different at $\alpha = 0.05$.
[2]W-Z: compares significant differences between initial and different storage times.

In the case of the samples covered with DS-CG/carvacrol films, the microbial count significantly decreased with storage time. After 8 h, the microbial count of the sample was reduced by 0.36 log cycle (from 4.24 log CFU/mL to 3.88 log CFU/mL). Increasing the storage time to 24 h further decreased the microbial count by 0.79 log cycle which corresponds to 83.6% reduction. This result further validates the positive effect of prediffusion on the efficacy of the films with carvacrol. The longer the films are in contact with the sample, the more carvacrol is able to diffuse into the sample; hence, inhibiting microbial growth. Figure 2 shows the decrease in number of *S. aureus* colonies with increase in storage time. This indicates the potential of DS-CG/carvacrol film as an antimicrobial packaging material.

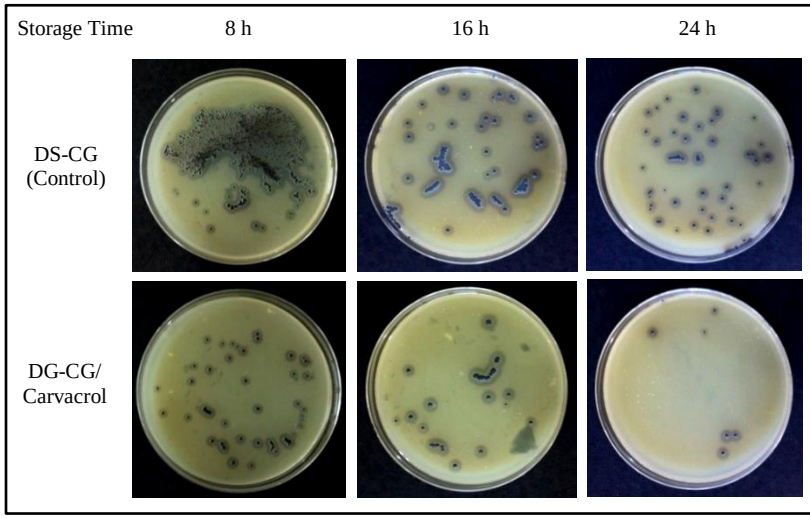

Figure 2. *S. aureus* colonies on Baird Parker agar from the durian candy covered with DS-CG/carvacrol films at different storage times at 4 °C

4. Conclusion and Recommendation

Aqueous treatment of durian seeds with 0.5% $NaHSO_3$ yielded 11.42% starch with 42% purity. The DS-CG blend films showed greater inhibitory effect against *S. aureus* at higher carvacrol concentration. DS-CG films containing 8% carvacrol were effective in reducing the *S. aureus* count in spike-inoculated durian candy. After 24 h storage at 4 °C, the microbial count was reduced by 0.79 log cycle or 83.6%. Findings of this study suggest the potential of the DS-CG/carvacrol films as an active packaging material to prevent the growth of Gram-positive pathogens. Due to the biodegradable nature of the films, these do not pose environmental concern as compared to traditional petroleum-based plastics. Future studies are recommended to investigate the effectiveness of the films against other foodborne pathogens in different food products. Furthermore, the physical properties (mechanical properties, gas barrier, and water vapor permeability) of the film must be determined to assess its suitability as a packaging material. It is also recommended to conduct proximate analysis of durian starch.

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