Antibacterial activities of biocomposite plastic-based phenolic acids-grafted chitosan and sugar palm starch (*Arenga pinata*)

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Abstract. Antibacterial activity of biocomposite plastics based sugar palm starch (SPS) and phenolic acid-grafted chitosan has been investigated. The various type of active compounds (ferulic acid-grafted chitosan, caffeic acid-grafted chitosan, and coumaric acid-grafted chitosan) was synthesis by free radical method and was evaluated by using FTIR and calculate the degree of substitution by using Folin-Ciocaltue procedure. The biocomposite plastics were made by combined SPS with glycerol, cellulose, zeolite, and active compounds (phenolic acid-grafted chitosan). Morphological structures were analyzed by using SEM, and antibacterial activities were evaluated by using agar diffusion method. It was found that the grafting reaction of phenolic acids was not improved the antibacterial activities. The biocomposite plastics impregnated with grafted products were showed no clear zone.

Keywords: phenolic acids, chitosan, biocomposite plastic, antibacterial activity

1. Introduction

The cross-contamination of foods by the transmission of foodborne disease via contact surface has paid attention in recent years. Several infectious bacteria, such as *Listeria monocytogenes* strains showed the initial adherence by cellular hydrophobic interaction of biofilm formation to polyvinyl chloride (PVC) [1]. Moreover, *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*) are viable to attach on the surface of polypropylene by biofilm formation [2]. Foodborne pathogens that patch on the plastic surface, potentially resulting in contamination of food products, could be reduced with antibacterial plastic by inhibiting the maximum population limit of microbes [3,4]. Antibacterial plastics are a class of active packaging in order to extend shelf life, maintain safety and quality of the products [5-7].

In recent years, the demand for antibacterial plastics is growing significantly in several areas, such as food packaging [8-9], medical devices [10], and water treatment [11]. Antibacterial plastics usually have been made from synthetic polymer based on petroleum. Due to the increasing problem of environmental pollution, the use of degradable plastics has attracted preferable interest. Starch has become one of biopolymer which has great potential as bio-based plastics. One of the promising starch produced in Indonesia is sugar palm starch (SPS), which was reported to be used as basic material for hard capsule [12] and biodegradable film [13-14].
Despite sugar palm starch (Arenga pinata) incorporate with plasticizers have excellent film-forming properties [15], but no antibacterial properties have been reported. The addition of antibacterial agents such as nanoparticles [16-17], essential oil [18], protein [19], and chitosan [20-22], to the plastics could give antibacterial activity on it. However, small molecular weight incorporated on plastics could be risked due to their compatibility and leaching issue; thus, the use of such additive products in food packaging is limited [23].

To extend the stability of the small molecule can be achieved by grafting, such as phenolic acid, into a long-chain polymer. Some studies reported that the supplementation of oregano essential oil into cassava starch–chitosan film gave an antimicrobial activity against Salmonella enteritidis, Escherichia coli, Bacillus cereus, and Staphylococcus aureus [24]. Another research reported that conjugates of chitosan-p coumaric acid compounds enhanced solubility in water [25]. This research aimed to study the antibacterial activities of several phenolic acids (caffeic acid, coumaric acid, and ferulic acid)-grafted chitosan compared with its supplemented into biocomposite plastics from sugar palm starch.

2. Materials and methods

2.1. Materials

Native sugar palm starch was obtained from PT. Aren Mulya, a sugar palm starch industry in Klaten, Central Java, Indonesia, was washed and dried overnight at 40 °C and dried starch was sieved at 100 mesh. Ferulic acid (Merck), coumaric acid (Merck), caffeic acid (Merck), chitosan from Aldrich (Mv 60000-120000 D, >93% of DD), glycerol (Merck), cellulose (fiber microcrystalline, Aldrich), zeolite 100 mesh (Natural zeolite powder from Gunungkidul, Yogyakarta, Indonesia, was obtained from CV Mountain Stone), hydrogen peroxide (Merck), ascorbic acid (Merck), Folin-Ciocalteu reagent (Merck), acetic acid (99%-w/w) (Merck), hydrogen peroxide 30% (Merck), sodium hydroxide (Merck), ethanol (Merck), commercial Amoxicillin (PT Dankos Farma), dialysis tubing cellulose membrane with molecular weight cut-off 14 kDa (Sigma-Aldrich) and hydrochloric acid (37% w/w) (Merck) were used without any purification. The products were prepared and characterized by using UV-Vis spectrophotometer (Dynamica Scientific Halo RB-10), freeze dryer Buchi L-200, infrared spectrophotometer (FTIR, Shimadzu-8201PC) over a wavenumber range of 400–4000 cm⁻¹ on KBr pellet, and Scanning Electron Microscopy (Hitachi SU3500).

2.2. Synthesis of phenolic acid grafted-chitosan

The synthesis of phenolic acid grafted-chitosan was conducted by using free radical method following Anwar et al. (2019) [26], with some modification. Chitosan (0.5 g) was dissolved in 50 mL of acetic acid solution (1% v/v) in a 250 ml Erlenmeyer glass. The mixture of ascorbic acid (0.8599 g) in 0.25 ml hydrogen peroxide 30% was dropped slowly into a chitosan solution. After stirring for 15 minutes, the three variations of phenolic acids, i.e., coumaric acid (0.4576 g), caffeic acid (0.5023 g) and ferulic acid (0.5416 g) in 5 mL ethanol were added into the flask, and the mixture was continuous stirring at room temperature for 24 h. Finally, NaOH 2 M was added into the mixture, and pH was adjusted to 10, followed with dialyzed (MWCO 14 kDa) in distilled water for 48 h, and lyophilized to produce the phenolic acid-grafted chitosan.

2.3. Determination of phenolic acid grafting on chitosan

The estimation of the total phenolic group (ferulic acid, caffeic acid, and coumaric acid) on the grafting products by Folin-Ciocalteu procedure according to the literature with minor modifications [27]. Briefly, 25 mg phenolic acid-grafted chitosan was dissolved in acetate buffer pH 5.6 up to 25 ml in a volumetric flask. Folin-Ciocalteu reagent (0.5 ml) was added to 0.5 ml sample solution, mixed vigorously, and followed by the addition of 8 ml of sodium carbonate (2.5%w/v). The mixture was allowed to stand for 2 h, and absorbance of phenolic content was measured at wavelength 765 nm against control. The standard curves of phenolic acid were drawn by plotting the concentrations to 20, 45, 60, 80, 100, 125, and 150 ppm versus absorbance. The total phenolic of phenolic acid grafted-chitosan was expressed as milligrams of phenolic acid per gram grafted product.
2.4. Preparation of biocomposite plastics

Three different plastic solutions based on active compounds were prepared into films using a casting method following Poeloengash et al. (2016) [15] with some modification. 0.03 g of active compounds (FA-g-Chi; Co-g-Chi; Ca-g-Chi) was dissolved in 10 ml water with the addition of 10 drops of acetic acid 1% to help solubility. Sugar palm starch (3 g), glycerol (0.9 g), zeolite (0.006 g), cellulose (0.006 g) was dispersed in water and maintained under vigorous stirring (400 rpm) at room temperature for 15 min. The same amount of chitosan (Chi), Amoxicillin (Amox), and no addition of active compounds were used as a control. The mixture was heated to 90 °C under constant stirring at 400 rpm for 30 min. The mixture solutions were degassed under vacuum, casting into Teflon mold (20x20 cm) and dried overnight at 50 °C.

2.5. Antibacterial activity of the active compounds and biocomposite plastic

The antibacterial assay was performed by agar diffusion method (Kirby Bauer test) based on Kun and Marossy (2013) [28] with modification in medium. The suspension of Gram-positive bacteria (Bacillus subtilis and Staphylococcus aureus) and Gram-negative bacteria (Escherichia coli, Salmonella typhimurium, and Pseudomonas aeruginosa) prepared under fresh stock conditions on Nutrient Broth (NB) medium (1%, v/v) with seed density was approximately around 10^5 CFU/ml. Cellulose paper disks with a diameter of 6 mm were used for antibacterial assay for active compounds and bioplastic samples disk was cut in the same contact surface area (diameter 6 mm) and wiped with ethanol 70% before the tests. Each bacterial test culture (100 µL) was inoculated on Nutrient Agar (NA) medium by spread plate technique. After the infection, agar plates were incubated for half an hour, before putting the disks on them. Cellulose paper disks are impregnated into each active compound solution (phenolic acids-grafted chitosan). All the test samples (paper disk contained active compound and biocomposite plastic) are placed on an infected agar plate and incubated at 37 °C for 24 h. The antimicrobial activities were evaluated by the appearance of an inhibition zone below or around the disk measured by a Digimatic caliper.

3. Results and discussions

3.1 Synthesis of phenolic acids grafted-chitosan

The grafting reaction of phenolic acids on the chitosan chain was done by free radical method using hydrogen peroxide and ascorbic acid as a radical initiator. The reaction was carried out at ambient temperature, and atmospheric for 24 h with the molar ratio of chitosan and phenolic acids was 1:1. The reaction initiated by ascorbate radical (Asc−) [29] to produce chitosan radicals and followed the addition of phenolic acids to form grafted products (FA-g-Chi; Co-g-Chi; Ca-g-Chi). Precipitation of the grafted products was done by the addition of dilute sodium hydroxide into alkaline (pH) conditions. Afterward, the precipitation products were washed with absolute ethanol followed by dialysis with cellulose membrane (MWCO 14 kDa) for 48 h to remove the residue of phenolic acids.

The grafted products were insoluble in alkaline and acidic conditions but dissolved at pH 6-7. The solubility of Co-g-Chi and Ca-g-Chi at pH 6-7 was easier than FA-g-Chi might due to related to the higher degree of substitution of the products. The higher degree of substitution of the phenolic acids on chitosan chain, the easier of the products to dissolve in neutral conditions.

The estimation of phenolic acids on chitosan chain was determined by using the Folin-Ciocalteu method. The Folin-Ciocalteu reagent can be used to quantify the number of phenolic acid compounds that were grafted onto chitosan. The highest degree of substitution was found in the grafted product Ca-g-Chi i.e.,37.186 mg caffeic acid equivalent/g product. However, the degree of substitution of Co-g-Chi and FA-g-Chi was 31.86 mg/g and 17.175 mg/g polymer, respectively. This result supported the solubility of grafted products.

The Fourier transform infrared spectra of chitosan, FA-g-Chi, Co-g-Chi, and Ca-g-Chi were shown in figure 1. Infrared spectra of chitosan exhibited broad peaks at around 3460 cm⁻¹ (-O-H st) and 2900-3000 cm⁻¹ (C-H st). New bands appeared at 1637-1651 cm⁻¹, corresponding to NC=O vibrations that were characteristic of amide group formation between the amino groups of the chitosan pyranose cycle and phenolic acid moieties (figure 2). FTIR spectra of Co-g-Chi (c) and Ca-g-Chi (d) exhibited
specific NC=O tertiary amide bond at 1651 cm$^{-1}$ (Amide I) and no peak at amide II. However, FA-g-Chi (b) spectra gave a different band compare to Co-g-Chi and Ca-g-Chi, especially on amide I (1636 cm$^{-1}$) peak and appeared peak at amide II ((1582 cm$^{-1}$). These spectra suggested that ferulic acid grafted to chitosan by secondary amide bond. Furthermore, the absence peak at 1730 cm$^{-1}$ (C=O st ester) suggested that no ester bond formed in the grafted products [29].

**Figure 1.** FTIR spectra of Chitosan (a), FA-g-Chi (b), Co-g-Chi (c) and Ca-g-Chi (d).

**Figure 2.** The purposed reaction of chitosan with phenolic acids initiated by ascorbic acid/H$_2$O$_2$.

3.2 Biocomposite plastics

The biocomposite plastics were made by combining sugar palm starch (3 g) with 30% (w/w SPS) of plasticizer (glycerol), according to Sanyang et al. (2015) [30]. The addition of natural zeolite (0.2% w/w SPS) as a reinforcement and controlling the humidity. Natural zeolite is well known as a porous material that has the capability to absorb water [31]. Cellulose has also reported as a reinforcement for biocomposite of carboxyl methylcellulose/starch [32], whereas the supplementation phenolic acids-grafted chitosan aimed to improve the antibacterial activity of biocomposite plastic.

The surface morphology images were captured by using a scanning electron microscope with a working distance of 5.4 to 7.7 mm, voltage acceleration 5 kV, and magnification at 500x. The morphology of the biocomposite plastic exhibited as a compact surface (figure 3a and 3b) due to degasting steps can be eliminated the soluble air in the preparation of biocomposite mixture. Mixing
prosses for 30 minutes in the constant stirred at 400 rpm gave the homogenous dispersion of the zeolite in the plastic film. The backscatter (BSE) detector generated the brighter images for a larger atomic number than those with a lower atomic number. Zeolite particles appeared with a brighter spot than others. The spread out of zeolite indicated that phenolic acids-grafted chitosan was dispersed well in the biocomposite plastics.

![Image](image_url)

**Figure 3.** The morphology images of biocomposite plastic of phenolic acids-g-Chi (a), the cross-section of the film (b) and biocomposite plastic with BSE detector (c)

### 3.3 Antibacterial activity of biocomposite

Three types of active compounds (phenolic acids-grafted chitosan) revealed antibacterial activity against Gram-negative (*S. typhimurium, P. aeruginosa, and E. coli*) and Gram-positive (*B. subtilis and S. aureus*) bacteria by using the agar diffusion method. Antibacterial activity of the phenolic acids-grafted chitosan and positive control test (amoxicillin and chitosan) against these pathogenic bacteria was expressed in terms of inhibition zone (table 1).

Amoxicillin, as a broad antibacterial drug, exhibited the largest clear zone against all pathogenic bacteria (figure 4). Whereas, another positive control, chitosan, also resulted in the most significant inhibition zone against Gram-positive bacteria compared to Gram-negative bacteria. This result implies that chitosan’s antibacterial inhibitory efficiency varies for different species of pathogenic bacteria. This may partly be explained by differences in the cell wall and expression of different surface molecules, in addition to differences in size, molecular weight, and deacetylation of chitosan itself [33]. The reason might be the chitosan forms a film which inhibits nutrient adsorption [34]. Gram-negative bacteria have a more complex cell wall structure than Gram-positive bacteria. Gram-negative bacteria have a cell wall containing a monolayer of peptidoglycan. Gram-negative bacteria are composed of a cell envelope in the outside of the cell wall, called the outer membrane. In the outer membrane of the Gram-negative bacteria, lipopolysaccharides, which serve as endotoxins, are found and act as a barrier to the entry of several substances, whereas in Gram-positive bacteria, the cell walls contain none polysaccharides content, so cells are easier to lysis [35]. There are two known mechanisms of antimicrobial action of chitosan. First, the interaction between positively charged of amine (NH$_2$) groups of the chitosan and negatively charged of microbial cell wall [36] resulted in ionic interaction which interference with peptidoglycan in the bacterial membrane [37] and trigger imbalances of internal osmotic [38]. The electrostatic interaction mechanism suggests that the greater positive charge of the nitrogen atom of amine groups in the chitosan, the higher antimicrobial activity [39]. Another mechanism involves the binding of chitosan to DNA, which can suppress the production of bacterial mRNA [40].

Caffeic acid and coumaric acid-grafted chitosan showed a bigger clear zone to Gram-positive than Gram-negative bacteria, however ferulic acid-grafted chitosan gave the opposite result. It might be due to the different grafting position of ferulic acid to chitosan. Based on FTIR spectra, grafting product of ferulic acid to chitosan is a secondary amide, whereas caffeic acid and coumaric acids, both are tertiary amides. The positively charged of cationic secondary amides is greater than tertiary amides might due to the number and types of substituents to the nitrogen atom. The higher substitution of electron-
withdrawing groups (such as carbonyl) to a nitrogen atom of chitosan will reduce the basicity and induced positively charges of the grafted products in acidic condition.

**Figure 4.** Antibacterial test done in Petri dishes with paper impregnated (a and c) and biocomposite plastics supplemented with grafted products (b and d).

**Table 1.** Antibacterial assay of phenolic acids impregnated into the paper disk and biocomposite plastic

| Microorganism     | Clear zone (mm) | Amox | Ca-g-Chi | Co-g-Chi | FA-g-Chi | Chi | Control |
|-------------------|-----------------|------|----------|----------|----------|-----|---------|
|                   | Paper Plastic   | Paper Plastic | Paper Plastic | Paper Plastic | Paper Plastic |
| *B. subtilis*     | 39.96 14.83     | 23.2 (+) | 20.34 (+) | 4.09 (+) | 22.05 (+) |
| (Gram +)          | (+++) (+++)    | (+++) (+) | (+++) (+) | (+) (+) | (+) (+) |
| *S. aureus*       | 36.48 17.63     | 8.69 (+) | 11.08 (+) | 4.85 (+) | 16.19 (+) |
| (Gram +)          | (+++) (+++)    | (+++) (+) | (+++) (+) | (+) (+) | (+) (+) |
| *S. typhimurium*  | 33.98 8.74      | 16.23 (+) | 17.35 (+) | 2.76 (+) | 12.21 (+) |
| (Gram -)          | (+++) (+)      | (+++) (+) | (+++) (+) | (+) (+) | (+) (+) |
| *P. aeruginosa*   | 34.98 6.17      | 1.3 (+)  | 1.58 (+)  | 9.66 (+) | 5.49 (+) |
| (Gram -)          | (+++) (+)      | (+) (+)  | (+) (+)  | (+) (+) | (+) (+) |
| *E. coli*         | 27.41 1.7       | 1.49 (+) | 4.09 (+) | 2.92 (+) |
| (Gram -)          | (+) (+)        | (+) (+)  | (+) (+)  | (+) (+) | (+) (+) |

- -, exhibit bacteria on plastic; +, no bacteria on plastic; ++, moderate inhibition (0-10 mm); ++++, strong inhibition (10–20 mm).

The antibacterial activity of native chitosan is relatively the same with Co-g-Chi and Ca-g-Chi, but higher than FA-g-Chi. Radical initiated radical grafting reaction to produce amide groups between carboxylic acid groups of phenolic acid [41] with amine groups of chitosan that both functional groups responsible for antibacterial activity. These explained the fact that grafting reaction only improved solubility, stability, and antioxidant activities [26], but not in antibacterial activities.

In the antibacterial assay of biocomposite plastics, the control plastic had no antibacterial activity against pathogens and plastic disk films supplemented with grafted products resulted no bacteria found below the plastic disk and also no inhibition zone around the plastics. This result indicated that the clear zone only occurred by direct contact with the plastic disk, due to the small / no diffusion of the active compounds. Jin and Zhang (2008) [42] reported the presence of a zone of inhibition around polyactic acid polymer (PLA)/nisin film against *L. monocytogenes* which indicates that nisin molecules were released from the PLA polymer and diffused from the film surface into the solid phase. This study result was accordance to Kun and Marossy (2013) [28] who reported that cellulose disk with biocide performed inhibition zone around the disk against *S. aureus*, whereas the plastic disk with biocide resulted no bacteria growth below the plastic and no migration ability of the biocide plastic around the BHI agar plate. Bideau *et al.* (2016) [43] also stated that a clear inhibition effect
against *B. subtilis* growth on agar only observed in direct contact with a composite film based the TEMPO-oxidized cellulose nanofibers (TOCN), polyvinyl alcohol (PVA) and polypyrrole (PPy) which indicated a minimum release or diffusion of PPy particles. This agar diffusion method provides information about the migration ability of the active compound in the plastic material. However, in this method the weak migration rate of the antimicrobial agents may limit their efficiency.

4. Conclusion

Phenolic acids-grafted chitosan is not improved antibacterial activities of chitosan. It suggested that the addition of grafted products into biocomposite showed no clear zone, and no bacteria were grown in the plastics.

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