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Physical characteristics of nanoemulsion from chitosan/nutmeg seed oil and evaluation of its coating against microbial growth on strawberry

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Abstract

The objectives of this research were to characterize nanoemulsion from chitosan/nutmeg seed oil and to evaluate its coating on fresh strawberry which stored at 10°C for 5 days. The ultraturrax and high-pressure homogenizer were used to prepare 1.34±0.25 - 5.79±1.61 nm of nanoemulsion size which confirmed by particle size analyzer. The morphology observed by SEM that exhibited the oil globules were covered by chitosan. They were aggregated and rough droplets. Interactions among the materials were observed using FTIR which led to the presence of a new peak at 1736 cm^{-1}. The coated strawberry by high-pressure homogenizer-emulsion showed the best result suppressing microbial (2.41±0.01) and mould-yeast (2.78±0.10) growth at the end of storage compared to control which were 3.37±0.02 and 3.69±0.14 for microbial and mould-yeast count respectively.

1. Introduction

Strawberry fruit *(Fragaria ananassa)* is non-climacteric and highly susceptible to fungal decay, mechanical injury, and water loss during storage (Gol *et al.,* 2013). Post-harvest loss of strawberry during handling, transportation, and storage is a serious problem. Development of edible coating has been studied continuously to serve an inhibition of fresh fruit decay and deterioration. It is an eco-friendly breakthrough applied to various food including postharvest commodities (Aulakh and Regmi, 2013). Among many kinds of fruits, strawberry is one of the most appropriate to be coated by edible coating because the customer generally consumes it without peeling (ready to eat).

The materials for making edible coating are classified into hydrocolloids (starch and protein), lipids (fatty acids and waxes) and composites. Carboxymethyl cellulose, starch, gelatine, alginate, and pullulan have been studied as coating matrix to maintain the quality of strawberry (García *et al.,* 1998; Li *et al.,* 2017; Aitboulahsen *et al.,* 2018; Yan *et al.,* 2019). Another potential polymer of hydrocolloid is chitosan, an edible, biocompatible, and non-toxic amino polysaccharide made from deacetylation of chitin (Romanazzi *et al.,* 2013). The use of chitosan in edible layers also provides a barrier effect on both gas and water vapor (Butler *et al.,* 1996). Previous studies revealed that chitosan coating suspension extended the shelf-life of guava and strawberry fruit for 7 and 8 days respectively (Petersen *et al.,* 1999; Hernández-Muñoz *et al.,* 2008). Amino groups of chitosan are able to interact with the electronegative charges of microbial cell surface resulting in the leakage of intracellular components (Rabea *et al.,* 2003). However, single chitosan coating has limited inhibition to particular microorganism and poor barrier properties to the gas (Ravi, 2000).

The application of chitosan coating needs to combine with other substances such as resins, essential oils, polysaccharides and proteins to improve its functional properties. Kittur *et al*. (2001) and Zhu *et al*. (2008) utilized chitosan coating to inhibit respiration rate, maintain hardness, prevent discoloration, prevent *Colletotrichum gloeosporioides* invasion and reduce the alteration of ascorbic acid of mango. The incorporation of essential oils in chitosan-based material has been also applied as an alternative such as lemon (Perdonesa *et al.,* 2012), peppermint (Picard *et al.,* 2013), and *Curcuma longa L.* (Yusof *et al.,* 2018) essential oil to keep quality of fresh fruits. Spices essential oil have been also utilized as both food and flavouring agent since a long time ago, and as functional substances and food preservatives in recent years (Lai and Roy, 2004; Thanoon *et al.,* 2013; Nabavi *et al.,* 2015). Nutmeg is one of the Indonesian

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local natural resources that can be further processed into oil which has antimicrobial activity. The concentration (5%) exhibited a maximum inhibitory zone (16.8 mm) against *Staphylococcus aureus* (Zheng *et al.,* 2016). Essential oil contained nutmeg (5-15%) are mostly consisted of camphene or sabinene, d-pinene, dipentene, d-linalool, d-borneol,i-terpineol, geraniol, myristicine, safrole, eugenol, and isoeugenol which antimicrobial activity was primarily caused by pinene component (Takikawa *et al.,* 2002; Lai and Roy, 2004).

Nowadays, nanotechnology has gained great attention as a new generation of microtechnology, with one potential use being nanoemulsion. Nanoemulsion provides a potential carrier applied for food commodities due to its ability to optimize the dispersion of active substances (Meindrawan *et al.,* 2018; Wardana *et al.,* 2018; Yuliani *et al.,* 2018). The formulation of various emulsion types can be applied as food coatings, particularly for postharvest products such as papaya, strawberry, and mango (Zhu *et al.,* 2008; Perdonesa *et al.,* 2012; Picard *et al.,* 2013). It might offer a breakthrough method in maintaining the shelf life and quality of strawberry. The aims of this study were to characterize nanoemulsion from chitosan/nutmeg seed oil and to evaluate its coating on fresh strawberry stored at 10^{o}C for 5 days.

2. Materials and methods

2.1 Materials

Fresh strawberry was obtained from a local store (Total Fresh Fruit) in Tangerang, Indonesia. Chitosan and nutmeg seed oil were obtained from M & H Farm, Bogor, Indonesia. The emulsifier and chitosan solvent used was tween 80 and acetic acid respectively purchased from Merck (Germany). Total aerobic microbial and mould-yeast count were conducted using Plate Count Agar (PCA) (Sigma-Aldrich, Germany) and Potato Dextrose Agar (PDA) (Sigma-Aldrich, Germany), respectively.

2.2 Preparation of nanoemulsion and samples

Chitosan 3.5 g, 2.5 mL nutmeg seed oil, 100 mL acetic acid 1% and 5 mL Tween 80 were blended in with constant stirring using magnetic stirrer at temperature 40^{o}C for 2 hrs which is further stated as conventional emulsion (CE). Then, it was cooled at 29±1^{o}C. UTR-Emulsion was produced by further homogenizing of CE using ultraturrax 24000 rpm for 3 mins at 29±1^{o}C. While, HPH-Emulsion was made by advanced processing which was homogenized with extremely high -pressure using instrument High-Pressure Homogenizer (HPH), GEA Niro Soavi (Italy) with a pressure 200 bar, 1 cycle. Subsequently, the fresh strawberries were dipped for 30 s in the different coating suspensions and drained following Hernández-Muñoz *et al.* (2008) with slight modification. After being air-dried, the fruits were placed on the sterile disposable petri dish, One Med, diameter 90 mm. All samples were stored at 10°C for 5 days.

2.3 Characterization of nanoemulsion

The size distribution of the emulsion system was done to evaluate the emulsion type of each suspension (UTR-Emulsion and HPH-Emulsion) based on their size of emulsion droplets formed. The size distribution of droplets was characterized using Particle Size Analyzer (PSA), Zetasizer (model Nano ZS series Malvern Instruments, UK). The 1 mL emulsion suspension was placed into polystyrene micro cuvet with the detector angle 173^{o}. The measurement was performed with three replications at temperature 25^{o}C for 50 s to observe the emulsion stability of edible coating suspension.

The morphology of the emulsion droplets contained in the edible coating suspension was observed using a scanning electron microscopy (SEM), JEOL 6010 LA Benchtop. Beforehand, all kinds of emulsion suspension were poured on the sterile disposable petri dish, One Med, diameter 90 mm. Then, they were dried in an oven at temperature 40^{o}C for 24 hrs to form thin films. The films were coated with gold and confirmed by using SEM under an acceleration voltage of 20 kV, at a magnification of 1000x and 2000x.

Fourier-Transform Infrared Spectroscopy (FTIR) analysis was performed to detect the functional groups of each sample used to produce nanoemulsion and to study the interactions among them. The samples were subjected to FTIR (Thermo Fisher Scientific, iN10) in the scanning range of 4000-500 cm^{-1}.

2.4 Evaluation of nanoemulsion against microbial growth on strawberry

Aerobic plate count and mould-yeast count were detected following Frazier and Westhoff (1998) method with slight modification, by diluting 5 g of samples in 45 mL of 0.1% peptone water, followed by homogenization in a vortex mixer. Further decimal dilutions were made from this 10^{-1} dilution. Subsequently, serial dilutions were performed using 0.1% peptone water and then samples were plated in duplicate. Aerobic plate count and mould-yeast count were performed using aerobic PCA and PDA, respectively. All plates were then incubated at 35-37°C and colonies of total aerobic microbial and mould-yeast count were calculated after 48 and 72 hrs respectively. The results were expressed as CFU/g.

2.5 Statistical analysis

The data obtained in this study were evaluated by T-test for particle size distribution and analysis of variance (ANOVA) for total aerobic microbial and mould-yeast count using SPSS (Statistical Product and Service Solutions) version 16.0 and followed by Duncan's Multiple Range Test (DMRT) at a significance level of $p<0.05$.

3. Results and discussion

3.1 Characterization of nanoemulsion

Nanoemulsion manufactured from chitosan and nutmeg seed oil blends has successfully formed a particle size in the range of 1.34 - 5.79 nm. Figure 1 exhibits the size distribution spectra of emulsion system determined by measuring the PSA.

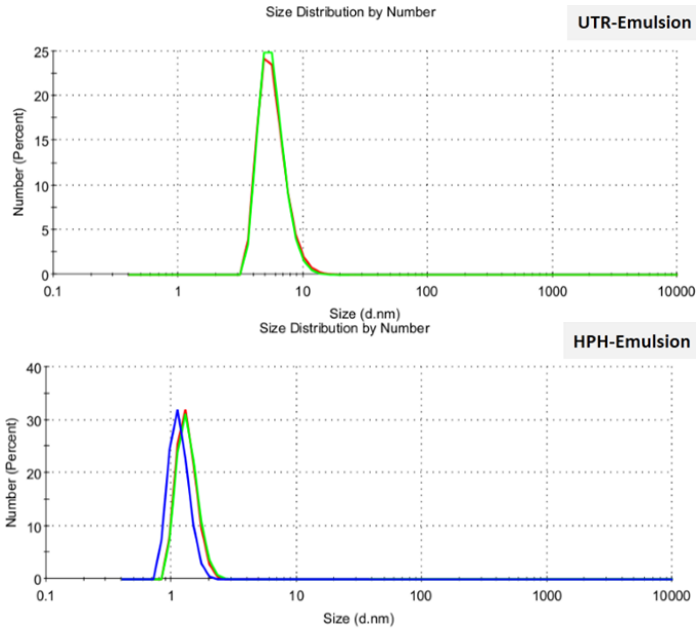

Figure 1. Size distribution spectra of chitosan/nutmeg seed oil blends using PSA

HPH-Emulsion has the smallest diameter average (1.34±0.25 nm) compared to another suspension. Moreover, the visual appearance of HPH-Emulsion appeared brighter than UTR-Emulsion (Figure 2a) because nanoemulsion could enhance the dispersion of formulation so that a larger specific surface area formed offering a brighter colour (Çinar, 2017). In the HPH system, a blend of oil, water and surfactant is pushed passing a small gap, typically 1 - 10 mm, where droplets undergo extremely shear and elongational stress resulting enhancement of pressure drop up to a few thousand bars (Mason *et al.,* 2006; Floury *et al.,* 2009; Helgeson *et al.,* 2012). Due to the high level of pressure inside the instrument, droplets undergo deformation and alteration into smaller form compared to UTR-Emulsion (5.79±1.61nm). While UTR-Emulsion is based on the moving of the rotor with a very high circumferential speed (24000 rpm). The suction produced pulled the sample into the rotor and pushed it to the outside. This process crushed the liquid droplets resulting in the sample's dispersion with smaller size. A previous study stated that the most dominant factors affecting the particle size reduction which were the time and speed of homogenization (Silva *et al.,* 2011). Another method has been reported by Ghaderi-Ghahfarokhi *et al.* (2017) in which nanoparticles with the size distribution of 235.6 nm were produced by incorporating cinnamon essential oil in chitosan matrix using an ultrasonic water bath for 1 hr.

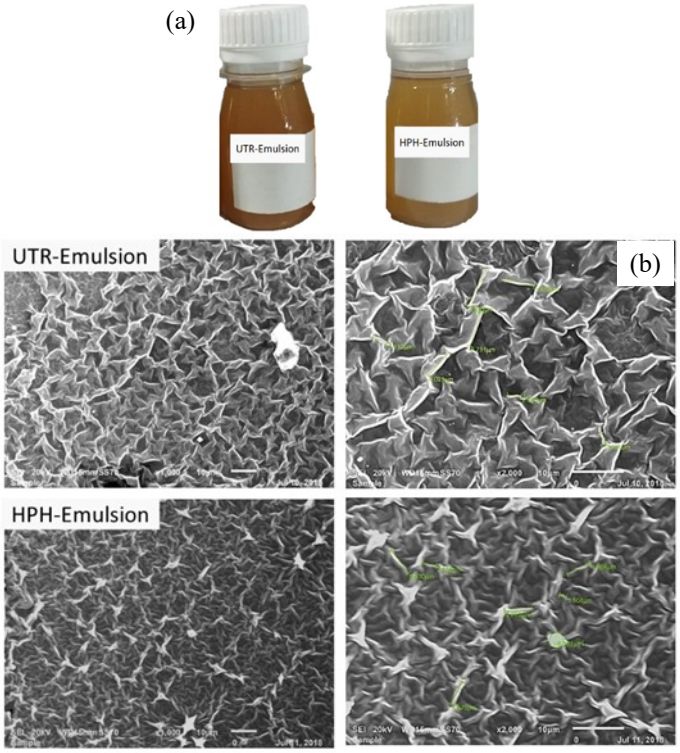

Figure 2. (a) Visually comparison of nanoemulsion between UTR-Emulsion and HPH-Emulsion and (b) the morphology of the emulsion droplets contained in the thin film at 1000x and 2000x magnification

SEM was used to confirm the particle size distribution of nanoemulsion. Morphology images of the thin film in this study (Figure 2b) show unclearly emulsion droplets shape which is generally exhibited by spherical droplets and smooth in nature of oil globules in edible coating suspension. That result was presumably that oil globules in nano-scale are covered by chitosan. They aggregated in the form of thin fused and rough droplets. In comparison, a previous researcher who developed nanoemulsion sample containing β-carotene; nanodrops were observed using SEM with spherical shape and smooth surface (Hasani *et al.,* 2015). Another study found that an atomic force microscope analysis clearly exhibited the spherical shape and nanosized structure of individual particles of cinnamon essential oil in the chitosan matrix (Ghaderi-Ghahfarokhi *et al.,*

2017). Another study also revealed that encapsulation containing repaglinide did not affect the morphology of nanoemulsion (Nanjwade *et al.,* 2013).

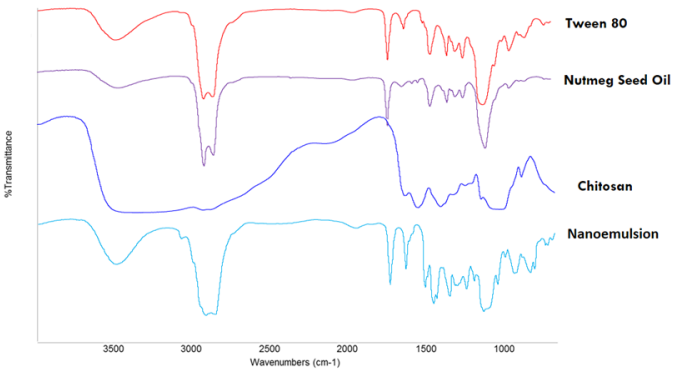

Figure 3. FTIR spectra

FTIR spectroscopy was used to observe the interactions among materials of nanoemulsion. Figure 3 shows FTIR spectra of chitosan, Tween 80, nutmeg seed oil and nanoemulsion. It could be seen that the peak of N -H bending in chitosan reached at 1552 cm^{-1} is shifted to 1633 cm^{-1}. The bands arising at the range of 2700 - 3000 cm^{-1} in the spectrum of nanoemulsion are attributed the stretching vibrations of $-CH_2$ (2924 cm^{-1}) and $-CH_3$ (2857 cm^{-1}) from tween 80 and nutmeg seed oil. The peak 3498 cm^{-1} of nanoemulsion is inferred to O-H stretching vibration which is shifted from 3949 cm^{-1} (Tween 80) and 3350 cm^{-1} (chitosan). Furthermore, the nanoemulsion leads to the presence of a new peak at 1736 cm^{-1}. It is in accordance with the previous study when the essential oil was incorporated into chitosan films (Cerqueira *et al.,* 2012; Sugumar *et al.,* 2015).

3.2 Evaluation of strawberry coating with nanoemulsion

Table 1. Total aerobic microbial and mould-yeast count of samples for 5 days

Sample	Microbial count (log CFU/g)	Mould-yeast count (log CFU/g)
Pre treatment (day 0)	4.76±0.04[a]	4.67±0.01[a]
Control	3.37±0.02[b]	3.69±0.14[b]
UTR-Emulsion	2.49±0.07[c]	2.93±0.04[c]
HPH-Emulsion	2.41±0.01[c]	2.78±0.10[c]

Application of nanoemulsion edible coating led to a reduction of the microbial counts at the end of storage compared to control as shown in Table 1. The microbial and mould-yeast counts of fresh strawberry reached 2.41 - 3.37 and <0.17 - 3.69 log CFU/g, respectively. However, those are still below the safe threshold for consumption which was regulated by Indonesia National Agency of Drug and Food Control (BPOM) that is the maximum of microbial contamination on fruit is 5.00 log CFU/g (BPOM, 2009). On day 5, nanoemulsion coating treatments on strawberry suppressed both microbial and mould-yeast growth compared to control. This is due to the nanoemulsion from chitosan combined with nutmeg seed oil filler which has antibacterial and antifungal abilities, respectively. Several mechanisms of nutmeg seed oil as antimicrobial have been reported, such as by inactivating microbial adhesion, enzymes, and cell envelope proteins (Gupta *et al.,* 2008). While, the specific antimicrobial activity of chitosan has been many previously studied, such as relating to its molecular weight, deacetylation degree, and an alteration in cell permeability (Devlieghere *et al.,* 2004). Chitosan which has low molecular weight could pass into the hypha of *Fulvia fulva* observed clearly using confocal laser scanning microscopy of fluorescein-labelled chitosan (Li *et al.,* 2011). Furthermore, the previous author revealed that interactions occurring between its amino groups and the electronegative charges on the surface of microbial cell tend to lead to the leakage of intracellular constituents (Rabea *et al.,* 2003). The colour of strawberry changes from light red to dark red. On day 5 of storage, HPH-Emulsion coating could still maintain the skin rigidity and colour (Figure 4).

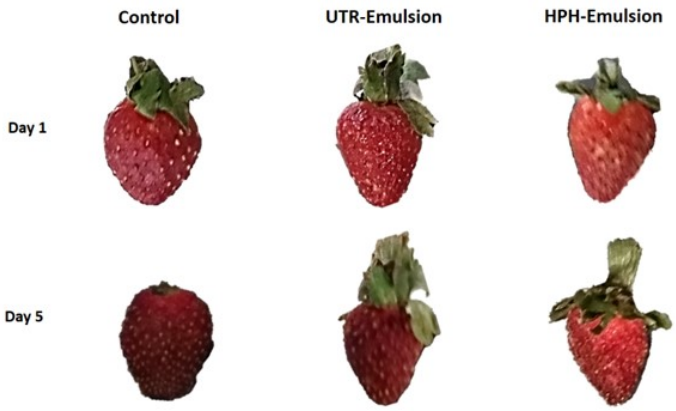

Figure 4. Strawberry appearance during storage at 10°C for 5 days

The droplet size of nanoemulsion also may affect in inhibiting microbial growth as demonstrated in Table 1. The coated sample by HPH-Emulsion has the highest inhibition of microbial growth. However, all formulations exhibit a beneficial effect against total microbial and mould-yeast growth compared to control. It is in line with the previous study which reported microbial growth inhibition in beef patties by applying nanoemulsion of cinnamon essential oil/chitosan as a coating (Ghaderi-Ghahfarokhi *et al.*, 2017). Several mechanisms have been reported by previous studies including that essential oil encapsulation at the nanoscale could enhance the antimicrobial activity. It is because there is an increase in the content of the bioactive compounds in the area which microorganisms are located (Weiss *et al.,* 2009; Donsì *et al.,* 2011).

4. Conclusion

Chitosan and nutmeg seed oil had potential as an

antimicrobial compound. They could apply on strawberry in the form of nanoemulsion. Nanoemulsion made by using HPH had a smaller particle size confirmed by PSA. SEM observation shows unclearly emulsion droplets which presumably oil globules are covered by chitosan matrix. Also, it led to the presence of a new peak at 1736 cm^{-1} confirmed by FTIR. Smaller particle size of the emulsion has a larger surface area which increased the effectiveness in inhibiting microbial and mould-yeast growth on strawberry. This finding could be an alternative to maintain the quality of post-harvest commodities during the 5 days storage.

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